Bioactive Marine Natural Products

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Springer

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Foreword

The chemistry of marine natural products has grown enormously in the last fifty years. On land, communication between insects is largely by pheromones. Because these must be volatile, their chemical structures are often simple and many are easy to synthesize. In contrast, in an aqueous environment communication between living organisms depends on solubility in water. As a consequence, the chemical compounds used in the communication can have complex structures and large molecular weights as long as there is adequate solubility in water.

Since all forms of life are subject to perpetual competition, it is not surprising that the organisms that live in the sea produce an enormous range of biological activity. Besides the compounds that repel predators by their toxicity, there are those which are attractive to make reproduction more probable.

In addition, there is a complex food chain from the simplest organisms to the most complicated. What is edible and what is not is also determined by the secondary metabolites of the life process.

Given all these factors it is not surprising that marine organisms are a wonderful source of biologically active natural products. It has taken half a century for this to be fully appreciated. In this time the means of collection have been developed so that marine diving, at least in shallow coastal waters, is relatively simple. Also, more sensitive biological tests are available and can be carried out on board ship. The result of all this is that there is an avalanche of new and biologically exciting marine natural products. However, there is one negative aspect to this work. It is that the compounds isolated are often available in minute amounts only. Therefore, if the structure is complex, it is an arduous, and often impossible, task to isolate enough of the natural material for clinical trials. This is where synthetic chemistry can come to the help of the clinician. Marine natural products are often wonderful challenges to synthetic chemists.

The present book by Dr. D.S. Bhakuni, a distinguished expert on natural products chemistry, [and Dr. D.S. Rawat] will serve as an excellent introduction to the scientific methods involved in marine natural products chemistry. It includes a description of the compounds and their biosynthesis. Of course, there can be no clinical discovery without prior and extensive biological testing so these

procedures are also described in some detail. But before any clinical tests can be carried out, the compound must be isolated. Even if there is never enough for clinical testing, the isolation and determination of structure must take priority.

All these aspects of marine natural products chemistry are treated with authority in this book. It is certain to become an internationally accepted and widely read volume on an important subject.

D.H.R. Barton (deceased)
College Station
Texas A&M University
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Preface

Marine natural products have attracted the attention of biologists and chemists the world over for the last five decades. To date approximately 16,000 marine natural products have been isolated from marine organisms and reported in approximately 6,800 publications. In addition to these publications there are approximately another 9,000 publications which cover syntheses, reviews, biological activity studies, ecological studies etc. on the subject of marine natural products. Several of the compounds isolated from marine source exhibit biological activity. The ocean is considered to be a source of potential drugs.

Marine organisms not only elaborate pharmaceutically useful compounds but also produce toxic substances. One of the most important societal contribution of marine natural products chemists has been the isolation and identification of toxins responsible for seafood poisoning. Outbreaks of seafood poisoning are usually sporadic and unpredictable because toxic fish or shellfish do not produce the toxins themselves, but concentrate them from organisms that they eat. Most marine toxins are produced by microorganisms such as dinoflagellates or marine bacteria and may pass through several levels of the food chain. The identification of marine toxins has been one of the most challenging areas of marine natural products chemistry.

The major occupation of marine natural products chemists for the past two decades has been the search for potential pharmaceuticals. It is difficult to single out a particular bioactive molecule that is destined to find a place in medicine. However, many compounds have shown promise. Marine organisms produce some of the most cytotoxic compounds ever discovered, but the yields of these compounds are invariably so small that natural sources are unlikely to provide enough material for drug development studies.

The art by which marine organisms elaborate bioactive molecules is fascinating. Marine environment provides different biosynthetic conditions to organisms that live in it. Marine organisms generally live in symbiotic association. The pathway of transfer of nutrients between symbiotic partners is of much importance and raises questions about the real origin of metabolites produced by association. A recent trend in marine natural products chemistry is the study of symbiosis. Biosynthesis of bioactive marine natural products provides many challenging problems.

The biological activity of an extract of marine organisms or isolated compounds could be assessed in several ways. Due to limited amount of the material generally available initially and high cost of biological testing, it is impossible in any laboratory to examine all permutation of drug-animal interaction, to unmask the drug potential of a material. Besides, the candidate drug has to pass through rigorous toxicological evaluation and clinical trials before it reaches the clinician's armamentarium. A fair understanding of biological, toxicological and clinical evaluation is essential to those interested in searching potential drugs from marine organisms.

Marine natural products chemistry has passed through several phases of development. The scuba diving made the collection of materials from deep seas easy. Effective methods of isolation provided many potent compounds in pure form. Advancement in instrumentation methods such as nuclear magnetic resonance, mass spectrometric techniques and X-ray diffraction have helped to solve many intricate structural and stereochemical problems. The present text is an effort to fill up the void in bioactive marine natural products. It would be inappropriate to claim that a complete coverage of all bioactive compounds has been made. Attempts have nevertheless been made not to leave out any of the major class of bioactive compounds.

The chemistry and biology of the bioactive metabolites of marine algae, fungi and bacteria and of marine invertebrates; separation and isolation techniques; biological, toxicological and clinical evaluation; bioactivity of marine organisms; biosynthesis of bioactive metabolites of marine organisms; bioactive marine toxins; bioactive marine nucleosides; bioactive marine alkaloids, bioactive marine peptides; and marine prostaglandins are dealt with in separate chapters so that the book may be adopted at any stage by any practicing organic chemist and biologist working in the academic institutions and R&D organizations. Each chapter in the beginning provides highlights of the main points discussed in the text with concluding remarks at the end. References of books, monographs, review articles and original papers are given at the end of each chapter. Considerable progress has been made in the biological evaluation. Thus, marine natural products have drawn organic, medicinal and bioorganic chemists, pharmacologists, biologists and ecologists to work in this area.

The book is dedicated to the late Sir Derek Barton, FRS, Nobel Laureate, Texas, A&M University, USA, who encouraged Dr. Bhakuni to write a book on bioactive marine natural products. The authors are grateful to him for writing the foreword before his sad demise. Thanks are due to the authorities of Central Drug Research Institute, Lucknow, for providing library facilities, and to Dr. S. Varadarajan, FNA, former President, Indian National Science Academy, New Delhi and Prof. John W. Blunt, Department of Chemistry, University of Canterbury, New Zealand for sending interesting information about marine organisms. Thanks are due to Prof. R.S. Verma, Lucknow University, for his valuable suggestions. We thank the publishing staff members of M/s Anamaya Publishers, especially Mr. M.S. Sejwal, who handled the project and offered splendid cooperation.

Finally, one of us (DSB) expresses his sincere thanks to the Council of Scientific and Industrial Research, New Delhi and Indian National Science Academy, New Delhi, for financial support.

D.S. Bhakuni
D.S. Rawat

Contents

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Bioactive Metabolites of Marine Algae, Fungi and Bacteria

Abstract

The chapter deals with the bioactive metabolites of marine algae, bacteria and fungi. The chemistry and biological activities of the bioactive brominated compounds, nitrogen heterocyclics, nitrogen-sulphur heterocyclics, sterols, terpenoids and sulfated polysaccharides isolated from marine algae, fungi and bacteria have been reviewed.

1. Introduction

About 30,000 species of algae are found the world over which occur at all places where there is light and moisture and are found in abundance in sea. They supply oxygen to the biosphere, are a source of food for fishes, cattle and man. Algae are also used as medicine and fertilizers. A few algae that excrete toxic substances pollute marine water.

A majority of red algae and almost all the genera of brown algae except *Bodanella, Pleurocladia* and *Heribaudiella* occur in salt water. Many macroscopic green algae like *Codium, Caulerpa, Ulva* and *Enteromorpha* grow in shallow waters. The species of some genera, for example *Prasiola, Enteromorpha* and *Cladophora* grow both in fresh water and sea water. In sea water, many algae grow as phytoplankton (especially the dinoflagellates and certain blue-green algae). Other marine algae grow as benthos, epiphyte on other algae, parts of higher plants, rocks, stones, gravels, sand and mud. A small group of algae occurs in brackish water.

2. Secondary Metabolites of Marine Algae

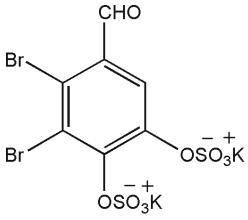
Extensive work has been done on the secondary metabolites of marine algae.[1] The work carried out on *Laurencia* species,[2] blue-green algae[3] and dinoflagellates[4] have been reviewed. Reports are available dealing with amino acids from marine algae,[5] guanidine derivatives,[6] phenolic substances,[7] bioluminescence,[8] carotenoids,[9] diterpenoids,[10] biosynthesis of metabolites,[11] indoles,[12] bioactive polymers[13] and halogenated compounds.[14,15]

3. Bioactive Metabolites

Chemically the bioactive metabolites of marine flora include brominated phenols, oxygen heterocyclics, nitrogen heterocyclics, sulphur nitrogen heterocyclics, sterols, terpenoids, polysaccharides, peptides and proteins. The chemistry and biological activities of the compounds isolated have been reviewed.[16]

3.1 Brominated Phenols

The green, brown and red algae had been extensively analyzed for antibacterial and antifungal activities. The active principles isolated from *Symphyocladia gracilis, Rhodomela larix* and *Polysiphonia lanosa* were: 2,3-dibromobenzyl alcohol, 4,5-disulphate dipotassium salt (**1**), 2,3-dibromo-4,5-dihydroxybenzaldehyde (**2**), 2,3-dibromo-4,5-dihydroxybenzyl alcohol (**3**), 3,5-dibromo-p-hydroxybenzyl alcohol (**4**) and the 5-bromo-3,4-dihydroxybenzaldehyde (**5**). Virtually nothing is known about the physiological importance and the mechanism of biosynthesis of the bromo phenols. Their antialgal activity suggests that they may play a role in the regulation of epiphytes and endophytes. The bromo phenols may be biosynthesised through the shikimate pathway, and bromination may occur in the presence of suitable peroxide.[17]



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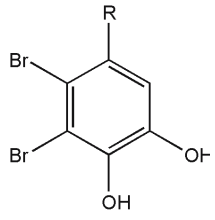
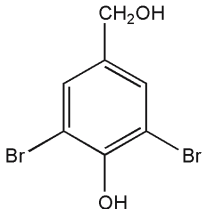


2, R = CHO
3, R = CH_2OH



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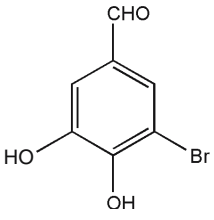

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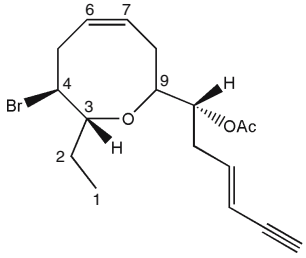
3.2 Brominated Oxygen Heterocyclics

The red algae *Laurencia* sp. have produced the diverse class of natural products.[18–22] *L. glandulifera*[19] and *L. nipponica*[23] had furnished two brominated oxygen heterocyclic compounds, laurencin (**6**)[22] and laureatin (**7**)[23], respectively. Laurencin ($C_{17}H_{23}BrO_3$), m.p. 73–74°C; $[\alpha]_D$ + 70.2° ($CHCl_3$) was isolated from the neutral fraction from methanol extract of the algae. The IR of the purified compound suggested the presence of a terminal methine (ν_{max} 3285 and 2180 cm^{-1}), an acetoxyl (1735 and 1235 cm^{-1}) and an ether (1168 and 1080 cm^{-1}) functions and *trans* and *cis* double bonds (3040, 950 and 750 cm^{-1}). The UV (in EtOH), λ_{max} 224 nm (ε 16,400) and 232 nm (ε 11,000) showed the presence of a conjugated diene or enyne. The NMR spectrum of the compound indicated the presence of four olefinic protons and an acetoxyl and ethyl groups. The presence of ethyl group was confirmed by isolation of CH_3—CH_2—CHO on ozonization of laurencin.



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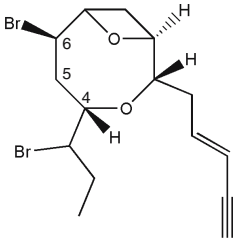

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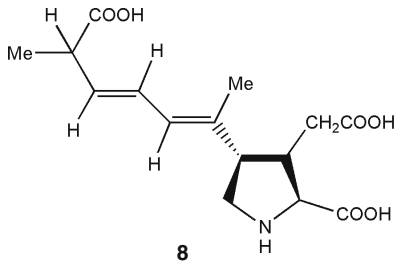
Laurencin consumed four moles of hydrogen over platinum in ethyl acetate to yield octahydrolaurencin ($C_{17}H_{31}BrO_3$). On mild hydrolysis with KOH laurencin gave deacetyl laurencin ($C_{15}H_{21}BrO_2$) which was reconverted into original ester in good yield by treatment with acetic anhydride/pyridine. Reduction of octahydrolaurencin with $LiAlH_4$ afforded a debromoalcohol ($C_{15}H_{30}O_2$). Extensive NMR studies and spin decoupling experiments of the parent compound and the degradation products established structure **(6)** for laurencin.

Laureatin ($C_{15}H_{20}Br_2O_2$) m.p. 82-83°C; $[\alpha]_D$+ 96° (CCl_4) has been isolated from the Japanese seaweed.[18] UV absorption λ_{max} 223 nm (ε12,800), 229 nm (ε10,400) and IR peaks at ν_{max} 3300, 2100, 1140, 1045, 975 and 965 cm^{-1} indicated that laureatin is an ether having a conjugated enyne group and contains neither hydroxyl nor carbonyl functions. NMR and spin decoupling experiments confirmed the presence of —CH_2—CH=CH—C≡CH and —$\underset{|}{CH}$—CH_2—CH_3 groups. NMR spectrum of the compound also contained peaks for 6 protons at τ 5.0, 6.5; three one-proton septets at τ 5.12 and 5.87, a broad quartet at 5.62 and two multiplets centered at 6.2 and 6.35. These absorptions were ascribed to protons on carbons bearing an ether oxygen or a bromine atom. Laureatin consumed three moles of hydrogen over platinum catalyst in ethanol to yield hexahydrolaureatin. On treatment with zinc in refluxing acetic acid and then with dilute alkali hexahydrolaureatin

gave an unsaturated glycol. Laureatin was finally assigned structure (**7**) on the basis of chemical degradation studies and NMR spectroscopic data. Other brominated metabolites which have been isolated from *Laurencia nipponica*, are prelaureatin, laurallene, isolaurallene, bromofucin, and chlorofucin. The total syntheses of (+)-prelaureatin and (+)-laurallene have been achieved recently.[24] Laureatin (**7**) and isolaureatin exhibit significant larvicidal activity (IC_{50}) 0.06 and 0.50 ppm, respectively, in mosquitos. Brominated compounds isolated from marine algae, particularly bromophenols, are toxic and due to this they are not of clinical value.

3.3 Nitrogen Heterocyclics

Marine algae had yielded nitrogen containing heterocyclic compounds. Of these the most interesting compounds are domoic acid (**8**) and the kainic acid.



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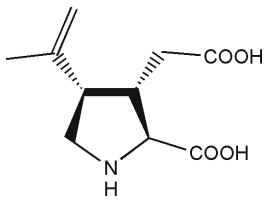
Domoic acid (**8**) ($C_{15}H_{21}NO_6$), m.p. 217°C (dec.): $[\alpha]_D$ – 109.6° [H_2O] an anthelmintic agent was first isolated from the alga *Chondria armata*.[25-29] The acid had UV λ_{max} 242 nm (log ε 4.42). Catalytic reduction of the compound with Pt-O_2 gave tetrahydrodomoic acid. Acetylation of the compound gave N-acetyl derivative, m.p. 121°C; $[\alpha]_D$–56° [H_2O]; λ_{max} 242 nm (log ε 4.48). Domoic acid showed marked anthelmintic activity. It was found to be very effective in expelling ascaris and pinworms without any observable side effects.

3.4 Kainic Acids

In Asia, the dried red alga *Digenea simplex* is widely used as an anthelmintic. It is found very effective in the treatment of ascariasis.[30] In the Mediterranean, extract of the alga *Corallina officinalis* is also used for the same purpose. Kainic acids as the active principles had been isolated from these algae. Of the kainic acids, α-kainic acid was the most active constituent. The structure (**9**) for α-kainic acid had been assigned by degradation studies[31] and confirmed by its synthesis.[32] The stereochemistry of α-kainic acid is shown in (**9**).[33]



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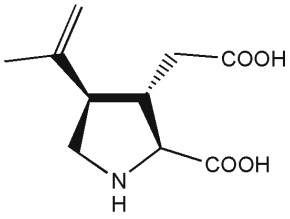
Isomers of α-kainic acid had been isolated from alga *Digenea.* The isomers isolated are γ-allo-kainic acid (**10**)[34] and γ-kainic acid lactone (**11**).[35] L-α-kainic acid and L-α-allo-α-kainic acid are configurational isomers. In α-kainic acid the substituents at C-2 and C-3 and at C-3 and C-4 are *trans* and *cis*, respectively. In α-allokainic acid configurations at both the centres are *trans*. α-Kainic acid lactone was considered to be an artifact.[36] α-Kainic acid had been found effective in the treatment of ascariasis, with a single dose of 5 to 10 mg per adult resulting in a 40 to 70% reduction in the population of instestinal parasitic worms. α-Allokainic acid was found to have far less anthelmintic activity. Several preparations of kainic acids are available in the market, including 'Digenin' and 'Helminal' (The Merck Index, 1968). This represents one of the few instances in which clinically useful pharmaceutical product has been isolated from marine source.



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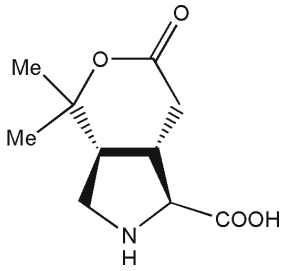

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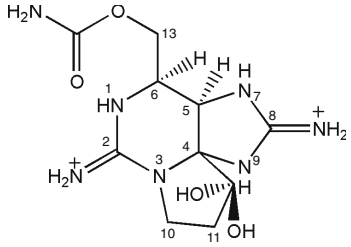
3.5 Guanidine Derivatives

Certain shellfish periodically become poisonous to humans. It is now well established that the substance responsible is produced by a marine plankton, *Gonyaulax catenella*. At certain unpredictable time the red plankton multiply and cause "red tide". Although many fishes are killed by this "red tide", mussels and clams survive and concentrate the toxic principles, thus becoming poisonous to humans. The toxin isolated from the Alaskan butter clam, *Californian mussel*[37] and the alga *Gonyaulax catenella*[38-40] is called saxitoxin (**12**).



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Saxitoxin ($C_{10}H_{19}N_7O_4$) when heated with P/HI in acetic acid, gave weakly basic compound I, $C_8H_{10}N_2O$ (m.p. 100-102°C), NMR analysis of I indicates

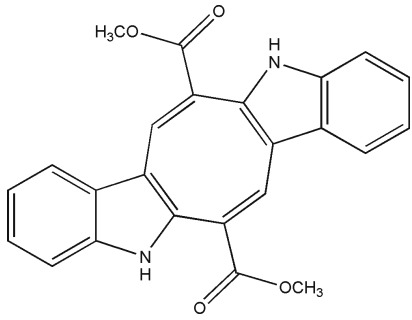
the presence of one C—CH_3 group.[41,42] On oxidation with potassium permanganate, urea and guanidinoacetic acid were obtained. Hydrogenation of I in the presence of platinum oxide (200 mole % hydrogen absorption) gave II, $C_8H_{14}N_2O$ (m.p. 129-131°C) which also contained one C—CH_3 group. Strong acid hydrolysis of II led to the strongly basic, and highly hygroscopic oily diamine III, $C_7H_{16}N_2$ and on heating with Pd-C, III resulted in the formation of a substance which readily gave a positive Ehrlich test for pyrroles. On the basis of these data, it was concluded that III was a pyrrolidine and II was a saturated cyclic urea. This conclusion was fully supported with its ultraviolet absorption and its strong infrared absorption at 3410 and 1635 cm^{-1} in chloroform. The structure (**12**) to saxitoxin was assigned on the basis of degradation studies and spectroscopic analysis. Saxitoxin blocks nerve conduction by specifically interfering with the intital increase in sodium permeability of the membrane. The symptoms caused by the toxin include peripheral paralysis. In extreme cases, complete loss of strength in the muscles and finally death occurred which is caused due to respiratory failure.[43] Saxitoxin is absorbed from the gastro-intestinal tract. It produced no major vascular action. The oral LD_{50} for toxin in various species of animals is reported. In man death had occurred following ingestion of as little as 1 mg of toxin.[44] The toxic compounds from marine algae appear to have biomedical potential. The compounds with neurotropic effects may yield important drugs.

3.6 Phenazine Derivatives

The marine alga *Caulerpa lamourouxii* is widely distributed in the Phillippines. The upper branches are eaten as a 'salad', despite their peppery and astringent taste. However, the alga is found toxic to some individuals. Chemical investigation of the alga had furnished caulerpicine, caulerpin, cholesterol, taraxerol, β-sitosterol and palmitic acid.[45] Caulerpin had also been isolated from *Caulerpa sertularioides, C. racemosa* var. *clavifera*[46] and caulerpicin from *C. racemosa*.[47]

Caulerpin (**13**) ($C_{24}H_{18}N_2O_4$) (M^+ 398) red prisms m.p. 317°C had λ_{max} 222, 270, 292, 317 nm (ε 50,000, 27,000, 29,000 and 35,000); IR bands at 1684, 1631 and 1613 cm^{-1} suggesting the presence of carbonyl functions in conjugation with aromatic system. The NMR spectrum of the compound indicated the presence of 18 protons τ 6.17 (6H, 2 OMe), 2.4-3.0 (8H, m), 1.76 (2H, s) and 1.36 (2H, s). Aromatic protons signal at τ 2.4-3.0 and 1.79 and the IR bands at 730 and 920 cm^{-1} suggested the presence of two identically substituted aromatic ring systems. This was substantiated by elimination of 26 mass units (CH=CH) in the mass spectrum of caulerpin, caulerpinic acid and decarboxy caulerpin acid. Caulerpin contained two methoxy groups in the form of α,β-unsaturated methyl ether group [ν_{max} 1685 cm^{-1}; NMR τ 6.17 (6H)]. Its mass spectrum supported the assignment m/z 398 (M^+), 366 (M^+–MeOH), 338 (366–CO), 306 (338–MeOH), 339 (M^+–CO_2Me) and 280 (M^+–$2CO_2Me$). The M^+ peak in the mass spectrum was the base peak.

Saponification of caulerpin with alcoholic KOH yielded caulerpinic acid ($C_{22}H_{14}N_2O_4$) (M^+ 370). The two exchangeable protons at τ 1.36 were due to secondary amino groups. The functions were conjugated with the two methoxy carbonyl groups as indicated by the low frequency carbonyl absorption (1685 cm^{-1}). The methoxy carbonyl groups were placed at the two α-positions of the two naphthalene rings conjugated with the NH groups at the β-positions. This arrangement accounted for the strong hydrogen bonding of the –NH protons. Caulerpinic acid when heated with copper bronze in quinoline at 200-210°C yielded a decarboxylated compound m.p. >300°C, (M^+ 282). On the basis of these studies caulerpin was assigned the structure α,β-dihydrodibeno[b,i]phenazine-5,12-dicarboxylate (**13**).[48] The stability of the compound was stated to favour the linear structure rather than the geometrical isomer. Caulerpin caused a mild anesthetic action when placed in the mouth, which resulted in numbness of the lips and tongue. In some people it produced toxic effects. The toxic syndrome had been reported to be some what similar to that produced by ciguatera fish poisoning.



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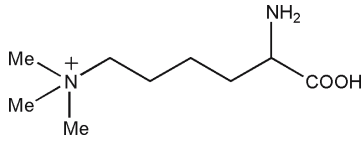
3.7 Amino Acids and Amines

Extracts of the marine algae *Laminaria angustata* and *Chondria amata* are reported to contain agents with hypotensive and other pharmacological properties. Laminine (**14**), a choline like basic amino acid had been isolated from a number of marine algae.[49,50] The compound had been characterised as trimethyl(5-amino-5-carboxypentyl)ammonium oxalate (**14**). Several syntheses of laminine are reported.[51]



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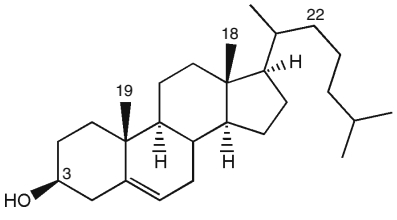
Laminine was isolated from water extracts of *Laminaria angustata* by amberlite ion exchange resin, IR-120 in acidic form and subsequent formation of reineckate and oxalate salts. The other amino acids isolated from this

source were: L-lysine, L-arginine, ethanolamine and choline. Laminine monocitrate was found to have a transitory hypotensive effect. Laminine, in general, depressed the contraction of excited smooth muscles. Laminine monocitrate had an LD_{50} in mouse, 394 mg/kg. It is considered to be a potentially useful pharmacological agent. Steiner and Hartmann[52] had reported the widespread occurrence of volatile amines, such as methylamine, dimethylamine, trimethylamine, ethylamine, propylamine, isobutylamine, isoamylamine, 2-phenylethylamine and 2-methylmercapto propylamine in red, green and brown algae. It is mentioned that biological activities of some of the extracts of the marine algae may be due to the presence of these amines.

3.8 Sterols

The presence of sterols in algae was first established by Heilbron et al[53] and later by Tsuda et al.[54] Gibbons et al[55] established the presence of 22-dehydrocholesterol and demosterol in red algae. However, later investigations showed that the sterol content of red algae were more varied than had been believed.[56] Idler et al[57] examined some species of red algae and found that the three species contained C_{27}, C_{28} and C_{29} sterols. An interesting feature of their result was the considerable variation in sterols content of four different samples of the alga *Rhodymenia palmata*. The percentage of demosterol, for example, varied from 97.2 to 7.7% in the mixture of sterols. Similary, cholesterol was detected in the concentration as high as 97.3% and as low as 2.1%. Cholesterol was again found the major sterol of Rhodophyta. Four species of algae, *Rhodymenia palmata, Porphyra purpurea, P. umbilicalis* and *Halosaccion ramentaceum* were found to contain desmosterol as the main sterol. However, *Hypnea japonica* was the only alga having 22-dehydrocholesterol as the major sterol. Of the 34 algae investigated by the Japanese and British investigators, only one sterol was detected in 25 species, while nine were found containing two sterols. Meunier et al[58] had given a comparative data of 14 species of Rhodophyta. All the species examined were found to contain cholesterol (**15**) as the major sterol except *Hypnea musciformis* in which 22-dehydrocholesterol (**16**) was detected in the highest concentration. *Hypnea japonica* was another example in which 22-dehydrocholesterol was present as the major sterol.



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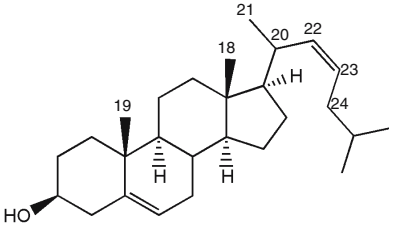

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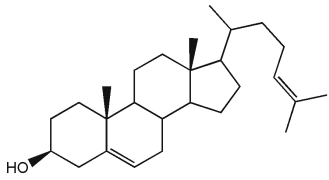
The distribution of sterols in algae had been reviewed.[58,59] Red algae contained primarily cholesterol (**15**). Several species contained large amount of demosterol (**17**), and one species contained primarily 22-dehydrocholesterol. Only a few rhodophyta contained traces of C_{28} and C_{29} sterols. Fucosterol (**18**) was the dominant sterol of brown algae. Most phaeophyta also contained traces of cholesterol and biosynthetic precursors of fucosterol.



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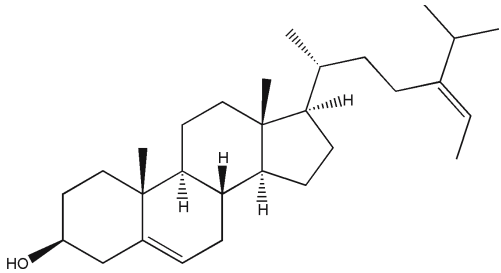

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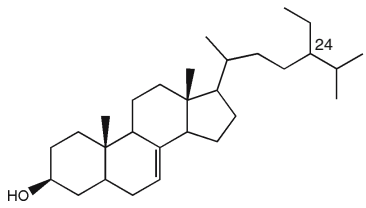
The sterols of green algae were much more varied. The green algae contained chondrillasterol (**19**), poriferasterol (**20**), 28-isofucosterol, ergosterol and cholesterol.



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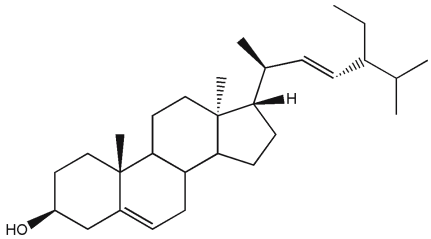

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24-Methyl cholesterol and sargasterol differ from fucosterol (**18**) in that the double bond is shifted to the C-28 position and is saturated at position 24. Sargasterol and fucosterol are isomers. The methyl group at position 20 in the former is α-oriented, whereas it is β-oriented in the latter. The sterols from marine algae are reported to be non-toxic and have the ability to reduce blood cholesterol level. They are also reported to reduce the tendency to form a fatty liver and excessive fat deposition in the heart.[60]

3.9 Sulfated Polysaccharides

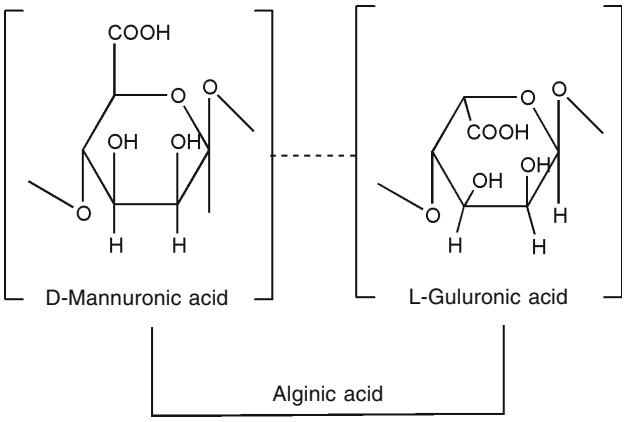
The sulfated polysaccharides obtained from seaweeds are economically most important products due to their extensive use in food and medicine. Of the four major seaweed classes, the rhodophyceae (red algae), the phaeophyceae (brown algae), the cyanophyceae (blue-green algae) and the chlorophyceae (green algae), the first two classes produce polysaccharides of main interest. The red algae produce carrageenan, agar, agarose, furcellaran or Danish

agar. Alginic acid is obtained from brown algae. The use of seaweed extracts in food and medicine is reviewed.[61] Carrageenan are produced by species of *Chondrus, Eucheuma, Gigartina* and *Iridea.* There are different views on the structure of red seaweed polysaccharides.[62] It is generally suggested that carrageenans be defined as a polysaccharide comprising D-galactose units and derivatives linked alternatively α (1→ 3) and β (1→ 4). The ι, κ, λ and μ and other carrageenans represent variations of this primary and general form in which the galactose units are sulfated in a definite pattern and/or are present in the 3,6-anhydro form expressed generally as an A-B-A polymer. Pernas et al[63] however, do not agree on the validity of the above simplified structural approach to carrageenan. These workers believe that carrageenan is a continum of potassium precipitable material of continuously variable structural form. The ester sulphate groups are distributed randomly on all available hydroxyl groups in κ, in support of this hypothesis. The chemical structure of κ and λ carrageenans are still a matter of discussion. κ Carrageenan is precipitated from dilute solution with potassium ions, and is believed to consist primarily of alternating anhydrogalactose and sulphated galactose units linked α 1,3 and β 1,4. λ Carrageenan contains little anhydrogalactose. It consists chiefly of mono- and disulphate galactose units with, perhaps, the same alternating 1,3 and 1,4-linkages. Both κ and λ carrageenans are reported to be strong antigens.[64] The latter is more potent than the former. In general, they behave as typical carbohydrate antigens. λ Carrageenan is also reported to stimulate the growth of connective tissues.[64]

Chondrus crispus and *Gelidium cartilagineum*, the well-known sources of carrageenan and agar, respectively, had been found to possess antiviral properties attributed to the galactan units in the polysaccharides of both. The specific antiviral activity had been shown against influenza B and mumps virus in embryonated eggs even after 24 h inocubation. Carrageenan was also found as anticoagulant and antithrombic agent. The use of carrageenan in ulcer therapy had been studied intensively. It was thought at first that the polysaccharide inhibits the activity of pepsin and that its action in preventing ulcers was due to this property.[65] However, subsequent studies revealed that the polysaccharide plays a much more active role than enzyme inhibition. In fact, it was found that *in vivo*, pepsin was not inhibited by carrageenan. The polysaccharide reacts with the mucoid lining of the stomach and gives a protective layer through which pepsin and acid have difficulty in passing. The treatment of gastric and duodenal ulcers by carrageenan was enjoying considerable popularity in France and Great Britain. In many cases of ulcer carrageenan proved an effective cure.[66]

Alginic Acid

This polysaccharide is obtained from the brown seaweeds, especially from species of *Fucus* and *Macrocystis*. Chemically alginic acid (**21**) is made up of two monomers, the D-mannuronic acid and L-guluronic acid. Both these



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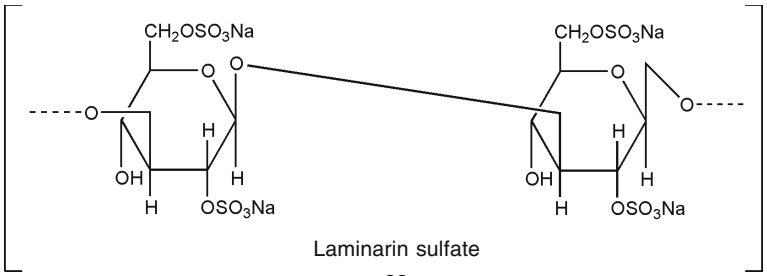
sugar acids are stereoisomers and differ only in the configuration of the carboxyl group. The two uronic acid moieties in alginic acid are linked though β-1,4-glycosidic linkage in such a way that the carboxyl group of each unit is free, while the aldehydic group is sheilded by the glycosidic linkage. Biosynthesis of alginic acid is not yet known. An attractive hypothesis of its formation from guanosine diphosphate mannose has been proposed.[67]

Commercially, sodium alginate is extracted from giant brown seaweed (*Macrocystis pyrifera*), horsetail kelp (*Laminaria digitata*) and sugar kelp (*Laminaria saccharina*). Sodium alginate has been used mainly in the manufacture of ice cream where it serves as a stabilising colloid. It is also used in cosmetics and pharmaceuticals.[68] Calcium alginate is reputed to be a hemostatic agent which stimulates the clotting of blood *in situ* which is subsequently absorbed in the tissue.[69] Sodium alginate is reported to be a useful adjuvent in immunisation against two strains of influenza virus. Sodium alginate is also found effective in diminishing hyper calciuria in urolithiasis, and found useful in the treatment of esophagitis.

The most significant property of sodium alginate is the ability to remove strontium 85 and strontium 87 from the body without seriously affecting the availability of Ca, Na or K in the body.[70] This selective action of sodium alginate is of great potential to remove Sr-90 contamination due to fall out from atomic explosions.

Laminarin

It is essentially a linear polymer of β-1,3-glucan, with occasional branching points at C-6 and with a variable proportion of the glucose chains terminated at the potential reducing end with a molecule of mannitol, which can be sulphated to produce a compound with anticoagulant properties. Laminarin (**22**) occurs at certain times of the year to the extent of 35% of the dry weight of *Laminaria cloustoni*. It has been found that laminarin sulphate formed with two sulphate groups by glucose unit gives maximum stability and



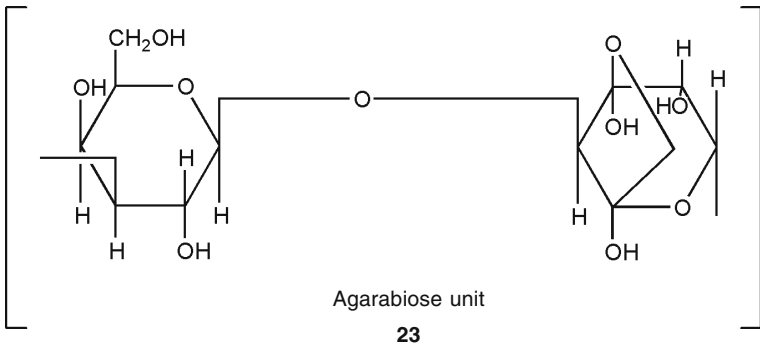
Laminarin sulfate

22

anticoagulant activity. Two lower sulphated laminarins are also reported to have antilipidemic activity like that of heparin.[71,72]

Agar and Agarose

The red algae are the source of agar and agarose. Although these polysaccharides have no direct medicinal use, their use in biomedical research is well known. The genera *Gelidium, Gracilaria, Acanthopeltis* and *Pterocladia* of the Rhodophyceae are the main producers of these materials. Commercial agar generally contains 50-90% recoverable agarose. The structure of agarose was determined by Araki[73] and substantiated by others. Chemically, agarose is a linear polymer made up of repeating units of agarobiose (**23**) which, in turn, consists of a molecule of β-D-galactopyranose attached $1 \rightarrow 4$ to a molecule of 3,6-anhydro-L-galactose. These repeating units are linked $1 \rightarrow 3$ to form the agarose polymer. The presence of traces of sulphate and uronic acid residues have, thus far, been attributed to contamination by agaropectin. Many uses of agarose are described.[74] However, its use in immunology is most interesting.



Agarabiose unit

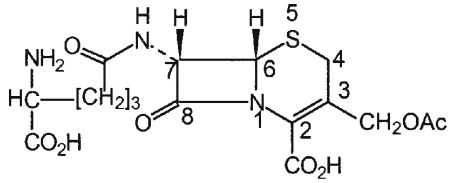
23

The interest to investigate the role of the polysaccharides in the body is growing. The sulphated seaweed polysaccharides are, in some ways, very much like the sulphated mucopolysaccharides of the body and yet, in some ways, are quite different. It is believed that, in some cases, the body may not distinguish the seaweed polysaccharides from those natural to it. In some other cases, they may be so much alike that reactions are started with them, but not finished in the normal manner, which may allow their use as inhibitors.

4. Marine Bacteria and Fungi

Bacteria and fungi are prime producers of the antagonistic substances in terrestrial environment. A similar role in the oceans from these organisms is expected. Indeed, this had been found true. Antibiotic, antiviral, antifungal, and antiyeast activities of these organisms had been reported.[75] Besides, a few growth stimulant properties which may be useful in studies on wound healing, carcinogenic properties, and in the study of cancers are reported.

Among the many bacteria showing antimicrobial activity, a variant of the ichthyotoxic *Pseudomonas piscicida*[75] exhibited marked antagonism to various micro-organisms. A red coloured bacterium obtained from Puerto Rico was found to excrete vitamin B and antibacterial substances into the sea water.[76] The bacteria and fungi from sea are also reported to produce substances which affect central nervous system (CNS), respiratory system (RS), neuromuscular system (NMS), autonomic nervous system (ANS), cardiovascular system (CVS) and gastrointestinal system (GI).[77] Some of the substances are known to produce local effects such as pain, necrosis, edema, parasthesias, pruritis etc. A marine isolate of the fungus *Cephalosporium acremonium* obtained from the sea near a sewage outfall of the coast of Sardina had been reported to produce a number of antibiotic substances.[78] A penicillinase sensitive antibiotic substance named antibiotic N active against Gram-negative bacteria, had been isolated from this source. This material was reported to be cephalosporin C (**24**)[79-81] which was different from cephalosporin N.



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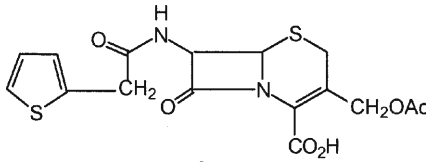
Other antibiotic substances isolated from *C. acremonium* were found to be penicillinase resistant and active against Gram-positive bacteria.[78] These substances were named cephalosporin P. This organism was also found to be a source of cephalothin (**25**), a semisynthetic derivative of cephalosporin C, having antibiotic action similar to that of benzylpenicillin but insensitive to penicillinase. It was active against a number of penicillin resistant *Staphylococcus* and some Gram-negative species of bacteria. A number of chemically-related antibiotic substances named cephalosporins P_1, P_2, P_3, P_4 and P_5 had been isolated from the marine species of fungus *Cephalosporium acremonium*.[82]

Cephalosporin P_1 ($C_{33}H_{52}O_8$) m.p. 147°C; $[\alpha]_D$ + 28° [$CHCl_3$] was a mono-basic triterpenic carboxylic acid. Methylation of the acid with CH_2N_2 at 0°C gave a monomethyl ester m.p. 196°C, while methylation at room temperature with CH_2N_2 produced a product containing nitrogen. The acid



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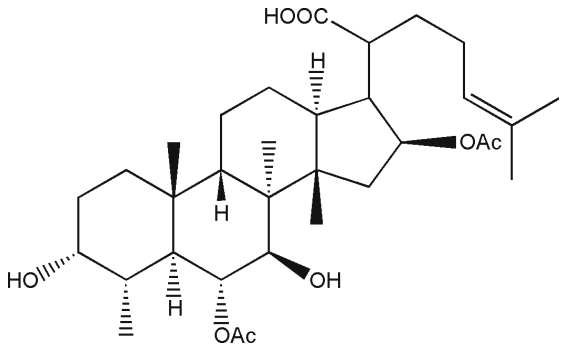
and its methyl ester rapidly absorbed one mole of hydrogen in the presence of PtO_2 to give dihydrocephalosporin P and dihydrocephalosporin methyl ester, respectively. On standing in 1N NaOH at 37°C cephalosporin P lost an acetyl function to give a hydroxy acid m.p. 220°C. Besides, hydrolysis with alkali yielded a product which rapidly lactonised on acidification to give a neutral compound, m.p. 186°C. The chemical studies when combined with NMR and mass spectral data, structure (**26**) was assigned to cephalosporin P_1.[83] It exhibited good activity against *Bacillus mesentericus, Mycobacterium phlei* and *S. aureus in vitro*.[83]



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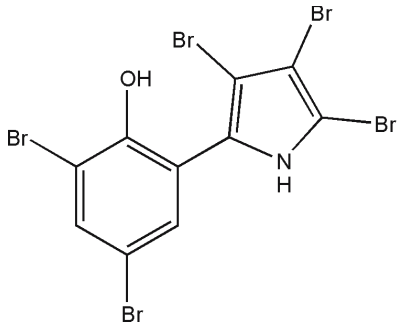
Several bacteria, which produce antibiotic substances, had been isolated from the shallow water near Puerto Rico. A bromo pyrrole antibiotic has been isolated from *Pseudomonas bromoutilis*,[84] which showed activity against many Gram-positive bacteria (at levels of 0.06 mg/mL in broth assay test), but was not active by the subcutaneous route in mouse protection tests. The bromo compound (**27**) ($C_{10}H_4Br_5NO$) was unique in that over 70% of its weight consisted covalently bonded bromine. The mass spectrum of the compound suggested a molecular weight 553.5 and the presence of five bromine atoms from the clasture of isotope peaks. A preferential and sequential loss of one, two and three bromine atoms from the molecular ion together with loss of HCN was observed. Metastable ion peaks corresponding to a simple cleavage of the phenol and pyrrole portions were also discernible. The structure (**27**) for the antibiotic was finally established by X-ray crystallographic analysis[84] and confirmed by its synthesis.[85] Pyrrolnitrin (**28**), a chloropyrrole had been isolated from *Pseudomonas pyrrocinia*. Pyrrolnitrin (**28**) exhibited high antibiotic activity against dermatophytic fungi, particularly



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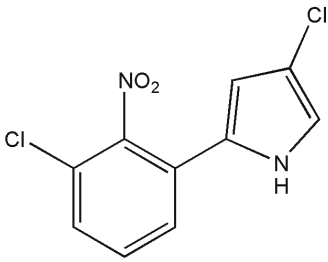

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against members of the genus *Trychophyton* and against many soil borne fungal plant pathogens like *Rhizoctonia solani* and *Fusarium sambucinum* and against foliar fungal pathogens like *Fusarium graminearum, F. culmorum* and *Pyrenophora tritici repentis*.[86] This compound was marketed in Japan under the name PYRO-ACE for the treatment of superficial dermatophytic infections.[86h] Its light sensitivity prevented the use of pyrrolnitrin (**28**) as a fungicide in plant protection. Recently, antimycobacterial activity was reported for (**28**) and related compounds.[86i] Biological activity of (**28**) at low concentrations was demonstrated to be due to the uncoupling of oxidative phosphorylation in *Neurospora crassa* and at higher concentrations due to inhibition of electron transport both in the flavin region and through cytochrome coxidase.[86*l*] However, recently it was reported that (**28**) leads to glycerol accumulation and stimulation of triacylglycerol synthesis resulting in leaky cell membranes and concomitant break down of biosynthetic activity followed by cessation of cell growth.[86m] It had been characterised as 3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole. A synthesis of the antibiotic is reported.[87]

The formation of antibiotic substances of the types mentioned above gives the indication that the marine microbes are capable of producing new and unusual types of antibiotic substances than the terrestrial ones. Some of these bioactive substances would, undoubtedly, be found useful in medicine and pharmacology, while others could become of even greater interest than native product.

Serratia marcescens, a widely distributed non-pathogenic bacterium, had furnished a red coloured antibiotic named prodigiosin.[88-96] It exhibited high order of antibiotic and antifungal activities. The high toxicity of prodigiosin precluded its use as a therapeutic agent.

Studies on the marine phytoplanktons are few because of difficulty of growing the organisms and the low yield of secondary metabolites. However, several toxins related to saxitoxin are isolated from *Gonyaulax* species.[97-103] The cultured cells of the dinoflagellate *Ptychodiscus brevis*, yielded brevetoxin B, C and dihydrobrevetoxin B.[103-107] A unique feature of their structure is a chain of eleven, continuous *trans*-fused ether rings in the form of a flat ladder. *P. brevis* yielded two phosphorus containing toxins[108] GB-4 and

GB-1 which do not appear to be natural products since attempts to incorporate ^{32}P into GB-1 gave ambiquous results.[109,110] Two new polycyclic ethers, GB-5 and GB-6 closely related to okadaic acid, a toxin that was first found in sponges and later in dinoflagellate have been isolated from the cultured cells of *G. breve*.[111] The dinoflagellate *Dinophysis*, produces and transmits shellfish, toxins that are responsibe for diarrhoetic shellfish poisoning.[112] *Lyngbya majuscula* known to cause swimmer's itch has furnished several class of compounds.[113-126] Pukeleimides (A-F) showed activity against *Mycobacterium smegmatis* and *Streptococcus pyrogenes*.[113,127,128] Cyclic depsipeptide, majusculamide-C isolated from the organism inhibits the growth of fungal plant pathogen.[129] Aplysiatoxins and oscillatoxins isolated from blue-green algae *Schizothrix calcicola* and *Oscillatoria nigroviridis* possess antileukaemic activity but their high toxicity precludes their medicinal use. Cytotoxic and fungicidal nucleosides have been isolated from a variety of blue-green algae.[130] Anatoxin-a, an exogenic toxin of blue-green alga *Anabaena flosaquae*[131] is one of the most potent nicotinic receptor agonist. It is suggested that the analogues of anatoxin-a may be of clinical value for treating disorders associated with defects in cholinergic regions of the central nervous system.

Several species of green-algae of the genus *Halimeda* produce an ichthyotoxin which exhibits diverse biological activities.[132-134] It inhibits the growth of marine bacteria and fungi, cell division of fertilized sea-urchin eggs and the motility of sea-urchin sperms at 1 μg/mL. Avrainvilleol, a brominated metabolite of green algae, *Avrainvillea longicaulis* exhibits high order of antifeedant activity in reef fish and also inhibits the growth of microorganisms. The genera *Halimeda, Penicillus* and *Udotea* are found to contain highly active but unstable sesquiterpenoids and diterpenoids. Some of these diterpenoids exhibit cytotoxic and antimicrobial activities.[135,136] Prenylated aromatics with small side chains are relatively common in brown algae.[137] Several highly unsaturated C_{11} hydrocarbons are isolated from *Dictyopteris plagiogramma* and *D. australis*.[138,139] The function of these hydrocarbons have been studied in detail.[140,141] It has been observed that the sperm cells aggregate around the female gametes of brown algae which exude C_{11} hydrocarbons that attract the former and cause them to remain in the excited state in the vicinity of the latter. The sex attractants that have been identified are: ectocarpene from *Ectocarpus siliculosus*,[142] fucoserratene from *Fucus serratus*, multifidene from *Culteria multifida*, n-butyl-cyclohepta-2,5-diene from *Dictyota dichotoma*, desmarestene from *Desmarestia viridis* and tinavarrene from *Ascophyllum nodosum*. Tracing the origin of arsenic in lobsters and in fish, it has been found that the brown algae *Ecklonia radiata* concentrates arsenic in the form of arseno-sugars.[143,1444] Hydroxydictyodial from *Dictyota spinulosa* inhibits feeding in the omnivorous fish *Tilapia mossambica*.[145] Three ichthyotoxic and phytotoxic diterpenes are isolated from *Dilophus fasciola*.[146,147] Several diterpenes from *Dictyota* species exhibit significant cytotoxicity.[147] Two phlorotannins from *Ecklonia kurome* exhibit

antiplasmin inhibitory activity.[148-152] The pharmacological properties of laminine has been studied. It has been found that the compound at high doses does have a hypotensive action as a result of a ganglion blocking effect. Marine red algae have yielded a vast array of halogenated lipids, some of these exhibit CNS-depressant and hypotensive activities. Three brominated acetylenic compounds active against mosquito larvae have been obtained from *Laurentia nipponica*.[153] Trihydroxy benzyl methyl ethers having antibacterial activity against *Bacillus subtilis* are isolated from *Grateloupia filicina*.[154,155] Enantioselective synthesis of (–)-kainic acid possessing anthelmintic, insecticidal and neuroexcitatory activities, have been achieved. The symmetrical bisbenzyl ether from *Symphyocladia latiuscula* showed antifungal activity. 5-Iodo-5′-deoxytubercidin, an unusual nucleoside has been isolated from *Hypnea valentiae*.[156] The nucleoside caused pronounced relaxation of muscles, hypothermia in mice and blocked polysynaptic and monosynaptic reflexes.

5. Micro Algae

Micro algae represent a subset of single cell microorganisms that generally grow autotropically using CO_2 as the sole carbon source and light as energy. These algae are ubiquitous in nature. Aquatic micro algae have been isolated in areas ranging from hot springs to glacial ice flows. There are over 50,000 different species of micro algae of which only a few have been characterised. Micro algae represent a major untapped resource of genetic potential for valuable bioactive agents and biochemicals. Phycocyanin and phycoerythrin are produced by cyanobateria (*Spirulina*), and recently have been used as fluorescent labelling agents. They are proteinaceous in structure and exhibit a high extinction coefficient. One future commercial application of micro algae could be in the production of special lipids. The omega-3-fatty acids found in the oils of certain cold-water marine fish are considered to be responsible to reduce incidence of coronary heart disease. These fatty acids are likely to originate from the phytoplankton in food chain. Many of these phytoplankton species are found to be rich in reserves of oils containing various amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Exploiting autotrophy, micro algae are being used for the production of labelled biochemicals. This involves the substitution of tritiated water [^3H_2O] for ^1H_2O or $^{14}CO_2$ for $^{12}CO_2$ and results in the production of radioactively labelled biochemicals. Deuterium labelled compounds and also compounds labelled with ^{13}C are made using heavy water [D_2O] and $^{13}CO_2$, respectively. One can produce enrichment levels upto 100% depending on the isotope enrichment of the culture medium. These labelled biochemicals are of high value. Uses of the stable isotopically labelled compounds include production of very high stability deuterated lubricants. The most attractive source of the ^{13}C and ^2H-labelled compounds are autotrophic micro algae. If diagnostic tests are developed using these compounds, the market will increase

dramatically. Micro algae can also provide a "designer oil", specially tailored to the food industry. Further, commercial scale production of EPA from micro algae is an attractive proposition. Micro algae are also expected to furnish potent antiviral, antiAIDS, antibiotic and other bioactive agents.

The extract of cyanobacterium *Planktothrix* sp. exhibited embryotoxicity.[157] Changes in the culture conditions of *Lyngbya majuscula* had the greatest effect on production of its secondary metabolites.[158-165] Several leptosins were isolated from the marine alga *Leptosphaeria* sp. and their biological activity evaluated. Of these leptosin M exhibited significant cytotoxicity against human cancer cell lines.[166] Two new antiinflammatory macrolides, lobophorin A and B were isolated from a marine bacterium.[167] Water extract of marine diatom *Haslea ostrearia* exhibited anticoagulant activity.[168] Brominated anisoles and cresols were detected for the first time in the red marine alga *Polysiphonia sphaerocarpa*.[169] The sulfated polysaccharide of the red microalga *Porphyridium* sp. showed high order of antiviral activity against herpes simplex virus (HSV-1 and 2) both *in vitro* and *in vivo*.[170] Ten new sesquiterpenoids were isolated from the fungus *Drechslera dematioidea*. Of these drechserine E and G exhibited antiplasmodial activity against *Plasmodium falciparum* strains K1 and NF54.[171] Fucoidan, a sulfated polysaccharide from brown seaweed displayed anticoagulant and antithrombotic activities. It also had inhibitory action in the growth of Lewis lung carcinoma and B16 melanoma in mice.[172] Antitumor and immunomodulation activities were found in different molecular weight α-carrangeenans from *Chondrus ocellatus*.[173] 1-Hydroxy monocyclic carotenoid 3,4-dehydrogenase from a marine bacterium that produces myxol was identified.[174] This unique type of crt D is a valuable tool for obtaining 1′-HO-3′,4′-didehydromonocyclic carotenoids. Antarctic bacteria inhibited growth of food-borne microorganisms at low temperature.[175]

6. Concluding Remarks

Marine algae have yielded a large variety of bioactive metabolites. Some of them have biomedical potential. Marine bacteria produce some of the most potent toxins such as saxitoxin and tetrodotoxin. The sulphated polysaccharides obtained from seaweeds are economically most important products. These are extensively used in food and medicine. The red algae are the source of agar and agarose. Although these polysaccharides have no direct medicinal use, however, their use in biomedical research is well known. Alginic acid obtained from brown seaweeds has several uses. The largest use of sodium alginate is in the manufacturing of ice cream. However, the most significant property of sodium alginate of biomedical value is that it has the ability to remove strontium 85 and strontium 87 from the body without seriously affecting the availability of Ca, Na or K in the body.

The treatment of gastric and duodenal ulcers by carrageenan is enjoying considerable popularity. Domoic acid and kainic acids have anthelmintic properties. Several preparations of kainic acids are available in the market. This represents one of the few instances in which clinically useful drugs are being manufactured from marine algae. Micro algae represent a major untapped resource of genetic potential for production of valuable bioactive agents and biochemicals. There are over 50,000 different species of micro algae of which only a few have been characterised. They are expected to furnish potent antiviral and antiAIDS agents.

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2

Bioactive Metabolites of Marine Invertebrates

Abstract

The chapter deals with the bioactive metabolites of the marine invertebrates. The chemistry and biological activity of the bioactive steroids, terpenoids, isoprenoid and non-isoprenoid compounds, quinones, brominated compounds, nitrogen heterocyclics and nitrogen-sulphur heterocyclics from marine invertebrates have been discussed. The chapter also reviews the bioactive secondary metabolites isolated in recent past from the marine sponges, jelly fish, sea anemones, corals, bryozoans, molluscs, echinoderms, tunicates and crustaceans.

1. Introduction

Several metabolites of unusual structure and exhibiting biological activities have been isolated from marine animals.[1-21] Some of these bioactive metabolites have biomedical potential. The bioactive metabolites that are of interest have been mainly isolated from marine sponges, jelly fish, sea anemones, corals, bryozoans, molluscs, echinoderms, tunicates and crustaceans.

2. Bioactive Metabolites

The bioactive metabolites isolated from marine animals could be divided into steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics, and nitrogen sulphur heterocyclics.

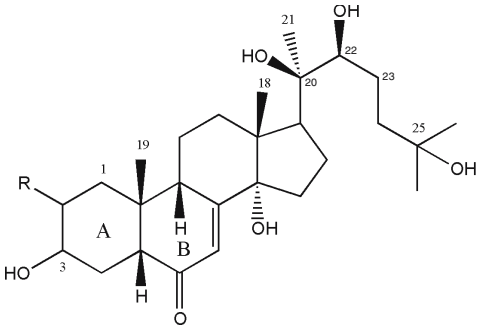
2.1 Steroids

The bioactive compounds isolated from marine animals, which have steroidal nucleus are insect moulting hormones, sterols and saponins. Karlson[22] found

that an extract of crustacean (*Cragon vulgaris*) was active in the insect (*Calliphora*) test. Horn et al[23] isolated crustecdysone (**1**, R = OH)[23,24] from crayfish (*Jasus lalandei*) collected in Australia.

Crustecdysone ($C_{27}H_{44}O_7$), m.p. 240-242°C; λ_{max} (EtOH) 240 nm (ε 12, 670); NMR (pyridine-d_5) δ1.07 (H-27), 1.20 (H-18), 1.36 (H-26) and (H-27), 1.56 (H-21) and 6.17 (H-7), the first crustacean moulting hormone has one oxygen more in its molecular formula than α-ecdysone and showed very similar chemical and physical properties. The chemical shift and splitting pattern of two methyl groups in the NMR spectrum of crustecdysone differed markedly from those of the C-18 and C-21 methyl signal in the spectrum of α-ecdysone. This difference was interpreted as generating from the presence of an additional hydroxy group at C-20 in crustecdysone. This assignment was supported by the mass spectrum of crustecdysone, which showed the same side chain fragment peak at *m/z* 99 and 81 as in α-ecdysone but had nuclear fragment peaks at *m/z* 363 and 345, one mass unit less than the corresponding ion in the mass spectrum of α-ecdysone. This indicated that the side chain C_{20}-C_{22} bond cleavage had taken place without hydrogen rearrangement, which was expected of vicinal diol. Finally, the structure (**1**, R = OH) was assigned to crustecdysone.[25]

20-Hydroxyecdysone and ecdysone obtained from different sources were shown to have same structure (**1**, R = OH) on the basis of similar evidences [26-30] and later on confirmed to be identical with β-ecdysone. New insect-moulting harmone 2-deoxy-20-dydroxy ecdysone has been isolated in 0.016% from *Zoanthus* sp. The compound exhibited promising oxytocic activity in guinea pig uterus assay.[31] The stereochemistry of crustecdysone was shown to be the same as that of α-ecdysone. The A/B *cis* ring fusion was confirmed by the ORD curve, which exhibited a positive cotton effect.[32] The stereochemistry of the tetracyclic nucleus was established by conversion of β-ecdysone into the known ketone (**2**).[33]



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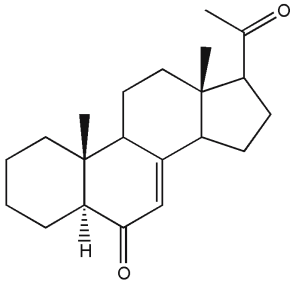


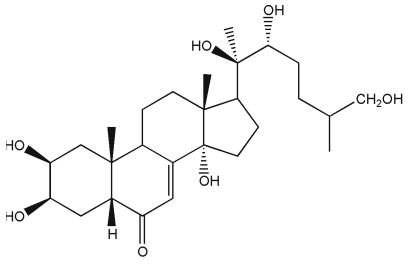
2

Since biological hydroxylation was known to proceed with the retention of configuration, the configurations at both the positions C-20 and C-22 were assigned *R* in α-ecdysone.[34,35] Chemical proof for C-20 (*R*) configuration was, however, presented by the Grignard reaction on pregnan-20-one.[36,37]

The C-20 (*R*)-configuration was also deduced by Grams rule. A convenient synthesis of crustecdysone (**1**) had been achieved.[36] An alternative synthesis of **(1)** had also been reported.[37,38]

Deoxycrustecdysone (**1**, R = H), m.p. 232-235°C, was another crustecean hormone. The UV spectrum of the compound suggested the presence of 7-en-6-one chromophore. In the NMR spectrum, the signals for five methyl groups were observed, and their chemical shifts were closely similar to those of β-ecdysone, indicating that deoxycrustecdysone has a structure very similar to that of β-ecdysone, as well as the presence of the hydroxy groups at C-15, C-20 and C-25. The MS spectrum revealed that the side chain of the new hormone and β-ecdysone were the same and that of the tetracyclic nucleus of the former had one oxygen less than the latter. The remaining unassigned hydroxyl group was placed at C-3β by comparing the rate of acetylation of the hydroxyl group at the ring A of deoxycrustecdysone with the rates of acetylation of several model compounds. The rate of acetylation of the ring hydroxyl of deoxyecdysone was found to be closer to that expected for an axial hydroxyl group. Deoxycrystecdysone was, thus, assigned structure (**1**, R = H).

The extract of female marine crab *Callinectes sapidus*,[39] when examined just before and after moulting, revealed an interesting phenomenon. In the early premolt state, callinecdysone A (**3**) was the only hormone present. At the later premolt stage callinecdysone A (**3**) was accompanied by β-ecdysone. Finally, after moulting β-ecdysone was the major hormone present along with traces of callinecdysone B (**4**). This was the first report of the presence in animal kingdom of the active hormone originally found in the plant kingdom.



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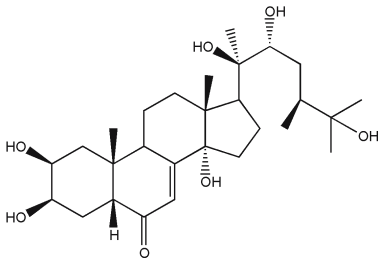


4

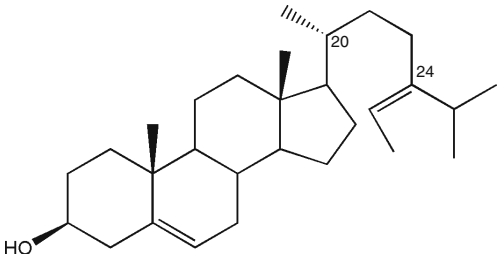
Accumulated information on the biosynthesis of steroids in nature revealed that plants with the exception of microorganisms are capable of synthesizing phytosterols. Higher animals, while being unable to utilise C_{28} and C_{29} phytosterols in their diets, are capable of synthesising C_{27} sterols. Insects, however, appear incapable of synthesising sterols essential to life, and therefore, depend on exogenous sources. Insects dealkylate C_{28} and C_{29} phytosterols to C_{27} sterols. α-Ecdysone and β-ecdysone are shown to be biosynthesized in *Calliphora erythrocephala* larvae from cholesterol.[35]

Steroid Hormones

The gonads of many fish species are known to contain testosterone, progesterone, estradiol-17β-esterone, estriol and androstenedione. The plasma of various fish species contains cortisol, cortisone, 11-ketotestosterone, 20β-dihydro-17-α-hydroxy-progesterone, testosterone, 16-ketoestradiol, estradiol 16β-estriol, epiestriol, estradiol-17β, and estrone.

Sterols

A large number of sterols have been isolated from marine animals. Zoosterols generally differ from sterols of plant sources, in the degree of unsaturation in ring B and the side chain. Some of them are positional isomers. The configuration of methyl group at C-20 in some cases is α, while in others it is β. The substituents that vary at C-24 are methyl, ethyl, and methylene. Some of the sterols, such as fucosterol (**5**),[40] isolated from marine sources have been reported to be nontoxic and have the ability to reduce blood cholesterol levels and also exhibit antidiabetic activity.[40] The sterols also appear to reduce the tendency to form a fatty liver and excessive fat deposition in the heart.



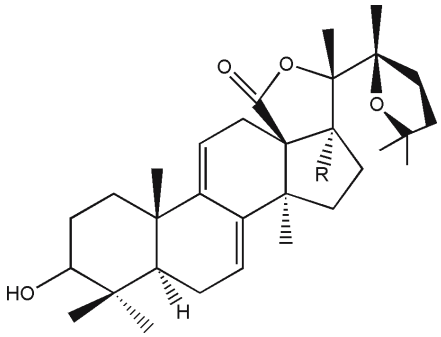
5

Saponins

Several species of sea cucumbers (Holothuroidea) are eaten in many parts of the world. Occasionally these cucumbers become poisonous. A peculiar gland found in these animals, named after the zoologist Cuvier, is particularly rich in toxins. Sea cucumbers probably use this poison for their defence against predators.

Chanley et al[41-43] isolated a number of saponins from the water extract of Cuvier gland of *Actinopyga agassizi*, a holothuria found in Bahama Islands. It is reported that although these saponins are poisonous, they are not responsible for the occasional poisioning caused by sea cucumbers.

The saponin holothurin A was a mixture of several glycosides. Hydrolysis of the saponin gave one mole of sulphuric acid and D-glucose, D-xylose, D-glucomethylose (quinovose) and 3-O-methylglucose as sugar moieties. The sapogenins obtained from holothurin A were named holothurinogenins. The two products isolated in pure forms were characterised as 22,25-oxidoholothurinogenin (**6**) and its 17-deoxyholothurinogenin (**7**).[41] Although



6, R = OH
7, R = H

the chemical defence mechanism of holothurians is believed to function through the Cuvier glands, toxic saponins have also been isolated from the body wall of *Halodeima grisea* and *H. vagabunda*.[44]

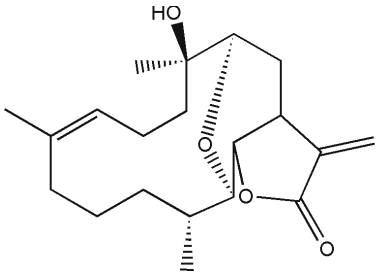
Habermehl et al[45] have studied a number of Mediterranean echinodermata. They have reported variation in holothurin content due to changes in ecological conditions. Holothurins has been found to be toxic to many animal species including mammals.[46,47] These compounds did not exhibit antibiotic property. However, they showed hemolytic activity *in vivo* and *in vitro* and neuromuscular and cytotoxic activities.[46,47] The saponin fraction from *Stichopus japonicus* showed antifungal activity against *Trichophyton asteroids, Candida albicans* and other fungal species *in vitro* at concentrations of 2.78-16.7 mg/mL.[48] The crude holothurin from *Actinopyga agassizi* was found to be active against Sarcoma-180.[49] It was also toxic to Kerbs-2-ascites tumour cells *in vitro*,[49] and inhibited the growth of Sarcoma-180 and adenocarcinoma in mice.[50] Both the crude holothurin and holothurin A were found to be cytotoxic to human epidermal oral carcinoma (KB) cell lines.[51]

Holothurins from the sea cucumbers are considered to have good pharmacological potential as neuromuscular and anticancer agents. Since the biological action of holothurins was vested in the steroid moiety of the molecule, it is hoped that one may find some useful chemotherapeutic agents from sea cucumber species.

2.2 Terpenoids

The extracts of gorgonians, which showed antibiotic activity, have furnished a number of bioactive terpenoids.[52] A diterpene, eunicin (**8**) m.p. 155°C; $[\alpha]_D - 89°$ exhibiting antibacterial activity has been isolated from the gorgonian *Eunicea mammosa* Lomouroux.[53-56] Horny corals, *Pseudoplexaura porosa* and *P. wagenaari* have furnished crassin acetate (**9**).[57]

Crassin acelate (**9**) is reported to be toxic to *Entamoeba histolytica* at 20 μg/mL *in vitro*. Both crassin acetate (**9**) and eunicin (**8**) also inhibited the growth of *Clostridium feseri* and *Staphylococcus* species.[58]



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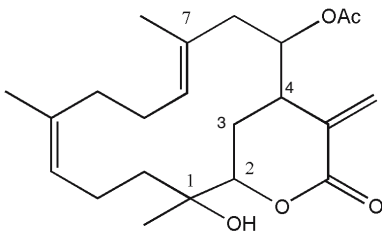


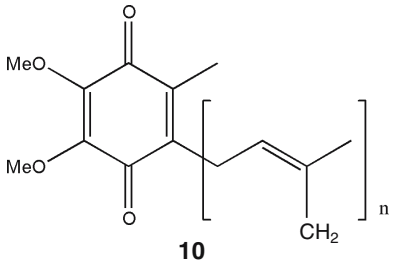
9

Although it has been suggested that the terpenoid lactones and the sesquiterpenoids may be functional antibiotics in the living gorgonians, the relatively high concentrations of some antibiotic substances present in these organisms and their potency suggest that further studies on their biomedical potential are warranted.

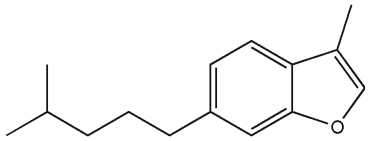
2.3 Isoprenoids

The terpenoid derived from ubiquinones such as **(10)** as well as vitamin D, vitamin A and carotenoids are widely distributed in marine organisms.

Furoventalene (**11**), a C-15 benzofuran possessing isoprenoid but having a non-fernesyl derived skeleton, have been isolated from sea fan *Gorgonia ventalina*.[59,60] The carbon skeleton of this compound could be formed by the union of the tails of two isoprene units with the head of the third isoprene unit.



10

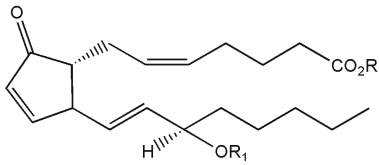


11

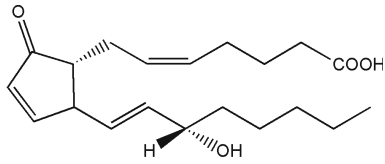
2.4 Prostaglandins

Prostaglandins are a group of naturally occurring hydroxy fatty acids found to be widely distributed in mammalian tissue. They exhibit a broad spectrum of biological activity. Their hormone like activity in fertility control, labour induction and renal physiology had attracted considerable attention. Two prostaglandins, 15-epi-PGA_2 (**12**) and its diester (**13**) and $PGA_{2\alpha}$ (**14**) have been isolated from the air-dried cortex of the gorgonian *Plexaura homomalla* (Esper) in high yield 0.2 and 1.3%, respectively.[61]

Although 15-epi-PGA_2 and its diester did not display the blood pressure lowering and smooth muscle relaxant effects as shown by PGA_2, the availability of these prostaglandins in high yield is of interest since 15-epi-PGA_2 could be converted into the active prostaglandins.



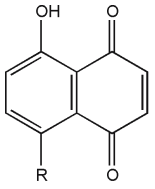
12, R = R_1 = H
13, R = Me; R_1 = Ac



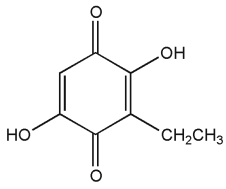
14

2.5 Quinones

Marine fauna are capable of synthesising quinones. A number of benzoquinones have been isolated from marine organisms. The predominant benzoquinones are structurally related to naphthazarin (**15**) and juglone (**16**). In addition, 2,5-dihydroxy-3-ethylbenzoquinone (**17**) and anthraquinones, especially rhodocomatulin (**18**) analogues have been isolated.[62]



15, R = OH
16, R = H



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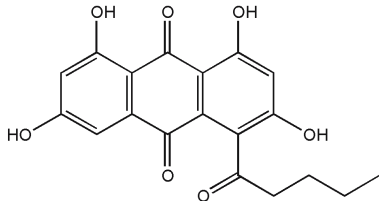


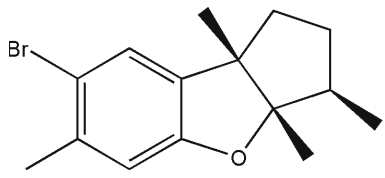
18

Naphthaquinones from higher plants and fungi generally have a one carbon side chain, whereas naphthaquinones from marine sources frequently possess a two carbon side chain.

2.6 Brominated Compounds

Many species of sponges are long lived and are resistant to the bacterial decomposition. They are suspected to produce antimicrobial substances. Indeed, this was found to be true, the extracts of a large number of sponges showed broad spectrum antibiotic activity. Some of the extracts were specially active against *Staphylococcus pseudomonas*, acid fast bacteria, and pathogenic yeasts, such as *Monila*. A variety of compounds containing bromine and having broad antibiotic activity have been isolated from these sources.

The sea hare, *Aplysia kurodai*,[63] has furnished aplysin-19, m.p. 85-86°C, $[\alpha]_D$–85.4° and aplysinol-20, m.p. 158-60°C; $[\alpha]_D$–55.5° which has been assigned structure **(19)** and **(20),** respectively, on the basis of spectroscopic data and degradation studies.



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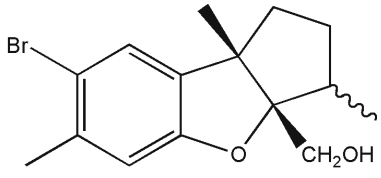
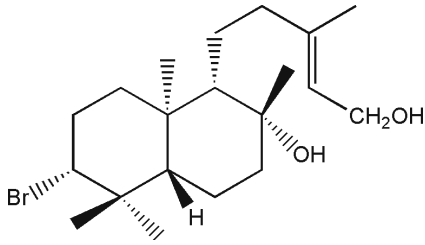


20

Aplysin-20, m.p. 146-47°C; $[\alpha]_D$–78.1°, a bicyclic diterpene containing bromine, have been isolated from *A. kurodai*.[64] The chemical evidences for the structure **(21)** for aplysin-20 were in agreement with the results of X-ray analysis. An interesting feature about aplysin-20 was that it had an axial hydroxyl function at C-8 and an equatorial bromine atom located at C-3.



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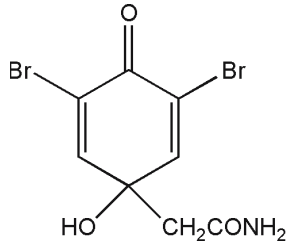
The sponge *Dysidea herbacea* from the western Caroline islands contained antibacterial compounds active against both Gram-positive and Gram-negative organisms. However, all these compounds are brominated products of 2-phenoxyphenol.[65] The compound isolated from the marine sponge *Verongia cauliformis*[66] have been characterised as 2,6-dibromo-4-acetamido-4-hydroxycyclohexadienone (**22**). Several closely related compounds have been obtained from *V. cauliformis*.[66] Methanolic extract of the sponge *V. fistudaris*[67] yielded compound **(23)**. The structure of this broad spectrum antibiotic was assigned on the basis of spectroscopic data.



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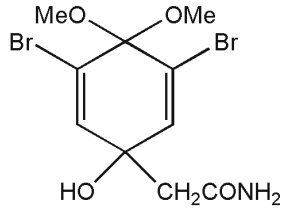
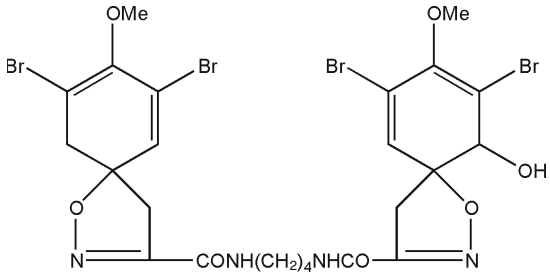


23

The sponges *Aplysia aerophoba* and *Verongia thiona* have yielded the antibiotic substance, aerothionin (**24**) having a spiro-cyclohexadienylisoxazole skeleton.[68-70] Aerothionin is probably formed by condensation of 3,5-dibromotyrosine with a C_4-N_2 unit, possibly derived from ornithine. The spiro structure could arise either by nucleophilic attack by an oxime function on an arene epoxide or conversion of the latter into a phenol followed by intramolecular phenol oxime coupling.

The sponge *Agelas oroides*[71] had yielded 4,5-dibromopyrrole-2-carboxylic acid (**25**), the corresponding nitrile (**26**), the amide (**27**), orodin (**28**), a novel bromopyrrole derivative and a substituted 2-aminoimidazole. The marine



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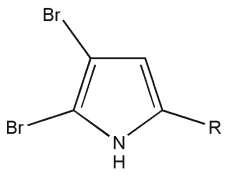
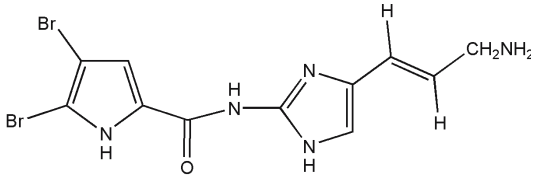
sponge *Phakellia flagellata* found in the Great Barrier Reef has furnished an interesting bromine containing alkaloid, dibromophakellin (**29**) m.p. 237-45°C (decomp), $[\alpha]_D$–203° and 4-bromophakellin (**30**).[72-74]



25, R = CO_2H
26, R = CN
27, R = $CONH_2$



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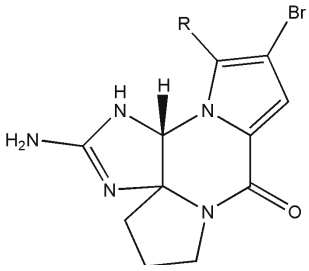


29, R = Br
30, R = H

Although these alkaloids have a guanidine unit in the five membered ring D, it did not exhibit the usual high basicity of guanidinium compounds. The X-ray structure revealed that the five-member ring D, containing the guanidine moiety was almost perpendicular to the plane of the other three rings. Simple brominated phenols, such as 2,3-dibromophenol have been isolated from the marine hemichordata, *Balano glossus*.[75] These materials are reported to be responsible for the iodoform like odour of the animal.

The brominated compounds isolated from marine animals generally show a broad spectrum antibiotic activity. However, most of them are toxic. Aplysin-21 given to mice via the stomach tube produced immediate hyperventilation, hypersalivation, atoxia, loss of motor coordination, respiratory

paralysis and death. No antibiotic substance from a marine sponge has ever reached the stage of clinical verification and none of the agents so far reportedly seem to have such a potential.

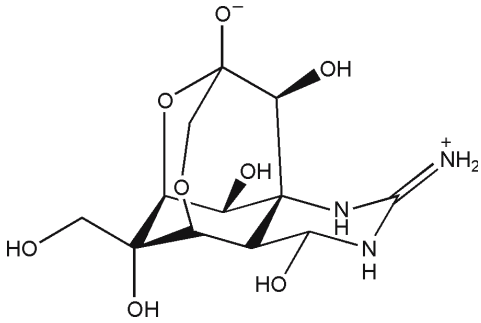
3. Marine Toxins

A variety of organic compounds containing nitrogen as part of a heterocyclic system have been isolated from marine animals. These compounds are mostly toxic. The toxicology, isolation, pharmacology and in some cases the chemistry[76-80] of these compounds have been reviewed.

3.1 Tetrodotoxin

The toxin has been known for many years. Its origin until recently was pufferfish of the family tetraodontidae. In recent years several derivatives of tetrodotoxin have been isolated from crabs, an octopus, a goby, molluscs, flatworms and even a terrestrial amphibian, all suggesting its origin from microbial source.

The structure elucidation of tetrodotoxin (**31**) has been a fascinating exercise. The chemistry of tetrodotoxin has been reviewed.[76-80] Tetrodotoxin is readily absorbed from the gastrointestinal tract. It altered the initial increase in sodium permeability of the membranes, which resulted in nerve block.



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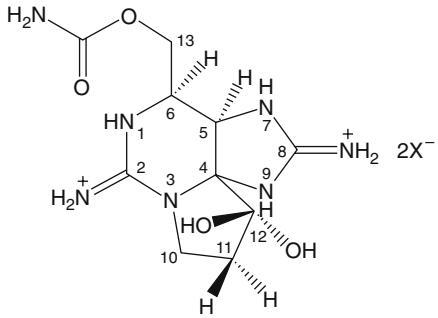
Tetrodotoxin was also considered a good hypotensive agent. It is a useful tool for the analysis of events which occur in the nerves. Tetrodotoxin was also found to be a potent respiratory inhibitor. It has been used clinically as a pain relieving agent in cases of patients suffering from neurogenic form of Hansen's disease (leprosy). Tetrodotoxin and a number of its derivatives have been examined for local anaesthetic action. The parent compound was found to be far superior than its derivatives. Tetrodotoxin itself has only limited pharmacological potential. However, it may provide useful information in understanding the mechanisms of selective membrane permeability.

3.2 Saxitoxin

Saxitoxin[79] has been isolated from several marine organisms such as the Californian mussels *Mytilus californiananus,* Alaskan butter clams *Saxidomus*

giganteus, Bay of Fundy scallops *Pecten gradis*, marine dinoflagellate *Gonyaulax catenella*, exoskeleton and the muscle of the appendages of the toxic crabs *Zosimus aeneus* and *Platypodia granulosa*. The chemistry of saxitoxin is reviewed.

Saxitoxin (**32**) is absorbed through the gastrointestinal tract. It blocks nerve conduction by specifically interfering with the initial increase in sodium permeability of the membrane. Saxitoxin produced effects on the cardiovascular system, and a marked hypotensive effect at a very low concentration, 2 μg/kg.



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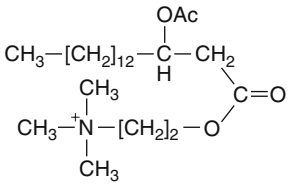
3.3 Pahutoxin

Pahutoxin[81] has been isolated from the skin secretion of the Hawaiian boxfish, *Ostracion lentiginosus*. It contained a quaternary nitrogen, an ester function and choline moiety. Hydrolysis under mild basic conditions furnished choline chloride, 2-hexadecanoic acid, and 3-acetoxyhexadecanoic acid. Acid hydrolysis with 2N-methanolic H_2SO_4 yielded methyl-3-hydroxy hexadecanoate. Pahutoxin (**33**) was, thus, a choline chloride ester of β-acetoxy palmitic acid.



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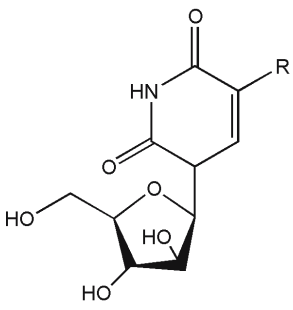
4. Marine Nucleosides

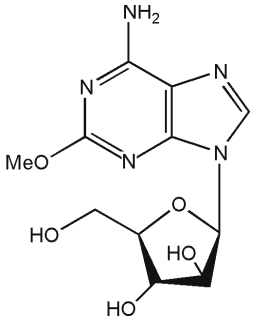
Three unusual nucleosides, spongothymidine (**34**), spongouridine (**35**) and spongosine (**36**) were isolated from the marine sponge *Cryptotethia crypta.* Chemistry and biology of nucleosides have been reviewed.

4.1 Nitrogen-Sulphur Heterocyclics

Bioactive compounds, having nitrogen-sulphur heterocyclic system, have been isolated from marine animals. The marine annelide, *Lumbriconereis heteropoda* have afforded nereistoxin (**37**)($C_5H_{11}NS_2$), b.p. 212-13°C and its oxalate m.p. 168-69°C (decomp). Nereisotoxin (**37**) was found to be toxic to insects, fish, mice, rabbits, and monkeys.[81] It affects the nervous system and the heart. It has been found to be a rapid acting anaesthetic for insects.



34, R = Me
35, R = H



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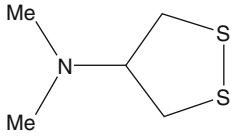

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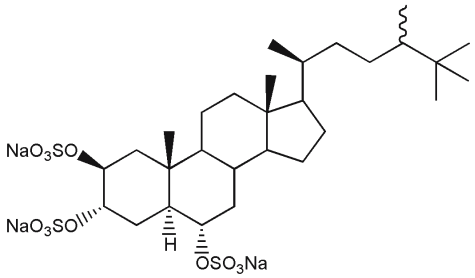
5. Bioactive Metabolites of Marine Sponges

Marine sponges are a good source of unusual sterols. Some of these sterols have phylogenetic significance. These sterols are also of interest to understand the function of biological membranes. The sulphated and alkaloidal sterols exhibit antimicrobial activity. Halistanol (**38**)[82] from *Halichondria mooriei* and the sterols (**39**-**41**) from *Toxadocia zumi*[83] inhibited the growth of *Staphylococcus aureus* and *Bacillus subtitis* at 100 µg/disc and 50 µg/disc. A hydroxy sterol (**42**) with unusual features is isolated from *Dysidea* species.[84] Two steroidal alkaloids, plakinamine A (**43**) and plakinamine B (**44**) as antimicrobial metabolites, were obtained from *Plakina* spp.[85] The compound (**43**) and (**44**) inhibited the growth of *Staphylococcus aureus* and *Candida albicans*.

The occurrence of terpenoids in sponges is widespread. The unusual terpenoids that are found most often in these animals are linear furanoterpenes, isoprenyl quinols, linear sesqui and sesterpenses and diterpenes. Most of these compounds exhibit biological activities. Furanoid sesquiterpenoids have been isolated from *Dysidea, Eurypongia* and *Siphonodictyon* species. Nakafuran-8 and nakafuran-9 from *D. fragilis*[86] exhibited antifeedant properties against fish *D. amblia, D. fragilis* were reported to provide different metabolites depending on the site of collection.[87]

Sesquiterpenes having a phenolic or quinoid moiety are common in sponges. The antimicrobial agent siphonodictyal-A (**47**) and siphonodictyal-B (**45**) and (**46**) have been isolated by Sullivan et al.[88,89] Arenarol (**48**) arenarone (**49**) and illimaquinone (**50**) from *Dysidea arenaria*,[90] puupehenone (**51**)



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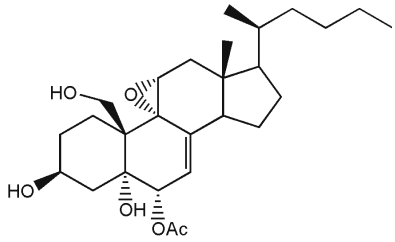

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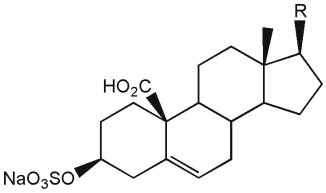
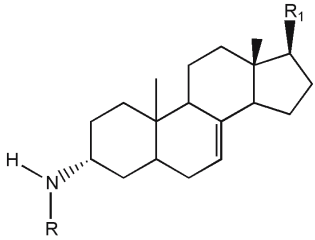
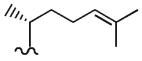




39, R =



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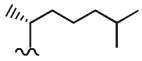
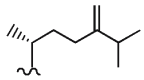
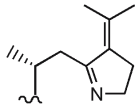


41, R =



43, R = H; R_1 =



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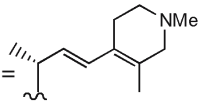

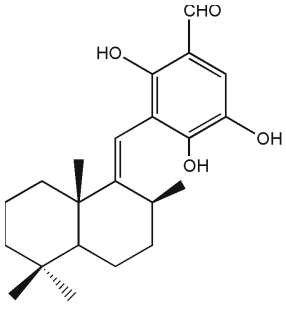

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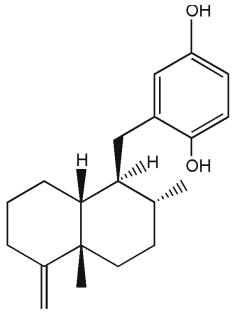

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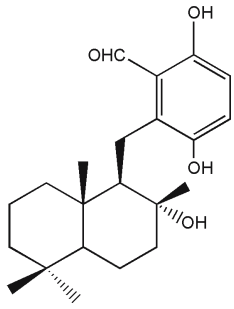

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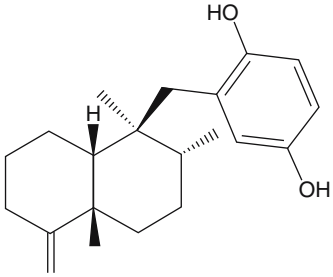
from *Hyrtios eubamma*[91]and sesquiterpene phenol (**52**) from *Smenospongia echina*[92] have been obtained. Biologically active sesquiterpenoid, avarol (**55**), which exhibited antimicrobial activity and also found active against "AIDS" virus was first isolated from a Mediterranean sponge *Disidea avara*[93] and later on from an Australian sponge *Disidea* spp.[94] The structure and stereochemistry of avarol, which represents the first 'Friedo' structure in sesquiterpene, is firmly established.[95] The ^{13}C NMR spectra of avarol dimethyl



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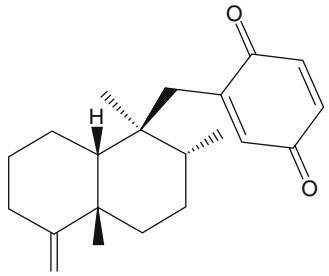

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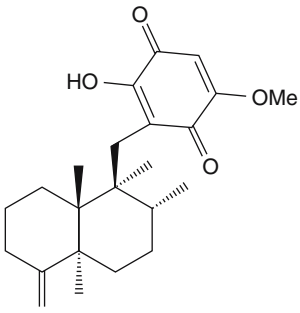

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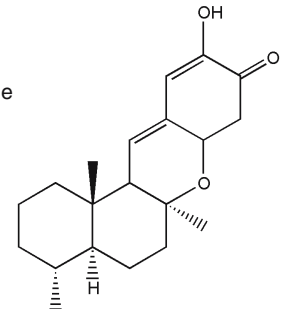

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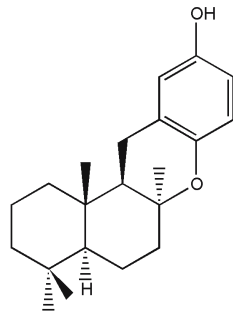
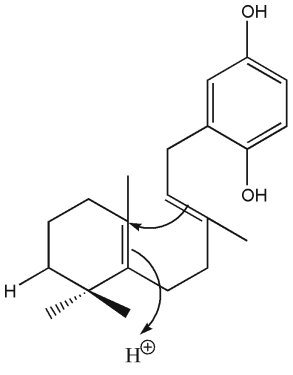


52

ether and its dihydro derivative have been compared with a series of *cis*- and *trans*-clerodane diterpenoid model compounds. It is suggested that avarol (**55**) could be derived in nature from a farnesyl precursor (**53**) by cyclisation to an intermediate cation (**54**) involving a drimane skeleton, followed by a 'Friedo' rearrangement, and finally deprotonation.[96] Ent-chromazonarol (**56**), yet another interesting compound biogenetically related to avarol (**55**) have been isolated from the marine sponge *Disidea pallescens*.[97,98] It is worth mentioning that the brown alga, *Dictyopteris undulata*,[98] provided the antipodal isomer, chromazonarol (**57**) along with its phenolic isomers, zonarol (**58**), isozonarol (**59**) and corresponding zonaroic acid.[99]



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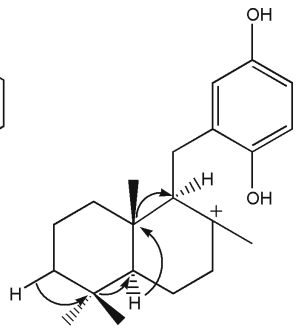

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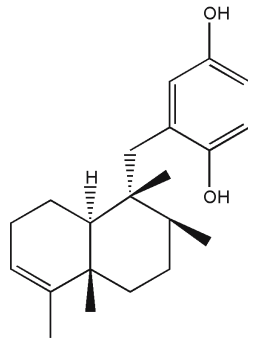

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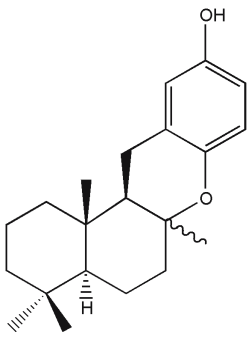

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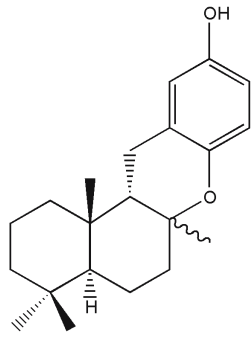

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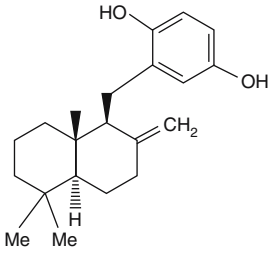

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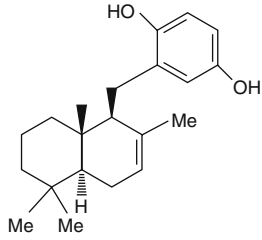

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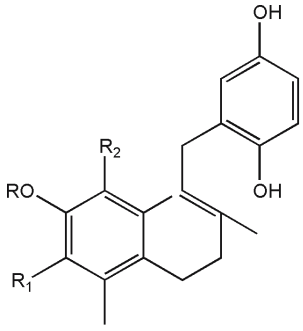
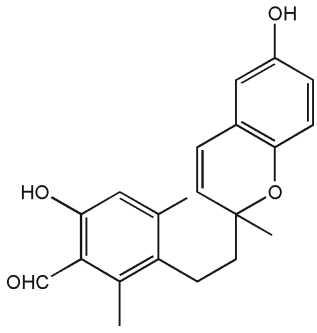


60, R = R_1 = Me, R_2 = H
61, R = R_2 = H, R_1 = CHO

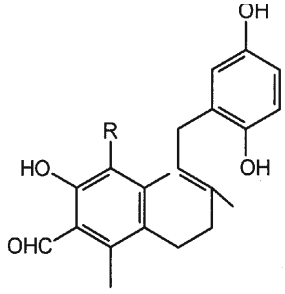


62

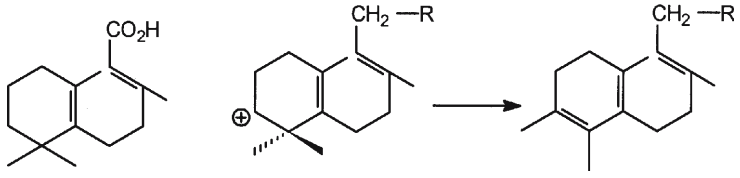
An interesting group of triprenyl phenols have been isolated from the red sponge *Halichondria panicea*. They are named as panicein A (**60**), B_1 (**61**), B_2 (**62**), B_3 (**63**) and C (**64**).[100] It is suggested that these compounds may be derived in nature from the monocyclofarnesyl derivative (**66**) by 1,2-methyl migration.[101] Co-occurrence of methyl *trans*-monocyclofarnesate (**65**) and paniceins in *H. panicia*[100] supports the intermediacy of a monocyclofarnesyl precursor.

Agelasidine-A (**67**), an interesting compound containing a guanino and a sulphone unit was isolated from the methanolic extract of Okinawa sea

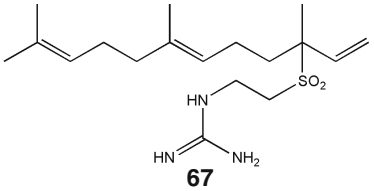
sponge *Agelas* spp.[102,103] The same compound was isolated from an unidentified *Agelas* species collected at Palau, Western Caroline Islands. *Spongia officinalis*, the common bath sponge is a rich source of terpenoids. Antifungal and antimicrobial activities have been reported in the tetracyclic furanoditerpenes isolated from sponge *S. officinalis*. Spongia-13(16),14-dien-19-oic acid (**68**),[106] spongia-13(16)-14-dien-19-al (**69**) and spongia-13-(16)-14-diene (**70**) are isolated by Capelle et al[104] from the same species diterpenes (**71**-**73**) have also been isolated.[105] *Agelas* species from the Pacific and the Caribbean have provided diterpenoids containing a purine or a 9-methyladenine unit.[106] These compounds exhibit antimicrobial and Na, K-ATPase inhibitory activities. Agelasine A (**74**), Agelasine B (**75**), from an unidentified *Agelas* species[106] were active against *Bacillus subtilis, Staphylococcus aureus, Candida albicans* and the marine bacterium B-392. These metabolites also show mild ichthyotoxic



63, R = H
64, R = OH



65 **66**



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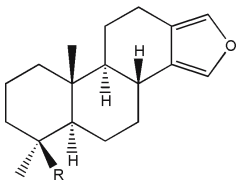
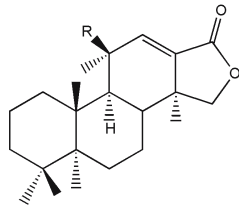
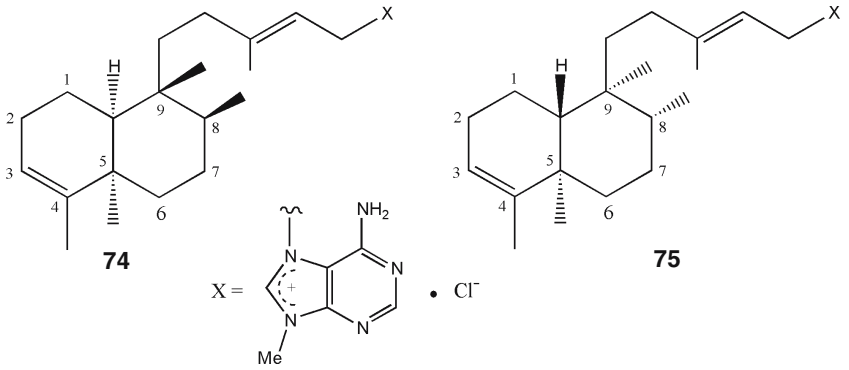




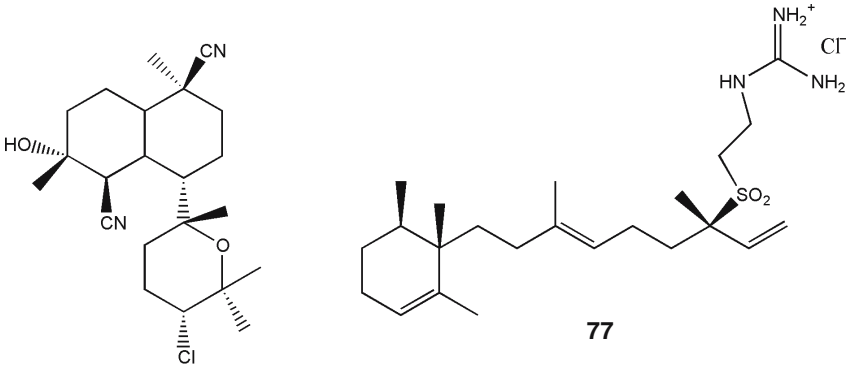
68, R = CO_2H
69, R = CHO
70, R = CH_3

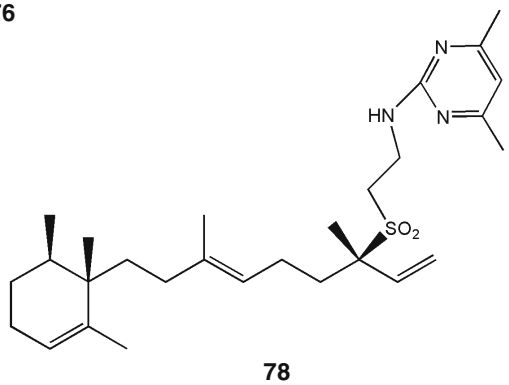
71, R = H
72, R = OH
73, R = OAc

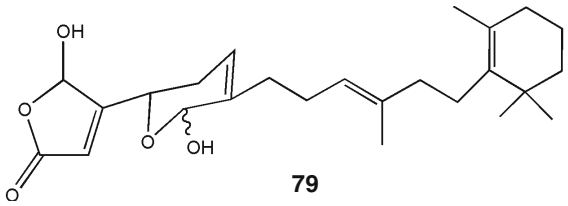


properties. Novel bicyclic and monocyclic[107] diterpenoids with a 9-methyladenine unit possessing inhibitory effect on Na, K-ATPase have been isolated from sea sponge *Agelas nakamurai*.

A series of tricyclic diterpenes having isocyano, hydroxyl, tetrahydropyranyl and chlorine functions and exhibiting antibiotic activity were isolated from *Acanthella* species.[108,109] Kalihinol A (**76**) is active *in vitro* against *Bacillus subtilis, Staphylococcus aureus* and *Candida albicans*. Two new diterpenoids agelasidine B (**77**) and agelasidine C (**78**) exhibiting antimicrobial activity







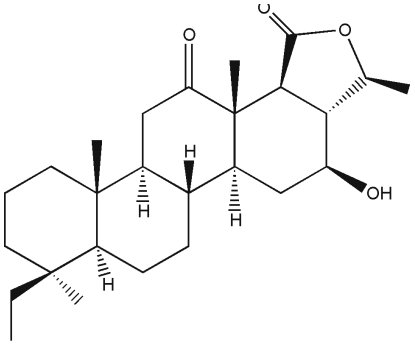
79

are obtained from the Okinawan sponge *Agelas nakamurai*.[108-110] These compounds also show inhibitory effect on contractile response of smooth muscles and enzymatic inhibitory action on Na, K-ATPase from pig brain. Many species of genus *Spongia* contain biosynthetically intriguing C_{21} difuranoterpenes probably derived from linear sesterterpene tetronic acid. *Luffarella variabilis*[111] has furnished four sesterterpenoid antibiotics. Manoalide (**79**), which contains an α,β-unsaturated γ-lactone function, had antiinflammatory activity and was found to be an inhibitor[111–113] of phospholipase A_2. Several tetracarbocyclic sesterterpenes have been isolated from *Cacospongia scalaris*.[114] 20,24-Bishomoscalarane sesterterpenes, such as phyllofolactore A (**80**) and phyllofoloctone (**81**) have been isolated from pacific sponge *Phyllospongia foliascens* compound **80** shows cytotoxicity against the P-388 cell lines (IC_{50} = 5 μg/mL).[115–119] The compounds from this sponge also exhibit antifungal and antiinflammatory activities.[119] Phyllofenone A (**82**) a 20,24-diethyl-25-norscalorane sesterterpene with antifungal activity from sponge *Phyllospongia foliascens* (Pallas) have been isolated.[120,121]

Several nor-sesterterpene peroxide antibiotics have been isolated from Red Sea sponges. Sokoloff et al[122] have investigated norsesterterpenoid peroxide antibiotics from the Red Sea sponge *Prianos* spp. The peroxides (**83-85**) strongly inhibit the growth of Gram-positive bacteria.[123] Suvanine (**86**),[124,125] a novel sesterterpene containing a guanidinium bisulphate and a furan functionalities, exhibited more than 90% inhibition of sea urchin egg cell division at 16 μg/mL.

Although sesqui-, di- and sesterterpenes are very common in sponges, triterpenes are very rare. Further, several carotenoids have been isolated from marine sponges.[126-130] Sponges also elaborate unusual compounds from tyrosin and tryptophane. Brominated tryptamines from *Smenospongia* spp.[131]exhibit antimicrobial activity. Methyl-aplysinopsin (**87**) from *Aplysinopsis reticulata*,[132] is a short acting inhibitor of monoamine oxidase.

Several peptide alkaloids and proteins have been isolated from marine sponges. Purealin (**93**), a novel enzyme activator from the Okinawan marine sponge. *Psammaplysilla purea*[133] modulates enzymatic reaction of ATPases. *Dysidea herbacea* has furnished a toxic metabolite (**88**). *D. herbacea*[134-139] from different location provides different polychloroamino acid derived metabolites. *Cliona celata* has yielded a series of linear peptide alkaloids.[140,141] Three diketopiperazine derivatives have been obtained from *Tedania ignis.*



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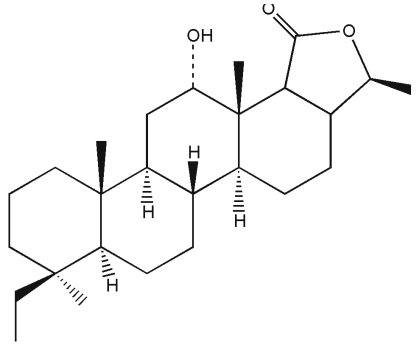

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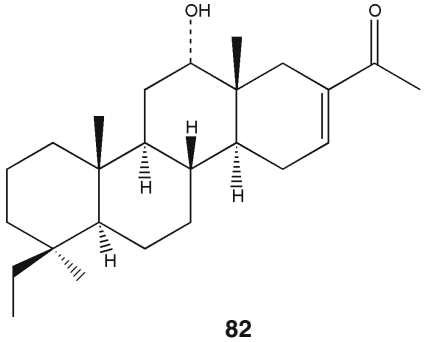

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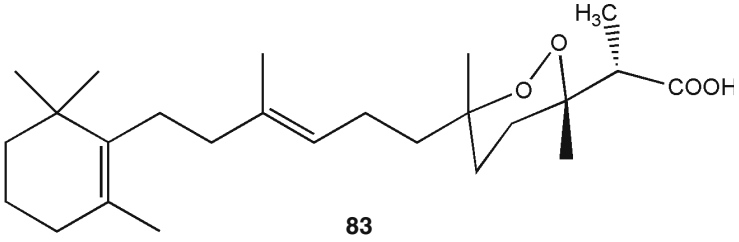

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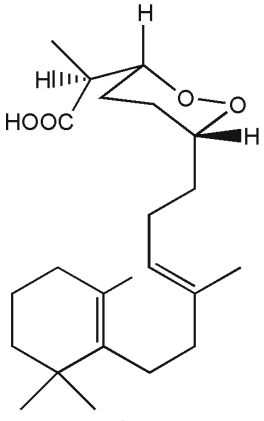

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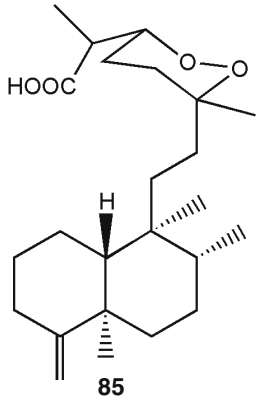
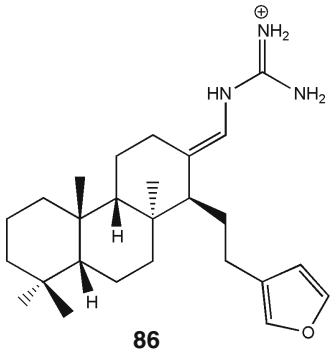


85

Matsunaga et al[142-144] have isolated bioactive polypeptides from *Discodermia kiiensis*. A glycoprotein (MW 27,000), from *Geodia mesotriaena*[145] is found active *in vitro* in 9KB test and *in vivo* in the murine P-388 and lymphocytic leukemia (PS). Geodiatoxin which contained a minor amount of silicon had LD_{100} at 6 mg/kg in mouse. Marine sponges are also a good source of bioactive unusual nucleosides. 1-Methylisoguanosine (**89**) first isolated from a nudibranch and subsequently from sponge *Tedania digitata* has been synthesised by two routes.[146,147] The nucleoside exhibits several pharmacological activities.[148] It interacts directly with adenosine receptors in guinea-pig brain slices to stimulate adenylate cyclase[149] and in contrast to adenosine it was resistant to deamination. Further, compound (**89**) was effective in displacing diazepam bound to rat brain membrane. Two unusual nucleosides, mycalesine A (**91**) and mycalesine B (**92**) have been isolated from the lipophilic extract of marine sponge *Mycale* spp.[150,151] Both the nucleosides inhibit cell division of fertilized starfish eggs. Isopropyl alcohol extract of the sponge *Tethya aurantia* showed chronotropic and inotropic activities on isolated guinea-pig atria. Subsequently, adenosine was found responsible for the



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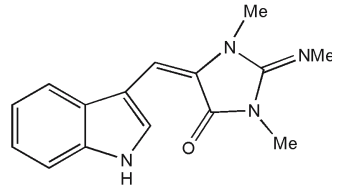

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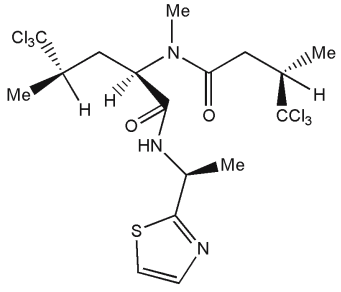

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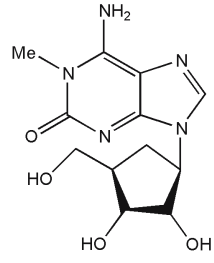
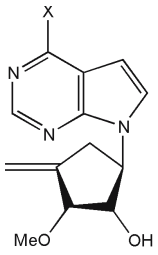


89

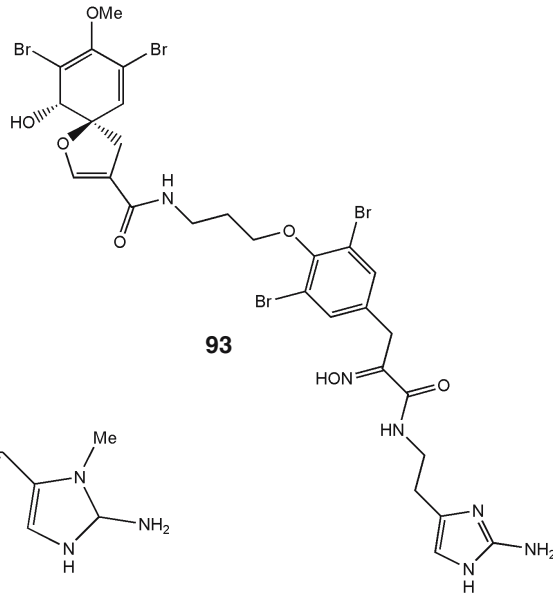
90

biological activities.[152] The dichloromethane and methanol extracts of *Echinodictyum* spp. exhibited high order of activities on the isolated guinea-pig trachea and 4-amino-5-bromopyrrolo-2,3-dipyridine (**90**) is found to be the active principle.[153]

Several alkaloids and other nitrogen heterocyclic compounds have been obtained from marine sponges. Keramadine (**94**),[154] a bromine containing alkaloid, antagonist of serotonergic receptor has been isolated from the Okinawan sea sponge *Agelas* species. Several bioactive metabolites containing a guanidine moiety have been obtained from marine sponges. Ptilocaulin (**95**) and isoptilocaulin (**96**) isolated from *Ptilocaulistaff* and *P. spiculifer*[154-156] exhibit high order of antimicrobial activity against Gram-positive and Gram-negative bacteria, and also inhibit cell growth against L 1210 leukemia cells. Aaptamine (**97**) from *Aaptos aaptos*[157] possesses α-adrenoceptor blocking activity in the isolated rabbit aorta. Amphimedine (**98**), a fused pentacyclic yellow aromatic alkaloid from a Pacific sponge



91, X = NH_2
92, X = OH



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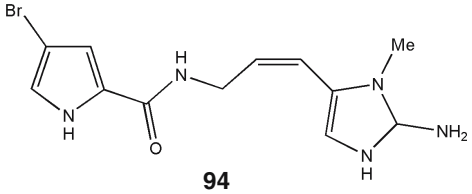

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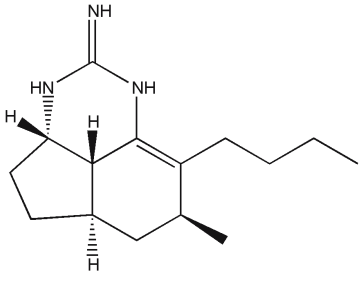

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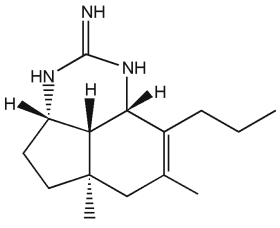

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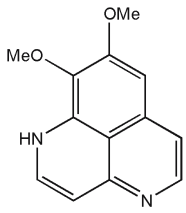

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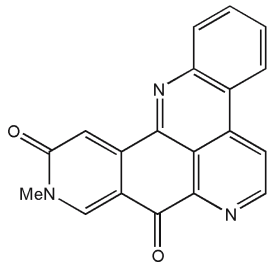
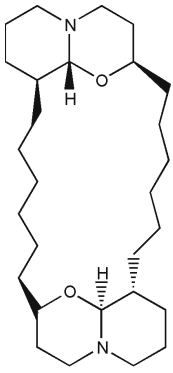


98

Amphimedon spp.[158] is a cytotoxic agent. The Xestospongin A, B, C and D from Australian sponge *Xestospongia exigua*[159-163] represent a new class of macrocyclic alkaloid incorporating two 1-oxaquinolizidine rings and are vasodilative compounds which induce relaxation of blood vessel *in vivo.* Xestospongin A and C have structures **(99)** and **(100)**, respectively.[163]



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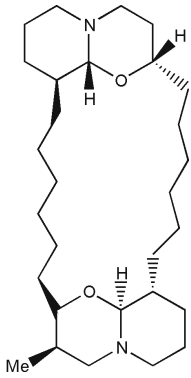

100

Marine sponges of the genus *Reniera*[159] have furnished a series of antimicrobial isoquinoline and isoindole derivatives. Renierone and N-formyl-1,2-dihydrorenierone from the sponge inhibit cell division of the fertilized sea-urchin eggs assay. Several bioactive macrocyclic alkaloids have been isolated from marine sponges. Of these the alkaloids latrunculin A to D from *Latrunculia magnifica*[164-170] are toxic. This sponge is not eaten by fish, and when squeezed into an aquarium causes death of the fish within 4 to 6 min. These toxic compounds excite the fish in a few seconds, followed by hemorrhage, loss of balance and finally death. Immunofluorescence studies with antibodies specific to cytoskeletal proteins revealed that the toxins cause major alterations in the organisation of microfilaments without obvious effects on the organisation of the microtubular system. The Red Sea sponge *Theonella swinhoei*[171] furnished a novel 22-member macrolide with antibacterial and antifungal activities. Extract of *Tedania ignis*[172] showed cytotoxicity *in vivo* and tedanolide, a potent cytotoxic macrolide had been

isolated from the sponge *Halichondria okadai*[173] which provides several antitumor macrolides. Norhalichondrin A is the major component in the series of halichondrins.

Marine sponges are also a good source of halogenated phenols and aromatic ethers. It is believed that some of the bromo derivatives from the sponges are presumably the constituents of symbiotic blue green algae[174] which are found in the ectosome of the sponge. The marine sponges have also furnished several saturated, unsaturated, methoxy and methyl-branched fatty acids as well as phospholipid bound fatty acids.[175-178] The fatty acids have unconventional pattern of unsaturation and substituents. Several high mol. wt. polyacetylenes have been isolated from the sponge *Petrosia ficiformis*,[179] found in dark caves. These compounds are related, but different from the polyacetylenes from the same sponge living in its unusual habitat. Several fatty acids derived metabolites of sponges exhibit high order of biological activities. Acanthifolicin, an episulfide containing acid from *Pandaros acanthifolium*[180] exhibits high order of activity against P388, KB and L 1210 cell lines. It is suggested that the isolation of this typical bacterial metabolite from a sponge strengthens the assumption that bacteria are the sources of some of the products isolated from the sponges. Several high mol. wt. polyacetylenes have been isolated from marine sponges. C_{30} linear polyacetylene alcohol from the marine sponge *Tetrosia* species inhibits mitotic cell division of the fertilised sea urchin eggs assay and is also active against *Penicillium chrysogenum*.[181] Glycerol ethers are widely distributed in marine sponges. It is speculated that the glycerol ethers, perhaps, act as growth factors.

6. Marine Invertebrates of the Andaman and Nicobar Islands

About 50 species of marine sponges occur in these Islands and only a few of them have been investigated. Even their classification is in a confused state. Several bioactive metabolites of considerable interest have been isolated from *Agelas* species[182,183] found in the Andaman and Nicobar Islands. *A. nata* the only species reported to occur remains to be investigated. Four species of *Cliona, C. ensifera, C. lobata, C. mucronata* and *C. quadrata* occur in the Andaman Islands. However, none of these appear to have been investigated. Two species of genus *Craniella, C. cranium* and *C. dactyloides* that are found in the Islands have remained uninvestigated. *Phyllospongia foliascens* Pallas which occurs in the Islands has furnished foliaspongin,[184] an antiinflammatory bishomosesterpene. An antiallergic and antiinflammatory glycolipid has been isolated from *P. foliascens*.[185] Chemical investigation of *Petrosia testudiana* is expected to furnish compounds of biological interest. Two species of genus *Tedania, T. nigrescens* and *T. brondifera* are found in the Islands and so far these are not investigated. However, these species may yield metabolites of biomedical interest.

6.1 Coelenterates

There has been much interest in the metabolites of jelly fish, sea nettle, the Portuguese man-of-war and the sea wasp which are widely distributed in warm tropical seas. The organisms release a nematocyst venom from the tentacles which causes painful injuries. The venoms are generally a complex mixture of enzymes and pain-producing factors. The nematocyst venom of *P. physalis* is a mixture of toxic proteins and enzymes which show multiple action including dermonecrosis, neurotoxicity, hemolysis and cardiotoxicity. The cardiotoxin from the wasp has been purified by immunochromatography.

6.2 Sea Anemones

Several species of sea anemones occur in the sea coasts of the Andaman and Nicobar islands. The toxins produced by these organisms are polypeptides or proteins. The toxins are found very useful tools for studying the voltage dependent Na^+ channels in nerve and cardiac muscle cells. It has been suggested that coelenterate toxins would be suitable for studies of tumor cell cytolysis *in vitro* and *in vivo*.

6.3 Corals

Some of the soft corals that occur in the Andaman and Nicobar Islands are horny gorgonians, sea fans, the red organ-pipe coral and the blue coral, *Helipora*. The mushroom coral *Fungia* is the most common solitary coral that occur in the Andaman and Nicobar islands. The coral *Clavularia viridis* has yielded cytotoxic steroids, stoloniferones A-D.[186] *Muricea* species such as *M. californica* and *M. fruticosa* have provided four new esterified aminogalactose saponins called muricins[187] which inhibit the growth of the diatom *Phaeodactylum tricornum* at 100 ppm concentrations. It is suggested that this activity may play a significant role in reducing fouling due to the diatoms. Soft corals elaborate a large variety of sesquiterpenoids and diterpenoids. Several of these are found to be toxic. Guaiazulene from the gorgonian *Euplexaura erecta*[188] exhibits mild activity against *Pseudomonas aeruginosa*. Subergorgic acid, a cardiotoxin is obtained from the pacific gorgonian coral *Subergorgia suberosa*.[189] The toxin inhibits neuromuscular transmission at 0.16 μg/mL in isolated guinea-pig heart assay. Pseudopterolide, an unusual diterpene with a 12 member ring from the gorgonian *Pseudopterogorgia acerosa*[190] shows unusual cytotoxic properties.

6.4 Bryozoans

Marine bryozoans constitute one of the important groups of fouling communities. *Bugula* spp occur abundantly on the ship bottoms. *B. neritina* has furnished several new macrolides called bryostatins.[191-197] Bryostatin-4 was isolated from *B. neritina*, bryostatin-8 from *Amanthia convoluta*.[192] The origin of bryostatin-5 and bryostatin-6 is uncertain. The marine bryozoan, *Flustra foliacea*[197] has yielded several brominated alkaloids called flustramines

A, B, C, flustraminol A, B and dihydroflustramine C, which inhibits cell division of the fertilized sea urchin eggs. The bryozoan *Phidolopora pacifica* has yielded phidolopin, a purine derivative largely responsible for high order of antifungal and antialgal activities.[198,199] Several macrolides like bryostatin-1 and bryostatin-2 were isolated from *Bugula neritina*.[200-202] Some of these metabolites show high order of antineoplastic activity. [2-Hydroxyethyl]dimethyl sulfoxonium ion acts as an allergen. These compounds are isolated from marine bryozoan *Alcyonidium gelatinosum*.[203] The bryozoan causes an eczematous allergic contact dermatitis called 'Dogger bank itch'.

6.5 Molluscs

Several steroidal, terpenoids, and acetylenic compounds isolated from nudibranchs were also found in sponges which they feed upon.[204] The bioactive nucleoside characterised as 1-methyl-isoguanosine has been found in the sponge *Tedania digitata*[205,206] as well as in the nudibranch *Anisodoris nobilis*.[207-209] Isoguanosine isolated from marine nudibranch *Diaulula sandiegensis*[210] produced hypotension and relaxation of smooth muscles in mammals. Hexadecylglycerol isolated from *Archidoris montereyensis* [211] showed high order of activity *in vitro* against *Staphylococcus aureus* and *Bacillus subtilis*. Sea-hares accumulate large quantities of metabolites in their digestive gland and skin. These compounds are believed to originate from the algae which they take as food. Aplysiatoxin, a toxic metabolite has been isolated both from the Hawaiian sea-hare *Stylocheilus longicauda* and also from blue-green alga *Lynbrya majusticula*[212] on which it feeds. Aplysistatin is a well known antileukemic metabolite from the sea-hare *Aplysia angasi.*[213] The metabolites of *A. dactylomela* are reported to have cytotoxic and antitumor activity *in vivo*.[214] *Dolabella auricularia* has yielded several antineoplastic and cytotoxic compounds named dolastatins.[215-219]

Marine snails of the family conidae synthesize potent toxins which they inject into their prey by means of a hollow tooth in order to immobilise their prey. Some species are known to cause injuries to humans and have proved to be fatal. The venom of *Conus geographus* is most dangerous to man. Striatoxin, a cardiotonic glycoprotein obtained from *C. striatus*[220] was found to have long lasting inotropic action on guinea-pig left atria. Its minimum lethal dose in the fish *Rhodeus ocellatus* was 1 μg/g body weight. Kelletinin-I and II isolated from marine mollusc, *Kelletia kelletii*[221] inhibit the growth of *Bacillus subtilis* and L1210 leukemia cells. Surugatoxin and neosurugatoxin are isolated from *Babylonia japonica* (Japanese ivory shell).[222,223] The antinicotinic activity of the latter is found to be 100 times that of the former. *Siphonaria diemensis* has furnished the antibiotic diemensin A and diemensin B.[223] The former inhibits the growth of *Staphylococcus aureus* and *Bacillus subtilis* at 1 μg/disc and 5 μg/disc, respectively. It also inhibits cell divisions in the fertilized sea-urchin egg assay at 1 μg/mL.

6.6 Echinoderms

The metabolites of Echinoderms mainly responsible for the biological activity are saponins. The chemistry and biological activities of bioactive saponins have been reviewed.[224] Asterosaponins are reported to have hemolytic, antineoplastic, cytotoxic, antitumor, antibacterial, antiviral antifungal and antiinflammatory activities.[225,226] Saponins of sea-urchins and starfishes differ considerably. Asterosaponins are sterol derivatives, whereas sea cucumber saponins are terpenoid in nature. Both groups have sulphate esters and quinovose sugar moieties. However, in asterosaponins the sulphate function is attached to an aglycone, whereas it is attached to the carbohydrate moiety in some sea cucumber saponins. The saponins from other sources lack sulphate functionality. To date, the saponins of over 50 sea cucumbers have been studied.[227] Many cerebrosides, pyrimidine nucleosides, thymine deoxyriboside and uracil deoxyribose have been isolated from the starfish *Acanthester planei*.[228]

6.7 Sea-urchins

Pedicellaria of some species of sea-urchins contained toxic substances. Extract of *Toxopneustes pileolus* causes histamine release from isolated smooth muscles.[229] The extract of the organism produced contraction of the longitudinal muscles of isolated guinea-pig ileum at a concentration of 3×10^{-8} g/mL. *Lytechinus variegatus*[230] and *Strongylocentrotus droebachiensis*[231] have yielded antineoplastic glycoproteins.

6.8 Tunicates

The most interesting compounds of Tunicates are the cyclic oligopeptides. *Lissoclinum patella* has furnished several cyclic peptides.[232,233] Of these ulicyclamide, ulithiacyclamide, and patellamide A, B, and C exhibit antitumor activity against L1210 murine leukemia culture *in vitro*. A new class of depsipeptides, some of them exhibited high order of antiviral (against RNA and DNA viruses) and antitumor (against L1210; P388 leukemia and B16 melanoma) activities, are obtained from *Tridemnum* species of the family Didemnidae.[234,235] The compounds designated as eudistomins contain substituted condensed oxathiazepine ring system. These compounds show high order of antiviral activity against *Herpes simplex* virus type (HSV-1) and have been isolated from the colonial caribbean tunicate *Eudistoma olivaceum*.[236] Other eudistomins having substituted β-carboline system and displaying modest activity against HSV-1 and *Bacillus subtilis* have been isolated from the same species.[237]

Dolastatin 10, a novel pentapeptide isolated from the marine mollusk *Dolabella auriculata* is in phase II clinical trials. Further studies revealed that it is unlikely to have substantial activity in the treatment of melanoma.[238] Aurantosides D, E and F exhibiting high order of antifungal activity against *Aspergillus fumigatus* and *Candida albicans* were isolated from marine sponge

Siliquariaspongia japonica.[239] Two chemotypes of marine bryozoan *Bugula neritina* were identified.[240] A new polysaccharide exhibiting anti-HIV activity and made up of galactan sulfate was isolated from the marine clam *Meretrix petechialis*.[241] Dolastatin 15, a potent antineoplastic peptide from the mollusk *Dolabella auriculata* was undergoing clinical trials in Europe and North America.[242] Two sesquiterpenes active against phytopathogenic fungus *Cladosporium cucumerinum* were isolated from a marine soft coral of the genus *Heteroxenia*.[243] A stereocontrolled synthesis of the antitumor agent (-)-laulimalide was achieved.[244] Linckosides A and B, the new neuritogenic steroid glycosides were isolated from the Okinawan starfish *Linckia laevigata*.[245] Three alkaloids having an unusual decahydroquinoline skeleton and showing significant and selective antiplasmodial and antitrypanosomal activities, were obtained from a new tunicate species of the genus *Didemnum*.[246] These bioactive alkaloids may serve as lead structure for the development of new antimalarial drugs. Vitilevuamide, a bicyclic peptide was isolated from marine ascidians *Didemnum cuculiferum* and *Polysyncranton lithostrotum*. The peptide was cytotoxic in several human tumor cell lines with IC_{50} values ranging from 6 to 311 nM.[247] Several polyacetylenes exhibiting significant selective cytotoxicity against human tumor cell lines were isolated from the marine sponge *Petrosia* sp.[248] Plakevulin A, a new oxylipin inhibiting DNA polymerases X and Y was isolated from the sponge *Plakortis* sp.[249] A novel prodigiosin-like immunosuppresant was isolated from *Micrococcus* sp. It had considerable selective T cell immunosuppressive potential.[250] Dolabellanin B2, a novel peptide consisting of 33 amino acid residues was isolated from the body wall of the sea hare *Dolabella auricularia*.[251] It was effective against some pathogenic microorganisms at a 2.5-100 μg/ml.[251] A phase-II clinical study of bryostatin 1, in patients with advance lung cancer was carried out.[252] Two new tryptase inhibitors cyclotheonamide E_4 and E_5 were isolated from the marine sponge of the genus *Ircinia*. Cyclotheonamide E_4 showed potent inhibitory activity against human tryptase (IC_{50} 5.1 nM) and may be useful as a therapeutic agent in the treatment of allergic diseases including asthma.[253] Aplidin (R) (APL) a novel depsipeptide from the tunicate *Aplidium albicans* exhibiting high order of cytotoxic activity was under clinical studies. Sulfated polymannuroguluronate (SPMG), a marine sulfated polysaccharide has entered in phase II clinical trial in China as the first AIDS drug.[254] Two new cytotoxic compounds, viz. nakiterpiosin and nakiterpiosinone were isolated from the Okinawan sponge *Terpios hoshinota*.[255] Aplidine, a marine depsipeptide from Mediterranean tunicate *Aplidium albicans* was in phase-II clinical trial.[256] Aplidine in human Molt-4 leukaemia cells was found to be cytotoxic at nanomolar concentrations and induced both a G(1) arrest and a G(2) blockade. The drug induced cell cycle perturbations and subsequent cell death do not appear to be related to macromolecular synthesis (protein, RNA, DNA) since the effects occur at concentrations (e.g. 10 nM) in which macromolecule synthesis was not markedly affected. Although the

mechanism of action of aplidine is still not clear, the cell cycle phase perturbations and the rapid induction of apoptosis in Molt-4 cells appear to be a mechanism different from that of known anticancer drugs.[256] A profile of the *in vitro* antitumor activity of lissoclinolide, a small non-nitrogenous lactone isolated from the marine ascidian *Lissoclinum patella* is available.[257] Lissoclinolide had a moderate selective activity towards colon tumor cell lines. The sponges of *Acanthella* sp. are prolific source of highly functionalized diterpene antibiotics. Eight kalihinol type diterpenes were isolated from two *A. cavernosa*.[258] *Haliclona* sp. a marine sponge yielded kendarimide A, a novel peptide which reversed glycoprotein mediated multidrug resistance in tumor cells.[259]

Xestospongin C and D isolated from marine sponge *Haliclona exigua* inhibited rat brain nitric oxide synthase activity.[260] Lamellarin α-20 sulfate, an ascidian alkaloid inhibited HIV-1 integrase of virus in cell culture.[261] Phase-I clinical trials were carried out on dolastatin-10, a peptide isolated from the marine mollusk *Dolabella auricularia* in patients with advanced solid tumor.[262] Spongistatin 1, a macrocyclic lactone polyether from marine sponge *Hyrtios erecta* exhibited a broad range of antifungal activity against a variety of opportunistic yeasts and filamentous fungi.[263]

7. Concluding Remarks

The marine sponges, jelly fishes, sea anemones, gorgonians, corals, bryozoans, molluscs, echinoderms, tunicates, and crystaceans during the last two decades have furnished a large variety of metabolites with unusual structures. Several of these metabolites exhibit high order of biological activities and some have biomedical potential. Investigation of sponges, jelly fish, sea anemones and corals from worldover furnished bioactive unusual sterols, steroidal alkaloids, unusual terpenoids, isoprenyl quinols, furanoids, sesquiterpenoids, triprenyl phenols, compounds containing a guanino and sulphone units. *Agelas* species have provided diterpenoids containing a purine or a 9-methyladenine unit. These compounds exhibit antimicrobial and Na, K-ATPase inhibitory activities. Biologically active sesquiterpenoid, avarol from a Mediterranean sponge *Disidea avara* has been found active against 'AIDS'.

A series of tricyclic diterpenes having isocyano, hydroxyl, tetrahydropyranyl and chlorine functions exhibiting antibiotic activity have been isolated from *Acanthella* species. Many species of the genus *Spongia* contain biosynthetically intriguing C_{21} difuranoterpenes probably derived from linear sesterterpenoid antibiotics. Several nor-sesterpene peroxide antibiotics have been obtained from the Red sea sponges. Although sesqui-, di-, and sesterpenes are common in sponges, triterpenes are rare. Purealin, a novel enzyme activator from the Okinawan marine sponge, *Cliona celata* has yielded a series of linear peptide alkaloids. Marine sponges are also a good source of bioactive unusual nucleosides. There has been much interest in the metabolites of jelly fishes. The nematocyst venom of the organisms has been studied in several cellular

and subcellular tissue preparations. A lethal toxin from the *Chrysaora quinquecirrha* affected ion permeability in lipid membranes by producing monovalent cation channels. A cardiotoxin from the sea wasp has been purified by immuno-chromatography. The toxins of sea anemones are generally polypeptides or proteins. The sterol composition of several soft corals and gorgonians as well as the composition of their associated symbiotic dinoflagellates have been studied. In general, highly oxygenated sterols often exhibit pharmacological activity. Pseudoterolide, an unusual diterpenoid with a 12-membered ring system and having two isopropenyl functionality, from the gorgonian *Pseudopterogorgia acerosa*, shows unusual cytotoxic properties. *Palythora* spp. have furnished palytoxins, the most potent toxins known so far. *Palythoa liseia* has yielded several metabolites exhibiting antineoplastic properties. The zoanthid, *Gerardia savaglia* is found to be an unexpected new rich source of molting hormone ecdysterone. Several macrolides have been isolated from *Bugula neritina*. Some of these metabolites show high order of antineoplastic activity.

The marine life of the Andaman and Nicobar islands is rich. There is hardly any work reported on the metabolites of marine invertebrates of these islands.

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3

Separation and Isolation Techniques

Abstract

The chapter deals with the separation and isolation techniques of bioactive compounds of marine organisms. The ion-exchange chromatography, reverse-phase columns, high/medium pressure chromatography on porous materials, combination of ion exchange and size-exclusion chromatography, and bioassay directed fractionation have been discussed. Besides, isolation procedures of some bioactive amino acids, peptides, nucleosides, cytokinins, alkaloids and toxins have been given.

1. Introduction

Marine organisms elaborate a variety of bioactive secondary metabolites. Chemically, these bioactive metabolites could be divided into amino acids, peptides, nucleosides, alkaloids, terpenoids, sterols, saponins, polycyclic ethers etc. The ethanolic/methanolic extracts of marine organisms exhibiting biological activities could be a mixture of several class of compounds. Since the chemical nature of bioactive compounds of the complex mixture is not known, it is not possible to follow any specific technique for the separation of the constituents of the complex mixture. However, a broad separation of the mixture can be achieved by fractionation with organic solvents. The ethanolic/methanolic extract is successively extracted with hexane, chloroform, ethyl acetate and then divided into water soluble and water insoluble fractions. Each of these fractions is then subjected to biological assay. If the separation is good the biological activity may concentrate in a particular fraction. Sometime the biological activity may be in more than one fraction. Generally lipophilic compounds are present in hexane and chloroform soluble fractions. The isolation of pure compound from hexane and chloroform soluble fraction is comparatively easier than from the water soluble fraction. The non-polar

compounds that are extracted in hexane, benzene and chloroform are generally esters, ethers, hydrocarbons of terpenoids, sterols, fatty acids etc. The mixture of these compounds are resolved by standard chromatographic techniques over SiO_2, Al_2O_3, HPLC etc.

2. Separation Techniques

2.1 Water Soluble Constituents

There are a number of problems associated with the separation of water soluble compounds. The abundance of salts carried over from seawater into the water extracts, makes the separation of water solube compounds more perplexing. This and other problems have been dealt in detail by Shimizu.[1] In handling aqueous extract of marine organisms or aqueous fraction of the ethanolic/methanolic extract of the organism, an inevitable problem is bacterial and fungal growth, which often degrades the active constituents or gives false results in bioassays due to endotoxins produced by microorganisms. It is particularly disturbing in antitumour activity screening because many endotoxins, e.g. lipopolysaccharides (PS) exhibit antitumour activities. Additions of alcohols or small amount of immiscible organic solvent, such as n-BuOH and toluene, help to prevent the microbial growth. Enzymes, such as gycosidases, sulfatase, proteinase, and various oxidases are usually activated upon homogenisation which can bring changes in some bioactive compounds and thus, the activity may be lost. If heat does not destroy the activity, brief heating or autoclaving may alleviate the problem considerably. For example boiling the organisms prior to extraction was used in the case of the nereistoxin,[2] an insecticide from sea worms and antitumour glycoproteins from scallops.[3] The concentration of aqueous extract also creates problems because of the great heat of evaporation of water. Prolonging the evaporation process often leads to the destruction of activity. This problem, however, can be circumvented by freeze-drying. The aqueous extract, therefore, should be freeze dried.

Desalting

While working with the aqueous extract of marine organisms, desalting is probably the most important, and often the most difficut process. The presence of large amount of inorganic salts gives rise to false results in bioassays. It also interferes in all chromatographic separations including gel-filtration. The methods generally used in biochemistry for desalting are not applicable for the isolation of low molecular weight compounds from the aqueous extract of marine organisms. If the size of the inorganic ions and the low molecular weight compounds is not different, both the inorganic ions and desired compounds will appear almost at the same position on the desalting gels or membranes widely used in biochemical preparations. The desalting of the freeze dried residue of the aqueous extract of the marine organisms

can be done conveniently and effectively by using absolute methanol. The residue is extracted with absolute methanol and the solvent removed. The process is repeated three to four times. Thus, the majority of the salt present is removed and further desalting becomes easier. The material is then carefully filtered through gels with small matrices, such as Sephadex G-10 or Bio-Gel P-2. The small molecular weight substances are, thus, desalted and separated. If the desired compound is reasonably hydrophobic, one may try other ionic resins, such as XAD-2, XAD-7, polyethylene or polypropylene powder and porous polyether type resins. Elution of organic molecules is often retained or retarded by these resins. Filtration through small pore membranes usually gives imperfect separation of salt. Adsorption on active charcoal is also partially effective for desalting. Generally, bioactive component of the total water soluble organic portion and inorganic salts, is a very minor fraction. It is, indeed, a formidable task to isolate pure active compounds from this complex mixture. There is no established standard fractionation procedure for water soluble compounds, and one has often to rely on trial and error.

2.2 Ion-exchange Chromatography

Ion-exchange chromatography is the most effective method of separating water soluble compounds, if the ionic character of the compounds and their stability on the resin and in buffer solutions are known. Choice of resins depends on the ionic character, and stability of the target compounds on the resins. It should be noted that many compounds decompose on the H^+ form of strongly acidic resins or the OH^- form of strongly basic resins. Similarly, the strong pH of solutions used for elution often causes decomposition. The use of weakly acidic or basic resins or poorly buffered resins is preferred in such cases. Various forms of resins with medium acidity or basicity are available.

2.3 Reverse-Phase (RP) Columns

The compounds with a wide range of polarity can be separated by reverse-phase column with various hydrophobic stationary phase with the proper combination of organic solvents, such as methyl alcohol, acetonitrile, and buffers, successes are reported in biochemical analysis with almost all type of compounds. However, several problems are encountered when it is used for preparative purposes. The first problem is that the sample size is very limited. The injection of large amounts of crude material results in the incapacitation of an expensive column. For this reason, the separation on reverse-phase columns is usually done for the final purification or fine separation. The second problem with reverse-phase columns is the use of buffer solutions. For most polar or ionic compounds, to effect good separation and recoveries, the use of buffers with appropriate pH and ionic strengths is often unavoidable. In such cases, sometime the separation of minute components from the buffer may become a major problem. This can be

overcome by the use of volatile buffers, which can be removed by vacuum evaporation or freeze drying. A list of volatile buffers are given in Table 1. It should be noted that the volatile buffers are also useful in regular ion-exchange chromatography.

Table 1

Buffer	pH
Ammonium acetate	7.8
Ammonium bicarbonate	5.7
Pyridine-HOAc (16.1 : 278.5)	3.1
Pyridine-HOAc (161.2 : 143.2)	5.0
Pyridine-HOAc-Picoline (10 : 0.4 : 10)	8.0
Pyridine-HOAc-N-ethylmorpholine (7.5 : 10.5 : 12.5)	9.3

2.4 High/Medium Pressure Chromatography

Various new types of stationary phases have been developed and found to be more effective for preparative purposes than the traditional C_{15} or C_8 column. These materials are mostly porous matrices, which possess both molecular filtration and adsorption capabilities and withstand high pressure. TSK-125, TSK-250 and TS-400 are some of these materials which are bonded silica with various pore sizes for separation of various molecular sizes. Another popular material is rigid organic matrices, such as styrene-divinylbenzene co-polymers with absorption properties and pore characteristics. Examples are TSK Type H gels with different pore sizes and Hitachi Gel 3000 series.

2.5 Combination of Ion-exchange and Size-exclusion Chromatography

Attachment of ion-exchange capabilities of matrices of various pore sizes provides a very powerful separation capability. Examples are DEAE Sephadex, carboxymethyl cellulose etc. In most cases, the actual separation is due to the combintion of three principles: ion-exchange, size-exclusion, or hydrophilic/hydrophobic interactions. Thus, the selection of a proper matrix may be the key for the successful separation.

A list of some chromatographic supports often used to separate water soluble substances are given in Table 2. Generally, compounds with basic characters are separated on cation-exchange resins, and those with acidic functional groups on anion-exchange resins. Strongly acidic or basic resins are also widely used to separate neutral and amphoteric compounds. A classical fractionation method for water-soluble compounds is suggested by Shimizu.[1] The key to a good fractionation method is the use of bioassay to monitor the activity of all fractions produced, so that subsequent work is done only on

Table 2

Water soluble compounds	Support
Mono- and oligo-saccharides	Sephadex G-10, G-15, Bio-Gel P-2, strong cation exchange (SO_3H) resins, weakly basic anion exchange resin, e.g. —$(CH_2)NH_2$
Polysaccharides	Sephadex G-50-200, Bio-Gel P-2, hydroxy appatite, DEAE bounded gels
Oligopeptides	Sephadex G-10, -15, Bio-Gel P-2, P-10, Sephadex LH-20, RP HPLC (C_8, C_{18})
Amino, guanidino, amino acids	Strong cation exchange resins (—SO_3—) weak cation exchange resins (—CO_2H) RP HPLC (C_8-C_{18} CN) etc.
Nucleic acids	Anion exchange resins, RP HPLC (C_8, C_{18}).
Polar carboxylic acids	Strong or weak anion exchange resins RP HPLC (C_{18}).
Glycosides	Sephadex G-10, LH-20, RP (C_{18}, C_8), XAD-2, XAD-7.

the active fractions, and the entire work is, thus, directed towards purification of the active material. This is essential since in most cases the active compound makes up a very small percentage of the crude extract. Examples have been seen where the active material is present in concentrations of less than a mg of the dried material.

3. Bioassay Directed Fractionation

The selection of an assay system to monitor fractionation is based on the original activity of the extract. An effort is made whenever possible to use *in vitro* systems to monitor activity since the test results can be obtained much more rapidly than with *in vivo* testing, and also the costs of bioassay are lower. For example, to follow anticancer activity, KB, L 1210 (LE) and P388 (9 PS) cell lines are used to follow progress of fractionation studies. The PS system *in vivo* is used only when the extract is not active in any of the *in vitro* systems. In most cases, the *in vitro* activity parallels with the *in vivo* activity so that extracts, which were originally discovered as *in vivo* actives, can be followed by cell culture assay.

4. General Fractionation

The active extract at early stages is fractionated by solvent partitions which eliminates much of the weight of inactive material, although the active fractions from these partitions are still exceedingly complex chemically. A typical solvent partitions of a active extract is suggested by Suffness and Douros.[4] The broad fractions, thus obtained, are further fractionated by column chromatography of several types (absorption on silica gel or alumina, ion exchange, partition, gel permeation) using a wide variety of solvent systems adapted to the polarity of the active fraction. Multiple chromatographies are necessary before the active fraction can be concentrated to a state of purity.

Other techniques, such as preparative thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), counter-current distribution, electrophoresis, and fractional crystallization, may be required in the final phases of isolation of pure compounds. The processes involved in isolation of active principles are complicated by lack of knowledge of the chemical nature of the active material. This makes the design of isolation procedure aimed at a particular chemical entity impossibe in many cases. Besides, the active principles may have reactive functional groups which can readily undergo reaction to yield inactive by-products. Thus, the activity is lost. This requires many modifications of the isolation procedures so that activity can ultimately be concentrated in a single fraction, and a pure material or materials can be isolated. The presence of multiple active compounds, which are closely related and are extremely difficult to separate also often complicates the isolation procedures.

Characterization of Active Compounds

The elucidation of the structure of a natural product with high biological activity is both stimulating and challenging. The first step in structure elucidation is to ascertain what the skeleton of the molecule is, and this can often be narrowed down by reference to phytochemical literature on related genera and species. A knowledge of biosynthesis of secondary metabolites is very helpful in deducing the most logical substitution patterns once the basic structural nucleus is established. Spectral data, such as ^{1}H NMR, ^{13}C NMR, infrared (IR), ultraviolet (UV) and mass spectra, are determined and compared with those reported for compound which may be related on the basis of chemical and biosynthetic reasoning. Unfortunately, not all the compounds isolated by activity-directed fractionation are novel and new. Some known compounds are often detected. The classical method of structure determination requires degradation of the molecule to establish the nucleus, and various transformation reactions combined with rigorous analysis of spectral data of the derivatives. X-Ray crystallographic studies are finally undertaken either on the compound itself or a heavy atom containing derivative to establish the structure and stereochemistry.

5. Isolation Procedures

5.1 Amino Acids and Simple Peptides

Several amino acids, amino sulphonic, iodoamino acids and simple peptides have been isolated from marine algae.[5-8] Analysis of algal amino acids is generally carried out, as is done in the analysis of terrestrial plants. The algal material is homogenized with ethanol furnishing a concentrate of about 70% aqueous ethanol. The extract contains nitrogen containing compounds, such as amino acids, amides, small peptides, amino sulfonic acids, amines, chlorophylls, low-molecular weight nucleotides, inorganic salts, and traces

of proteins. It is generally deeply colored because of the presence of chlorophylls and carotenoids. The alcohol from the ethanol extract is removed under reduced pressure. The aqueous concentrate, thus obtained, is partitioned with ether. The lipophilic compounds soluble in ether layer are discarded. Although the aqueous phase may be directly examined for amino acids, it is advisable to carry out a preliminary separation of the total amino acid fraction by adsorption on a strongly acidic ion-exchange resin (e.g., Dowex 50; H^+ form) and subsequent elution with aqueous ammonia. Ammonia is removed under reduced pressure at low temperature and the residue is analyzed by standard methods. Two-dimentional paper (or thin layer) chromatography has been used extensively. Automatic amino acid analysis and the gas chromatography allow accurate quantitation of known amino acids and easy detection of new compounds. Since amino acids are non-volatile compounds, gas chromatography requires prior derivatization. However, this additional step is not cumbersome or time consuming. It must be noted that preparation of certain derivatives may lead to modifications of a particular component of the amino acid mixture other than those that involve the amino and carboxyl functions. When trifluoro acetamides, n-butyl esters are used as derivatives, compounds such as glutamine and asparagine are converted to esters during butylation with butanol and hydrogen chloride and, thus, become indistinguishable from the corresponding acids. During the same derivatization process methionine sulfoxide is reduced to methionine by hydrogen chloride, and possibly other sulfoxides undergo analogous reactions. However, these difficulties are overcome by passing the amino acid fraction through a column of Dowex-3 that is washed with water to remove neutral amino acids and then eluted with 0.5 N formic acid to recover dicarboxylic amino acids and methionine sulfoxide. The fractions, thus obtained, are then individually derivatized and analyzed by gas chromatography.

The most versatile and efficient method for the isolation of amino acids is, undoubtedly, ion-exchange chromatography. Many variations of the method have been used with success. Generally, the amino acid fraction is adsorbed onto a strong acid cation-exchange resin in H^+ form, which is then eluted with dilute ammonia or a linear gradient of hydrochloric acid. The separation of acidic amino acids is more readily carried out on column of Dowex-1 resin in acetate form; neutral and basic amino acids are washed out with water, and the acidic amino acids are then fractionated with a linear gradient of acetic acid. Basic amino acids are usually isolated by adsorption on a strongly acid cation-exchange resin (Dowex 50) in ammonium form and subsequent elution with dilute (0.5 M) ammonia, after complete removal of neutral and acidic amino acids by washing with water. Compounds isolated by the use of one of these methods often require further purification. This can be achieved by other chromatographic techniques (e.g. preparative paper chromatography) or by crystallization. Basic amino acids are usually purified *via* appropriate salts, among them are flavianates, reineckates, and oxalates.

The algal extracts, in addition to amino acids, often contains nitrogenous compounds that are not adsorbed on strongly acidic cation-exchange resins. From the eluate of the column 'acidic' compounds (amino sulfonic acids) may be recovered by adsorption on, and elution with 2N ammonia from a strongly basic resin (Dowex-1), whereas "Neutral" N-compounds (e.g. N,N,N-trimethyltaurine) are not adsorbed on acidic or basic resins and are, therefore, found in the final eluate. Although no general method has been described in the literature, column chromatography on carbon-celite is probably the best method to obtain individual components from mixture of amino sulfonic acids. The other effective methods could be column chromatography on cellulose powder, preparative paper chromatography and preparative thin layer chromatography on silica gel.

5.2 Peptides

Haas and Hill[9] initiated the investigation of peptides from marine algae. They isolated an octapeptide of glutamic acid from the brown alga *Pelvetina canaliculata*.[9] Since then several simple peptides had been isolated from marine algae.[5] Some of them in good yield. In recent years several peptides of interest have been isolated from marine organisms.[10] Discodermin-A, the first bioactive peptide was isolated from the marine sponge *Discoderma kiiensis*,[11] which contained the rare *tert*-leucine and cysteic acid and several D-amino acids. The rapid progress in the chemistry of sponge peptides has been due to development of reversed phase HPLC for the isolation of peptides and advances in spectroscopy, essentially 2D NMR and FAB mass spectrometry for the structural study, because sequence analysis of unusual peptides cannot be accomplished by Edman degradation due to the presence of blocked N-termini and β- and γ-amino acid residues. The assignment of absolute configuration of amino acids with small amounts of material is now possible due to progress in chiral chromatography. The isolation procedures of a number of peptides are available in literature. For example, jaspamide, from *Jaspis* spp.,[12] cyclotheonamide-A from marine sponge,[13] onnamide-A from Okinawan sponge of the genus *Theonella* spp.[14]and discodermin-A from sponge have been isolated.[15] The peptides isolated from marine sponges were usually cyclic and lipophilic. It is likely that the linear or more polar peptides have been missed since the chemists were using a specific bioassay in the isolation of the peptides from the sponges.

5.3 Nucleosides

Bergmann (1950) first isolated three unusual nucleosides, spongothymidine (Ara-T), spongouridine (Ara-U) and spongosine from the marine sponge *Cryptotethia crypta* by soxhlet extraction with acetone.[16] Spongothymidine was obtained by repeated crystallisation of the mixture. Spongoadenosine and its 3′-O-acetyl derivative were obtained by chromatography on silica gel column followed by silica gel preparative TLC.[17] Chromatography on Sephadex

LH-20 column followed by preparative TLC furnished adenosine and deoxyadenosine.[18] Chromatography on Bio-Gel P-2 column followed by chromatography on SiO_2 had yielded doridosine.[19,20] The 5′-deoxy-5′-dimethylarsenyl-adenosine had been isolated by gel permeation and buffered ion exchange chromatography of the aqueous methanol extract of the kidney of giant clam.[21] Adenosine and 2′-deoxyadenosine are common components of the nucleic acids, perhaps it is the first report of their occurrence in the free state in sponge. It is unlikely that the mild isolation procedure that has been used would liberate these nucleosides from their polymeric forms. The pyrrolo[2,3-d]pyrimidine nucleosides mycalesine A and mycalesine B were obtained by extraction of the marine sponge *Mycale* spp. with ethanol followed by fractionation of the extract with ethyl acetate and subsequent SiO_2 flash chromatography. The active fractions were subjected to low pressure column chromatography on Kiesel gel.[22] Methylthioadenosine was isolated from the nudibranch *Doris varrucosa*[23] by extraction with acetone. Fractionation of acetone extract with ether and n-butanol followed by chromatography of the active fraction on Sephadex LH-20 column.[23]

5.4 Cytokinins

Cytokinins are known to be involved in cell division and differentiation in plants. The commercially available seaweeds extracts which are used to improve the yield in agriculture, show high order of cytokinin activity. The cytokinins from marine organisms are generally purine derivatives isolated by ion-exchange chromatography and purified by preparative TLC on silica gel HF-254. The spots are visualized by exposure to I_2 vapor/or UV light. The solvent systems generally used for TLC are : n-BuOH-HOAc-H_2O (12:3:5). Zeatin, 1-methylzeatin, zeatin riboside, dihydrozeatin and isopentinyl adenine had been isolated by following the above procedure from marine green alga *Caulerpa texifolia*.[24]

5.5 Alkaloids

Of the several types of alkaloids isolated from marine organisms, there is currently great interest in marine pyridoacridine alkaloids because of their significant biological activity particularly antiHIV activity, Ca^{2+} releasing activity, metal chelating properties and the intercalation of DNA.[25,26] Pyridoacridine alkaloids are typically isolated as refractory microcrystalline solids with melting point above 300°C. In most cases, they have been isolated as hydrochloride salts. Only a few are optically active. Because of variability in oxidation states of the heterocyclic nucleus, these alkaloids exhibit facile redox reactions. For example, the iminoquinone system present in many alkaloids is easily reduced by sodium borohydride. Partially saturated nitrogen containing rings in these alkaloids are easily aromatized by air oxidation (autoxidation) upon storage or heating in solution. Pyridoacridine alkaloid 2-bromoleptoclinidone exhibiting toxic properties in cell culture against

lymphocytic leukemia cells (PS) is isolated from the ascidian *Leptoclinides* spp. by Schmitz et al.[27] The ascidian *Eudistoma olivaceum* is an extraordinary rich source of tryptophan derived alkaloids. Several alkaloids named eudistomins having a β-carboline system had been isolated from this source.[28,29] Eudistomin-A and other eudistomins exhibited significant antiviral activity. Hexacyclic pyridoacridine alkaloids, segoline-A and isosegoline-A are isolated from the Red sea tunicate *Eudistoma* spp.[30]

6. Marine Toxins

The majority of marine toxins are produced by microalgae, especially dinoflagellates. Some of the toxins are also produced by bacteria and a few by macroalgae.

6.1 Saxitoxin

Schantz et al[31] first isolated pure saxitoxin from the Alaskan butter clam using weakly basic Amberlite IRC 50 and alumina chromatography. The Alaskan butter clam is still considered the best source of saxitoxin. The isolation procedure is fairly simple. However, this procedure is not applicable to the isolation of other shellfish toxins, since these are not strongly basic. A general procedure which is now commonly used, had been developed.[32,33] The mixture of toxins is broadly resolved by selective adsorption on Bio Gel P-2 or Sephadex G-15. The toxin fraction is eluted with a dilute acetic acid solution. The mixture of toxins is then applied on a column of weakly acidic carboxylic resin, Bio-Rex-70 in acid form. The acetic acid gradient elution furnish pure toxins in the reverse order of the net positive charge of the molecule. However, they cannot be separated by either preparative thin layer chromatography or careful chromatography on Bio-Gel P-2.

6.2 Brevetoxins

The dinoflagellate *Gymnodinium breve* has yielded several toxins named brevetoxins. Chemically they are highly oxygenated polyethers. Of the isolated toxins, brevetoxin-A is most potent toxin. It is of interest not only because it is the most potent toxin of the family but also because it uniquely binds to sodium channels of the excited membrane. Brevetoxin-B is the first member of this class of toxins which is isolated from the cultured cells of *Gymnodinium breve*.[34–37] Unialgal cultures of *G. breve* isolated during an outbreak at Florida were grown in an artificial sea-water medium. The medium containing the cells were acidified to pH 5.5 and extracted with diethyl ether to give 90 mg of crude brevetoxins. Repeated flash chromatography of the crude toxin mixture with 5% methanol in diisopropyl ether (v/v) gave brevetoxin A (0.8 mg) brevetoxin B (5 mg) and brevetoxin C (0.4 mg). Purity of the various toxins was checked by HPLC.[34] Brevetoxin-B crystallized from acetonitrile as colorless needles, m.p. 270°C (dec.); UV λ_{max} (MeOH); 208 nm (ε 1600); FT IR (KBr, pellet) 1735 and 1691 cm^{-1}.

6.3 Tetrodotoxin

Tetrodotoxin is the best known marine toxin, because of its frequent involvement in fatal food poisoning, its unique chemical structure and its specific action of blocking sodium channels of excitable membranes. Tetrodotoxin is a colourless crystalline compound. It is virtually insoluble in all solvents but is soluble in acidic media and in weakly basic medium. The remarkable feature of tetrodotoxin is that the number of oxygen and nitrogen atoms are equal to the number of carbon atoms. The three nitrogen atoms of tetrodotoxin are present in the molecule as a guanidine moiety. It was surprising that in spite of the presence of the guanidine function in the molecule, the toxin was only weakly basic (pka 8.5) and attempts to prepare crystalline salts did not succeed. However, treatment of the toxin with 0.2N HCl did yield a crystalline, O-methyl-O,O′-isopropylidene-tetrodotoxin hydrochloride monohydrate. About 1-2 g of crystalline tetrodotoxin had been obtained from 100 kg of puffer ovaries by Hirata's procedure.[38] Tetrodotoxin derivatives occur in puffers, newts, and a frog. These toxins can be detected by a highly sensitive tetrodotoxin analyzer which separates them on a reverse-phase column and detects fluorescent products formed upon heating the toxin with sodium hydroxide solution.[38]

6.4 Ciguatoxin and Its Congeners

Ciguatera, a kind of food poisoning which endangers public health and hampers local fisheries in tropical and subtropical regions of the world, is caused by ciguatoxin and its congeners. Ciguatoxin (0.35 mg) was isolated from the visera of moray eel, *Gymnothorax javanicus* (125 kg).[39,40] A less polar congener (0.74 mg) of ciguatoxin was isolated from the causative epiphytic dinoflagellate, *Gambierdiscus toxicus*. Ciguatoxin was obtained as a white solid, no UV maximum above 210 nm; IR (film) 3400, 1111 and 1042 cm^{-1}. Ciguatoxin was first isolated in 1980 by Scheuer's group at the University of Hawaii and characterized to be a polyether compound.[41] The structure of ciguatoxin was finally elucidated by Yasumoto's group in 1989.[42] Ciguatoxin congeners have been isolated either from toxic fish[42] or from cultured *G. toxicus*.[43,44] Dozens of ciguatoxin analogs have been found in fish and in dinoflagellates.[45] However, only a few of them have been identified.

6.5 Maitotoxin

Maitotoxin (MTX) had been isolated from cultured cells of *Gambierdiscus toxicus*.[46,47] Approximately 25 mg of maitotoxin was obtained from 5000 L of the culture. Maitotoxin had attracted much attention because it had molecular formula $C_{165}H_{258}Na_2O_{67}S_2$ and molecular weight of 3421.6 Da (as the disodium salt), it had extremely potent bioactivity. The lethality against mice, LD_{50} was ca 50 ng/kg (ip) and it plays a role in diversifying ciguatera symptoms, particularly in the poisoning caused by hervivorous fish. Maitotoxin is a polyether containing two sulphate ester functions, 32 ether rings,

29 hydroxyl and 21 methyl groups. The one-half of the molecule of maitotoxin, which includes fragment A, is relatively hydrophilic, while the other half, comprising mostly continuous fused rings is hydrophobic, thus accounting for the duel polarity of the toxin.

6.6 Palytoxin and Its Congeners

Palytoxin is an extremely poisonous substance found originally in the genus *Palythora* of marine coelenterates. Its intravenous lethality (LD_{50}) was 0.025 mg/kg in rabbit and 0.45 mg/kg in the mouse. Palytoxin and its congeners have not only been found in *Palythora* soft corals but in wide variety of other marine organisms, such as seaweed *Chondria armata*, crabs belonging to the genus *Demaria* and *Lophozozymus*, a triggerfish *Melichthys vidua* and a file-fish *Alutera scripta*. Continuous interest in the toxins of *Palythora* spp. led to the isolation of four minor toxins characterised as homopalytoxin, bishomopalytoxin, neopalytoxin and dideoxypalytoxin from *P. tuberculosa*.[48]

Moore and Scheuer[49] were the first to isolate palytoxin from *T. toxica*. The toxin was completely extracted with 70% ethanol-water from the unground wet animal. Reverse-phase chromatography of the defatted extract on powdered polyethylene separated the toxin from inorganic salts and other polar organic materials. Elution of the column with 50% aqueous ethanol gave readily the toxic fraction. Successive ion exchange gel fitration of the toxin material first on DEAE-Sephadex at pH-7 and then on CM Sephadex at pH 4.5-5, yielded pure palytoxin in 0.027% yield based on the wet weight of the animal. A similar procedure was used by the Japanese investigators to isolate palytoxin from *P. tuberculosa*.[50] The reverse-phase chromatography, however, was carried out on polystyrene gel instead of powdered polyethylene. Separation of neopalytoxin from palytoxin was not feasible by HPLC, but was possible by HPTLC. Palytoxin was a colourless, water soluble, amorphous hygroscopic solid. Attempts to crystallize the toxin and its derivatives did not succeed. The toxin had $[\alpha]_D + 26°$ in water.

6.7 Gambierol

Gambierol, a ladder-shaped polyether compound, was isolated from *Gambierdiscus toxicus* (RGI-strain) cultured cells.[51] The cultured cells of the dinoflagellate were extracted with MeOH. The extract was partitioned between CH_2Cl_2 and $MeOH/H_2O$ (6:4). The toxin was extracted into the organic phase and was further purified by guided mouse bioassay. 1100 L culture furnished 1.2 mg of gambierol, as an amorphous solid. UV λ_{max} (MeOH) 237 nm (ε 15,000).

6.8 Okadaic Acid and Its Congeners

Okadaic acid and its congeners are responsible for most human diarrheic shellfish poisoning (DSP) related illnesses. The acid was first isolated from the sponge *Halichondria okadai*,[52] and was subsequently found in

dinoflagellates *Prorocentrum lima* and *Dinophysis* spp. The sponge *H. okadai* was extracted with MeOH. The methanolic extract was repeatedly chromatographed on polystryrene gel. Sephadex LH-20:SiO_2, crystallized from MeOH and recrystallization from dichloromethane/hexane furnished okadaic acid, as colorless needles, m.p. 171-175°C (10^{-4} % yield); $[\alpha]_D$ + 21° (c, 0.33, $CHCl_3$); UV (end absorption) and IR (3450, 1740, 1080, 880 cm^{-1}).

6.9 Miscellaneous Toxins

Screening of microalgae for toxin production led to the isolation of a wide variety of toxic metabolites from dinoflagellates. The cultivated dinoflagellate *Amphidinium* spp. had furnished amphidinolides-A, and C.[53] Amphidinol as pale yellow amorphous solid : $[\alpha]_D$ - 250° (c, 0.18, MeOH); UV λ_{max} (MeOH) 259 (37, 500), 270 (41, 900) and 282 nm (27, 500), had been isolated from cultured cells of the dinoflagellate *Amphidium klebsii*.[54] Goniodomin-A, a novel antifungal polyether macrolide had been obtained from dinoflagellate. *Goniodoma pseudogoniaulax* by column chromatography on silica gel followed by reverse-phase HPLC.[55] Neosurugatoxin (4 mg) was isolated from midgut glands of the Japanese ivory shell *Babylonia japanica* (20 kg) as a causative agent of intoxication resulting from ingestion of the toxic ivory shell.[56] Midgut glands of the shellfish were extracted with 1% AcOH. Gel filtration on Sephadex 825 column and then on to a CM-Sephadex ion exchange column and finally by reverse phase HPLC yielded neosurugatoxin. It was found extremely unstable in alkaline medium and fairly heat labile. A toxic glycoside, named polycavernoside A, had been isolated from the red alga *Polycavernosa tsudi*.[57] The red alga (2 kg) was extracted with acetone. The toxic material was purified by column chromatography, guided by mouse bioassay.

The procedure for the isolation of fucan sulfates from the body wall of sea cucumber *Stichopus japonicus* and their ability to inhibit osteoclastogenesis were described.[58]

7. Concluding Remarks

There is no specific technique that could be followed for the separation of the constituents of the complex mixture present in the ethanolic/or methanolic extract of a marine organisms since the chemical nature of the bioactive compounds is not known. However, a broad separation of the mixture can be achieved by fractionation with organic solvents. If the separation is effective, the biological activity may concentrate in a particular fraction. The abundance of salts carried over from seawater into the water extracts of the marine organisms makes the separation of water soluble compounds more perplexing. The concentration of aqueous extract also creates problems because of the great heat of evaporation of water, while working with aqueous extract desalting is probably the most important, and often the most difficult process. There is no established standard fractionation procedure for isolation of water soluble

compounds, and hence one has often to rely on trial and error. Ion-exchange chromatography, reverse-phase columns, high/medium pressure chromatography on porous materials, combination of ion-exchange and size-exclusion chromatography, are effective techniques for the separation of compounds. The broad fractions, thus obtained, are further subjected to column chromatography of several types. Multiple chromatography is necessary before the active fraction can be concentrated to a state of purity. Other techniques, such as preparative thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), counter-current distribution, electrophoresis, and fractional crystallisation, may be required in the final phases of isolation of pure compounds.

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4

Biological, Toxicological and Clinical Evaluation

Abstract

The chapter deals with the methods of biological, toxicological and clinical evaluations of the extracts and/or pure compounds from marine organisms. The screening models for evaluation of antibacterial, antifungal, antileishmanial, antihookworms, antitapeworms, antimalarial, antiviral and anticancer activities and problems of screening have been discussed. Besides, the methods of evaluation of analgesic, antiallergic, antiarrhythmic, antithrombotic, hypolipidaemic, hypoglycemic, hypotensive, antihypertensive, diuretic, adaptogenic, immunomodulatory, hepatoprotective, choleretic and anticholestatic activities have been described. Finally, evaluation of acute and regulatory toxicity and studies needed for clinical trials have also been discussed.

1. Introduction

The biological activity of an extract of marine organisms or of isolated compounds could be assessed in several test systems. Due to limited amount of material generally available initially and high cost of biological testing, it is impossible in any laboratory to examine all permutations of drug-animal interactions, to unmask the drug potential of a material. The basic premises of a screening programme are : (i) drugs operate in a dose response manner and produce toxicity in higher doses; (ii) each class of drug has a characteristic dose response profile; (iii) for majority of drugs, route of administration produces only a quantitative change in action; (iv) absolute potency is not of major importance in therapeutics and (v) it is possible to predict usefulness and toxicity of a new compound by utilizing a dose response spectra library of various prototype drugs. The criteria of a good screening programme is

that it should be simple, economical, reliable, pick up new unexpected or unique activity, unbiased and comprehensive.[1] Finally, it should have in-built safety mechanisms. The primary screening of an extract or a compound is mainly descriptive and qualitative. In-depth evaluation is carried out at the secondary screening.[2-10]

2. Types of Screening

2.1 Individual Activity Screening

Individual activity screening is used to detect a particular biological property of an extract or compound, such as antibacterial,[8] anticancer[9] and antifertility[10] etc.

2.2 Broad Biological Screening

Broad biological screening is employed to know whether an extract or compound has any exploitable biological potential. A broad biological screening is being done at the Central Drug Research Institute, Lucknow, India, for several years for evaluation of terrestrial plant extracts, extracts of marine plants and animals and of synthetic compounds. About 125 test systems are available for evaluating the biological activities of the test material. Extracts of over 4,000 terrestrial plants, and about 450 of marine plants and animals have been tested so far. The results of biological screening of extracts of terrestrial plants[11-24] and marine organisms[25-32] have been reported in a series of papers.

3. Screening Models and Activity

Excellent accounts of various models are published[6,25] for screening antibacterial, antifungal, antileishmanial, anthelmintic, antitapeworm, antimalarial, antiviral, antiinflammatory, analgesic, antiallergic, antiarhythmic, hypolipidaemic, hypoglycemic, antihypertensive, diuretic adaptogenic, immunomodulatory, hepatoprotective, choleretic, anticholesteric, CNS, activities and toxicity.

3.1 Antibacterial and Antifungal Activities

The need for effective antibacterial and antifungal drug has been realized more acutely with the emergence of Acquired Immunodeficiency Syndrome (AIDS) and AIDS-Related Complex (ARC), which are often associated with opportunistic infections. The *in vitro* antimicrobial testing can be carried out by several methods, viz. poison food technique; disc diffusion method; tube dilution method, and microtitre technique. The details of these testing methods are described.[6,25]

3.2 Antileishmanial Activity

Leishmaniasis is caused by invasion of the hemoflagellate protozoan parasite,

Leishmania donovani. The parasite is transmitted by the sand fly of the genus *Phlebotomus* sp. Humans are the main reservoir of the infection. In leishmaniasis, peculiar situation occurs where the invader amastigote thrives and multiplies within the macrophages, the cells which normally destroy invaders. This adaptation not only deranges host's defense systems but even defeats man's ingenuity in directing and delivering drug at the appropriate site. Both promastigotes and amastigotes are used as test parasites for *in vitro* testing. *In vivo* testing is conducted in mouse and hamster. The details of the method of testing are described.[25]

3.3 Anthelmintic Activity

The most prevalent helminth parasites of man are hookworms, ascarids, oxyurids and filarids. Tapeworms, though not very common, produce severe pathology in infected children, and are notoriously resistant to anticestode drugs.

Antihookworm Screening

N. americanus and *Ancylostoma ceylanicum* are the hookworm parasite of man and both can be maintained in hamsters. *N. americanus* has been forced to parasitize rodents.[26] On the other hand, *A. ceylanicum*, being a parasite of dog, cat and man, has a wider host range and is easy to maintain in hamsters without the use of immunosuppressants.[27,28] The host infectivity is almost 100%.[29] The test extract and standard drug in appropriate doses are fed to groups of infected hamsters in single or multiple doses by keeping separate infected group as untreated control. The efficacy assay is made on autopsy of animals on day two of the last medication, and the worm count of the treated group are compared with those of the untreated group. The efficacy is expressed either in terms of host clearance or of percent worm reduction in respect to untreated controls. The secondary screening is conducted in dogs and cats.[27] Besides, testing is carried out against human filarial worm *Brugia malayi* in mastomys/cats.[28]

Antitapeworm Screening

Hymenolepsis nana is the only tapeworm which can be cycled directly in experimental host.[29] The mice are infected by oral inoculation of 200 viable ova. Since the infectivity is not 100%, ovoscopic examination of infected mice is mandatory to select parasitised mice for testing. After appropriate grouping and drugging of infected mice, the autopsy of treated and untreated animals is done on day two of the medication to examine tapeworms in the lumen and the scolices in the intestinal scrappings. The criterian of efficacy is 100% clearance of parasites from treated animals. Secondary screening is carried out in naturally infected dogs, cats and poultry with their respective cestodes.[30]

3.4 Antimalarial Activity

Blood schizonticidal activity of the test extract is evaluated in swiss mice infected with *Plasmodium berghei* in primary screening. The secondary screening is conducted in rhesus monkey infected with *P. cynomolgi* Chloroquine (5 mg × kg × 7 days) is used as a standard drug. Absence of any recrudescence upto the end of 50 days indicate complete cure. Radical curative activity of the test substance is evaluated in rhesus monkey infected with *P. cynomolgi*. Causal prophylactic activity of the test material is also evaluated in rhesus monkey infected with *P. cynomolgi* sporozoite. The details of the methods of testing are described.[25]

3.5 Antiviral Activity

Theoretically, the test material can be evaluated for antiviral activity in all test systems by combination of virus and susceptible host system. It is suggested that the potential antiviral agents must not be evaluated in inert medium but in a living cell or animal host. The primary antiviral testing involves a simple schedule of treatment, and is assessed by comparing the response of the treated and untreated host systems to viral infection. The secondary antiviral screening involves multiple doses of the test substance against several doses of the virus. The time and route of both treatment and infection are varied in relation to each other. Also experiments are conducted in more than one host virus systems. The purpose is to arrive at the optimum dose and schedule of treatment and to assess the consistency of antiviral activity. Testing for antiviral activity is usually conducted in cell culture; chicken eggs and animal models. Each test method has its advantages and disadvantages. *In vitro* antiviral testing using cell cultures involves the virus of interest and a primary or permanent cell line which can support its multiplication. The cells are infected with the virus and then exposed to the test substance. If the substance has antiviral activity the multiplication of the virus will be inhibited which will be evident from the morphology of the cell monolayer. It is important to assess the toxic effect of the test substance on cells at each dilution. This can be done by examining the uninfected cell monolayers exposed to the test substance only. From the observed ED_{50} and LD_{50} of the test substance, its therapeutic index is calculated. Several viral targets are studied to estimate the antiviral effect of test substance in a cell culture system. Some of these are viral DNA polymerase activity; ribonucleotide diphosphate reductase; mRNA polyadenylation and RNA dependent RNA polymerase; terminal deoxynucleotidyl transferase; thymidine kinase; uracyl DNA-glycolase; d-UTPase and reverse transcriptase. Testing for antiviral activity in chicken eggs is very simple, effective as well as economical. Here prophylactic and therapeutic assays may be carried with different test substances since a wide choice of routes and timing of application of both virus and antiviral agents are possible. There are three main routes through which the virus or the test substance could be administered into embryonated eggs. These are allantoic

cavity inoculation, amniotic cavity inoculation and choric allantoic membrane (CAM) inoculation. The virus and the test substance may be given through the same route or different routes. The test substance can be given before, along with or after virus infection. Testing in animal models has relatively the maximum predictive value among the various methods employed for detecting antiviral activity. Testing in these model systems can identify both antiviral activity as well as antiviral agents. The ideal animal model should have three features : (i) use of a human virus with minimal alteration by adaptation; (ii) use of the natural route of infection and size of inoculum as in man, and (iii) similarity of infection, pathogenesis, host response, drug metabolism and drug toxicity. Animal models exist for both local and systemic virus infection. Antiviral activity of a test substance can also be assessed by titrating the virus in blood and other target organs. The details of these models are described.[25]

3.6 Antiinflammatory Activity

The knowledge about the inflammatory process is still fragmentary. The drugs that are being used for its treatment have been found either by accidental clinical observation or by screening in animals. A number of *in vivo* screening models are described in the literature. Many older methods have been replaced by new and better models.[25] However, none of the tests reported so far have achieved the ideal.

(*A*) *Acute Models*

Carrageenin-induced Oedema in Mice

The model was developed by Srimal et al[31] Carrageenin solution in normal saline (0.025 ml of 1.0%) is injected subcutaneously into the hind paw of mice with the help of a Hamilton microsyringe after one hr of oral feeding of the test material. The mice are killed after three hours with an overdose of ether. Both the hind paws are cut identically at the ankle joint and weighed. The difference between the weight of the two paws gives the amount of oedema developed in that particular animal. The mean of the group is calculated and compared with the mean oedema developed in the control group. Percent inhibition of the oedema, if any, by the test substance is calculated and compared with the group receiving a standard compound, like phenylbutazone (50 mg/kg) or hydrocortisone (30 mg/kg) or indomethacin (4 mg/kg). Test material showing less than 20% activity are rejected.

Carrageenin-induced Oedema in Rats

Carrageenin (0.1 ml of 1% solution) is injected in the hind paws. Volume of the paw is measured plethysmographically immediately and three hours after the injection of the irritant. The difference in the volumes give the amount of oedema developed. Percent inhibition of the oedema between the control

group and the test substance treated group is calculated and compared with the group receiving standard drug.[32]

(*B*) *Sub-acute Models*

Cotton Pellet Test
Test autoclaved pellets of cotton or sponge (50° + 1 g) are implanted on the shaved back of rats aseptically, one on each side of the midline incision. The test material is fed once a day from day one to seven of the experiment. On the eighth day the rats are sacrificed by a overdose of ether. The pellets surrounded by granuloma tissue are dissected out carefully and dried in a hot oven at 60°C till a constant weight is obtained. Finally, percent inhibition compared to the control group is calculated.[33]

Granuloma Pouch Test
Pouch on the back of the rats is produced by injecting air (20 ml) in the subcutaneous tissue followed by 1.0 ml of 1.0% croton oil in sterile olive oil. The test substance is fed from day one to day 13 of the experiment, and rats are sacrificed on day 14. The exudate formed in the pouch is aspirated and measured. The pouch itself is dissected out carefully and dried at 60°C to constant weight. The percent inhibition compared to the control group is calculated.[34]

Formaldehyde-induced Arthritis
Formaldehyde (0.1 ml or 2.0% formaldehyde solution) is injected into the hind paws of rats on day one, and day three of the experiment. Volume of the paw is measured before the injection of the irritant and once daily for the next ten days. The test substance is fed once a day from day one to day 10 of the experiment. The mean increase in the paw volume of each group over a period of ten days is calculated and compared with the control group to find the difference.[35]

(*C*) *Chronic Models*

Adjuvant-induced Arthritis
Killed *Mycobacterium tuberculosis* (0.5 mg) suspended in liquid paraffin (0.1 ml) are injected into one of the hind paws of the rat. Volume of both the paws is measured plethysmographically daily for the next ten days. The test substance can be administered either from day one of the experiment to study the effect on the acute as well as the chronic phase of the arthritis or it can be given from day 14 of the experiment to study its effect on the established arthritis.[36]

In vitro Models
A large number of tests have been developed to evaluate potential antiinflammatory compounds *in vitro*,[25] but no single test is satisfactory.

Gastric Irritation Test
Gastric irritation is the most important side effect of antiarthritic drugs and therefore, any potential drug must be subjected to this test. There are several tests described in the literature. However, test by Thuiller et al[37] is considered most convenient and accurate.

3.7 Analgesic Activity
Tests for analgesic activity could be divided into two categories : (a) centrally acting analgesics, and (b) peripherally acting drugs. The details of the tests for evaluating some peripheral analgesics are described.[25]

3.8 Antiallergic Activity
Allergic conditions such as bronchial asthma, atopica eczema, allergic rhinitis, conjunctivities urticaria etc. affect about 20% of the population and are increasing in prevalence and severity. In spite of significant progress in the field of new drug development in recent years, still there is no satisfactory antiallergic drug available for therapeutic use. A number of experimental tests models for primary and secondary screening are discussed.[25] Of these, the mouse and rat passive cutaneous anaphylaxis (PCA) tests are convenient and reliable.

3.9 Antiarrhythmic and Antithrombotic Activities
In spite of recent advances in diagnostic and investigative cardiology, the life threatening ventricular arrhythmias still present a common therapeutic problem. Although the available antiarrythmic drugs are useful in reducing the incidence of cardiac arrhythmias, the successful treatment with minimal side effects is still far from satisfactory. Evaluation of antiarrythmic potential drugs are made difficult because animal models do not necessarily mimic those seen in humans and further we still have poor understanding of mechanism of clinical arrhythmias. However, there are a number of animal models[25] of wide diversity for testing extracts or compounds for evaluating antiarrhythmic activity.

3.10 Hypolipidaemic Activity
There is no single model for testing lipid lowering activity of test substance. Several experimental models for primary screening *in vivo* are employed for evaluating hypolipidaemic activity. The tests are conducted in triton induced hyperlipoproteinemia in male rats. Antilipolytic activity *in vitro* models is measured in fat cells isolated from epididymal fat pads of normal and test substance treated male Charles Foster albino rats.

3.11 Hypoglycaemic Activity
In vivo hypoglycaemic activity is done in Charles Foster albino rats in single and multiple doses. A fall of more than 30% in blood sugar is taken as active. Once the blood sugar lowering activity of a test substance is established in

normal rats, it is further confirmed in different species of animals, such as guinea pigs, albino rabbits, dogs and rhesus monkey.[25]

3.12 Hypotensive Activity

The test for hypotensive activity are normally conducted in dogs, cats and rats even though theroretically any mammal can be used. The test animal is generally anaesthetised with sodium pentobarbiton (3540 mg/kg, iv or ip) or a chloralose (7080 mg/kg, iv). The blood pressure is recorded. The test extract is given at 25 and 50 mg/kg (iv), and the effect noted. The experiment put up not only evaluates the activity of the test extract but also gives an idea about its neuromuscular blocking property (respiratory failure), respiratory stimulant activity apart from the possible alpha or beta, adrenergic antagonist, antihistaminic and anticholinergic activities.

3.13 Antihypertensive Activity

Ideally a test substance should be tested in hypertensive test models, but in practice it is more time consuming and difficult. There are many methods described to make the animal hypertensive.[25]

3.14 Diuretic Activity

Rats are convenient animal model for evaluating diuretic property of a test substance because of cost and ease in maintaining large number of animals. However, dog is the most reliable animal model for the evaluation of diuretic activity because of the close resemblance of renal physiology of the dog with man.[25]

3.15 Adaptogenic and Immunomodulatory Activities

Much before the concept of immunity was known, a large number of plants were being used in the traditional medicine of Europe, China and India for rejuvination therapy and treatment of chronic ailments.[3840] Immunomodulatory substances of varying chemical structures and molecular sizes have now been isolated from these plants. Immunostimulants offer promise in enhancing antigen specific (vaccines) and non-specific immune response against infection and malignancy, immunotherapy of viral infection and potentiating the efficacy of drugs in immuno compromised host.

Models and Parameters for Evaluation

In preliminary evaluation, effect of test material is studied on certain parameters of humoral and cell-mediated immune response in mice taking sheep red blood cells (SRBC) as antigen. The materials found active in this model are further studied for their effect on non-specific immune response taking certain factors of macrophages and lymphocytes as parameters. The extracts showing high order of immunostimulant activity in these models are studied for non-specific protection against certain infections in animal models.

Antigen Specific Response
Albino mice are infected each with 1×10^8 sheep red blood cells (SRBC), intraperitoneally. Four days later blood is collected from retro-orbital plexus for haemagglutinating antibody (HA) titre. The animals are then sacrificed and spleen is taken out for plaque forming cells (PFC) assay.

Non-specific Immune Response
The non-specific immune response is studied in macrophages and lymphocytes. Details of evaluation for adaptogenic and immunomodulator activities are described.[25]

3.16 Immunomodulation Activity

Immunomodulators are substances which have potential to modulate an immune response *in vivo* and/or *in vitro*. Since development of a particular immune response is a multistep process and is regulated at several levels through various mechanisms, immunomodulators may act at different steps/levels of the overall immune network. Therefore, a large battery of assays may be required to evaluate the immunomodulators.

Functional Assays for T-cells
Human and monkey mature T-cells have an affinity for sheep erythrocytes (E) and, thus, bind with them spontaneously. The binding can be observed visually as 'rosette' in which E are seen to form a cluster around a central lymphocyte. Erythrocytes bind with T-cells through a receptor known as CD_2 receptor. Since only mature T-cells have this receptor, its expression is regarded as a marker of T-cell maturation.

Functional Assays for B-cells
B-cells are involved in the humoral immunity. Their antibody producing function is established. Therefore, their number and functional capacities may have a direct bearing on the outcome of the immune response.

B-cells and their subsets can be identified by (i) immunoglobulin (Ig) determinants, (ii) FC component of Ig and (iii) C-3 portion of the complement. Ig determinants can be demonstrated on the surface of cells by peroxidase conjugated or fluorescence tagged anti-Ig sera. The peroxide labelled protein is histochemically localised and can be examined using electron or light microscopy. Fluorescein isothiocyanate or rhodamine conjugated anti-Ig sera can also be used to visually localise surface Ig determinants by using a fluorescence microscope. FC receptors can be identified by their binding to antibody complexed antigen. C_3 Receptors bearing B-cells can be demonstrated by their ability to form rosettes with sheep erythrocytes (E) having activated C_3 receptors on their surface.

Interleukin-1 *Assay*
Interleukin-1 (IL-1) is one of the most important cytokines. It is produced by various cells like monocytes, macrophages and B-cells, and acts upon various cells. It can induce T-cells to produce interleukin-2 (IL-2) and, thus, has co-mitogenic effect on T-cells (along with antigen or mitogen). Its functions as an amplification signals in the induction of immune response and thus its modulation may serve as an important indicator for the evaluation of an immunomodulator.

Interleukin-2 *Assay*
Interleukin-2 (IL-2) is produced by T-cells and can stimulate the production of various other cytokines. It enhances IL-2 receptor expression on T-cells, and increases antibody production. In the development of immune response, it acts as the ultimate extracellular mitogenic signals and, therefore, provides a key target for the modulation of the expression of immune response. Hence, a substance that can modulate IL-2 production may function as potential immunomodulator.

3.17 Hepatoprotective Activity
It is known that the biochemical and physiological functions of liver and the activity of a wide range of hepatic enzymes are altered when the animals are exposed to a variety of chemicals such as carbon tetrachloride, D-galactosamine, thioacetamide, heavy metals and drugs such as paracetamol, fungal toxins and parasitic infections. The available animal models for evaluating the hepatoprotective activity are : carbon tetrachloride, D-glactosamine, *Plasmodium berghei* infection, paracetamol, thioacetamide, monocrotaline and aflatoxin B_1.[25]

3.18 Choleretic and Anticholestatic Activities
The liver occupies a central position in body metabolism. Its size, softness, and relative homogeneity makes it a favourite organ for pharmacological and biochemical studies. Liver is also exposed to a variety of xenobiotics and therapeutic agents, thus, the disorders associated with this organ are numerous and varied. For the study of intact liver functions under controlled conditions, the isolated perfused liver has been extensively used by employing collagenase as a liver dispersing enzyme. The two steps *in situ* collagenase perfusion method is now routinely used for the isolation of viable adult hepatocytes from various animal species. The test substance is administered orally to animals in pre-selected doses. Study on bile flow is conducted 24 hours after the last dose of the test substance. Activity is assessed by observing the increase in bile flow and its contents (bile salts and bile acids). For anticholestatic activity, animals are treated separately with test substance and cholestatic agent. Study on bile flow is carried out 24 or 48 hour post-treatment with toxic agent. The details of test methods are described.[25]

3.19 Acute Toxicity and CNS Activities

LD_{50} is determined in mice using the method of Horn et al.[41] Doses of the test substance are given to groups of four to five mice each in a geometrical progression or regression starting with a dose of 464 mg/kg (ip) and mortality in 24 hour recorded. The LD_{50} with fiducial limits is read out from the table.

Gross Effects

After administration (ip) of the test substance in groups of five mice each, the animals are observed continuously for three hours after administration of the test substance, then every 30 min for next three hours and finally after 24 hours. CNS Stimulation is judged by increased spontaneous motor activity (SMA), piloerection, exophthalmos, clonic and/or a tonic convulsions, CNS depression by reduced SMA, a sedation, ptosis, crouching, catelepsy and autonomic effects by piloerection, urination, defaecation, salivation, lachrymation etc.

Effect on Hexabarbital Sleeping Time

Hypnosedatives and neuroleptic agents are reported to increase the barbiturate sleeping time in mice. Graded doses of the test substance are administrated (ip) in groups of five mice each and hexobarbitone (75 mg/kg, ip) is administered one hour later. Saline treated controls and hexobarbitone adminstered mice are run concurrently. The sleeping time for each animal is the period for which the righting reflex is absent. The prolongation of sleeping time with respect to the control is then calculated. The significance of drug-induced prolongation of sleeping time is judged using student's t' test.

Effect on Conditioned Avoidance Response in Rats

Amphetamine toxicity test in aggregated mice; anti-reserpine test in mice; swimming performance test in rats; anticonvulsant activity in mice; pentylene tetrazole seizure threshold test in mice, and anorexigenic activity in the mice are studied with the test substance to evaluate the CNS activities.[25]

3.20 Isolated Tissues

The advantages with the isolated system is that it is easy to set up from a single animal, small amount of the test material is required and the drug effect is tested directly without the factors of absorption, metabolism, excretion or interference due to nerve reflexes. The water insoluble substances are not recommended for test in the *in vitro* system. In case the substance is dissolved in a solvent other than water/saline, a control should be run with the solvent. The common sources of isolated tissues are usually rabbit, guinea pig and rat, cat and dog. Mice are also used sometimes for testing. The procedure for taking out organs and tissues from animals, carrying out tests on guinea-pig ileum, mouse vas deferens, rat uterus, rat hind limb, perfusion perfused rabbit heart, guinea-pig right ventricular papillary muscles and rat phrenic nerve diaphragm are described.[25]

4. Anticancer Screening

The Cancer Chemotherapy National Service Centre (CCNSC), National Cancer Institute, Bethesda, MD 20205, USA has the biggest organisational capability for evaluating the extracts of plants both marine and terrestial, marine animals and synthetic compounds.

The original objective of the CCNSC was the acquisition and screening of chemical substances for anticancer activity together with conducting the necessary pre-clinical developmental studies needed to bring active compounds to clinical trials. The programme was intended to give service to academic institutions, research institutes and pharmaceutical industry which lacked resources to follow up their investigations. The CCNSC rapidly developed into a large highly targeted drug development programme covering all areas of drug development from acquisition of substance of plant materials to clinical trials. Since there was not enough information available for extensive rational drug design based on biology and chemistry of tumor cells, the CCNSC decided to follow an empirical approach based on screening large number of materials.[42] It was soon realised that natural products were an excellent source of complex chemicals with a wide variety of biological activities. A number of animal extracts, mainly of marine origin, were tested.[42] The status of plant and animal extracts by the NCI till January 1981 was as follows: total plant extracts screened 1,14,045 and activity confirmed 4,897 (4.3%) which include 1,551 genera and 3,394 species. Total animal extracts screened, 16,196 activity confirmed 660 (4.1%) which include 413 genera and 561 species. The decision by the CCNSC to include natural products extracts proved to be very wise. Many active compounds with unusual structure were found active and some of them entered in clinical trials. This opened up new areas for investigation of related compounds as anticancer agents, and also provided new biochemical tools for cell biology. The inclusion of natural products of plant, animal, and microbial origin in the screening programme resulted in the discovery of different substances from each type of natural product. Although many compounds of both natural and synthetic origin were discovered which had good activity in the experimental models, only small number proved useful in the clinic. Hence, there is continuing need for searching active compounds with novel structures.

4.1 Selection of Materials

At the NCI in the early years pure natural products or species of novel botanical or zoological groups were selected for screening. The collection of plants relied heavily on taxonomy, in both the selection of plants for collection, screening and identification of active compounds in the crude extract. An example of the advantages of the use of toxonomy in identifying known active compounds are as follows: a collection of *Merrilliodendron megacarpum* (Icacinaceae) showed high activity against the P 388 leukemia, and a literature survey revealed that the plant has not been chemically investigated. The only

highly active compound previously isolated from Icacinaceae was camptothecin which was isolated from *Nothapodytes foetida* (syn. *Mappia foetida*). Comparison of the crude extract of *M. megacarpum* with a sample of comptothecin led to tentative identification of camptothecin in the crude extract of the plant. The identity of the compound was finally confirmed by isolation, thereby saving considerable time and expense over bioassay directed isolation.

The screening programme of the CCNSC since its inception was based on 'random' screening of plants, that is, plants that have no reason for being screened other than novelty. There was fairly extensive literature on folklore and traditional medicine in the treatment of cancer.[43] Spjut et al[44] did a retrospective correlation of several groups of medicinal and poisonous plants with the NCI screening data and found substantially higher activity for plants used as anthelmintics, arrow poisons and fish poisons when compared with plants selected randomly. Further examination of the data indicated that the highest correlation was between poisonous or toxic plants and *in vitro* activity (cell toxicity), and that the correlation between these plants and activity *in vivo* was not high. It was not surprising since poisonous plants are toxic to cells in culture.

4.2 *In vitro* and *in vivo* Activity

There is a great deal of loose usage of terminology with reference to *in vitro* and *in vivo* anticancer activity as pointed out by Suffness et al.[42] Many compounds are cited as anticancer or antitumour agents, which are, in fact, only cytotoxic to tumour cells *in vitro*. This crops of compounds contains a wide variety of toxic substances which display no particular selectivity towards tumor cells as opposed to normal cells and have no hope of being useful anticancer agents. Suffness et al[42] has suggested the following terminology to eliminate the confusion. The term cytotoxicity means toxicity to tumour cell in culture. The term anticancer, antitumour or antineoplastic, therefore, should not be used to express *in vitro* testing results. For *in vivo* activity in experimental system, it is suggested to use the term antitumour or antineoplastic in reporting results.[42] Besides, the term anticancer should be reserved for reporting clinical trials data in man.

4.3 Screening Methods

The quality of the screening methodology employed is the most important factor in drug discovery for any type of biological activity. The predictability of the screens for clinical activity is absolutely critical, since if the screens identify compounds which are clinically inactive, all the efforts which help the development of such compounds are wasted. The correlation of screening 'actives' with clinical activity is an extremely difficult process since it takes a number of years from when active compounds are tested until results of clinical trials are available, and the data base is very small since few compounds ultimately reach clinical trials.

New screens are usually validated by testing a wide variety of agents which have been under clinical trials and comparing the detection of known clinically active compounds (true positives) with the detection of compounds which show activity in experimental screens, but not in man (false positives) and compounds which are clinically active but undetected in experimental screens (false negatives). The data thus, obtained can be used to judge the quality of the new screen.

4.4 Screening Problems

There are special problems encountered in screening extracts of plants and animals. Generally, the active principle present in the crude extracts are present in very low concentration, therefore, the screen for their detection should be very sensitive, in general, *in vitro* test methods are more sensitive than *in vivo* method. Besides, screen should be selective, and specific. The methodology must be adaptable to materials which are highly coloured, tarry, poorly soluble in water and chemically complex. The second problem in screening crude extracts is that the pre-screen, screen or bioassay used must be insensitive to the inactive compounds which are potential interfering substances. The assay must also be insensitive to ubiquitous compounds which will give false actives. The NCI dropped the Walker 256 model while screening extracts of plants since it was highly sensitive to tannins. The assay also must be highly selective. The assay chosen must still meet the other requirements of any good assay, including validity, predictability, correlation, reproducibility, and the cost should be reasonable.

4.5 Current Approach and Status

The current approach at the NCI for screening crude extracts is to use a primary *in vivo* assay, the P 388 leukemia, and to use *in vitro* systems as bioassay to guide isolation of *in vivo* active compounds. The advantages of a battery of pre-screens are that (i) there is a greater chance to detect novel compounds; (ii) compounds with differing mechanisms of action can be selected and (iii) identification of known compounds is simplified since only certain classes of compounds will show activity in particular pre-screens. The biologically active compounds are generally present in low concentrations (1 to 100 mg per kg of dried plant), and the chances of finding these compounds by the standard phytochemical method of purifying and characterising the most abundant compounds present in an active extract are quite rare. The bioassay guided isolation method is essential in these cases. The major bioassays currently in use in the NCI programme are the KB and P 388 cell lines. While these *in vitro* systems are excellent bioassays, they are poor screens because of their sensitivity to cytotoxic substances which are devoid of *in vivo* activity. All novel compounds which are reproducibly active in the P 388 leukemia pre-screen then put to tumour panel testing. There are eight systems in the panel; of which five are mouse tumour lines and three human

tumour lines carried in athymic mice. There are two activity levels for each tumour in the panel, a level of statistically significant activity (minimal activity) and a level of biologically important activity. The NCI screens about 10,000 new synthetic compounds, and 400 pure natural products per year, and about 14,000 crude extracts (8,000 fermentation, 5,000 plant and 1,000 marine animal). From 8 to 12 compounds that pass Decision Network 2A as pre-clinical candidates, about 6 to 8 compounds enter clinical trials each year of which slightly less than half are natural products. Didemnin-B, a marine natural product, is one of the several natural products that are in clinical trials.[45] Didemnin-B was isolated by Rinehart's at the University of Illinois.[46] It is an extremely potent agent which exhibited activity against the B 16 melanoma system in μg/kg dose, and has also shown a broad spectrum of activity against stem cells in culture. A closely related compound, didemnin-A, is of interest because of its antiviral activity. Several other peptides have been isolated from marine organisms[47,48] and these also have shown antitumor activity. There are several non-peptidyl compounds isolated from marine organisms which show high order of activity in P 388 leukemia and which appear to be likely prospects for further development. It is expected that the NCI programme will continue to discover novel compounds of natural origin with antitumour activity. It is hoped that these new compounds will ultimately become clinically useful in cancer treatment as well as being novel biochemical probes for the study of tumour cell biology.

5. Testing Methods

There are a number of methods that are used for evaluating the biological activity of a pure compound or of crude extracts of marine organisms. The details of some of these methods are described in a series of papers.[11–24]

6. Toxicity Evaluation

Toxicity of the test substance is determined by the oral or intraperitoneal route in two or three adult albino mice of either sex and usually 15 to 20 g in weight. The test material is suspended in 0.1% agar or in 10% gum acacia in distilled water. Concentrations are so adjusted that a 20 g mouse receives a volume of 0.2 ml. The initial dose is at a level of 400 or 500 mg/kg going up or down by a factor of 2. Occasionally, an interval of 1.5 is used for closer approximation. Doses higher than 1000 mg/kg are not generally used. Control animals are administered only the vehicle. The animals are observed for 5 to 6 hours after dosage for toxic symptoms. If death occurs during this time, the cause of dealth is recorded. The approximate LD_{50} is estimated and the maximum tolerated dose is also recorded for use in subsequent investigations.

6.1 Regulatory Toxicity

Regulatory toxicity studies of the test substance are carried out only after the

pharmacological activity is confirmed, and baseline data is generated regarding the effective dose, lethal dose and maximum tolerated dose. These studies are carried out with the potential drug before it is passed on to clinicians to Phase I and II clinical trials. The studies needed are : (i) acute toxicity; (ii) repeated dose study; (iii) subacute toxicity study; (iv) reproductive studies, (v) teratological study; (vi) prenatal and post natal study; (vii) carcinogenic study and (viii) mutagenic study.

6.2 Reproductive Studies

Reproductive studies important for clinical Phase II trials are : (i) study of fertility and general reproductive performance; (ii) teratological study, and (iii) prenatal and postnatal studies. The study is needed to determine the effect of a given drug on gonadal function, estrus cycles, mating behaviour, conception rates and early stages of gestation. The observations recorded are: number and distribution of embryos in each uterine horn, presence of empty implantation sites, embryo undergoing resorptions, abnormal condition in the uterus, gestation period, litter size, stillborn, litter weight, gross anomalies.

6.3 Teratological Study

Teratological study of test substance is needed to determine the potential of a test substance for embryotoxicity and/or teratogenic effects. The observation recorded are : number of corpora lutea in each ovary, number of implantation in each horn, correlation of the foetal placement in each horn with the number of corpora lutea in each ovary, number of foetuses, number of live foetuses, number of dead foetuses, number of resorptions, early/late, weight of foetus, external anomaly in the foetus, and visceral/skeletal anomaly in the foetus.

6.4 Pre- and Postnatal Study

Pre- and postnatal study is needed to observe the effects of test substance on the newborn when administered to the mother during the last trimester of pregnancy and through the period of lactation. The observation recorded are: duration of gestation, type of labour and delivery, litter size, litter weight at birth day four, day 21 and at weaning, effect on lactation, litter morbidity and mortality.

6.5 Carcinogenic Study

Carcinogenic study is needed to determine the carcinogenicity of the test agent in both sexes of two species and is designed to cover the greater part of the animal's life span.

6.6 Mutagenic Study

A variety of short-term tests are conducted on test substance to predict the mutagenic as well as carcinogenic activity of chemicals. The test carried out are: (i) DNA damage tests in bacteria, (ii) gene mutation tests in bacteria,

(iii) chromosomal aberration tests *in vitro*, (iv) micronucleus test in mice, and (v) dominant lethal tests in rodents.

7. Use of Animals in Experiments

Use of animals in experimental work has been in practice for long, but in the recent past this has become a major consideration all over the world to create alternatives to the use of laboratory animals. Although animal models are not the end point in predicting toxic hazards for man, there is yet little evidence that it will be possible in the near future to dispense entirely with animal testing. However, it is possible to reduce their number by better experimental design and analysis, better use of available data on the toxicity of previously investigated compounds. Efforts are continuing to replace animal experiments by other methods. Alternations to animal research can be divided into three categories; (i) modified animal use; (ii) use of living systems, and (iii) use of non-living systems. *In vivo* studies can be modified by combining them with some *in vitro* methods. However, there are several lacunae in *in vitro* test methods, such as physiological dissimilarity, interpretation of data and acceptance by regulatory agencies.

8. Clinical Trials

Clinical trials of new drug candidates for safety and efficacy evaluation are mandatory before a drug candidate is cleared for marketing. However, the need to carry out clinical trials does not justify experimentation in man, without a number of conditions being fulfilled, aimed at making their study in man as safe as possible. The candidate drugs are approved for clinical practice, after they have been evaluated in different phases of clinical trials. Clinical development of a new drug progresses from phase I, performed on healthy human volunteers, to studies in patients during phase II which is further expanded into multicentric phase III trials. Finally, if necessary, field studies are also undertaken to have large number of patients treated in life situation. Even after marketing the performance of a drug has to be followed in post-marketing surveillance for efficacy and rare adverse drug reactions. Thus, by following the phased approach for clinical trials, minimum number of human subjects are exposed to candidate drug, and adequate data can be generated with minimal risk.

In phase I, clinical trials the tolerability of the test compound in different doses in healthy volunteers is first assayed. As soon the dose reaches to the range of the anticipated therapeutic dose, blood of the volunteer is withdrawn and the level of the drug in the blood is estimated, with these data the half-life of the compound can be calculated. In order to get more insight into kinetics and metabolism in human beings, experiments with radiolabelled compounds are performed. For these studies, safety experiments in animals, particularly its distribution in organs, are undertaken with radio label test compound. Side effects during the phase I studies are carefully monitored. If

possible, dynamic studies are performed. For example, influence on blood pressure in healthy volunteers after application of an antihypertensive drug. In several cases, the therapeutic effects can not be measured in healthy volunteers. The therapeutic effects of the test drug are carefully monitored in phase II studies in patients. Usually the toxicological data, available at this time, include one or three month toxicity data in two species. In order to extend the studies in patients beyond four weeks, long-term toxicity studies including histopathology, are done in two animal species. Additional toxicity studies are carried out during this period, such as nephrotoxicity, teratology, antigenicity and mutagenicity. At the same time, kinetic and metabolic studies in animals are continued in order to identify the metabolites of the test compound, both qualitatively and quantitatively.

Phase II clinical trials are only started when data on human volunteers on tolerability, kinetics and metabolism and also its therapeutic efficacy are available. Phase III cinical trials include dose-finding studies in order to achieve dose-response curves and very carefully planned double-blind studies against placebo or a standard drug. Furthermore, the dosage regimen is estabished, including food interaction studies. During this period additional paraclinical studies, such as, chronic toxicity in two, eventually three animal species, carcinogenicity in two animal species and further studies on teratology and fertility are conducted. If the results of these studies are positive, clinical trials are expanded into phase III. In phase III clinical trials, data on several thousands patients in various well-defined indications are collected. Studies in patients with impaired renal and hepatic function are performed as well as interaction studies with other drugs. The side effects of the compound are carefully monitored at every stage. During this period the long term toxicity and carcinogenicity including histopathology studies are continued. Only after very careful evauation of the analytical expert report indicating the quality requirement of the new drug, the pharmacological, toxicological expert report indicating the benefit/risk ratio from the experimental point of view and the clincial expert report indicating the benefit/risk ratio from the clinical point of view, the data are submitted to the health authorities, for example, the Drug Controller in India and to the FDA in the USA.

It is unethical to carry out any experiment in human subjects without their written information consent. Any attempt to violate the WHO guidelines for experimentation on humans may lead to risks which may end up in medicolegal situation involving the investigators. Therefore, it has been emphasised that no investigator should involve a human being as a subject in research unless the investigator has obtained legally effective written informed consent of the subject or his/her legally authorised representative. The information that is given to the subject shall be in the language understandable to the subject. It is required that an investigator makes certain that the subject ready to participate in the clinical trial understands the nature of the risk and benefits that may occur from the treatment offered, and willingly gives his/her informed

consent signing a consent form. This implies that the subject entering the trial is an adult and is capable of understanding what is told to him/her.

8.1 Clinical Trials Protocol

It is essential to design a well-defined protocol which should provide a written description of all issues related to the conduct of a clinical trial. The protocol should clearly define aim of the study, clinical and laboratory parameters required to characterise the status (health or disease) or clinical trial subjects who should be included in the study only after the execution of an informed consent and satisfying inclusion and exclusion criteria. The subjects should be advised to avoid taking drugs for atleast two weeks prior to entering into the trial to eliminate possible drug induced effect on hepatic enzyme system. These volunteers should be recommended on overnight fast prior to dosing and should not eat until a standard meal is provided to them two to four hr post-dosing. Clinical trial methodology should clearly state the assignment of subjects to treatment, personal interviews and examination, method of assessment during follow up, blood sampling schedule, procedure and conditions for storage of plasma-serum samples. The sensitivity, reproducibility and specificity of the technique recommended for drug level determination and method for analysis of time concentration data should also be contained in the protocol. In trial experiments, the patients are randomised in order to overcome bias and near equal distribution of various parameters. Randomization is justified when the treatments compared are nearly similar according to available state of knowledge. Double-blind technique is used to overcome physician and patient bias. Sometimes a ‘single-blind’ technique is also used. Use of placebo has generated a lot of discussion, because an inert placebo (dummy tablet) will deprive the patient of treatment. Use of placebo would be objectionable if the placebo is pharmacologically inert; and deprives the patient of necessary treatment. But even in some severe illnesses where there are no effective drug or the available drug have serious hazards, use of placebo could be debatable. Sometimes an inert placebo produces marked relief, as patient believes that he/she is getting new effective treatment. Not informing the patient about placebo is a medical lie and is not acceptable. But if the patient is informed that there is possibility that he may get placebo treatment, and if this is accepted, then there should be no objection to the use of placebo. It is said that deception declared is acceptable, but deception concealed is unethical. In evaluating anagesic, use of placebo in a double-blind treatment could be used, if provision is made for giving additional known analgesic drug ‘on demand’ for patient whose pain becomes severe.

8.2 Duplicating Trials

If one drug in a comparative clinical trial has shown significantly better results, both in effectiveness and safety over other drug, administering the

less effective drug to some patients, in another comparative trial, with same two drugs raises ethical problems. However, if doubts arise about the validity of first trial, about methodology, analysis of results, or professional integrity, then duplicting the trial is necessary as well as ethical. While conducting this duplicating trial, participating patients have to be informed about the background and some of them getting the so called łess effective' treatment. A protocol designed, usually a clinical pharmacologist, should always consider the regulatory requirement, ethical issues and the human and financial resources, needed from the management of the sponsoring agency while drafting a protocol, leading to proper volunteer/patient selection for clinical trials and would enable the investigator to generate valuable scientific data.

8.3 Ethical Considerations

The ethical conduct of research involving human subjects requires a balancing of society's interest in developing beneficial medical knowledge and in protecting human subjects in research. However, investigators and researchers can not achieve the balance all by themselves. There is, thus, a need of a committee involving outside people at institutional and also outside the institutional level. Various funding agencies have now introduced the practice of getting documentary evidence of clearance from institutional ethics committee along with the research project proposals involving human subjects. With increasing consciousness in the society, there is now much concern about safety in human experimentation. The World Health Organisation (WHO) has given serious attention to it and given guidelines for the consideration of the clinical investigators to protect them against the risk due to ethical issues. In addition to the WHO guidelines, it is still safer to have an independent, competent ethics committee at the institutional level which must scrutinise each project and give their approval for conducting the trial. Ideally, an ethics committee should consist of about five persons. At least one professional member should be an outsider. Either a lawyer or social worker may represent the society. Composition of ethics committee varies from country to country, but it should be competent and independent.

Aplidin (APL) a novel depsipeptide from the tunicate *Aplidium albicans* exhibiting high order of cytotoxic activity was under clinical studies. A sensitive and highly specific liquid chromatographic method with electrospray ionisation tendem mass spectrometric detection (LC-ESI-MS/MS) was described for its estimation in human plasma, blood and urine.[49] Phase II study of LV 103793 (dolastatin analogue) in patients with metastatic breast cancer was carried out. LU 103793 is a synthetic analogue of dolastatin 15 and inhibits tubulin polymerisation. The patients were given a dose of 2.5 mg/m^2/day of the test compound over 5 min for 5 consecutive days every 3 weeks. The results did not support the further evaluation of LU 103793 in metastatic breast cancer patients using this dose and schedule.[50] Human immunodeficiency virus (HIV) replication requires integration of viral cDNA

into the host genome, a process mediated by the viral enzyme integrase. A new series of HIV integrase inhibitors, thalassiolins A-C were isolated from the caribbean sea grass *Thalassia testudium*. Thalassiolin A, the most active of these molecules displayed *in vitro* inhibition of the integrase catalyzed strand transfer reaction (IC_{50} = 0.4 μM) and an antiviral IC_{50} of 30 μM.[51] A rapid and sensitive assay for paralytic shellfish poison (PSP) toxins using mouse brain synaptoneurosomes was developed.[52] The assay relied on the ability of PSP toxins to block veratriodine induced depolarization of synaptonerosomes. The adrenergic and cholinergic activities contribute to the cardiovascular effects of lion fish *Pterois volitans.*[53]*Laminaria digitata*, a marine kelp is used in tents' for carvical dilation. *Laminaria* hypersensitivity reaction, including anaphylaxis, are exceedingly rare. However, a patient developed anaphylaxis after insertion of a laminaria tent.[54] *In vivo* and *in vitro* studies provided strong evidence of osteogenic activity of nacre obtained from bivalve mullusk *Pinctada maxima* in three mammallian cell types: fibroblasts, bone marrow stromall cells and oesteoblasts.[55] Calcium dependent smooth muscle excitatory effect was elicited by the venom of the hydrocoral *Millepora complanata*.[56] The venom of *Conus geographus* converted angiotensin II into angiotensin.[57] The constitutive androstane receptor cDNA in northern fur seal *Callorhinus ursinus* were identified.[58] A reversed-phase high-performance liquid chromatographic method was developed and validated for quantification of the novel anticancer drug ecteinascidin 743 in human plasma.[59] It was a potent drug against human ovarian carcinoma.[60]

9. Concluding Remarks

There are special problems encountered in biological screening of extracts of marine plants and animals. Generally, the active principles in the crude extract are present in a very low concentration (1:1000), therefore, the test system for their detection should be very sensitive. The screening should also be selective and specific. The methodology must be adaptable to materials which are highly coloured, tarry, poorly soluble in water and chemically complex. The assay must be insensitive to ubiquitous compounds. The candidate drugs are approved for clinical practice after they have been evaluated in different phases of clinical trials. Field studies are undertaken, if needed, in large number of patients. The performance of the new drug is also followed in post marketing surveillance for efficacy and adverse drug reaction. National Cancer Institute (NCI), Bethesda, U.S.A., has developed a highly targetted drug development programme covering all areas of drug development. It is expected that NCI programme will discover novel compounds of natural origin with antitumour activity. It is hoped that these compounds will ultimately become clinically useful drug in cancer treatment, as well as novel biochemical probes for the study of tumour cell biology.

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5

Bioactivity of Marine Organisms

Abstract

The chapter deals with bioactivity of marine bacteria, fungi, phytoplanktons, seaweeds and marine animals. The antimicrobial activity of bacteria, the effects of toxins and other bioactive substances produced by bacteria, fungi, dinoflagellates, seaweeds, and animals have been discussed. Besides, antibacterial, antifungal, antiprotozoal, antifertility, antiviral, anticancer, and a wide range of pharmacological, hypoglycaemic, antiimplantation and antimalarial activities of the extracts of marine red, green and brown algae, sea grasses and the marine species of phylum Coelenterata, Echinodermata, Porifera, Mollusca and Arthropoda have been reviewed.

1. Introduction

Marine organisms exhibit a wide range of biological activity.[1-12] Antifertility,[13] antiviral,[14] antibiotic,[15] antifungal and antimicrobial activities of marine organisms have been reported.[16-24] The extracts of seaweeds also have nutritional value.[25] Besides a few growth stimulant properties which may be useful in studies on wound healing and carcinogenic properties in the study of cancers have been mentioned. Many toxins involved in highly fatal poisoning, such as paralytic shellfish poisoning (PSP) are produced by a number of dinoflagellates.[26] Ciguatera, a seafood poisoning, is caused by ingestion of coral reef fish that have become toxic through diet. It is now established that the toxins are produced by the epiphytic dinoflagellate and transferred to herbivorous fish and subsequently, to carnivores through the food chain.The extracts of marine tunicates exhibit high order of antitumour, antiviral and immunosuppressive activities.[27]

2. Bacteria and Fungi

Several marine bacteria exhibit antibiotic activity. Among the many marine bacteria showing antimicrobial activity,[17,28] a variant of *Pseudomonas piscicida*[28] showed marked antagonism to various microorganisms. A red coloured bacterium[29] from Puerto Rico is found to excrete into sea water medium antibacterial substances.

The bacteria and fungi from sea are also reported to produce substances, which affect central nervous system, respiratory system, neuromuscular system, cardiovascular system, and gastrointestinal system. Some of the substances are known to produce local effects, such as pain, necrosis, edema, parasthesias, prurities etc.

Several marine bacteria produce toxins. Tetrodotoxin, one of the best known marine toxin, is frequently involved in fatal food poisoning. The toxin has specific action of blocking sodium channels of excitable membranes. It has now been traced that the primary source of the toxin is a symbiotic bacterium.[30] Saxitoxin and its derivatives are known for their involvement in highly fatal poisoning, called paralytic shellfish poisoning (PSP). The origin of saxitoxin in PSP is suggested to be bacterial.[31] However, the claim has been made on rather poor evidence and needs further confirmation.

3. Phytoplanktons

The little plants of the sea along with their benthic relatives constitute the primary producers of food to support life in the sea. Sometimes the marine environment becomes so favourable for their growth that they cause 'tide'. Along the Florida coast, the dinoflagellate *Gymnodinium breve* (*Ptychodiscus brevis*) often forms blooms, leading to mass mortality of fish. Large blooms of this organism (red tide) can kill hundreds of tons of fish a day. The blooms sometimes cause human irritation of eyes and throat in the coastal area.[32] The contamination of shellfish occasionally results in human poisoning. The toxic principle isolated from the dinoflagellate is called brevetoxin-B.[33] It is the most potent ichthyotoxin among the toxins produced by *G. breve*. Several dinoflagellates of the genus *Dinophysis* are the causative organisms of diarrhetic shellfish poisoning (DSP). Appearance of the dinoflagellates, even at a low density, leads to toxification of the shellfish. DSP has a wide distribution world over. The poisoning is a serious problem both to public health and to the shellfish industry. The prominent human symptoms are gastrointestinal disorders, such as diarrhoea, nausea, vomiting and abdominal pain. The dinoflagellates *Prorocentrum* and *Dinophysis* have yielded the toxins okadaic acid and its analogs. Okadaic acid is reported to be a non phorbol ester type cancer promoter. It inhibits dephosphorylation of proteins. The epiphytic dinoflagellate *Gambierdiscus toxicus* produces ciguatoxin, its congeners and maitotoxin. Ciguatoxin is regarded as the principle toxin responsible for human illness. The clinical symptoms are diverse. Neurological disturbances are prominent, reversal of thermal sensation, called 'dry ice

sensation." Other illnesses include joint pain, miosis, erathism, cyanosis and prostration. Gastrointestinal disorders are nausea, vomiting, and diarrhoea. Cardiovascular disturbances are low blood pressure and bradycardia. Maitotoxin presumably plays a role in diversifying ciguatera symptoms, particularly poisoning caused by hervivorous fish. It is, perhaps, the most potent non-proteinaceous toxin.

Gambierol is another toxic constituent produced by dinoflagellate *G. toxicus*. Its symptoms resemble those shown by ciguratoxins. From the culture medium of *G. toxicus* potent antifungal agents, gambieric acids A-D have been isolated.[34,35] The antifungal potency of gambieric acid-A exceeds that of amphotericin-B by a factor of 2000. Thus, it is the most potent antifungal agent known.

Three groups of macrolides named amphidinolides have been isolated from the dinoflagellate *Amphidinium* sp. that is symbiotic to the flatworm. Some of these macrolides exhibit extremely potent cytotoxicity against L1210, 100 pg/mL. The dinoflagellate *Amphidinium klebii* yielded amphidinol which shows potent antifungal and hemolytic activity. Goniodomin-A has been isolated from the tide pool of dinoflagellate *Gonyaudoma pseudogonyaulax*, as an antifungal agent.

Dinoflagellates, such as *Goniodoma,* produce antimicrobial substances which inhibit growth of certain kinds of bacteria and fungi. Anticoliform activity of sea water in the Rhode Island has been traced to its source in blooms of the diatom *Skeletonema costatum*.[36] There are many reports dealing with pharmacological activities of diflagellates and diatoms.[37] The blue algae, besides having antimicrobial and antibiotic properties,[38] are implicated in outbreaks of dermatitis among swimmers and cause toxicity both in fish and mammals.

4. Bioactivity of Marine Organisms

4.1 Seaweeds

The green, brown and red algae have been extensively screened for antibacterial and antifungal activity.[1] The active principles isolated from *Symphycladia gracillis, Rhodomela larix* and *Polysiphonia lanosa*[39-41] are 2,3-dibromobenzyl alcohol, 4,5-disulphate dipotassium salt, 2,3-dibromo-4,5-dihydroxybenzaldehyde, 2,3-dibromo-4,5-dihydroxybenzyl alcohol, 3,5-dibromo-*p*-hydroxybenzyl alcohol, and 5-bromo-3,4-dihydroxybenzaldehyde. Virtually nothing is known about the physiological importance of these bromophenols. However, their antialgal activity suggests that they may play a role in the regulation of epiphytes and endophytes.

4.2 Seaweeds of Indian Coasts

Several seaweeds have shown a wide range of bioactive properties.[1,42-49] Hemolytic and antimicrobial activities are reported in seaweeds collected

from Mandapam, Tamil Nadu, the Gulf of Manner and Back Bay, India.[50] The extracts of seaweeds, in general, are found active against Gram-negative microbes. The extract of *Enteromorpha compressa, Cladophoropsis zollinger, Padina gymnospora, Sargassum wightii* and *Gracilaria corticata* are found active against the Gram-positive cultures of *Bacillus*. High order of hemolytic activity is shown by the extracts of *Cladophoropsis zoolingeri* and *Grateloupia lithophila*.

Extracts of 25 seaweeds from the Indian coast have been put through a broad biological screen which includes tests for antiviral, antibacterial, antifungal, antiprotozoal, antifertility activities, and a wide range of pharmacological activities. Significant activity is found in 13 seaweeds, the most promising activity being 100% antifertility (antiimplantation) activity observed in three species.[51] The seaweeds which showed biological activities are: *Caulerpa racemosa* (Chlorophyceae) collected from Malvan in November exhibited hypotensive activity (90 mm, 5 mg/kg), LD_{50} 93.75 (mg/kg in mice), *Cladophora pinnulata* (Chlorophyceae) from Baga, Goa, in December was found toxic, LD_{50} 8.25 (mg/kg, in mice). *Codium elongatum* (Chlorophyceae) collected from Cabo de Rama, Goa, India, in February showed antiviral activity (50%, 1 mg), LD_{50} 681 (mg/kg, in mice). *Enteromorpha* spp. (Chlorophyceae) collected from Cabo de Rama, Goa, India, Showed diuretic activity (112%, 170 mg/kg), LD_{50} 681 (mg/kg in mice). The other green algae, *Caulerpa surtularioides* collected from Malvan, Maharashtra in November, *Chaetomorpha media* from Vengurla, Maharashtra in May; *Enteromorpha intestinalis* from Chaopora, Goa, in February and *Ulva fasciata* from Baga, Goa in December were found devoid of activity. The alga *Trichodesmium erythraeum* (Cyanophyta) collected from Mandovi, Goa, in March exhibited diuretic activity (104%, 170 mg/kg), LD_{50} 681 (mg/kg, in mice), whereas the alga *Trichodesmium erythraeum* (Cyanophyta) from Aguada, Goa, in April was found devoid of activity. The brown algae *Stoechospermum marginatum* collected from Dona Paula, Goa in December showed spasmolytic activity (50%, 50 µg/ml), LD_{50} more than 1000 (mg/kg, in mice), *Padina tetrastromatica* collected from Baga, Goa, in January exhibited spasmogenic activity (50 µg/ml) and antifertility activity (100%, 200 mg/kg), LD_{50} 464 (mg/kg, in mice); *Sargassum tenerrimum* from Baga, Goa, in December showed CNS depressant activity (89 mg/kg), LD_{50} 178 (mg/kg in mice). The brown algae *Chnoospora implexa* collected from Mandapam, Tamilnadu, in March, *Dictyota bartayresiana* from Anjuna, Goa, in January and *Spatoglossum asperum* from Baga, Goa, in December did not show any activity.

The red algae which exhibited activity are: *Acantophora specifera* from Baga, Goa, in February showed antifertility activity (100%, 200 mg/kg in mice), LD_{50} more than 1000 (mg/kg, in mice), *Chondria armata* from Baga, Goa, in December exhibited hypotensive activity (40 mm, 1 mg/kg, 200 min) LD_{50} 178 (mg/kg, in mice); *Corallina* spp. from Anjuna, Goa, in January

showed spasmogenic and oxytocic activities (50 µg/ml), LD_{50} 681 (mg/kg, in mice) and *Hypnea musciformis* from Baga, Goa, in December showed diuretic activity (130%, 250 mg/kg), LD_{50} more than 1000 (mg/kg, in mice), *Gelidiella acerosa* from Eravali, Tamilnadu, in December showed antifertility activity (100%, 100 mg/kg, in mice). The red algae *Gelidiella* spp. collected from Baga, Goa, in November, *Gracilaria corticata* from Baga, Goa, in November and *Hypnea musciformis* var. *cervicornis* from Malvan, Maharashtra in November did not show any activity. The sea grasses *Diplanthera universe* collected from Mandapam, Tamilnadu, in March and *Thalassia hemprichii* from Mandapam, Tamilnadu, in March did not show any activity.

Several members of the red, brown and green algae, which were screened, are reported to show a broad spectrum of biological activities. Some of the species that were examined were also tested earlier by other workers, but none of the activities were observed. For instance, extracts of *Chondrus crispus* and *Gelidium cartilagineum* have been found to be active against influenza B and mumps virus. This activity has been attributed to the presence of galactan unit in the polysaccharides, agar and carrageenan present in both the species. Based on this, one may expect *Hypnea musciformis, Gelidiella* spp. and *Gracilaria corticata* which are well-known sources of these polysaccharides to exhibit similar activity. Unfortunately, the extracts of these algae have not been tested against the above virus. However, they have been found inactive against *Ranikhet disease* virus, *Vaccinia* virus and *Semiliki forest* virus. The absence of the antiviral activity may be attributed to the varying solubility behaviour of the sulphated polysaccharide, responsible for the activity in aqueous ethanolic solutions or to the well known variation in phytoconstituents depending upon the ecological factors prevailing at the time of collection and upon the growth stage of the plant. The last reason could also be applicable to the differences in activity of two different samples of *Enteromorpha* spp. and *Hypnea musciformis* collected at different places. The extract of *Sargassum tenessimum* was not found to have any chemotherapeutic activity though the species of this genus have been reported to possess antibiotic effects due to the presence of antibiotic substances sarganin-A and sarganin-B. This activity, however, seems to be seasonal, and has been reported to decrease directly with plant vigour as a function of latitude.[52]

Acute poisoning from seaweeds has not been reported in the literature, but it has been mentioned that *Caulerpa*, one of the most popular edible algae in the Philippines is considered toxic during the rainy season. This toxicity has been attributed to the presence of caulerpicin which has been found to have neurotropic effects.[3] Unfortunately, the test for this activity has not been performed for the extract from *Caulerpa racemosa* which was collected during the pre-monsoon season. Since caulerpin is not reported to possess hypotensive activity, the effect observed in this species seems to be due to some other constituent. This inference is further supported by the

absence of similar activity in *Caulerpa sertularioides* considering that caulerpicin is a common constituent of both the species.

The most promising activity is, perhaps, the antiimplantation activity found in three species, viz. *Padina tetrastromatica*, *Acantophora specifera* and *Gelidiella acerosa*. Preliminary experiments conducted on mice have shown that these extracts are 100% active. The occurrence of such activity in seaweeds has not been reported hitherto in the literature.

Palytoxin, the toxin principle of the soft coral *Palythoa toxica*,[53] has also been found in seaweed *Chondria armata*.[26] Extensive pharmacological/ biochemical studies have been carried out on palytoxin, such as membrane depolarisation, Na^+ or Ca^{2+} influx, stimulation of arachidonic acid release, stimulation of neurotransmitter release, inhibition of Na^+/K^+-ATPase induction of contraction of smooth muscle and tumor-promising, while it is believed that palytoxin acts through Na^+/K^+-ATPase. However, detailed mechanism of its action is still unknown.[26]

Microalgae are frequently involved in various forms of seafood poisoning. However, poisoning due to macroalgae are rare. Human intoxication due to ingestion of the red alga *Polycavernosa tsudai (*formerly *Gracilaria edulis*) occurred in Guam in 1991. Thirteen people became ill, three of whom died.[26] A novel glycosidic macrolide, polycavernoside A[54-58] has been isolated from the red alga, and is believed to be responsible for the poisoning. The content of polycavernoside A in the alga was low, but it caused in mice symptoms comparable with those observed in human patients. The sudden and transient occurrence of polycavernoside A in the alga remains unexplained. Previous outbreaks of fatal poisoning caused by *G. chorda* and *G. verrucosa* also remain unexplained as to the nature of the toxin(s). The red alga *Chondria armata* is a folk medicine used as an anthlmintic. Two palytoxin analogs, domoic acid and its seven derivatives are isolated from the alga.[59-64] No incidence of poisoning due to this alga is known. However, domoic acid, produced by the diatoms *Nitzschia pungens* and *Pseudonitzschia australis,* has caused fatal food poisonings, after accumulating in shellfish. This recently described poisoning is termed *amnesic shellfish poisoning*. A related neurotoxic amino acid, kainic acid, also occurs in red alga but with no intoxication episode. Debromoaplysiatoxin has been isolated from the blue green alga *Lyngya majuscula* as a potent inflammatory agent, and is responsible for incidents of severe dermatitis among swimmers who have come in contact with the alga in Hawaii and Okinawa. Interestingly, debromoaplysiatoxin is reported to have tumour promoting activity which is identical to that shown by phorbol esters.[26]

Ethanolic extracts of the Indian marine algae have been tested for antiviral, antibacterial, antifungal, antifertility, hypoglycaemic and a wide range of pharmacological activities. Of 34 species screened 17 were found active. Six were diuretic, three hypoglycemic, three hypotensive, four exhibited antiimplantation activity. Cytokinin activity was observed in one, and one

extract showed adrenergic blocking action. The extracts neither had antibacterial, antifungal and antiviral activities nor were found toxic.[65] The green algae which exhibited activity are: *Caulerpa taxifolia* collected from Okha, Gujarat, in April showed hypotensive activity, LD_{50} 825 (mg/kg, in mice). *Halimeda macroloba* from Andaman in February showed diuretic and antiimplantation activities: *Monostroma* spp. from Goa exhibited antiimplantation activity, LD_{50} 681 (mg/kg, in mice); *Pseudobryopsis* spp. from Okha, Gujarat, showed hypoglycaemic activity, LD_{50} 681 (mg/kg, in mice); *Udotea indica* from Porbander, Gujarat, showed cytokinin activity, LD_{50} 1000 (mg/kg, in mice); *Dictyopteris australis*, the brown alga from Cabada, Gujarat exhibited diuretic and hypotensive activities, LD_{50} more than 1000 (kg/mg, in mice). The green algae *Acetabularia crenulata* from Andaman, *Chamaedoria auriculata* from Gujarat, *Ulva reticulata* from Andaman, *Valoniopsis* spp. from Tamilnadu and *Valoniopsis pachynema* from Gujarat did not show any activity.

The brown algae *Iyengaria stellata* from Gujarat showed adrenergic activity; *Padina gymnospora* from Gujarat exhibited hypoglycemic activity; LD_{50} 1000 (mg/kg, in mice); *Pocockiella variegata* from Tamilnadu showed the CNS stimulant activity, LD_{50} 1000 (mg/kg, in mice), *Sargassum cinereum*, Malvan, Maharasthra, also showed CNS stimulant activity and *Spatoglossum asperum* from Anjuna, Goa, exhibited antiimplantation and hypotensive activities, LD_{50} 1000 (mg/kg, in mice). The brown algae *Hydroclathrus clathratus* from Dwarka, Gujarat, *Sargassum wightii* from Gulf of Manner, Tamilnadu, *S. myriocystum* from the same locality and *Turbinaria decurrens* from Andaman did not show any activity. The red algae *Botryocladia leptopoda* from Gujarat exhibited antiimplantation and CNS stimulant activities, LD_{50} 825 (mg/kg, in mice); *Gracilaria* spp. collected from Andaman in February showed diuretic and hypotensive activities LD_{50} more than 1000 (mg/kg, in mice); *Laurencia papillosa* collected from Anjuna, Goa, exhibited diuretic activity, LD_{50} more than 1000 (mg/kg in mice); *Scinaria hatei* from Gujarat showed hypotensive activity, LD_{50} more than 1000 (mg/kg, in mice); *Solieria* spp. from Anjuna, Goa, showed diuretic and antiimplantation activities. LD_{50} 316 (mg/kg, in mice), and *Spyridia fusiformis* from Andaman showed diuretic and hypoglycemic activities LD_{50} 681 (mg/kg, in mice). The red alga *Chondrococus* spp. from Goa *Galaxaura oblongata* and *G. rogosa* from Andaman, *Gastroclonium iyengarii*, *Gracilaria edulis* and *Halymenia venusta* from Gujarat, *Jania adhaerens* from Mandapam, Tamilnadu and *Spyridia insignis* from Trivandrum, Kerala, did not show any activity.[65] Seaweeds extracts are known to exhibit inhibitory effects upon a number of Gram-positive and Gram-negative bacteria, but none of the extracts screened showed antibacterial activity. The most promising activity observed was the antiimplantation activity found in four species, viz. *Helimeda macroloba*, *Monostroma* spp., *Spatoglossum asperum* and *Botryoctadia leptopoda*.

Alcoholic extracts of 50 Indian marine algae collected from the coasts of Goa, Maharashtra, Tamilnadu, Gujarat, Lakshadweep, Orissa and the Andaman and Nicobar Islands during pre-monsoon and post-monsoon periods have been tested for a wide range of biological activities. Of these, two extracts exhibited antiamoebic and antiviral activity each three of them had antiimplantation activity, nine had hypoglycaemic activity while hypotensive activity was associated with 11 extracts, 14 extracts were found to be diuretic and one of them had antiinflammatory activity. Further, ten of these extracts exhibited two types of activities, while a combination of 3 and 4 types of activities was observed in one extract each.[66]

Caulerpa taxifolia collected from Porbunder, Gujarat, exhibited hypotensive and diuretic activities. Several species of *Caulerpa* have been investigated and bioactive principles, such as caulerpin and caulerpinin have been isolated from a number of them. Caulerpin produces mild anaesthetic action, difficulty in breathing, sedation and loss of balance. The toxic syndrome has been reported to be somewhat similar to that produced by ciguatera fish poisoning. The neurotropic activity of caulerpicin is thought to be of clinical value. Tropical green algae, and a few of their temperate relatives have yielded a number of bioactive metabolites and some of these are believed to be used by the algae as a chemical defence against herbivorous animals. The characteristic feature of these metabolites is the presence of a 1,4-diacetoxybutadiene moiety which has been found in more than half of the compounds reported so far. *Dictyota atomaria* collected from Okha, Gujarat, exhibited hypotensive activity. Several diterpenes have been isolated from *Dictyota* species. Of these, some are found to have cytotoxic activity. The sex attractant isolated from *Dictyota dichotoma* is n-butylcyclohepta-2,5-diene. Several highly unsaturated hydrocarbons have been isolated from marine algae, and the function of these hydrocarbons have been studied.[67] It has been found that the sperm cells aggregate around the female gametes of brown algae which exude C_{11} hydrocarbons that attract the former and cause them to remain in the excited state in the vicinity of the latter. Hydroxydictyodial isolated from *Dictyota spinulosa* inhibits feeding in omnivorous fish. *Enteromorpha intestinalis* collected from Chilka Lake, Orissa, exhibited antiamoebic, hypotensive and diuretic activities. No chemical investigation on this alga appears to have been carried out. It would be interesting to investigate this alga thoroughly. Alcoholic extracts of *Halimeda gracilis* collected from Lakshadweep, and *H. opunita* from the South Andaman Island, have shown diuretic and hypotensive activites. The alcoholic extract of *Halimeda tuna* from the South Andaman coast, exhibited only diuretic activity. The genus *Halimeda* has been found to contain highly active sesquiterpenes.[26] Some of the diterpenes from *Halimeda* species are reported to exhibit cytotoxic and antimicrobial properties. Extract of *Lyngbya confervoides* from Lakshadweep has shown hypoglycaemic activity, which has not been reported from any species of *Lyngbya.* Several bioactive compounds have been isolated from *Lyngbya*

majusculla[68] which causes swimmers itch. Of the compounds isolated from this species lyngbyotoxin and bromoaplysiatoxin are highly toxic. Alcoholic extracts of *Ulva faciata* and *Ulva lactua* from the Gujarat coast exhibited antiviral and antiimplantation activities, respectively. The most interesting finding is that the alcoholic extract of the roots of *Avicennia officinalis* from the South Andaman Island, exhibited antiamoebic, hypoglycaemic, hypotensive and antiinflammatory activities which need to be investigated thoroughly. *A. officinalis* leaves extract exhibited moderate diuretic activity which was localised in the glycosidic fraction. *Achrostichum aureum* (family Pteridaceae), a mangrove from Jhirkatang, South Andaman, exhibited antifertility activity in female rats. The water soluble fraction of the ethanolic extract of the mangrove prevented (100%) pregnancy when administered to female rats on 1-7 post coitum. This fraction was found devoid of both estrogenic and antiestrogenic activities.[69] These findings support the view that the antifertility activity of this plant may be due to certain properties other than estrogenicity/ antiestogenicity which need to be investigated.

There have been several reports of antiviral activity of seaweeds and seaweed extracts.[70] It has been indicated that agar, which is used as a culture medium for microorganisms, is itself a source of some active components against myxovirus. Caccomese et al[71] have studied the antiviral activity of marine algae from Eastern Sicily against tobacco mosaic virus, and Blunden et al[72] have surveyed British marine algae for antiinfluenza virus activity.

Ethanolic extracts of the Indian marine algae belonging to the Rhodophyceae, Phaeophyceae, and Chlorophyceae have been tested against *Semiliki forest* (SFV), *Ranikhet disease* (RDV) and *Vaccinia* viruses (VV). Of the 31 seaweeds screened 17 showed activity of which seven were found active against SFV and ten against *Vaccinia* virus (VV). None of them showed antiviral activity against RDV. The antiviral activity observed in *Codium elongatum* and the two species of *Hypnea* was attributed to the polysaccharides.[73] Of the active extracts, significant inhibition being exhibited by the green algae *Ulva fasciata* (70%, SFV) and *Codium elongatum* (50%, SFV and 45%, VV) and red algae *Chondria armata* (75%, SFV) *Hypnea cervicornis* (100% VV), *Hypnea musciformis* (50%, SFV and VV) and *Spyridia insignis* (50%, VV). Significant antiviral activity was also found in brown algae *Sargassum myriocystum* (75%, VV) and *S. weightii* (50%, VV). In the follow up studies, the activity was concentrated in water soluble fraction of the alcoholic extracts of *Codium elongation* and the two species of *Hypnea.* Subsequently it was found that the antiviral activity in the water soluble fraction was due to sulphated polysaccharides. Carrageenans from *Chondrus crispus* is known to be antiviral. The activity has been reported to be due to the sulphated galactose unit of the phycocolloid. Algal polysaccharides such as laminarin, fucoidan, and algin, which lack sulfated galactose unit exhibit no antiviral activity. It is interesting to note that in some cases, such as *Hypnea*, two different species belonging

to the same genus showed antiviral activity. In other cases not all species belong to the same genus such as *Sargassum* and *Galaxaura* exhibited activity.

Indian seaweeds belonging to Rhodophyceae. Phaeophyceae and Chlorophyceae have also been screened for their effects on central nervous system. Of the 69 extracts screened, activity was observed in eight extracts. Among the active extracts six exhibited the CNS depressant activity.[74] In the gross behaviour, most of the extracts exhibiting CNS activity with the exception of *Sargassum tenerrimum* and *Caulerpa sertularioides* showed a CNS stimulant action as evidenced by marked increase in spontaneous and locomotor activities, hyper sensitive to touch, pilo reaction, Straub' phenomenon and in certain cases (*Ulva fasciata*) preconvulsion clonic. Extracts of *Sargassum tenerrimum* and *Caulerpa sertulariodes* potentiated pentobarbital hypnosis and showed depression of both spontaneous and locomotor activities. *S. tenerrimum* also reduced to 41% the amphetamine induced hyperactivity. None of the five animals tested for forced locomotor activity (rotating test) could stay on the slowly rotating rod for two minutes when administered a dose of 89 mg/kg (ip) of *S. tenerrimum* extract. Thus, all the tests conducted indicated that *C. sertulariodes* and *S. tenerrimum* possess CNS depressant activity. The CNS simulant activity of *Hypnea cervicornis* was confirmed in a repeat collection when a dose of 76.6 mg/kg (ip) reversed, the fall in body temperature and completely counteracted aptosis and sedation. Similar effects were observed in benzene and water soluble fractions at a dose of 16, 5 and 232 mg/kg (ip), respectively. Its significant antireserpine activity added further interest this extract. Follow up studies of the water soluble fraction located the activity in the carrageenan component. In view of the CNS depressant activity exhibited by *Caulerpa sertularioides,* the extract was fractionated and the activity concentrated in the chloroform soluble fraction. Caulerpin, an ether soluble constituent of *Caulerpa sertularioides*,[75] could be responsible for the observed depressant activity, as caulerpin has been reported to produce sedation.[76-81] It is interesting to note that in contrast to depressant activity mostly observed in terrestrial plants, CNS stimulant activity predominates in seaweeds.

Marine algae from the French Mediterranean coast have been tested for antibacterial and antifungal activities.[82] Seaweeds of South Florida have been evaluated for antimicrobial and antineoplastic activities.[77] Carrageenan, a cell wall polysaccharide of marine red algae, exhibits several types of properties. It is co-internalised into infected cells with the *Herpes simplex* virus (HSV), and inhibits the growth of this DNA containing virus. Carrageenan interferes with fusion between cells infected with human immunodeficiency virus (HIV), an RNA-containing virus, and inhibits the retroviral enzyme (reverse transcriptase). The sulphated polysaccharides from red algae have a broad spectrum antiviral activity. There is considerable evidence to support that carrageenan bind to and modulate cell-cell interactions of various kinds, including sperm-egg fusion in the brown alga *Fucus*. It inhibits fertilisation in sea urchins, hamsters, and guinea pigs, embryogenesis in the green alga

Volvox, aggregation of isolated sponge cells. Degraded carrageenan is known to be a potent inflammatory agent *in vivo*. Hydrolysed carrageenans are used to induce intestinal inflammation in many animal models of inflammatory bowel diseases. Carrageenans are also reported to have long lasting effects on the immune system.[79] It also induces inflammation and ulceration.[80]

4.3 Marine Invertebrates of Indian Coasts

Twenty six species of marine invertebrates belonging to phyla Porifera, Echinodermata, Coelenterata and Annelida collected from Goa, Maharashtra, Tamilnadu, Lakshadweep and the Andaman islands have been screened for antiviral, antibacterial, antifungal, antifertility and a wide range of pharmacological activities. Of the 26 extracts tested, 16 showed biological activity. Of the active extracts two showed hypotensive, six antiimplantation, two spasmogenic, and two diuretic activities.[81] The species of Phylum Coelenterata which exhibited activity are: *Gemmaria* spp-I collected from Baga, Goa, exhibited hypotensive activity, LD_{50} 187.5 (mg/kg, in mice), *Gemmaria* spp-II from Malvan, Maharashtra, was found very toxic, LD_{50} 0.02 (mg/kg, in mice), *Anthopleura panikkar* from Anjuna, Goa also showed toxic properties, LD_{50} 68.1 (mg/kg, in mice), *Melitodes ornata* from the Andaman Islands exhibited spasmolytic activity, *Montipora divaricata* from Mandapam, Tamilnadu, also showed spasmolytic activity, LD_{50} more than1000 (mg/kg, in mice); *Pocillopora demicornis* from Mandapam, Tamilnadu, exhibited hypotensive activity, LD_{50} 68.1 (mg/kg, in mice). The extracts of corals that showed 100% antiimplantation activity in female mice are: *Porites lichen* from Malvan, Maharashtra, LD_{50} more than 1000 (mg/kg, in mice). *Acropora corymbosa* and *Lobophyllia corymbosa* both from Lakshadweep, and LD_{50} of both were more than 1000 (mg/kg, in mice) and *Concinarea monile* from Malvan, Maharashtra, LD_{50} more than 1000 (mg/kg, in mice) *Porites lutea* from Malvan, Maharashtra, exhibited 80% antiimplantation activity in female rats, LD_{50} more than 1000 (mg/kg, in mice). The species of the phylum Echinodermata which exhibited activity are: *Astropecten* spp. from Goa, showed spasmogenic activity, LD_{50} 68.1 (mg/kg, in mice); *Holothuria* spp. (body-wall) from Goa, showed diuretic activity, LD_{50} 15.63 (mg/kg, in mice); *Holothuria* spp. (Cuvierian glands) also from Goa, exhibited antiimplantation activity, LD_{50} 15.63 (mg/kg, in mice). The species of phylum Porifera which showed activity are; *Haliclona pigmentifera* from Malvan, Maharashtra, exhibited diuretic activity, LD_{50} 68.1 (mg/kg, in mice) and *Suberites carnosus* from Anjuna, Goa, showed spasmogenic activity, LD_{50} 68.1 (mg/kg, in mice). It is interesting to note that the extract of *Gemmaria* spp., collected in the month of October, showed hypotensive activity. However, when the material was again collected in June, it was found devoid of activity. The results suggest that there are distinct seasonal variations in the activity. Holothurins from sea cucumbers have been found to be toxic to various animal species, including mammals. While confirming these results, the

investigation unmasked their diuretic activity. The saponin fraction from sea cucumber *Stichopus japonica* has been reported to possess antifungal activity against *Trichophyton asteroid* and *Candida albicans* and other fungal species *in vitro*.[82] This activity was, however, not observed in the species examined. Some of these saponins are also reported to have sperm immobilising properties and cause induction of egg and sperm shedding effects.[83] The saponin fraction of the Cuvierian glands of the species showed antiimplantation activity. It is not possible to suggest the mechanism of this effect on the basis of available data. Extracts of sponges have been reported to show antibacterial and antifungal properties.[84] But none of the species examined exhibited similar activity. The most promising activity observed was, perhaps, the antiimplantation activity found in five species of corals. Preliminary experiments conducted on mice have shown that these extracts were 100% active.

Twentyeight extracts of marine animals collected from the coasts of Goa, Maharashtra, Tamilnadu, Gujarat, Lakshadweep and the Andaman islands have been tested for antibacterial, antifungal, antiimplantation, antiviral, hypoglycaemic and a wide range of pharmacological activities. Of 28 extracts screened, 15 extracts showed biological activity. Of these two showed antiimplantation, three CNS stimulant, two hypotensive, four diuretic, four hypoglycaemic, two spasmolytic, one CNS depressant and one was found toxic. Five extracts showed multiple activities.[85] The species of phylum Coelenterata which exhibited activity are: *Parazoanthus* spp. collected from Kanya Kumari, Tamilnadu, in March showed hypoglycaemic and spasmolytic activities, LD_{50} more than 1000 (mg/kg, in mice) and *Stoichactis giganteum* collected from Okha, Gujarat, in March showed hypoglycaemic activity, LD_{50} 68.1 (mg/kg, in mice). The species of phylum Echinodermata which showed activity are: *Acanthaster planci* collected from Kavaratti, Lakshadweep, in February exhibited antihistaminic and CNS stimulant activities, LD_{50} 100 (mg/kg, in mice) and *Astropecten indica* collected from Ratnagiri, Maharashtra showed CNS depressant activity, LD_{50} 6.81 (mg/kg, in mice). The species of phylum Mollusca which exhibited activity are *Aplysia benedicti* collected from Okha, Gujarat, in December showed hypoglycaemic activity, LD_{50} 6.81 (mg/kg, in mice); *Cellana radiata* collected also from Okha, Gujarat, in December showed hypoglycaemic activity, LD_{50} 1000 (mg/kg, in mice), *Ischnochiton campus* from Okha, Gujarat, in December showed antiimplantation activity, LD_{50} more than 1000 (mg/kg, in mice), *Melibe rangi* from Okha, Gujarat, in December exhibited CNS stimulant activity, LD_{50} 178 (mg/kg, in mice) and *Turbo intercostalis* from Malvan, Maharashtra in October showed hypotensive activity, LD_{50} 68.1 (mg/kg, in mice). The species of the phylum Porifera which showed activity are: *Aurora globostellata* collected from the Andaman island in February exhibited diuretic activity, LD_{50} more than 1000 (mg/kg, in mice); *Axinella* spp. also collected from Andaman in February showed diuretic activity, LD_{50} 825 (mg/kg, in mice), *Callyspongia* spp. collected from Ratnagiri, Maharashtra, in October showed

antiviral activity, LD_{50} 681 (mg/kg, in mice); *Dendrilla cactus* collected from Anjuna, Goa, in February exhibited hypotensive and spasmolytic activities, LD_{50} more than 1000 (mg/kg, in mice); *Dysidea fragilis* also collected from Anjuna, Goa, in February also showed diuretic activity, LD_{50} more than 1000 (mg/kg, in mice) and *Ircina ramosa* collected from Malvan, Maharashtra, in February showed antiviral and CNS stimulant activities, LD_{50} 1000 (mg/kg, in mice). It is surprising that the sea cucumber *Holothuria cinerascens* did not show any toxicity. This might be due to the fact that the species is always found concealed underneath the boulders and are never exposed as other *Holothurians* sp. The most promising activity observed was the antiimplantation exhibited by *Ischochiton camptus*. Preliminary experiments conducted on mice have shown that the extract was 100% active. The occurrence of such activity in this species has not been reported hitherto.

Twentynine marine animals belonging to phyla Arthropoda, Coelenterata, Echinodermata, and Mollusca have been collected from the coasts of Goa, Maharashtra, Tamilnadu, Gujarat, Lakshadweep and the Andaman islands during the pre-monsoon and post-monsoon periods. The extracts of these marine animals have been tested for antibacterial, antifungal, antifertility, antiviral, hypoglycaemic and a wide range of pharmacological activities. Of the 29 extracts screened, 14 extracts showed activity. Of these two had the CNS stimulant, three hypoglycaemic, 26 antiimplantation, two antimicrobial, two antiviral, one spasmolytic, one hypotensive, one CNS depressant, activities. Six extracts were found toxic. Six of the active extracts showed multiple activities.[86] The species of Arthropoda which showed activity is *Leptodius arassimanus* collected from Malvan, Maharashtra, in October. The extract of the animal showed CNS stimulant activity, LD_{50} 31.6 (mg/kg, in mice). The species of Coelenterata which exhibited activity are: *Echinomurica* spp. collected from Grand island, Goa, in November showed antiimplantation activity, LD_{50} more than 1000 (mg/kg, in mice); *Alcyonium* spp. collected from Mandapam, Tamilnadu, in May showed antiviral activity LD_{50} more than 1000 (mg/kg, in mice), *Epizoanthus* spp. collected from Veraval, Gujarat, in December exhibited hypoglycaemic activity, LD_{50} 0.38 (mg/kg, in mice) and *Lexo suberites* collected from Mandapam, Tamilnadu in February showed CNS depressant and hypoglycaemic activities, LD_{50} 26.1 (mg/kg, in mice). The species of Echinodermata which showed activity are: *Afrocucumis africana* collected from Agati, Lakshadweep, in October showed antiviral and anti-implantation activities. LD_{50} 681 (mg/kg, in mice), *Actinopyga mauritiana* collected from Lakshadweep in October showed hypotensive and antimicrobial activities LD_{50} 215 (mg/kg, in mice), and *Thelenata ananas* collected from Lakshadweep showed antimicrobial activity, LD_{50} 56.2 (mg/kg, in mice). The extracts of *Holothuria ichinitis* collected from Mandapam, Tamilnadu, in April and *H. impotium* collected from Lakashadweep in April were found toxic, LD_{50} 26.1 and 38.3 (mg/kg, in mice) respectively. The species of Mollusca which showed activity are : *Nerita* spp. collected from Mandapam,

Tamilnadu, in January showed CNS stimulant activity, LD_{50} 68.1 (mg/kg, in mice); *Octopus vulgaris* collected from Okha, Gujarat, in December exhibited spasmolytic activity, LD_{50} 1000 (mg/kg, in mice) and *Onchidium verruculatum* collected from Mandapam, Tamilnadu, in May showed hypoglycaemic activity, LD_{50} more than 1000 (mg/kg, in mice). The ability of Octopi to paralyse prey before ingestion, with secretions of posterior salivary glands has long been known. The active agents present in the salivary glands of the Octopus have been found to be a mixture of low molecular weight amines, peptides, and proteinaceous materials.[87] Ghiretti et al[88] isolated an active proteinaceous fraction from the salivary glands of *Octopus valgaris* and *O. macropus*. While these authors found that the toxins from Octopi have paralytic and hypotension action, the investigations on *Octopus vulgaris* indicated only spasmolytic action.

Forty marine invertebrates belonging to the Mollusca, Coelentrata, Echinodermata and Porifera have been tested for antibacterial, antifungal, antiamoebic, antimalarial, antiviral, antifertility, hypoglycaemic and a wide range of pharmacological activities. Of these, three extracts each exhibited antiimplantation and hypoglycaemic activities; four were found toxic, five had antiviral activity against EMCV and or RDV, seven gave evidence of hypotensive activity, while nine extracts were found to be diuretic. One extract had the CNS stimulant effect, and one had oxytoxic activity as well as low order of abortifacient activity. Besides eight extracts revealed more than one type of activity.[89] The species of Coelenterata which showed activity are: *Acropora corymbosa* collected from Kadmai Island, Lakshadweep, in January showed CVS activity, *Acropora formica* collected from the same locality showed diuretic activity, LD_{50} 2.15 (mg/kg, in mice), *Acropora humilis* collected from the Kadmai Island, Lakshadweep, in January exhibited antiviral activity against *Ranikhet Disease* virus, LD_{50} more than 1000 (mg/kg, in mice), *Alcyonaria* spp. collected from the same locality exhibited diuretic, and hypoglycaemic, activities, LD_{50} 3.16 (mg/kg, in mice); *Montipora divaricata* collected from Krusadai Island, Tamilnadu, in September showed hypoglycaemic activity, LD_{50} 1000 (mg/kg, in mice), *Paracondylacts* spp. collected from Chandipur, Orissa, in April showed antiimplantation activity in rats, LD_{50} more than 1000 (mg/kg in mice); *Zoanthus* spp. collected from Veraval, Gujarat, in April showed cardiovascular effects. The species of Crustacean which showed activity is *Eurythoe complanata* collected from Chandipur in April showed diuretic activity. The species of Echinodermata which showed activity are: *Pentaceraster regulus* collected from Rameswarm, Tamilnadu, in September showed diuretic and antiviral activity against *Encephalomyocaritis* virus; *Ischnochiton campus* collected from Orissa in March exhibited antiimplantation activity in rats and CNS activity, LD_{50} 26.1 (mg/kg, in mice); *Modiolus striatulus* collected from Krishnapuram, Orissa in February showed diuretic activity, *Terebralia palustris*, collected from South Andaman in April showed cardiovascular effects. The species of

Porifera which showed activity are: *Axinella andamanensis* collected from the Red Skin island, South Andaman, in April showed cardiovascular activity, LD_{50} 46.4 (mg/kg, in mice), *Callyspongia spinosissima* collected from Rameswaram, Tamilnadu, in September showed antiviral activity against *Ranikhet Disease* virus and *Encephalomyocarditis* virus; *Heteronema erecta* from Red Skin island, South Andaman in September exhibited hypoglycaemic activity, *Sigmadocia pumlla* from Rameswaram, Tamilnadu, in September showed, diuretic, antiimplantation activity in rats and cardiovascular effect; *Spirastrella inconstans* from Krusadai island, Tamilnadu, in September exhibited diuretic activity, LD_{50} more than 1000 (mg/kg, in mice and *Spirastrella inconstans* var. *digitata* from Rameswarm, Tamilnadu, in September showed antiviral activity against *Encephalomyocarditis* virus.

Many Coelenterates produce toxins or other biologically active metabolites not only for self-defence but at times to capture prey. Echinoderms are known to contain polyhydroxysteroids and saponins. Most of these saponins are reported to have haemolytic activity. Mostly the secondary metabolites isolated from hard crust Molluscs are venomous in nature, and are used by the animals to capture prey. Sponges are by far the most extensively studied marine animals. A variety of biologically active constituents have been isolated from sponges.

Extracts of marine organisms have been assayed for their cardiovascular[90] and toxic properties.[91] The results of screening of 118 marine organisms (Coral, Alcyoniarians, Molluscs, Echinoderms) found in coastal waters of India, for their toxicity on fish and mice fingerlings as well as their hemolytic activities are reported.[92] Echinoderms were found to be toxic to both fish and mice. They also exhibited high order of hemolytic activity. The Alcyoniarians were found toxic to fish and mice, but had weak hemolytic activity. The Molluscs, *Patelia chathalamus*, *Trochus* spp., *Conus* spp., *Siganus* spp. and *Conus textile* were found toxic to fish only, whereas *Drupa heptogonalis* was found lethal only to mice. The coral *Acropora formosa* and *A. millepora* exhibited hemolytic activity and were the only corals toxic to mice. *Subergorgia suberosa,* the only gorgonian collected showed lethal toxicity to fish as well as strong hemolytic activity. The sponges did not show toxic reaction to fish and mice, but exhibited weak hemolytic activity. Specimen of *Spirastrella inconstans*, *Porites lutea, Pocillopora damicornis, Favites abdita, Montipora digitata* and *Noditittoria pyramidalis*, collected from three different locations to determine whether their biotoxicity differs with change in ecological conditions, showed that their biotoxicities did not differ with ecological changes.

4.4 Search of Pharmaceutically Useful Compounds

Rinehert et al[93] have been searching pharmaceutically useful compounds from marine organisms. Their first effort invovled a survey of marine organisms for antibacterial and antifungal properties. The bioassay were carried out on

shipboard.[94] These were the first systematic shipboard assay for pharmaceutical activity, and had many advantages.[95,96] The most active antiviral extract in shipboard testing was from a tunicate identified as *Eudistoma olivaceum*,[97] but the extract was surprisingly inactive in the secondary assay. However, the extract of the recollected sample proved very active in both primary and secondary assays against *Herpes simplex* and other viruses, confirming the value of on site assay. Chemical investigation of the bioactive extract of *Eudistoma* yielded 17 eudistomins having variously substituted β-carbolines.[98,99] Although all the isolated compounds exhibited antimicrobial or antiviral activity, the most potent compounds were the tetracyclic eudistomins with the unique oxathiazepine ring. The *in vivo* testing of these compounds could not be carried out due to the scarcity of the tunicate and paucity of many of the eudistomins. Rinehart et al[100,101] have synthesised a number of these eudistomins. However, oxathiazepine eudistomins have been prepared in extremely poor yield.

The extract of the tunicate *Trididemnum solidum* was found to have potent antiviral activity in bioassays carried out on shipboard.[93] It was also found to be most cytotoxic. Moreover, these activities were confirmed in *in vivo* testing at Upjohn,[101] USA. Nine major and several minor didemnins have been isolated from the extract of *T. solidum*[102-104] which is widely distributed and didemnin-B was relatively abundant in it. Syntheses of some didemnins have been reported.[105,106] Didemnins are active *in vivo* against DNA and RNA viruses. Of these, didemnin-B is the most active. Besides, didemnin-B exhibits remarkable immunosuppressant activity.[107] It is 1000 times as potent as cyclosporin-A in inhibiting T-cell mitogenesis. It has also been shown *in vivo* activity in prolonging skin grafts.[96] Unfortunately, didemnin-B is toxic.

Ecteinascidia turbinata is reported to contain a potent antitumour agent.[108] An interesting immunoregulatory activity was subsequently observed in the extract of the organism.[109] Later on, cytotoxicity was found in the extract. Counter current chromatography and TLC bioautography on tissue culture plates resulted in the isolation of ecteinascidins in yields 10^{-4} to 10^{-6} percent.[96] All the compounds had either anti-bacterial activity or cytotoxicity against L 1210 leukemia cells.[96]

Over 300 marine organisms from Okinawan water have been screened for cytotoxicity, antiviral and antimicrobial activities.[110] An extract of a sponge *Theonella* spp. was found highly cytotoxic in the assay against P 388 murine leukemia cells. Bioassay guided separation led to the isolation of an active constituent designated as misakinolide A, which had IC_{50} 10 ng/ml in *in vitro* tests and T/C 145% at a dose of 0.5 mg/kg against P 388 in mice. The biology and ecology of tropical *Holothurians* have been reviewed.[111] Work has been conducted on the toxicity of sponges and holothurians.[112]

5. Actinomycetes

Actinomycetes play a major role in producing antibiotics and other metabolites

such as extracellular enzymes, pigments, and growth-promoting factors.[113] Marine actinomycetes have furnished new and unique antibiotics.[114] The marine environment could be a rich source of new actinomycetes and new antibiotics. Antagonistic properties of actionmycetes from Molluscs of the Porio Novo region, South India, have been reported.[115]

Twenty two ethanolic extracts of seaweeds collected from Karachi Coast, Pakistan, were screened for brine shrimp cytotoxic activity. Of these only six extracts showed significant activity.[116] Sixty extracts of marine algae and invertebrates from Bulgarian Black Sea Coast were examined for inhibitory activity on the reproduction of influenza virus in tissue culture.[117] The extracts of 10 ascidians collected from the northestern Brazilian Coast were tested for cytotoxicity using brine shrimp lethality assay, sea urchin egg development assay, hemolysis assay, and MTT assay using tumor cell lines and the extract of *Eudistoma vannamei* showed the highest toxicity in brine shrimp assay [LD_{50}=34.7 μg/ml).[118] Robust microtiter plate based assay method for assessment of bioactivity was developed.[119] The Pacific razor clam *Siliqua patula* was known to retain domoic acid, a water soluble glutamate receptor agonist produced by diatoms of the genus *Pseudonitzschia*. The mechanism by which razor clams tolerate high levels of domoic acid in their tissue while still retaining normal nerve function was unknown. This aspect was studied and the results suggested that razor clam siphon contains both a high and low affinity receptor site for kainic acid and may contain more than one subtype of glutamate receptor, thereby allowing the clam to function normally in a marine environment that often contains high concentrations of domoic acid.[120] The spotted dog fish *Scyliorhinus canicula* was found to have antioxidant efficiency and detoxification enzymes.[121] Bioactivity of extracts of marine sponge from Chinese sea was evaluated for biological activity.[122] The effect of novel cytotoxic marine macrolide amphidinolide H (Amp-H) on actin dynamics was investigated *in vitro*. It was found that Amp-H stabilizes actin in a different manner from that of phalloidin and serves as a novel pharmacological tool for analyzing actinmediated cell function.[123] Pharmacological properties and isolation procedures of bioactive marine peptides were reviewed.[124,125] A cDNA microassay technique for analysis of global gene expression profiles in tributylin-exposed ascidians was developed. *Ciona* is a cosmopolitan species and a genomic analysis of *Ciona* revealed that ascidians had approximately 15,500 protein-coding genes. This was the first large cDNA microassay of this animal.

6. Concluding Remarks

Marine organisms exhibit a wide range of biological activities. The activity in some cases varies with ecological variation and the time of collection. A number of marine organisms exhibit toxic properties. Marine toxins have drawn attention because of their involvement in human intoxication and the socioeconomic impacts brought by those incidents. Many of the toxins have

been found to be useful tools for probing biological or pharmacological phenomenon. Although the majority of marine toxins have been found to be produced by microalgae, especially dinoflagellates, it is now clear that bacteria are responsible for production of some toxins. Seaweeds extracts exhibit inhibitory effects upon a number of Gram-positive and Gram-negative bacteria. They also show a number of other useful activities. The high order of antiimplantation activity exhibited by some marine organisms from the Indian coast is noteworthy. It is interesting to note that in contrast to CNS depressant activity mostly observed in terrestrial plants, the CNS stimulant activity predominates in seaweeds. Although high order of useful activity has been shown by a number of marine organisms, no drug in clinical use has so far been developed from marine source. There appears good possibility of obtaining new antiviral and anticancer drugs from marine organisms.

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6

Biosynthesis of Bioactive Metabolites of Marine Organisms

Abstract

The chapter deals with the biosynthesis of bioactive metabolites of marine blue-green, green, red and brown algae, as well as bioactive metabolites of marine sponges, coelenterates, and molluscs. The problems of microbial contamination and the role of symbionts in the biosynthesis of some metabolites of marine organisms and the feeding techniques used in the biosynthetic studies of metabolites of marine algae and marine invertebrates have been discussed. The biosynthesis of carotenoids in halophilic bacteria, algal sterols, isocyanides and isothiocyanides of marine sponges, sponge sterols, cholesterol, sponge phospholipids brominated fatty acids and arsenic containing compounds of macroalgae are also described.

1. Introduction

Marine organisms synthesize a variety of secondary metabolites with unsual structure and interesting biological activities. The chemistry and biological activities of these metabolites have been reviewed by many workers.[1-13] Several hypotheses have been put forward to explain the biogenesis of these metabolites[9] and some experimental evidences have been procured for biogenetic theories.[14] It is generally believed that the origin and mode of formation of secondary metabolites of marine organisms do not differ substantially from the well documented biosynthetic pathways of the secondary metabolites of terrestrial plants and animals. However, biosynthetic experiments are yet to confirm this assumption.The marine environment provides different biosynthetic conditions to organisms living in it.[15] The buffering action of sodium carbonate and bicarbonate maintains the pH of the sea water between 8.2 and 8.5. Sea water contains upto 40% salt and has an osmotic pressure of

15-20 atm.[16] The cell structure, particularly the membrane composition of marine organisms, is expected to differ from their terrestrial counterparts. Besides, there are some striking differences between the metabolism of marine and terrestrial organisms. For example, halogens and isocyanide functions are frequently found in the metabolites of algae and sponges, whereas these are rarely observed in the metabolites of terrestrial plants and animals. Moreover, the absolute stereochemistry of the metabolites of marine organisms often differs from that of terrestrial organisms. It is not yet known whether these differences reflect the individuality of the producer organisms or are the outcome of evolution.

2. Problems of Biosynthetic Studies

The problems associated with the biosynthetic studies of the marine metabolites have been discussed by Garson.[14] For example, the rate of synthesis of metabolites in marine organisms is often slow, particularly if the organism is slow growing. A long term feeding experiment is, therefore, essential to get detectable level of incorporation of the precursor into the product. The time of feeding the precursor is yet another important factor, since some metabolites are produced only when the organism is fully developed or when the nutrient levels are high. Fungal and bacterial metabolites are generally produced during stationary growth periods, whereas other metabolites are synthesised during the period of active growth. Although in many cases, the metabolites produced in artificial environment are identical to those produced *in situ*, there are several organisms, the metabolites of which are highly affected by the temporal and environmental changes. The transport and uptake of precursors are some other problems involved. Nutrient levels in the oceans are low. The concentrations of small organic molecules, such as amino acids and sugars, are generally from 0 to 25 μg dm. Uptake of precursors, therefore, occurs against a concentration gradient. Further, the biosynthesis of metabolites may occur at specialised sites or cells and the precursors must be transported to the sites intact. Marine organism commonly live in symbiotic associations. For example, the association between sponges and microalgae or bacteria (or both) and that between coral (or dinoflagellates) and gorgonians is well known. The degree and exact nature of the association varies from species to species. The pathways of transfer of nutrients between symbiotic partners is of great importance, particularly when sessile animals are involved and raise questions about the real origin of the metabolites produced by the association. The understanding of the metabolic processes in the marine environment is still very limited.

3. Feeding Techniques

Feeding experiments have been carried out either *in situ* or on organisms cultured in the laboratory. Many marine invertebrates, such as sponges, are filter feeders, digesting bacterial and other particular debris from the seawater.

Soft corals are carnivorous, feeding on microorganisms. Corals contain photosynthetic algae and, therefore, also take nutrients through this source. Thus, marine invertebrates may be herbivorous, carnivorous even omnivorous. In several biosynthetic experiments, precursors are fed in aqueous solutions, using either physiological saline or sterile seawater, except where the precursors are insufficiently soluble. Sterol precursors are fed in alcoholic solutions. The addition of Tween 80, perhaps, facilitates the transport of precursors across cell barriers. Labelled precursors can be injected into corals, sponges, star fish, molluscs or fish. Slow release techniques, such as liposomes or implants (e.g. gelatin capsule and osmotic pumps), embedded directly in cell tissues, are now being employed. Organisms can be maintained in an environment that contains the precursor or is provided with a labelled food source, such as microalgae grown on $^{14}CO_2$. The nutrient levels in the oceans are normally of the micrograms per litre. The high concentrations of labelled precursors may overload the metabolic pathway. Besides, the concentration of precursor may prove toxic to organisms. The use of cell free extracts technique in marine biosynthetic studies is not very common, although it may resolve some of the problems such as uptake and transport of precursors, effects of symbiotic associations, etc. However, this technique is best suited where a reasonable rate of synthesis of metabolites is observed.

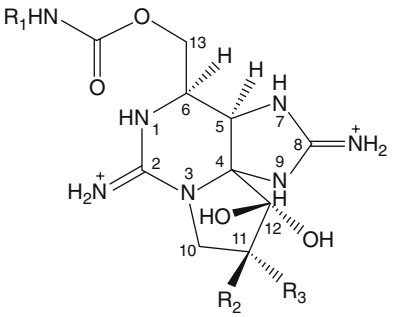
4. Biosynthesis of Metabolites of Algae

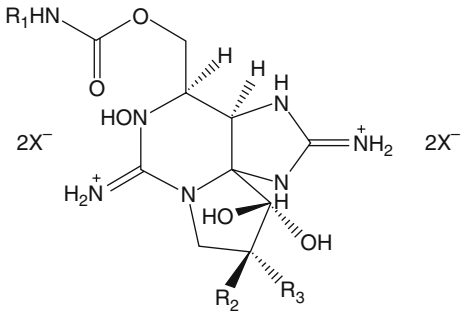
The algae could be divided into two major types. the macro-algae that grow in the littoral zone and the micro-algae found in both benthic and littoral habitats and also throughout the ocean waters as phytoplanktons (diatoms, dinoflagellates, green and yellow-brown flagellates and blue-green algae). As photosynthetic organisms micro-algae play a key role in the productivity of oceans. Marine algae have furnished a variety of bioactive metabolites.[17] However, biosynthetic studies have been carried out on a few metabolites only.

4.1 Saxitoxin and Related Compounds

Saxitoxin and its relatives from *Gonyaulax* species could be divided into four groups (a) saxitoxin (**1**) and neosaxitoxin (**7**) and toxins without sulphate functionality; (b) toxins with sulphated 11-hydroxy groups, (**2**), (**3**), (**8-11**); (c) toxins with an N-sulphated carbamoyl residue, e.g., (**7**) and (**8**); these are less toxic than (**1**) and (**2**), and (d) toxins with 2-sulphate functionalities e.g., (**6**) to (**11**).

Several biosynthetic mechanisms have been put forward to explain the formation of the perhydropurine ring system of saxitoxin and its relatives, and the involvement of arginine is confirmed experimentally in *G. tamarensis*.[18,19] *Guanidino*-^{14}C-arginine was incorporated (1.1% incorporation) into gonyautoxin-III which on treatment with Zn and HCl furnished labelled saxitoxin (**1**). Hydrolysis of (**1**) established 28% radioactivity in the





1, $R_1 = R_2 = R_3 = H$ (Saxitoxin)
2, $R_1 = R_2 = H$, $R_3 = OSO_3^-$
3, $R_1 = R_3 = H$, $R_2 = OSO_3^-$
4, $R_1 = OSO_3^-$, $R_2 = R_3 = H$
5, $R_1 = R_3 = OSO_3^-$, $R_2 = H$
6, $R_1 = R_2 = OSO_3^-$, $R_3 = H$

7, $R_1 = R_2 = R_3 = H$ (Neosaxitoxin)
8, $R_1 = R_2 = H$, $R_3 = OSO_3^-$
9, $R_1 = R_3 = H$, $R_2 = OSO_3^-$
10, $R_1 = R_3 = OSO_3^-$, $R_2 = R_3 = H$
11, $R_1 = OSO_3^-$, $R_2 = R_3 = H$
12, $R_1 = R_2 = OSO_3^-$, $R_3 = H$

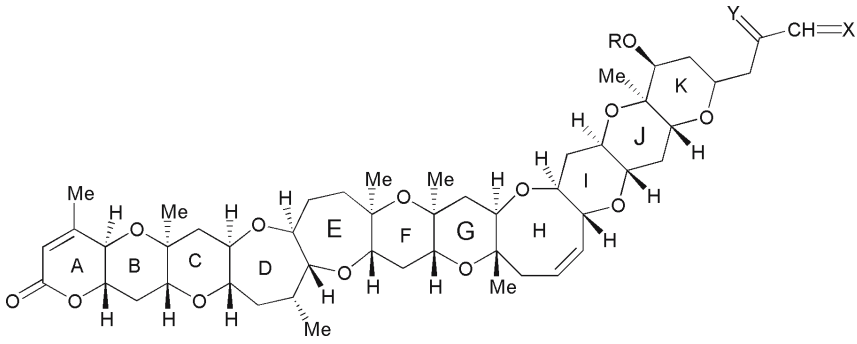
N-carbamoyl group. The experiments with [1,2,3,4,5-^{14}C] arginine established that over 90% of the radioactivity was located in the purine ring of the biosynthetic toxin. It is, suggested that the residual labelling of the carbamoyl group presumably arises from metabolism of the precursor, which is a common problem during biosynthesis of toxins in these marine organisms. Feeding with [2-^{13}C] glycine demonstrated[20] that the carbon atoms C-11 and C-12 of gonyautoxin were specifically labelled with ^{13}C, whereas the labels were found scrambled when [$^{13}C_2$]-acetate was fed to *G. tamarensis*. It was proposed that arginine combines with acetate such as at $C_{(5)}$-$C_{(6)}$ and to a lesser extent of C_{10}-C_{11}, while [2-^{13}C] acetate labelled C-6 and C-11 but not C-5. The results are consistent in which a Claisen condensation occurs at the α-carbon of arginine and decarboxylation takes place in the presence of the adjacent amino function. The incorporation of doubly labelled [2-^{13}C, 2-^{15}N] ornithine into neosaxitoxin revealed that the C_4-N bond remains intact in the biosynthesis of the toxin.[21] The hydroxymethyl function at C-6 has been assumed to be derived from acetate. Feedings of [^{13}C] carbon dioxide, of [^{13}C] formate, and of 3-hydroxy[1-^{13}C] propionate were unsuccessful, suggesting that this carbon atom is not derived via C_1 or C_3 unit. Feeding of [1,2-$^{13}C_2$] glycine, [3-^{13}C] serine, or [methyl-^{13}C] methionine led to labelled neosaxitoxin (**2**) enriched at C-13 with ^{13}C label, consistent with the operation of the C_1-tetrahydrofolate pathway.[19] Experiments have shown that (**2**) is converted into (**1**) in shellfish, demonstrating thereby that N-hydroxylated toxins are converted into N-nonhydroxylated ones.[22]

The origin of the saxitonins is precisely not known. Some believe that these toxins result from the bacterial contaminants within the dinoflagellates. Shimizu et al[21] have shown that the toxicity is an inherent character of each of these algae species and is not the result of symbiotic microorganisms. The

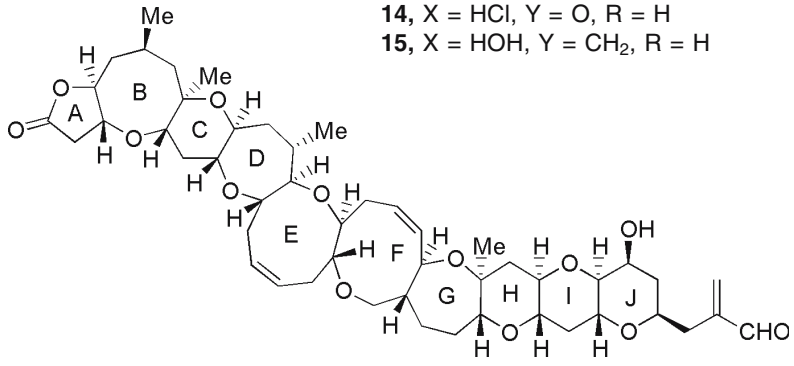
cyanobacterium *A. flosaquae*, used in the experiments, did not contain symbionts. The chemistry, biochemistry and biogenesis of saxitoxin (**1**), its relatives and tetrodotoxin (**23**) have been reviewed.[18]

4.2 Brevetoxins

The dinoflagellate *Gymnodinium breve* Davis (syn. *Ptychodiscus brevis* Davis) causes heavy fish mortality in the Gulf of Mexico. A number of toxins, known as the brevetoxins (GB-toxins), have been isolated from this dinoflagellate.[23-28] The brevetoxin-B (**13**), brevetoxin-C (**14**) and brevetoxin-3 (**15**) all contain 11 transfused ether rings and differ only in the substituents at C-39. Other toxins, GB-5 and GB-6, have acetoxy or epoxy functions at the periphery of the molecules. Brevetoxin-A (**16**) differs in the nature of the A,B ring systems.[29] The brevetoxins may be derived in nature from a transpolyene precursor by an epoxide-mediated cyclisation, giving the desired absolute configuration at every olefinic center.[30]



13, X = O, Y = CH_2, R = H
14, X = HCl, Y = O, R = H
15, X = HOH, Y = CH_2, R = H



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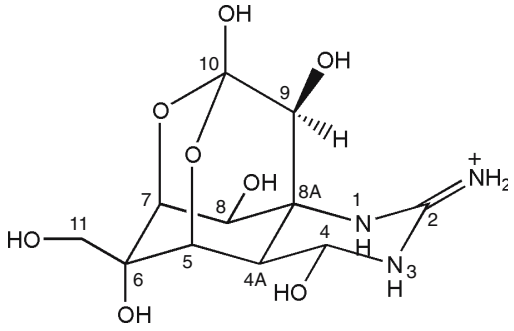
Feeding experiments have been carried out on neurotoxin brevetoxin-B (**13**) in the ten day old cultures of *G. breve*.[30,31] The organism was treated with antibiotics to remove bacterial contaminants and then supplied with the levelled precursors. During feeding with the precursors, it was observed that the

growth of cultures was often highly erratic. Acetates stimulated the growth in some experiments, while in some other there was a rapid deterioration too. The labeling pattern of brevetoxin-B (**13**) as obtained by feeding experiments with [1-^{13}C], [2-^{13}C] and [$^{13}C_2$] acetates and [methyl-^{13}C] methionine is as follows : 16 carbon atoms of (**13**) were found to be derived from [1-^{13}C] acetate; 30 carbons from [2-^{13}C] acetate and four carbons (the methyl groups at C-8, C-22, C-25 and C-36) from [methyl-^{13}C] methionine. The experiments further revealed that the carbon backbone is not a simple polyketide and contains six sets of adjacent carbon atoms that are both labelled by [2-^{13}C] acetate plus two sets of three adjacent carbons which are labelled. Similar results were obtained on the biosynthesis of (**13**) by Schimizu and Chou[32] who also found that prolonged incubation produced random, but differential labelling. Short-term incubation (for two days) with [2-^{13}C] acetate furnished brevetoxin-B in which the same 30 carbon atoms were labelled, but 18 carbon atoms showed splitting patterns due to the presence of an adjacent ^{13}C atom. Studies with [$^{13}C_2$] acetate revealed the presence of 5 intact acetate units. These data were rationalized by the involvement of succinate, 2-oxoglutarate and propionate. The remaining six-, five- and four-carbon fragments were rationalized as a unit that had been derived from 3-hydroxy-3-methylmalonate. Schimizu and Kobayashi[33] have suggested that the biosynthesis of brevetoxin-B is mixed type. The fragments may be formed by the Claisen-type condensation of dicarboxylic acids followed by decarboxylation.

4.3 Tetrodotoxin

Tetrodotoxin (**17**) a highly toxic compound, has been isolated from a variety of marine organisms, including pufferfish, newts and blue-ringed octopus. It is believed to be a product of bacterial metabolism.[21,34] Feeding experiments with ^{14}C-labelled citrulline, arginine, glucose and acetate to newts by injection or oral administration did not furnish labelled tetrodotoxin, although these precursors were metabolised by the organisms to produce sterols and amino acids. It is postuated that they are produced only in response to aggression or developmental needs. However, the biosynthesis of (**17**) remains an unsolved problem.



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Carotenes

The red or pink colours of halophilic bacteria are due to a wide variety of isoprenoid compounds (phytoene, phytofluene, lycopene and β-carotene). An unusual character of halophilic bacteria is that they need at least 15-25% salt for their normal growth. The biosynthesis of carotenoids of *Halobacterium cutirubrum*, carried out with cell-free preparation using labelled precursors, proceeds as follows: Isopentyl phyrophosphate-*trans*-phytoene-*trans*-phytofluene-carotene β-carotene.[35] It has also been demonstrated that the C_{50} red pigments bacterioruberins are formed from C_{40} carotene, lycopene.[36]

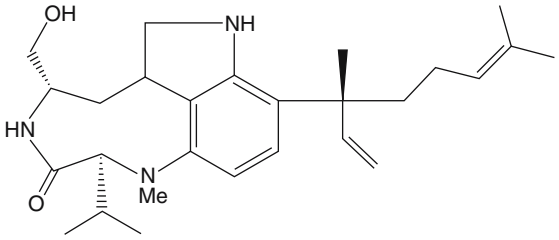
4.4 Sterols

Algal sterols play a key role in the marine environment because algae are at the bottom of the food chain. The status of marine sterols has been reviewed by Kerr and Baker.[37] The major sterol of blue green algae is stigmasterol which is accompanied by cholesterol and 24-ethylcholesterol. Dinoflagellates typically contain a complex mixture of sterols. The most characteristic sterol of this group is dinosterol characterized as 4,23,24(*R*)-trimethyl-5-cholest-22-en-3β-ol. Many invertebrates, particularly coelenterates and some molluscs, contain dinoflagellate symbionts known as zooxanthellae. They contain cyclopropyl sterols, gorgosterol and 23-demethylgorgosterol. Their unique structure raises intriguing questions to their biosynthetic origin and biological role. Although there has been much discussion concerning the biosynthesis of dinosterol and gorgosterol, the precise mechanism has not yet been established.[37] Diatoms comprise a significant portion of the phytoplankton. The sterols of diatoms, however, have not received much attention. The majority sterols of diatoms are conventional C-27, C-28 and C-29 phytosterols. Coccolithophorids are microscopic, unicellular algae widely distributed in the oceans. Sterol compositions of these micro-algae are generally very simple. The marine unicellular chlorophyta contain ergosterol, 7-dehydro stigmasterol, and sterols. Sterols of macro-algae have a structure similar to that of cholesterol with the exception of a methyl or ethyl group at C-24 and or unsaturation. The sterol of red algae are unusual, as they generally lack C-24 alkylation. Fucosterol is the predominant sterol of brown algae. The green algae typically have a complex mixture of sterols. Phytosterols of marine algae differ from animal sterols in having extra double-bonds in ring B or extra alkyl groups at C-24. The mechanisms of the side-chain modification have been elucidated by labelling studies with mevalonated or methionine.[38] The mechanisms of formation of 24-methyl-24β-sterol is common in both fungal and photosynthetic algae. Alkylation of a precursor and migration of hydrogen from C-24 to C-25 generates a intermediate which undergoes stereospecific reduction to a 24-methyl-24β-sterol. These algae have a similar mechanism for the production of 24-ethyl-24β-sterol. Chlorophyta follow a different biosynthetic pathway for the synthesis of 24-alkyl sterols. The final step in the biosynthetic process is the reduction of a substrate. Migrations of two hydrogen are involved in

the formation of 24-methyl-24β-sterols. In the biosynthesis of 24-alkyl sterols in chlorophytes, the original hydrogen at C-24 is retained. This hydrogen migrates to C-25 in the biosynthesis of sterols in other photosynthetic algae. In all the algae studied so far, poriferasterol is formed by the introduction of the bond prior to reduction of the σ-bond (in the chrysophyte, *Ochromonas malhamensis*[39] or bond (in a species of green alga of the genus *Trebouxia*.[40] Although these studies have been carried out on fresh water algae, it is expected that marine microalgae follow the same biosynthetic pathways. An unidentified chrysophyte[41] incorporated [methyl-^{13}C]- and [methyl-$^{2}H_3$] methionine into [24*E*]-25-propylidene cholesterol, and it was established that the propylidene group was not formed from the cleavage of a cyclopropane ring. It has been reported that cholesterol and 24-methylcholesta-5,22-dien-38-ol are biosynthesized from mevalonate in the chrysophyte *Pseudoisochrysis paradoxa*.[42] The 23-methyl group in dinosterol is derived from the methionine and also the methylene bridge in 4α-methylgorgostanol in *Peridinium balticum*.[9]

5. Metabolites of Blue-Green Algae

The blue-green algae show many structural features in common with bacteria, notably the absence of membrane bound organelles. They are called algae since they contain chlorophyll-a and related compounds. All prokaryotes can convert atmospheric nitrogen into ammonia and therefore, nitrogenous metabolites are frequently formed in the blue-green algae. Some of these metabolites exhibit potent biological activities. For example, a number of strains of *Lyngbya majuscula* which cause contact dermatitis known as Swimmer's itch', produce toxins, lyngbyatoxin and debromoaplysiatoxin. Hormothamnione (**20**) is an unusual chromone from *Hormothamnion enteromorphoides*, the biogenesis of which cannot be explained by the acetate or shikimate pathway.[43] No terpene appears to have been isolated from cyanobacteria, although compounds incorporating isoprenoid units, such as lyngbyatoxin A (**18**)[44-46] and hapalindole (**21**)[47-50] have been isolated. β-Cyclocitral (**19**) isolated from *Microcystis* species, is a degradation product of carotenoid[51]. The biosynthesis of hapalindole (**21**) has been investigated by Moore et al.[52] It has been shown that the indole portion of **(21)** is derived from tryptophan. A tetrahydrofolate origin of the isocyanide carbon is demonstrated by the feeding of [2-^{14}C] glycine, L-[3-^{14}C] serine, L-[methyl-^{14}C]-methionine, and [^{13}C]serine, [methyl-^{14}C]-methionine, and [^{13}C]formate incorporated into hapalindole A. [^{14}C]Cyanide was incorporated efficiently and also [2-^{13}C,^{15}N]glycine into the isocyanide carbon of **(21)**. Several monocyclic, aliphatic or glycosidic carotenoids occur in cyanophytes.[53] The biosynthesis of carotenoids, both *in vivo* and *in vitro*, has been studied in *Aphanocapsa* species.[54] Here, the cell-free extract of the cyanobacterium converts [2-^{14}C]-mevalonate into [^{14}C]-labelled phytoene or geranylgeranyl diphosphate and [^{14}C]-labelled phytoene into β,β-carotene. Results from



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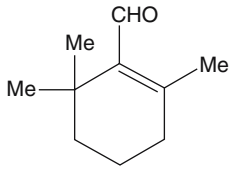

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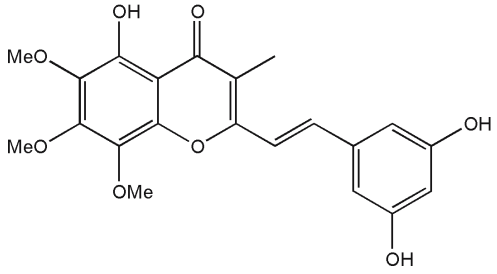

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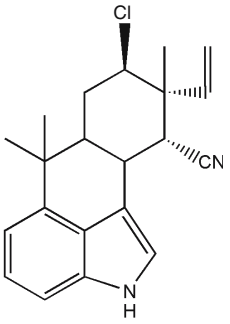

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time course experiments support the postulate that phytoene is converted into β,β-carotene via the normal pathways, while inhibitor studies showed that its biosynthetic conversion into β-cryptoxanthin proceeds via β,β-carotene. Phytoene is converted into myxoxanthophyll in experiments with disrupted *Aphanocapsa membranes*, while an intact membrane preparation synthesised all xanthophyllic components except xeananthin.

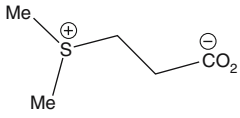
The fatty acid composition of cyanophytes is characteristic. These organisms contain many polyunsaturated C_{16} and C_{18} acids. Many marine cyanophytes use small organic molecules to maintain the osmotic balance with their surroundings. The biosynthesis of 1-α-D-glucopyranosyl-5n-glycerol in the cyanobacterium *Synechococcus* spp. has been studied by Mackay and Norton.[55] A rapid increase in glucosylglycerol follows hyper osmotic shock in the organism.

6. Metabolites of Macro Algae

Dimethylpropiothein (**22**) is produced by several species of green algae. Experiments on intact thalli of *Ulva lactua*[56] have shown that the sulphur atom and dimethyl group of methionine are incorporated into **(22)**. The uptake of acetate into *Ulva* species has also been studied.[57] The members of phaeophyta are a rich source of low-molecular weight carbohydrates of commercial and chemotaxonomic interest. These polyhydroxylated metabolites are also important substrates for respiration and are synthesised by the C_3 pathway, although the C_4 Hatch-Slack pathway is a prominent feature of

brown macrophytic algae.[58] However, these are less common in the chlorophyta and rhodophyta. The metabolites of brown algae exhibit seasonal variation. The diterpenes, such as eleganolone and related compounds are elaborated by *Cystoseira elegans* only during periods of active growth and, thus, play an antifeedant role.[59] These compounds may also serve as precursors for the production of sterols and carbohydrates when nutrient supply becomes limited.

A number of cyclic and acyclic C_8 and C_{11} hydrocarbons act as gamate-releasing or attracting factors during sexual reproduction of brown algae. For example (+)-(*S*)-1-[(*Z*)-but-1-enyl]-cyclohepta-2,5-diene (**23**) and (3*E*, 5*Z*)-octa-1,3,5-triene (**24**) are produced by *Ectocarpus siliculosus*[60] and *Fucus serratus*,[61] respectively. These metabolites appear to have been derived from acetate via polyunsaturated fatty acids. However, biosynthetic studies on such compounds occurring in minute quantities have limitations. Algae of the genus *Dictyopteris* produce volatile C_{11} hydrocarbons. The structure of these compounds is consistent with the biosynthetic intermediates of pheromones. Different quantities of the same compounds are found in different collections of the alga *Dictyopteris membranacea*. It is not yet clear whether this difference is due to genetic or environmental factors.[62,63] The alga *Giffordia mitchellae* elaborates a number of odoriferous $C_{11}H_{16}$ hydrocarbons derived from giffordene [(2*Z*, 4*Z*, 6*Z*, 8*Z*)-undeca-2,4,6,8-tetraene].



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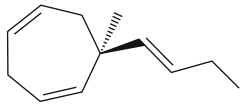

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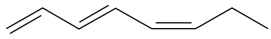

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Biosynthesis of fatty acids in marine algae at different temperatures using [1-^{14}C] acetate has been carried out.[64,65] It has been found that *Fucus vesiculosus* gives mostly labelled 18:1 fatty acids at 5°C, while *Polysiphonia lanosa* gives labelled 16:1 and 18:1 acids at 5°C and labelled 16.:0 and 18:1 acids at 15°C. A seasonal variation study has shown that more unsaturated fatty acids with shorter chains are found in algae in winter than in summer. Some of the brown algae produce organo-arsenic compound,[66] notably arsenosugars. The biosynthetic pathways of these compounds have not yet been traced. However, the incorporation of [^{74}As] into arsenic-containing phospholipids in the marine phytoplankton *Chaetoceros concavicornis*[67] has been shown. The inorganic arsenic appears to enter the normal biosynthetic pathway to phospholipids, and the resulting arsenolipids act as analogous of the normal substrates. The algae appear to detoxify inorganic arsenic by converting it into methylated arsenates and arsenolipids. The low levels of incorporation are a common problem in the biosynthetic studies of monoterpenes in red algae. It is believed that bromophenols are derived from tyrosine *via* intermediates, such as 4-hydroxybenzaldehyde,[68] and are stored in the cell as sulphated derivatives which are rapidly hydrolysed when excreted into the

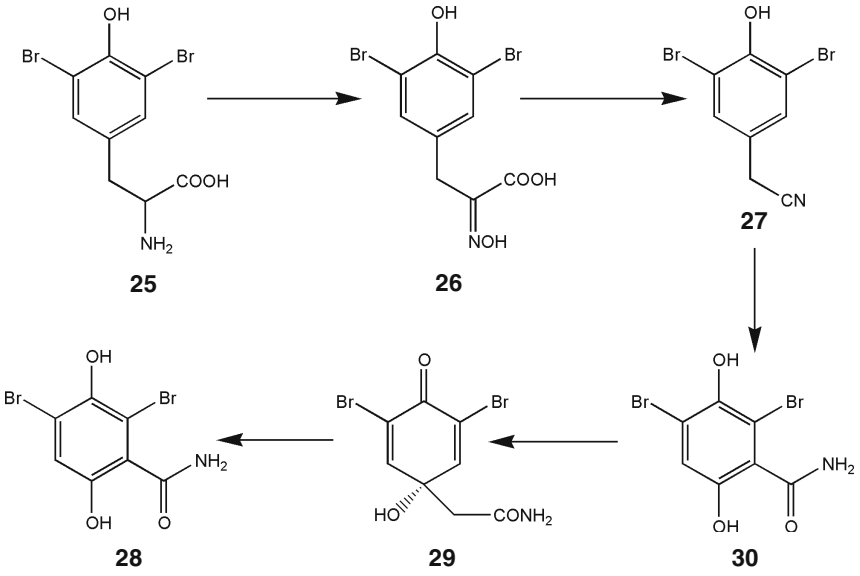
sea. The biosynthesis of tribromopeptene oxide in *Bonnemaisonia nootkana*[69] has been studied. Freshly collected healthy species were incorporated into a limited volume of sterile seawater, containing [^{14}C]-labelled acetate, malonate, butyrate or palmitate. The highest levels of incorporation achieved were 0.004% with acetate or palmitate. These low levels of activity precluded chemical degradation. Although both chloride and bromide ions are found in abundance in seawater, brominated metabolites occur in greater abundance than chlorinated and iodinated compounds in the marine algae and sponges. Bromoperoxidases, which can oxidize iodide or bromide ion but not the chloride ion, are commonly found in marine algae.[70] These enzymes catalyse the formation of bromochloro-derivatives from alkenes or alkynes in the presence of bromide and chloride ions and hydrogen peroxide.

7. Metabolites of Marine Invertebrates

7.1 Sponges

Over 500 metabolites have been isolated from sponges.[1-13,71-80] Several of these have unusual structures with interesting biological activities. Despite much speculation on the biogenesis of these metabolites, there has been only limited experimental evidence. The main difficulty in the biosynthetic studies is the slow rate of metabolism of these marine invertebrates. Accumulation of metabolites occur over a number of years, rather than in weeks or months. The turnover of metabolites may also be slow. The sponges cannot be maintained under aquarium conditions for longer periods for incorporation studies. Animals may become stressed resulting in loss of metabolites production or a switch over to a different biosynthetic pathway. Attempts to trace the biosynthetic pathways of aeroplysinin-1, aerothionin and the amide by feeding [1-^{14}C]acetate, [2-^{14}C]mevalonate, [methyl-^{14}C]methionine, L-[U-^{14}C]tyrosine and L-[U-^{14}C]ornithine to the sponge *Aplysina aerophoda*[81] and *A. fistularia*[82] provided more meaningful results. The suggested steps in the biosynthesis as shown below are : tyrosine → **25** → oxime (**26**) → nitrile (**27**) → amide (**30**) → dienone (**29**) which undergoes rearrangement to yield finally the bromo metabolite (**28**). This scheme is consistent with the isolation of oximes, nitriles and amides from the members of *Aplysia* genus. The use of randomly labelled precursors do not allow establishing the specificity of labels in the biosynthetic products. Use of specifically labelled precursors in the biosynthetic studies are expected to provide more respectable data.

Energy-dispersive X-ray microanalysis[83] located the site of biosynthesis of aerothionin and homoaerothionin in *Aplysia fistularia* (syn. *Verongia thiona*) as spherulous cells. These bromo metaboites have antibiotic properties and may function to exclude some type of bacteria or aggregate dietary bacteria. Alternatively, they may be involved in chemical defence.[83-85] The other bromo metabolites present in *Aplysia* species may derive from combination of bromotyrosine with other amino acids, such as cysteine,



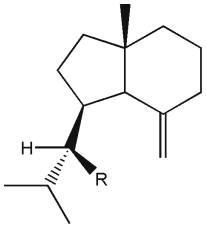
lysine, distamine etc. The origin of the butenylene side-chain in the rearranged dibromotyrosine derivative aplysinadiene is of interest[86] and is worth investigating.

Isocyanides and Isothiocyanides

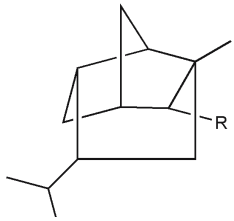
Marine sponges have furnished a number of isocyanoterpenoids.[87,88] Excellent reviews on naturally occurring isocyanides, cyanides and isothiocyanides both of terrestrial and marine sources have appeared recently.[88] The origin of terpenoids metabolites of sponges containing isocyanide, isothiocyanide, formamide, urea and amino functionalities are of considerable interest.[88,89]

It was believed that the formamide might be the biogenetic precursors of the isocyanides. However, it has not been proved by an experiment in which ^{14}C-labelled aximide-1 (**31**) was supplied to *Axinella cannabina*[90] which produced axisonitrile-1 (**32**). Scheuer et al[91] have studied the origin of isocyanide groups in 2-isocyanopupukeanane (**33**) in *Hymeniacidon* species. ^{13}C-Labelled precursors encased in double gelatin capsules were implanted in the sponge and were left in the natural habitat. After one or two weeks, the sponge was killed and the metabolites of interest were isolated. The data suggested that ^{13}C-labelled (**33**) was converted into the formamide (**34**) and isocyanate (**35**), whereas ^{13}C-labelled (**34**) was not converted into (**33**). The origin of the isocyanides in the metaboites of marine organisms is intriguing. The incorporation of sodium [^{14}C]cyanide into diisocyanoadociane in marine sponge, *Amphimedon* spp. has been studied.[92,93] The chemical degradation of labelled (**36**) revealed that the isocyanide carbons were equally and selectively labelled by cyanide. The results are of interest for a number of reasons. Many strains of bacteria are known to have developed cyanide-

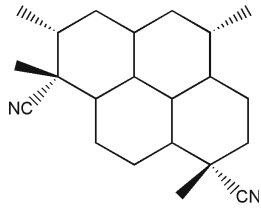
insensitive respiratory chains or to generate inorganic cyanide from amino acids. Incorporation of a number of inorganic and organic forms of cyanide has been investigated[93] in an attempt to develop conditions for the uptake of cyanide under non-saturating, non-toxic levels. The source of cyanide *in situ* is believed by analogy with terrestrial metabolism, to be an amino acid but may not be glycine, leucine, alanine or arginine since these did not appear to be incorporated into (**36**). Long chain aliphatic bisisothicyanates (**37**) or mixed aldehydo-isothiocyanate (**38**) have been isolated from a Pacific species of *Pseudaxinyssa*.[94] The absence of corresponding isocyanides supports the postulate that the biosynthetic pathways of these metabolites are different.



31, R = NHCHO
32, R = NC



33, R = NC
34, R = NHCHO
35, R = NCS



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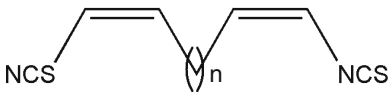
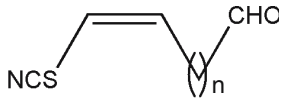
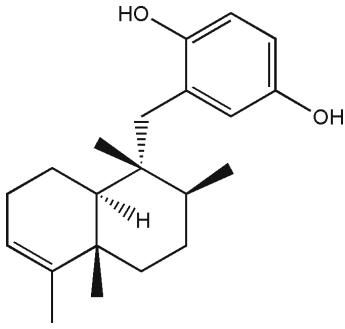


37, n = 8–15



38, n = 9; 15 or 16

Avarol (**39**), the prenylated aromatic metabolites of *Dysidea avara* and *D. fragilis*, is a potent cytostatic and antibacterial agent.[95-102] The metabolite also exhibits good activity against AIDS virus.[103] A study of the subcellular location of (**39**) in *D. avara* has also been undertaken.[104] Cutting the bacterial symbionts (*Alcaligenes* species) associated with this sponge did not produce avarol. The metabolite appeared to be synthesised by sponge cells and not by



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bacterial cells. The compound is probably compartmentalized in intracellular cytoplasmic vesicles (spherular cells) and, therefore, has no inhibitory effect on the sponge cells themselves. Avarol is released from these cells to assist in regulation of the bacteria with which the sponge is associated symbiotically.

The origin of metabolites of sponges has been a subject of discussion, as to whether they are products of bacterial, algal or sponge metabolism. Currently, it is believed that the brominated metabolites of the tropical sponge *Dysidea herbacea* are the products of symbiotic metabolism while terpene metabolites result from metabolism of the sponges themselves.[105]

Sterols

A bewildering variety of sterols is found in sponges.[106-113] The origin and biosynthesis of sponge sterols have been reviewed by Djerasi et al.[114] The tracer experiments have shown that some sponges made their sterols *de novo* and others did not. It seems that sponges containing novel sterols were not capable of *de novo* sterol biosynthesis, whereas common sterols are synthesised *de novo*. The sterols found in sponges differ from their terrestrial counterparts and sponge sterols, unlike plant sterols, usually have multiple alkylated side chains. In several sponge sterols, this side chain contains cyclopropanes and cyclopropene rings. Several sponge sterols have different conventional nuclei, some have A-nor and 19-nor nucleus. The sources of sponge sterols are: *de novo* biosynthesis, assimilation by host organism of sterols produced by symbiotic algae or other associated bacteria or fungi; assimilation of dietary sterols; and modification of dietary sterols. The water soluble precursors, such as acetate, methionine, and mevalonates, are poorly incorporated by sponges into sterols. However, squalene is converted *de novo* by sponges into their sterols.[115] The biosynthesis of the characteristic sterols of terrestrial fungi has been studied in sponges. It has been demonstrated that *Pseudaxinyssa* species,[116] convert precursors into sterols, 24β-sterols, such as codisterol and clerosterol, but not their 24α-epimer. The conversion of sterols by reduction was demonstrated in *Agelas* species. In contrast, [4-^{14}C] cholesterol was converted into cholesta-5,7-dien-3β-ol in an *Amphimedon* species containing symbiotic fungi or bacteria. The biosynthetic studies carried out so far were mainly concerned with the mechanism of side chain modifications. The studies on 24-isopropylsterol, the major sterols of *Pseudaxinyssa* species, showed that their biosynthesis from demonsterol proceed *via* 24-methylene cholesterol and fucosterol. However, there was no specificity in alkylation at C-28 of the (*E*)- and (*Z*)-isomers of fucosterol.[117] The steps involved in the biosynthesis of these sterols are: alkylation at C-28 of fucosterol, migration of a proton from C-28 to C-24 and proton abstraction yields (24*S*)-24-isopropenylcholesterol which is incorporated in preference to its (24*R*)-isomer. A regioselective hydrogen migration from C-28 to C-24 was confirmed by

feeding with 24-methylene [26(27)-^{3}H]cholesterol[118] and also established that the final alkylation at C-28 is on the α-face. A low incorporation of [2-^{14}C]mevalonate suggested that synthesis *de novo* is very inefficient compared to biochemical modification of dietary sterols.

It has been shown that the triply methylated sterol, xestosterol, results from double extension of the side chain at C-26 and C-27 of codisterol to give 25,27-didehydro-*epi*-aplysterol. Thus [24-^{3}H] (*epi*) codisterol gives ^{3}H labelled xestosterol[119] which was shown by chemical degradation to contain tritium at C-25. The homologous precursors, clerosterol and *epi* clerosterol, were not used by the sponge for the synthesis of the 26-methyl *epi* stronglylosterol or xestosterol analogues. This result of enzyme selectivity is important since it counters the earlier findings that all transformation of sterol side chains are non-specific and are mediated by non-specific enzymes. In an *Aplysia* species, the methyltransferase appears to be sensitive to the stereochemistry, or the substituents at C-24, C-26, C-27 and C-28 of a range of sterols yet are unable to methylate cholesterol, which is the most abundant sterols in these organisms. Since cholesterol plays an important role in membrane structure and function, it is suggested[14] that the sterols present in minute quantities may provide some protection to the organisms or act as key metabolic precursors. Study of the regulation of biosynthesis of sterols and the role of species specific symbionts are interesting problems for further investigations. It has been observed that [2-^{14}C] mevalonate is very poorly utilised by the sponges[120] to make sterols which suggets that the presence of abundant dietary sterols might be causing feedback inhibition. Low incorporation of [2-^{14}C] mevalonate into xestosterol may be due to poor uptake of water soluble precursors.[119] Poor uptake of methionine has also been noted.[117] It would, therefore, be of interest to determine if methionine is utilised by sponges for the synthesis of xestosterol and, if so, which additional methyl groups are labelled.

Phospholipids

An excellent review on sponge phospholipids has been published by Djerasi et al.[121] The exceptionally long chain fatty acids, mostly polyunsaturated one called demonspongic acids occur in high quantities (24-79% of the total fatty acids) in sponges. Of special interest is the 5X, 9Z-diene pattern of many C_{24}-C_{27} acids with or without methyl branching and the abundance of numerous odd-chain (C_{21}-C_{27}) analogues.[122] The double bond is unusual and occurs mainly in the C_{16}-C_{18} fatty acids. It is noteworthy that all demospongic acids, identified so far, predominate in the PE and PS fractions (ca.75% vs 25% in PC). The functional significance of demospongic acids has not yet been understood. The possible sources of occurrence of demospongic acids in sponges are *de novo* biosynthesis, dietary intake, and incorporation from symbionts with or without further modifications.

The biosynthetic studies of sponge lipids have been carried out using the whole cell system and the cell free extract. High incorporation of [1-^{14}C] acetate into C_{22}, C_{24} and C_{26} fatty acids of *Microciona prolifera* was achieved when the whole cell system was employed. High levels of radioactivity were incorporated into fatty acids 26:2 (5, 9) and 26:3 (5, 9, 19), and it was demonstrated that the activity was generally located near the carboxyl end of the chain, consistent with a chain elongation mechanism for their formation from 16:0 and 16:1 acids. Djerassi et al[123] have studied the biosynthesis of fatty acids in sponges. The sponge *Jaspis stellifera* contains four major very long chain fatty acids (VLFA) in its phospholipid fraction. These are hexacosa-5,9-dionoic acid, [26:2(5,9)], 24:5-methylpenta-cosa-5,9-dienoic acid, 25-methylhexacosa-5,9-dienoic acid and 24-methylhexacosa-5,9-dienoic acid. Carboxy-^{14}C-labelled palmitic acid, 13-methyltetradecanoic acid and 12-methyltetradecanoic acid were fed to *J. stellifera* over a period of 30 days. In each experiment, most of the radioactivity was found in the unchanged precursors. No incorporation of any precursor into the *iso*-26:2 acid was observed. Assuming that a short chain *iso*-acid generates only a long chain *iso*-acid, and not an *anti-iso* acid, these data suggest that there is a specific incorporation of each acid into its long-chain polyunsaturated homologue. Degradation of the biosynthetic normal 26:2, *iso*-27:2 and *anteiso*-27:2 acids by bis-epoxidation and cleavage with periodate ion yielded mono-epoxides having 84:6 to 92.3% of the radioactivity. The results, thus, supported that the major pathway of formation of these VLFAs is by chain elongation of the normal 16:0 precursor followed by 5-adenosyl-methionine-mediated alkylation. Besides, the results also indicated that there is only limited degradation and resynthesis from acetate of these fatty acids. The marine sponge *Aplysia fistularia*[124] produces 22-methyloctacosa-5,9-dienoic acid and triaconta-5,9,23-trienoic acid as its major VLFA in the phospholipid fraction. The unusual branched fatty acid may be formed by chain elongation of a short-chain branched precursor, such as 10-methylhexadecanoic acid or by methylation at the double-bond of an apppropriate trienoic precursor. Carboxyl-labelled (±)-10-methylhexadecanoic acid, palmitic and palmitolenic acids were supplied to the sponge for one month.[123] Again, major quantity of the radioactivity was associated with unchanged short chain fatty acids. Since palmitic acid was not utilised by the sponge to form 20:3 acid, it may be concluded that the sponge is unable to introduce a double bond into the middle of the chain. Both isomers of 10-Me-16:0 were incorporated into 22-Me-28:2 (5,9), although the chirality of the naturally occurring acid was demonstrated to be (22*R*), [^{3}H]. Methionine was incorporated rapidly into the branched short chain fatty acids, but only poorly into the 22-Me-28:2 acid. It is suggested[14] that the short chain fatty acids are likely to be of bacterial origin but are converted into the VLFAs by sponge cells. A species of the marine sponge *Petrosia*[125] produces brominated fatty acids, such as (*S*, *E*, 9*Z*)-6-bromo-25-methyl-hexacosa-5,9-dienoic acid and (*S*, *E*, 9*Z*)-6-bromo-24-methylhexacosa-5,9-

dienoic acid. The biosyntheses of these bromo fatty acids are of interest, specially the origin of bromine atoms in these acids. The marine sponge *Tethya aurantia* produces the ether lipids (2*S*)-1-(hexadecyloxy) propane-2,3-diol, (2*S*)-1-(16-methylheptadecyloxy) propane-2,3-diol. The biosynthesis of these lipid ethers has been investigated in whole animals and dissociated sponge cells using [1-^{14}C] hexadecanol.[126] It was found that in intact animals, the precursor was oxidised to the corresponding acid, and then converted into the acid components of phospholipids. In contrast, the precursor was utilised for the synthesis of unesterified glycerol monoethers by dissociated cells but not incorporated into phospholipids.

7.2 Coelenterates

The phyum coelenterata includes the corals, gorgonians (or sea pens), sea anemones, jellyfish and other related marine invertebrates. They form symbiotic associations with microalgae, the zooxanthelae. The metabolites isolated from these invertebrates are, therefore, products of algal, coelenterates or joint metabolism. A few biosynthetic studies have been carried out on the metabolites of soft corals. Generally, intact animals colonies are maintained in aquaria. The radioactive precursors are added in the water soluble form. Feeding of precursors to the animals by injection technique has been less successful, possibly because of lack of information about the site of biosynthesis of the metabolites. The soft coral *Sinularia capillosa*[127] was incubated in a beaker containing seawater and [2-^{3}H] mevalonolactone (1 μci cm^{-3}) and [^{14}C] bicarbonate (1 μci cm^{-3}) for 20 h at ambient temperature (26-28°C) and normal shaded light. The coral was harvested and worked up. The major metabolite, furanoquinol was isolated and degraded. The results supported the nonisoprenoid origin of the quinol. The soft corals, unlike sponges, are capable of rapidly synthesising terpenes *de novo*. Biosynthetic studies have been carried out on crassin acetate, a major metabolite of the gorgonian *Pseudoplexaura porosa*.[128-130] The incorporation of [2-^{14}C]acetate and sodium [^{14}C]carbonate into the tips of the gorgonian was investigated. It was found that the crassin acetate was more effectively labelled by acetate. However, positive evidence for the labelling of the cembrane ring was not obtained. Recent studies,[131] using cell-free preparation, have established that the cells of the symbiont alone are responsible for the production of crassin acetate. Biosynthesis of crassin acetate occurs *via* standard isoprenoid pathways. Animal cells are incapable of synthesising terpenes or to have any effect on their synthesis of zooxanthellae. It has been suggested that although the zooxanthellae contain the enzymatic mechinery for formation of terpenes, it is controlled by gorgonian partner.

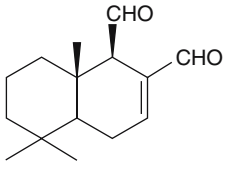
The biosynthesis of sterols in gorgonians is complex. Several novel sterols, such as gorgosterol containing a cyclopropane ring, are found in symbiotic gorgonians, whereas aposymbiotic coelenterates lack these sterols. Besides, in artificially cultured zooxanthellae, these sterols were found absent,[132]

though these organisms do synthesise 4-methyl sterols, such as dinosterols, which have been implicated in biosynthesis of gorgosterol. However, it is not clear at which stage of the biosynthesis the 4-methyl group is lost.[133] Interestingly, sea anemone *Aiptasia pulchella* are capable of synthesising the sterols in the absence of host tissue.[134] The biosynthesis of sterols in zooxanthellae, therefore, appears to be directed by specific animals host system. Attempts to demonstrate the synthesis of gorgosterol in cell free system from four different gorgonian species, using a wide variety of preparation techniques, incubation media, and co-factors, have been totally unsuccessful, in contrast to the studies on biosynthesis of diterpenes and triterpenes suggest that the gorgonian partner exerts a major influence on the biosynthetic steps from squalene to these sterols. There is yet no evidence that coelenterates are capable of synthesising sterols *de novo*. The fatty acid composition of zooxanthellae symbionts from corals and clams depends upon the nature of the host.[135]

Prostaglandins-PGA and PGA_2 are produced in high yield in the gorgonian *Plexaura homomalla*. Details of their biosynthesis, which differs from the endoperoxide route, have been reviewed by Garson.[14]

7.3 Molluscs

The dorid nudibranch *Dendrodoris limbata* synthesises the antifeedant dialdehyde polygodial (**40**)[136-140] in its mantle tissue and a variety of sesquiterpene esters in its digestive tissue.[141] None of these compounds was found in the diet of the animal, suggesting their *de novo* synthesis. Biosynthesis of the dialdehyde (**40**) has been studied by injecting [2-^{14}C] mevalonate into the hepatopancrease of the *D. limbata*. The biosynthetic polygodial (**40**) (incorporation 1.6%) contained ^{14}C label. The site of lebelling in the terpene portion has not been determined. However, the data clearly support the postulate that the anti-feedant metabolite (**40**) is synthesised *de novo* in the animal and, perhaps, is used as a chemical defensive agent. *Dendrodoris grandiflora*, a Mediterranean dorid nudibranch synthesises polygodial along with 6β-acetoxyolepupaune in its mantle tissue.[142] The digestive gland of the animal contained a mixture of sesquiterpenesters, terpenes, such as microcionin-1, fasciculatin and prenylated chromanols. Feeding experiments with [2-^{14}C] mevalonate revealed that *D. grandiflora* synthesises *de novo* these compounds. The remaining terpenoids are of dietary origin. The diterpenoid glycerides



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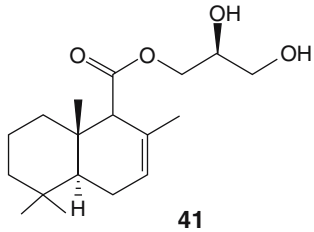

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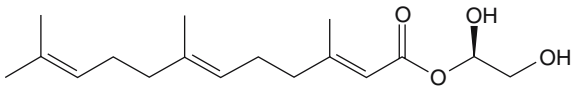
(**41**, **42**) are produced by the British Columbian nudibranch *Archidoris montereyensis* and *A. odhneri*.[143,144] The precursor feeding studies using [2-^{14}C] mevalonic acid revealed these to be true metabolites of the nudibranch. The opisthobranch mollusc *Navanax inermis*[145] deposits navenones-A, B and C (**43**, **44**, **45**) as a trail from the glandular tissue of animals, if irritated. Under normal conditions (**43**, **44**) are regenerated in about three days, while under stress conditions the regeneration of metabolites is slow. When [^{14}C]acetate was added into the food supply of *N. intermis*, the label was detected in (**45** and **44**) 0.05% and 0.28% incorporation, respectively. However, further biosynthetic experiments are needed to trace the biosynthetic pathways of these metabolites. The origin of pyridine moiety is of much interest in the metabolites (**43**). The sacoglossan *Placobranchus ocellatus* contains 9,10-deoxytridachione and deoxytridachione, which are photochemically interconvertible *in vitro*. The possibility that this transformation also occurred *in vivo* and the metabolites might be formed from intermediates obtained from the products of photosynthesis in dietary assimilated chloroplats[146] was tested by incorporation studies using [^{14}C] bicarbonate.[147]

There are conflicting results regarding the biosynthesis of sterols in molluscs. Some species appear to be capable of synthesizing sterols from precursors, such as acetate or mevalonate, while others utilised a deitary source and modify these sterols. In some cases, for exampe in *Patella unigata*,[148] two distinct pathways of biosynthesis of cholesterol operate. Cholesterol is synthesized *de novo* and is also made from phytosterols. It is suggested that



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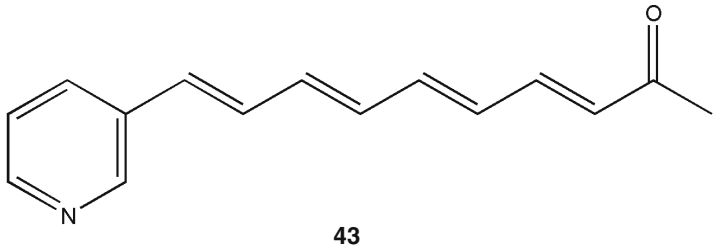
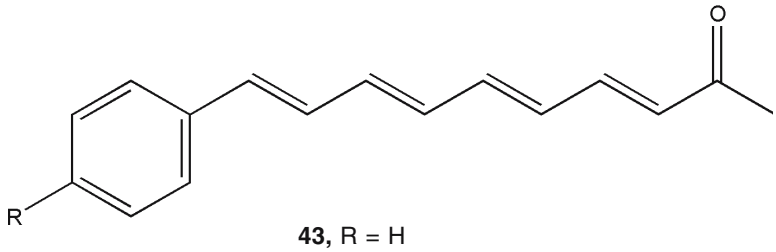


43



43, R = H
45, R = OH

possibly all molluscs originally had the capacity to synthesise sterols, but lost this capacity in favour of the energetically less demanding dealkylation of phytosterols. Limpets, such as *P. vulgata* would, therefore, represent an intermediate evolutionary stage.

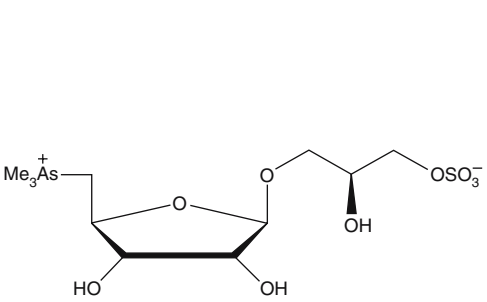
8. Cholesterol Biosynthesis

Cholesterol, once believed to be a typical animal sterol, is now one of the most widely distributed sterols in both terrestrial and marine plants and animals,[37] where it is biosynthesised from squalene. Several of the individual enzymes of the early steps of squalene biosynthesis have been isolated. The biosynthetic status of cholesterol has been reviewed.[149] Insects have no capacity for *de novo* sterol synthesis. They rely exclusively on exogenous sources, that is phytosterols. Sterols metabolism in insect has been discussed by Ikekawa et al.[150] Cholesterol in insects is formed by dealkylation of phytosterols. The analogous deakylation of sterols also occurs in sponges[151] and crustaceans.[152]

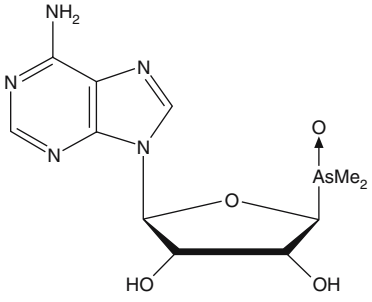
9. Biosynthesis of Arsenic-Containing Compounds

Arsenic containing compounds have been isolated from a number of macroalgae,[153,154] particularly brown algae, where arsenic occurs at levels of 10-40 mg/kg^{4} . Arsenic containing ribosides have been identified in some bivalve molluscs.[155] It is believed that unicellular algae, which are consumed by the molluscs, are the source of these compounds. The kidney of the giant clam, *Tridacna maxima*,[156,157] had yielded the unusual ribosides. The other compounds that have been isolated recently are characterised as: N-(5′deoxy-5′-dimethyl-arsinoyl-β-D-ribosyloxyglycine; (2*S*)-3-(5′-deoxy-5′-dimethylarsinoyl-β-D-ribosyloxy)-2-hydroxypropanoic acid; (2*R*)-3-(5′-deoxy-5′-dimethyl-arsinoyl-β-D-ribosyloxy)-2-hydroxypropanoic acid; 9-(5′-deoxy-5′-(dimethylarsinoyl)-9H-adenosine; N-[4-(dimethyl-arsinoyl) butanoyltaurine and (2*S*)-3(5′-deoxy-5′-trimethylarsonio-β-D-ribosyloxy)-2-hydroxypropyl) sulphate.[158]

The arsenic containing compounds are probably formed in marine organisms as follows. The reduction and oxidative methylation of absorbed oceanic arsenate by algae in two stages could give dimethylarsinic acid a known algal metabolite.[159] The reduction followed by oxidative adenosylation, yield the range of dimethylarsinoylribosides that have been identified from algal sources. Adenosylation may precede one or both, of the methylation stages. Trimethylarsonio ribosides (of which the zwitterion (**46**) is, so far, the only known naturally occurring) may also be formed by this pathway. The stage at which the third methyl group is transferred to arsenic is less certain. It is likely that methylation of arsenic proceeds in algae without adenosylation, thus yielding a tetramethylarsonium salt. Such an arsenic species is yet to be identified in algae, although it is a common constituent of bivalve molluscs feeding on unicellular algae.[160]



46



47

The presence of the nucleoside (**47**) in *Tridacna*, as a consequence of algal metabolism, supports the proposed pathway for the synthesis of arsenic containing ribosides by algae. The presence of (**46**) may represent the first example of donation, by *S*-adenosylmethionine, of all three of its alkyl groups to a single acceptor (arsenic) within one organism.

10. Problems of Microbial Contamination

Several metabolites of marine organisms of considerable biological interest have been obtained in trace quantities. For example, to isolate 100 mg of bryostatin-2, an antileukaemic agent from the bryozoan *Bugularia* species, one requires about 1500 kg of the appropriate bryozoan. These metabolites are probably produced by micro-organisms growing on the surface of the organism. Conflicting results regarding the chemical constituents and biological activity of different collections of the bryozoan *Chartella papyracea* have been noted.[161] The possibilities of microbial contamination are also not excluded. An autoradiographic study on feeding and transport of metabolites in the marine bryozoan *Membranipora membranaceae* has been made by Best and Thorpe.[162] The green alga *Platymonas convolutae* is a symbiont of the marine flatworm *Convoluta roscoffencis*. Tracer experiments with [^{14}-C] labelled precursors have shown that the fatty acids and sterols synthesised by the alga are provided to the host[163] which has the capacity to synthesise complex lipids. The biosynthesis of prostaglandins in the tissue homogenates of fish and other marine invertebrates, such as sea squits and clam, has been carried out by Ogata et al.[164] Several studies on the biosynthesis of wax esters and carotenoids in fish have been reported.

Phospholipid biosynthesis in the oyster protozoan parasite, *Perkinsus marinus* was studied.[165] The biosynthetic gene cluster for antitumor rebeccamycin, a halogenated natural product of the indolocarbazole family was characterized.[166] The cloned genes may help to elucidate the molecular basis for indolocarbazole biosynthesis and set the stage for the generation of novel indolocarbazole analogues by genetic engineering. Current status of biosynthesis of *pullulan* was reviewed.[167]

11. Concluding Remarks

The biosynthesis of the secondary metabolites of marine algae and marine invertebrates is fascinating and challenging. In spite of several problems involved in biosynthetic studies, the biosynthetic pathways of several classes of secondary metabolites have been traced using tracer experiments. Biosynthesis of metabolites exhibiting high order of biological activities or toxicity and produced in minute quantities is still a challenging problem. There is a lot of confusion regarding the origin of compounds of marine organisms living in symbiotic form or contaminated with bacteria. Feeding experiments are expected to resolve some of these problems.

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7

Bioactive Marine Toxins

Abstract

The chapter deals with paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), ciguatera, diarrhetic shellfish poisoning (DSP) and other miscellaneous toxins. The origin, chemistry, properties and mode of action of saxitoxin, neosaxitoxin, gonyautoxins I-VIII, tetrodotoxins and its congeners; brevetoxin-A and brevetoxin-B, ciguatoxin and its congeners; maitotoxin, palytoxin and its congeners, okadaic acid and its congeners; pectenotoxins, yessotoxin, amphidinolides, amphidinol, proprocentrolide, neosurugatoxin, surugatoxin and prosurugatoxin; goniodomin-A, polycavenoside-A, toxic substances of *Chondria armata,* aplysiatoxin, debromoaplysiatoxin, toxic peptides, nodularin and microcystin, lyngbyatoxin-A, and isodysiden have been discussed. Transfer of toxins between organisms and methods of detection of paralytic shellfish toxins have also been discussed.

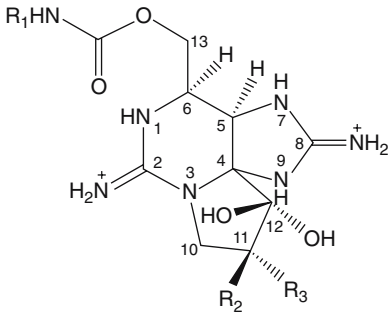
1. Introduction

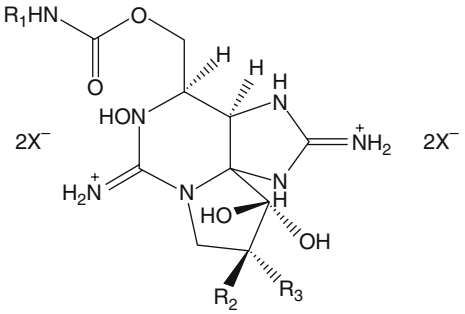
Marine toxins have drawn worldwide attention because of their involvement in human intoxication and the socio-economic impacts brought by those incidents.[1-15] The chemistry of the toxins is imperative because it helps in devising proper counter measures, such as detection, determination and therapeutic methods. It is important to understand their mechanism of action at molecular level. Many of the toxins have been found to be useful tools for probing biological or pharmacological phenomena, for example, use of tetrodotoxin in sodium channel studies,[1] and okadaic acid in protein phosphatase studies.[16] The chemical modification of toxins followed by structure-activity relationship studies provides an attractive target for chemists and biologists. The etiological studies of marine toxins are important since most marine natural products chemists are concerned about the origin of these toxic

compounds. Although the majority of marine toxins have been found to be produced by microalgae, especially dinoflagellates, it is now clear that bacteria are also responsible for production of some toxins. The historical aspects and details of marine toxin studies have been discussed in books, monographs[17–20] and reviews.[21]

2. Paralytic Shellfish Poisoning

Paralytic shellfish poisoning (PSP)[22–25] is one of the most severe forms of food poisoning caused by ingestion of seafood. It is acute and often fatal. There is no effective way to destroy the toxins or to treat the patients. Therefore, it poses serious health problems. Deterring shellfish consumption causes economic problems. The history of the poisoning goes back to prehistoric days and the incidents due to the consumption of toxic shellfish are well documented. The problem exists along both the East and West coasts, often leading to a total ban on shell fishing in a wide area with enormous economic loses. It is now known that the toxic principles responsible for toxic effects are produced by a marine plankton *Gonyaulax catenella* and some other dinoflagellates. At certain unpredictable times these red plankton multiply and cause "red tide." Although, many fishes are killed by this "red tide," mussels and clams survive but concentrate the toxic principles, thus becoming poisonous to humans. The toxin isolated from the Alaskan butter clam, California mussel and the marine microalga *Gonyaulax catenella* is called saxitoxin (**1**).[26–29] It is now established that saxitoxin (**1**), neosaxitoxin (**7**) and their congeners (**2-18**) are involved in paralytic shellfish poisoning (PSP). The dinoflagellate that produce saxitoxin and its congeners are *Alexandrium* spp. (formerly *Gonyaulax* or *Protogonyaulax*), *Gymnodinium catenatum, Pyrodinium bahamense* var. *compressum*.[30] The origin of saxitoxin

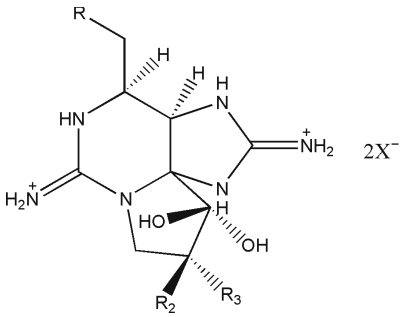




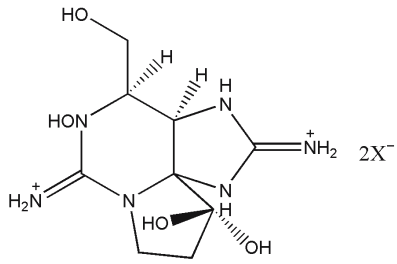
1, $R_1 = R_2 = R_3 = H$ (Saxitoxin)
2, $R_1 = R_2 = H$, $R_3 = OSO_3^-$
3, $R_1 = R_3 = H$, $R_2 = OSO_3^-$
4, $R_1 = OSO_3^-$, $R_2 = R_3 = H$
5, $R_1 = R_3 = OSO_3^-$, $R_2 = H$
6, $R_1 = R_2 = OSO_3^-$, $R_3 = H$

7, $R_1 = R_2 = R_3 = H$ (Neosaxitoxin)
8, $R_1 = R_2 = H$, $R_3 = OSO_3^-$
9, $R_1 = R_3 = H$, $R_2 = OSO_3^-$
10, $R_1 = R_3 = OSO_3^-$, $R_2 = R_3 = H$
11, $R_1 = OSO_3^-$, $R_2 = R_3 = H$
12, $R_1 = R_2 = OSO_3^-$, $R_3 = H$

(**1**) in PSP was found to be bacteria.[31] Saxitoxin (**1**) and neosaxitoxin (**7**) are also reported to be produced by the fresh water blue-green alga, *Aphanizomenen flos-aquae*.[32–34] Structurally the toxins of this class could be divided into two major groups, saxitoxin (**1**) and neosaxitoxin (**7**). The members of these two groups are further diversified by the presence of 11-O-sulphate or N-sulphate, the absence of carbamoyl group (**13**, **14**, **15**) and oxygen at C-13 (**15**, **17**).



13, R_1 = OH, R_2 = H, R_3 = OSO_3^-
14, R_1 = OH, R_3 = H, R_2 = OSO_3^-
15, $R_1 = R_2 = R_3$ = H
16, $R_1 = R_2$ = H, R_3 = OSO_3^-
17, $R_1 = R_3$ = H, R_3 = OSO_3^-



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2.1 Transfer of Toxins between Organisms

Originally it was thought that the shellfish accumulate toxins by filter feeding the toxic plankton during blooms. The toxins then enter into the hepatopancreas where most toxicity is normally found. It was expected that the toxicity of the shellfish will be lost over several weeks or months after the disappearance of the plankton. But this did not happen. In fact, the first source of saxitoxin, the toxic Alaska butter clam, *S. giganteus* was found in water where no noticeable bloom of the toxic plankton was seen. Moreover, the toxicity which is mostly localized in the siphons, did not disappear even after a year in uncontaminated sea water. Paralytic shellfish poisons were found in non-filter feeding snaits, crabs, and toxic macro algae where the secondary transfer of the toxins was not possible. Thus, the mechanism of toxification of these organisms is still not clearly understood.

2.2 Saxitoxin

2.2.1 Isolation

Schantz et al[35] first isolated pure saxitoxin (**1**) from the Alaskan butter clam using weakly basic Amberlite IRC 50 and alumina chromatography. The Alaskan butter clam is still considered the best source of saxitoxin. The isolation procedure is fairly simple. However, this procedure is not applicable for the isolation of other shellfish toxins, since these are not strongly basic.

A general procedure which is now commonly used had been developed.[36,37] In the procedure,[37] the mixture of the toxin is separated from the other constituents by selective absorption on Bio Gel P-2 or Sephadex G-15. The toxin fraction is eluted with a dilute acetic acid solution. The mixture of toxins is then applied on a column of weakly acidic carboxylic acid resin, Bio-Rex 70 in acid form, the acetic acid gradient elution furnishes pure toxins in the reverse order of the net positive charge of the molecule. The toxins with negative net charge are not separable by this technique. However, they can be separated by either preparative thin layer chromatography (TLC) or careful chromatography on Bio-Gel P-2.

2.2.2 Assay Methods

There are a number of assay methods for detecting saxitoxins.[17] In the mouse assay, each mice weighing 20 g are injected (ip) with 1 ml test solution of adjusted pH and toxicity. Time of death is measured, and the toxicity in mouse units (Mu) is found from the standard table and corrected by factor obtained from control mice injected with the standard saxitoxin dihydrochloride solution and expressed in micrograms equivalent of saxitoxin dihydrochloride. One mouse unit (Mu) is the amount of toxin needed to kill a 20 g mouse in 15 min. The assay is very reliable. However, it does not give the amount of individual toxins and latent sulphated toxins. The serious drawback of the method is the requirement of mouse of uniform size, which is sometimes difficult to obtain.

Chemical Assay: Bates et al[38] have developed a fluorometric assay of saxitoxin based on the degradation product formed by treatment with NaOH-H_2O_2. It is quite sensitive method for saxitoxin derivatives, but not for neosaxitoxin derivatives. The limitation of the method is that the other fluorescence products present in the crude extracts interfere in the measurements.

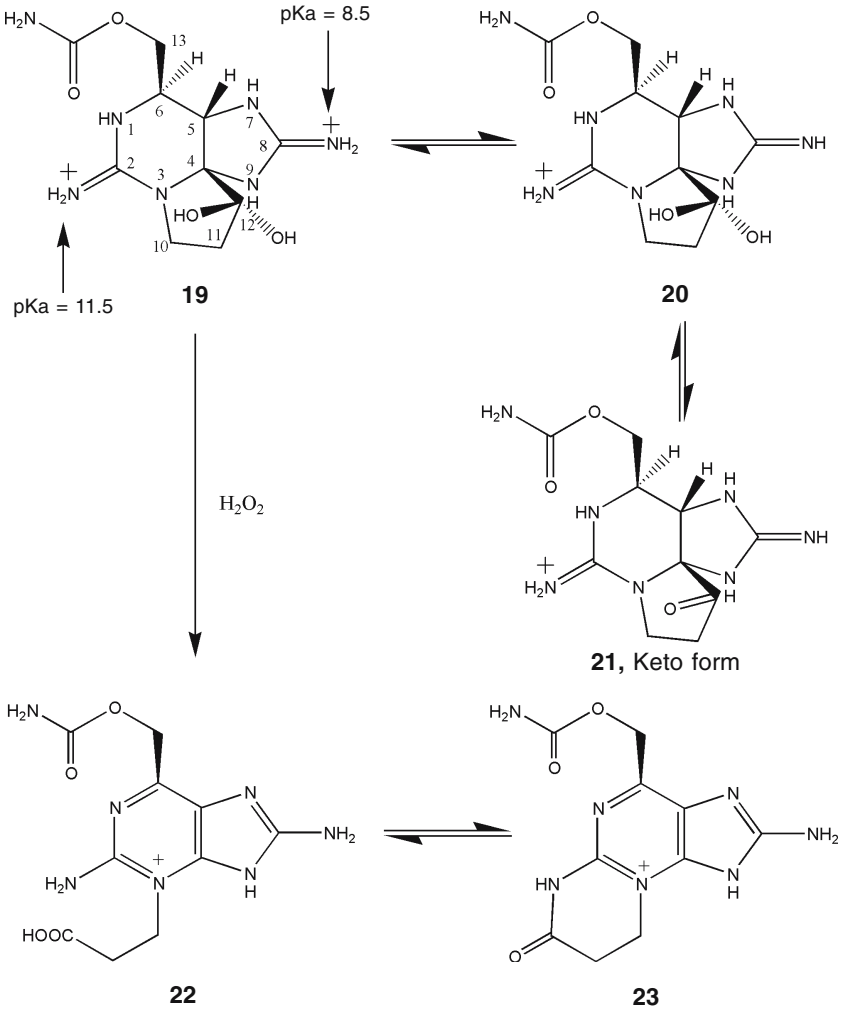
High-Pressure Liquid Chromatography (HPLC) Assay: The method developed by Sullivan et al[39,40] is used for routine analysis. All toxins, including latent toxins, can be quantified in a fairly short time. The method is very useful for metabolic studies of toxins.

Immuno assay: Davio et al[41] has developed a radioimmuno assay that is very sensitive in the detection of saxitoxin. However, the utility of the method depends upon the selection of an antibody with desirable cross reactivity to toxins with diverse structure variations.

Fly Bioassay: Ross et al[42] have developed a method to substitute the mouse assay. In the method flies are temporarily immobilized at low temperature and injected with a minute quantity of test solution using a micro syringe. However, the method has not been officially recognized.

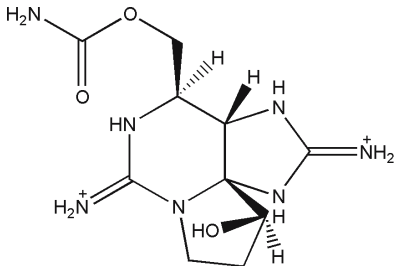
2.2.3 Chemistry

Chemistry of saxitoxin have been extensively studied.[43-47] A tentative structure was proposed in 1971.[45] The final structure (**1**) to saxitoxin was assigned by the X-ray crystallography of its p-bromobenzensulfonate salt[46] (**19**) and its hemiketal. Saxitoxin has several interesting structural features. Its perhydro purine skeleton with an additional five member ring fused at the angular position is unprecedented. It has a ketone hydrate at position 12 stabilized by two neighboring electron withdrawing guanidinium groups. The ketone (**21**) is also readily enolized to effect the rapid exchange of protons at position 11. The molecule has two pKa values, viz. 11.5 and 8.1. The proton and carbon nuclear magnetic resonance (NMR) chemical shift studies under different pH conditions indicated that the later pH value is associated with the imidazoline guanidinium group.[48,49] It is suggested that the abnormally low pKa for a

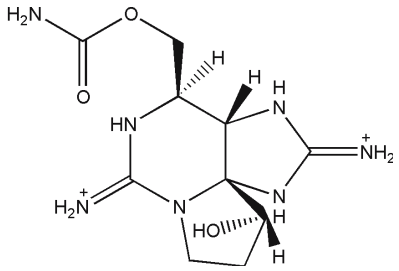


guanidinium group is a result of the insufficient participation of N-7 in the guanidinium resonance, probably due to the stereochemical strain of the five member ring. A high resolution NMR study[44] suggested that in the pH range of physiological condition, saxitoxin exist in an equilibrium of three molecular species; divalent cation (**1**) monovalent cation of the hydrated form (**20**), and monovalent cation in a keto form (**21**). Saxitoxin is very stable in acidic solution. For example, it can be kept in dilute hydrochloric acid solutions for a long time without loss of its potency. However, the toxin is extremely unstable under alkaline conditions, especially in the presence of oxygen, and undergoes facile oxidative degradation to yield the aromatized aminopurine derivatives (**22**) and (**23**) which can be more efficiently obtained by oxidation of the toxin (**1**) with H_2O_2.[50]

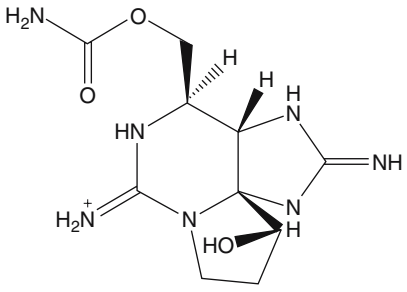
The oxidation products are highly fluorescent, and the reaction is used for quantitative estimation. The hydrolysis of the carbamoyl ester function of saxitoxin can only occur in concentrated acid solutions, such as 7.5 N HCl at 100°C. Structure activity relationship studies have also been carried out on saxitoxin. Hydrogenation of saxitoxin with platinum catalyst afforded the hydroxyl isomer of (12*R*) dihydro saxitoxin (**24**), whereas reduction with borohydride gave a mixture of (**24**) and the β-isomer (**25**).[44] Decarbamoylation of saxitoxin afforded the compound (**26**) which retains about 70% original toxicity.[50]



24, (α-isomer)



25, (β-isomer)



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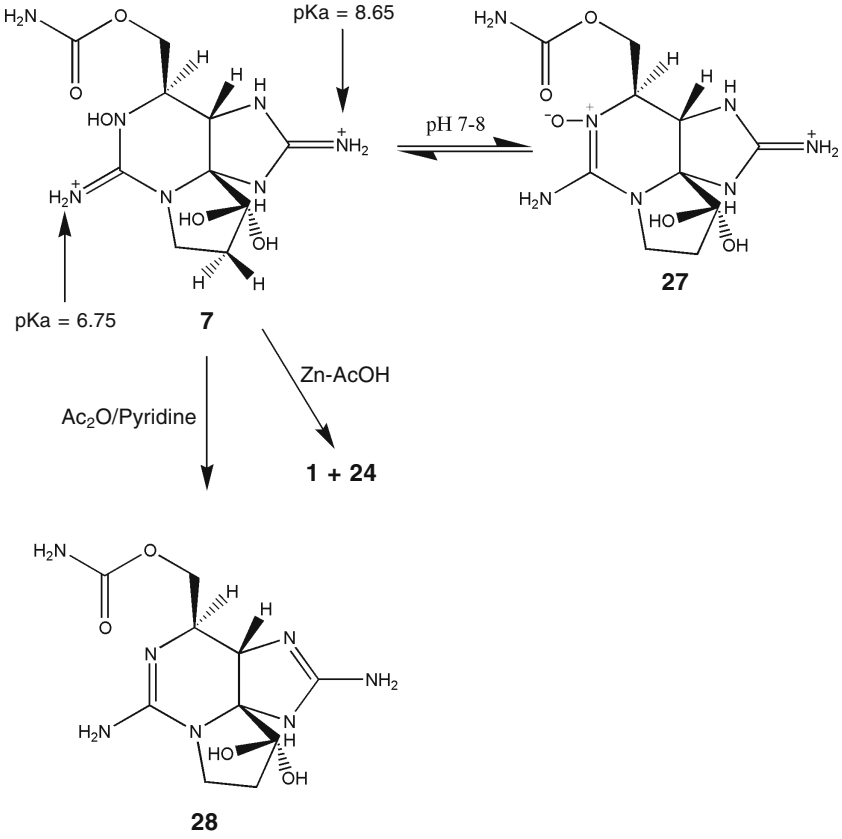
2.2.4 Neosaxitoxin

Neosaxitoxin (**7**)[37,51] was isolated as a minor product from an Alaskan butter clam. Later on it was isolated as a major constituent from most toxic shellfish, dinoflagallates, blue-green algae and the crab samples. Neosaxitoxin was characterized as 1-N-hydroxy-saxitoxin (**7**) by spectroscopic and chemical evidence by Shimizu et al[52] The structure was later confirmed by ^{15}N-NMR.[53] Treatment of neosaxitoxin (**7**) with zinc-acetic acid yielded saxitoxin (**1**) and dihydro saxitoxin. Neosaxitoxin was not as stable as saxitoxin in acidic conditions, and tends to decompose in hydrochloric acid solutions. The imidazoline guanidine ring of neosaxitoxin has a pKa value similar to saxitoxin (8.65). However titration and NMR studies indicated the presence of another pKa 6.75, which is suggested due to the hydroxy-guanidine group of purine ring in (**7**).[54] Electrophoretic study confirmed that the net positive charge of the molecule around physiological pH is reduced to about half of saxitoxin.[54]

2.2.5 Gonyautoxin-I

Gonyautoxin-1 (**29**) was first obtained from soft-shell clams exposed to *G. temerensis* blooms.[42] Subsequently, it was found to be a major component in



many PSP samples. Its structure (**29**) was established on the basis of spectroscopic data and chemical correlation with gonyautoxin-II, neosaxitoxin and saxitoxin.[55-58] Reduction of gonyautoxin-I (**29**) gives a mixture of neosaxitoxin (**27**) and gonyautoxin-II (**30**), which can be further reduced to saxitoxin (**1**). Similar reductive biotransformation was observed in scallop tissues.[56] Gonyautoxin-I and its stereoisomer gonyautoxin-IV are probably the most unstable among the PSP toxins.

2.2.6 Gonyautoxin-II

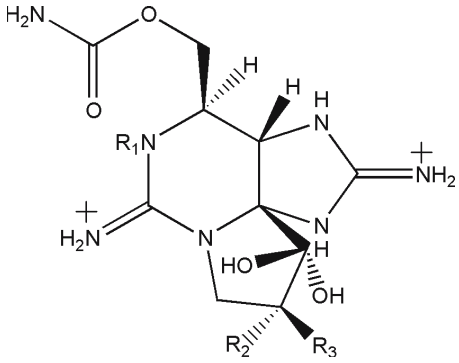
Gonyautoxin-II (**30**), as a major toxin, was first isolated from soft-shell clams from the New England coast.[36, 59] Subsequently, it was found to be a major toxin in a number of samples. The structure (**30**) of gonyautoxin-II was assigned by spectroscopic data and extensive chemical degradation methods.[60] It was first reported to be a free 11-hydroxy derivative of saxitoxin, but later amended to its sulphate ester.[61] Finally, structure (**30**) was confirmed by correlation with saxitoxin.[56]

2.2.7 Gonyautoxin-III

Gonyautoxin-III (**31**) is the 11-epimer of gonyautoxin-II (**30**).[60,61] It forms about 7:3 equilibrium mixture of (**31**) and (**30**) in a neutral or higher pH solution. It is believed that thermodynamically less stable gonyautoxin-III (**31**) is the parent form that exists in the living organism.

2.2.8 Gonyautoxin-IV

Gonyautoxin-IV (**32**) is the 11-epimer of gonyautoxin-I (**29**).

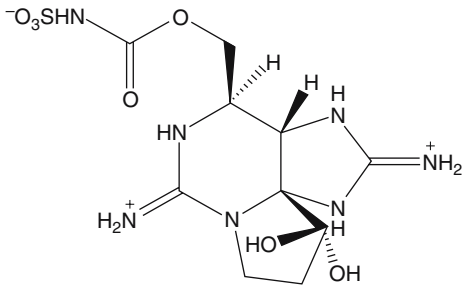


29, R_1 = OH, R_2 = OSO_3^-, R_3 = H
30, R_1 = H, R_2 = OSO_3^-, R_3 = H
31, R_1 = H, R_3 = OSO_3^-, R_2 = H
32, R_1 = OH, R_2 = H, R_3 = OSO_3^-

2.2.9 Gonyautoxin-V

Gonyautoxin-V was first isolated from the Japanese and Alaskan PSP samples. Subsequently, it was found in a number of other organisms.[55,62,63] It is the

major toxin in the tropical dinoflagellate, *P. bahamense* var. *compress*.[64] The structure was established by several groups under different names.[65,66] Gonyautoxin-V (**33**) is the carbamoyl-N-sulphate of neosaxitoxin (**27**), and almost nontoxic. Hydrolysis of (**33**) with weak acid yielded neosaxitoxin (**27**) a potent toxin.



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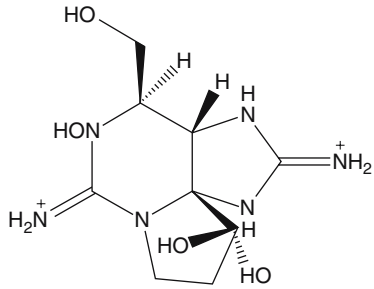
2.2.10 Gonyautoxin-VI

Careful chromatography of the mixture of toxins on Bio-Rex 70 yielded gonyautoxin -VI.[50, 58] The toxin was the carbamoyl-N-sulphate of saxitoxin (**1**) and was found to be identical with B_2 toxin.[65,66] Treatment of the toxin with dilute mineral acid afforded saxitoxin. A partial synthesis of gonyautoxin-VI was achieved by sulfonylcarbamoylation of saxitoxin and neosaxitoxin.[65]

2.2.11 Gonyautoxin- VII

Gonyautoxin-VII (**34**) was first found in a toxin mixture from the sea scallop, *Placopecten megallanicus*.[67] The toxin was subsequently found identical with decarbamoyl saxitoxin.[66, 68] An identical compound was later found in the little neck clam and considered to be a product of biotransformation in the shellfish.[69] A number of decarbamoyl derivatives of PSP toxins have been isolated from the tropical dinoflagellates *P. bahamense* var. *compressa*.[68]



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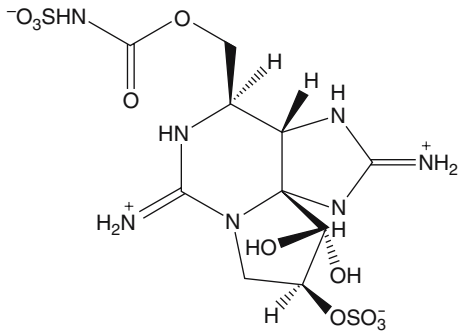
2.2.12 Gonyautoxin-VIII

Gonyautoxin-VIII is the first toxin that was found to have an N-sulfonyl group and a negative net charge on the molecule.[70,71] The toxin was characterized as carbamoyl-N-sulfonylgonyautoxin-III (**35**). It was easily isomerizes to epigonyautoxin-VIII. Treatment of the toxin with dil. mineral acid easily afforded gonyautoxin-III (**30**).



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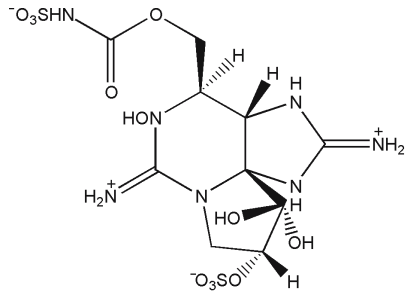
2.2.13 C_3 and C_4 Toxins

C_3 And C_4 toxins are called latent toxins.[63] They are not retained on a cation exchange column because of their net negative charge. The C_3 and C_4 toxins were assigned the structure (**36**) and (**37**), respectively.



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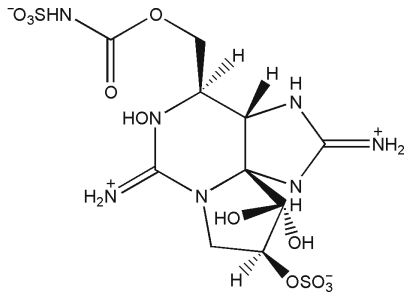

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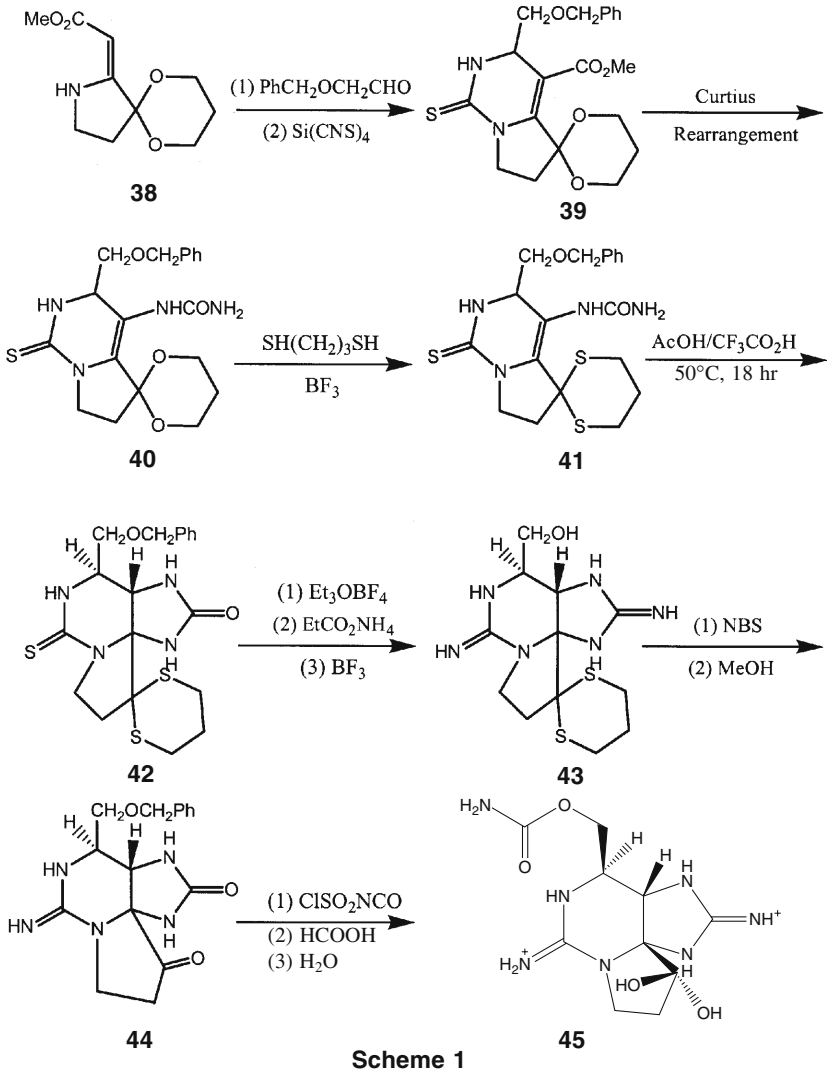
The structure of C_4 toxin was confirmed by X-ray crystallography. Treatment of the C_3 and C_4 toxins with dilute acids readily yielded gonyautoxin-I and IV, respectively. The presence of sulphate conjugation is characteristic of saxitoxin class of toxins. In the dinoflagellates, most toxins occur as 11-O-sulphate and/or N-sulphocarbamoyl derivatives, and saxitoxin (**1**) is a minor component. The occurrence of N-sulphated groups is rather rare among natural products. The N-sulphated groups are easily hydrolysed by weak acids and also possibly by the enzymes in the biological system. It is not yet clear whether the formation of the sulphated toxins precedes the unsulphated ones in the dinoflagellates. However, it has been proved that the reductive

cleavage of O-sulphate could take place in shellfish to give unsulphated toxins such as saxitoxin. Similarly, the N_1-hydroxy group of neosaxitoxin (**7**) series can be reductively removed. It is not yet known whether saxitoxin types of compounds are the precursors of neosaxitoxin types or *vice-versa* in the dinoflagellates.

2.2.14 Total Synthesis of (±)-Saxitoxin

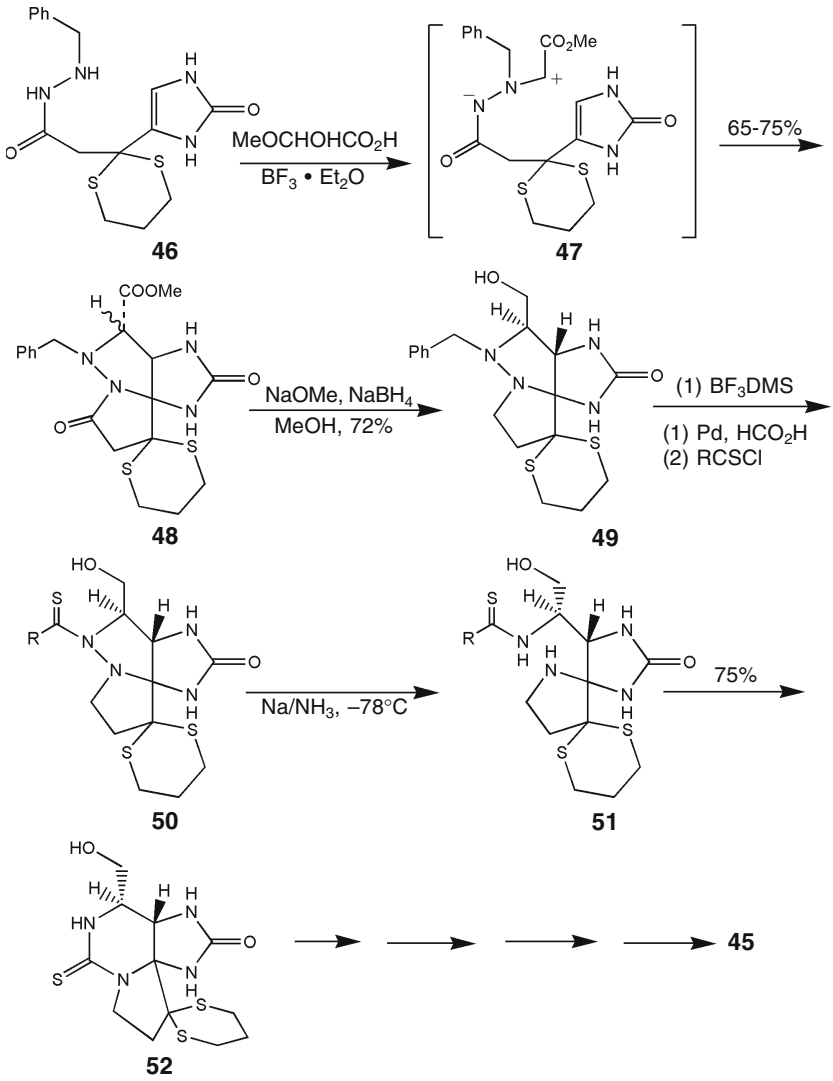
Synthesis of (±) saxitoxin has been achieved by two groups. Kishi's group[72] at Harvard was the first to report the synthesis of (±) saxitoxin. The strategy of the synthesis (Scheme 1) was to construct A/B ring first, and then attach the ring C (**38-45**).



Scheme 1

The key step in the synthesis was the acid catalyzed ring closure of the bicylic intermediate (**41**). Using a mixture of acetic acid and trifluoroacetic acid, the compound (**42**) was obtained in 50% yield. Removal of the protecting groups gave (**44**) which on treatment with chlorosulfonyl isocyanate followed by hydrolysis finally furnished (±)-saxitoxin (**45**).

Jacobi's group[73] reported a new synthesis of (±)-saxitoxin in 1984. The strategy in the synthesis (Scheme 2) (**46-52**) was to construct the C/D ring of saxitoxin first and then to add ring A. Thus the spirobicyclic intermediate (**51**) was cyclized to the tricyclic compound (**52**), which was then converted into (±)-saxitoxin (**45**) as in case of Kishi's synthesis.



Scheme 2

2.3 Detection of Paralytic Shellfish Toxins

Several human ailments, such as ciguatera, paralytic shellfish, and diarrhetic shellfish poisoning are caused by the ingestion of toxins produced by marine organisms. Initially, it was thought that PSP was restricted to temperate coastal areas and involved only filter feeding molluscs, recent evidence, however, indicates that the problem is widespread. It is now apparent that the toxins are present not only in molluscs and dinoflagellates but also in zooplankton, crab, red alga and a variety of interstidal organisms. It was imperative to develop qualitative and quantitative methods of detection of these toxins. The methods available now have been reviewed.[74-78]

2.3.1 Bioassays

The biological assays for marine toxins are most widely utilized method for their detection. A wide range of organisms are sensitive to the toxins and therefore are potential test organisms for a bioassay, but the mouse and housefly are the only species utilized to date. The mouse bioassay has been adopted by the Association of Official Analytical Chemists as an "Official Procedure," and is in use today as the primary analytical technique to support the majority of toxin-monitoring programmes in shellfish.

2.3.2 Sodium Channel Binding Assays

The pharmacological activity of the saxitoxins at the molecular level has been exploited in developing assay techniques. The toxins bind to sodium channels in nerve cell membranes, preventing the influx of sodium and subsequent depolarization of the membrane. A number of electrophysiological systems have been utilized for measuring the binding events. These are frog sciatic nerve,[79] voltage clamp of single nerve cell,[80] and blockage of sodium conductance through single-sodium channel isolated in lipid bilayers.[81] These techniques are useful for determining the pharmacological properties of the toxins. They are, however, unlikely to serve as routine assay techniques.

2.3.3 Immunoassays

Johnson et al[82,83] were the first to develop immunological techniques for assaying saxitoxins. Saxitoxin was coupled to bovine serum albumin (BSA) via formaldehyde treatment and antibody prepared from rabbit antiserum. Carlson et al[84] developed a radio-immunoassay (RIA) capable of detecting low level of saxitoxin. However, neosaxitoxin exhibited no cross reactivity. Chu et al[85] have developed an enzyme linked immunosorbent assay [ELISA] to the PSP toxins that is sensitive to about 2-10 pg STX. Since the toxicity of a shellfish extract is due to the collective effect of a number of different toxins present, the application of immunoassays for accurate detection of "Total Toxicity" is very difficult. However, immunoassay methods can be of much use as a rapid "Field Test" for detecting the presence of the PSP toxins.

2.3.4 Chemical Assays

Schantz et al[86] have developed a colorimetric assay based on the reaction of the saxitoxins with picric acid. The method, however, is not sensitive and prone to interferences. Gershey et al[87] have described a colorimetric test based on a reaction with 2,3-butanedione, but this was also subject to interferences. Bates and Rapoport[38] have reported a chemical assay for STX based on fluorescence of the 8-amino-6-hydroxymethyl-2-iminopurine 3(*2H*)-propionic acid a hydrogen peroxide oxidation product of STX. The method is extremely sensitive and fairly specific for the PSP toxins.

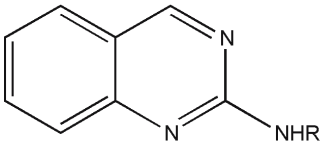
2.4 Tetrodotoxin

Tetrodotoxin (TTX) is the best known marine toxin because of its frequent involvement in fatal food poisoning, unique chemical structure, and specific action of blocking sodium channels of excitable membranes.[88] The toxin derives its name from the pufferfish family (Tetraodontidae) and occurs widely in both the terrestrial and marine animal kingdom.[89] The marked fluctuation of toxin concentration in TTX-containing animals from different regions, and seasons led to the belief an exogenous origin of the toxin in these animals. The primary source of the toxin was traced by Yasumoto et al[90] from fish to a dietary alga and finally to an epiphytic, or symbiotic, bacterium. The bacterium was first thought to be a *Pseudomonas* sp. then a *Alteromonas* sp. and finally *Shewanella alga*.[91] Subsequently, it was found that the toxin is produced by a broad spectrum of bacteria.[92-94] However, the identification of the toxin in bacterial cultures had been made on the basis of rather poor evidence.

2.4.1 Chemistry

Tetrodotoxin is a colorless crystalline compound. It is virtually insoluble in all organic solvents but soluble in acidic media. It is weakly basic having the composition $C_{11}H_{17}N_3O_8$. The molecule is small (mol. wt. 319), but possesses the remarkable feature that the number of oxygen and nitrogen atoms are equal to the number of carbon atoms. The chemistry and biology of tetrodotoxin has been extensively studied[95-101] and reviewed.[102-112] Woodward[95] demonstrated that the three nitrogen atoms of tetrodotoxin are present in the molecule as a guanidine moiety by isolating guanidine (as the picrate), following vigorous oxidation of the toxin with aqueous sodium permanganate at 75°C. Drastic degradations of the toxin by warm aqueous sodium hydroxide, pyridine-acetic anhydride followed by vacuum pyrolysis, phosphorus hydrogen iodide followed by potassium ferricyanide, and conc. sulfuric acid, gave closely related quinazoline derivatives of structure (**53**), where the nature of R depended on the exact mode of degradation. The formation of these key compounds indicated strongly that six of the 11 carbon atoms of tetrodotoxin are contained in a carbocyclic ring. It was surprising that in spite of the

presence of the guanidine function in the molecule, the toxin was only weakly basic (pKa 8.5) and attempts to prepare crystalline salts did not succeed. However, treatment of the toxin with 0.2 N hydrogen chloride in methanol-acetone did furnish a crystalline O-methyl-O′,O′-isopropylidene-tetrodotoxin hydrochloride monohydrate which was given structure (**54**) on the basis of X-ray crystallographic analysis.[95] If one element of acetone and methanol is subtracted from the molecular formula of the toxin derivative ($C_{15}H_{23}N_3O_8$) and adds two molecules of water one arrives at $C_{11}H_{17}N_3O_8$, the exact formula of tetrodotoxin.



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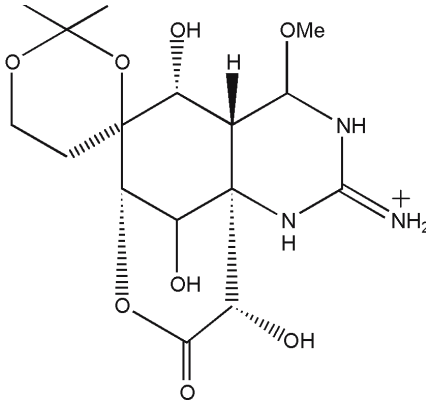
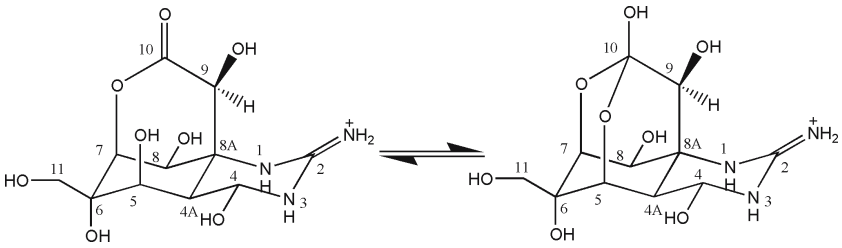


54

Comparison of the NMR spectra of compound (**54**) and tetrodotoxin further confirmed their close structural relationship. The two compounds, however, differ in one aspect. The compound (**54**) was a lactone having IR absorption band at 1751 $cm^{\pm}$ while the toxin itself lacked a lactonic infrared band. On the other hand, the IR bands assigned to the guanidine moiety (1638, 1605 $cm^{\pm}$) remained unchanged in the two compounds, thus demonstrating that the hydrochloride cannot be a guanidinium salt. The basicity of tetrodotoxin (pKa 8.5) was far too weak to be originating from the guanidine moiety. This fact, coupled with the observation that the pKa of the hydrochloride increased to 9.2 in aqueous dioxane, strongly suggested that the basicity of tetrodotoxin must be due to its Zwitterionic nature and that one of the hydroxyl groups is being titrated when the pKa is measured. Increased pKa is characteristic of hydroxyl ionization when one proceeds from a medium of high to one of low dielectric constant. That which of the hydroxyl groups in tetrodotoxin is sufficiently acidic to furnish a proton to nitrogen, was revealed by the NMR spectral measurements of heptaacetyl-anhydrotetrodotoxin. If the methylated precursor of (**54**) was to undergo acetylation, the product would exhibit three characteristic changes, resonances in the NMR arising from protons on carbon which also bear acetoxy groups, viz. C-5, C-7 and C-8. In fact only one such

resonance was present in the NMR spectrum of the heptaacetyl compound which forced the conclusion that two of the three groups cannot be present as free hydroxyls in tetrodotoxin, but must be combined in a new entity. If one of the hydroxyl groups combines with the lactones function to form a hemiacetal, only one characteristic proton should remain. Double resonance experiments proved that it is the C-5 hydroxyl in tetrodotoxin which is part of the hemilacetal (or a two-third orthoester) function. This consideration led to the assignment of structure (**55**) for tetrodotoxin. The presence of hemiacetal function in tetrodotoxin is unique (**55a, 55b**). This is the first example where a complex function of this nature is present in a natural product.

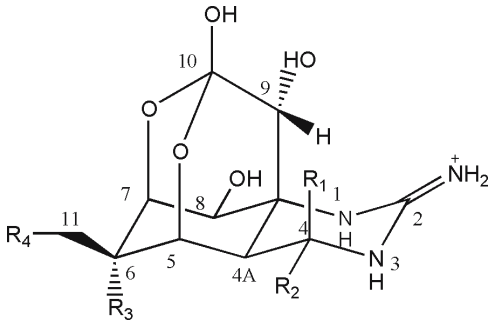


55a **55b**

The monomeric structure of tetrodotoxin was confirmed by single crystal X-ray diffraction studies by Woodward et al.[113] Measurement of the unit cell dimensions and the density of the crystals and consideration of symmetry requirements led to the unambiguous conclusion that crystalline tetrodotoxin is monomeric and contains two molecules per unit cell. The monomeric nature of tetrodotoxin in solution had been ascertained through a careful analysis of its titration curve.[99] About 1-2 g of crystalline precipitate (tetrodotoxin) was obtained from 100 kg of puffer ovaries by following Hirata's procedure.[99] Total synthesis of tetrodoxin was reported in 2004.[100]

2.4.2 Tetrodotoxin Derivatives

Detection of tetrodotoxin (TTX) derivatives occurring in puffers, newts, and a frog was facilitated by a highly sensitive TTX analyzer, which separates analogues on a reversed phase column and detects fluorescent products formed upon heating with sodium hydroxide solution.[114, 115] Yasumoto et al[116] have isolated tetrodotoxin (**55**), 4-epi-TTX (**56**), 6-epi-TTX (**57**), 11-deoxy-TTX (**58**) and 11-deoxy-4-epi-TTX (**59**) from newts collected in Okinawa, Japan, and assigned their structure mainly through NMR measurements. 11-Nortetrodotoxin-6 (*R*)-ol, 6-epi TTX (**57**) and 11-deoxy TTX (**58**) have been obtained from the puffers *Fugu niphobles*.[117] Chiriquitoxin,[118‡23] an unusual analogue of tetrodotoxin in which 11-CH_2OH of TTX had been replaced by a $CH(OH)CH(NH_2)$-CO_2H group, had been isolated from the Costa Rican frog *Atelopus chiriquiensis*.[118] The puffer *Arothron nigropunctatus* had



Compound	R_1	R_2	R_3	R_4
55, TTX	H	OH	OH	OH
56, 4-epi-TTX	OH	H	OH	OH
57, 6-epi-TTX	H	OH	CH_2OH	OH
58, 11-deoxy-TTX	H	OH	OH	CH_3
59, 11-deoxy-4-epi	OH	H	OH	CH_3
60, 11-oxo-TTX	H	OH	OH	CH_2OH

furnished 11-oxotetradotoxin hydrate (**60**) TTX, 4-epi TTX, 6-epi TTX, 11-deoxy TTX, 11-nor TTX-6(*R*)-ol.[115] Two epimers of 11-nor TTX are likely to be decarboxylation products of a hypothetical 11-CO_2H derivative.

Those analogues found in puffers and newts were not found in a Costa Rican frog, *Atelops chiriquiensis* which contained TTX and chiriquitoxin. Interestingly, l-oxo TTX (**60**) was more active than TTX in blocking sodium channels.[124,125] Other analogues were less potent than TTX. A pocket shaped model has been proposed for binding site in the sodium channel protein. The charge groups in a clevis of channel protein supposedly act as anchoring points by interacting with the toxin's functional groups orienting in different directions.[125]

2.4.3 Mechanism of Tetrodotoxin and Saxitoxin Action

Tetrodotoxin (**55**) and saxitoxin (**1**) are the most widely studied marine toxins by physiologists and pharmacologists. In spite of their structural dissimilarities, both are known to inactivate the sodium channel in the skeletal muscles and nerve tissues of various animals. The effect of these toxins is specific as both selectively block the transient Na^+ current without any effect on the steady state current by K^+ ions. Owing to this specific action of these toxins, many investigators are using them as tools in the characterization of ion channels. These toxins have become an extremely useful and popular chemical tool for the study of neurophysiology and neuropharmacology. Tetrodotoxin binds to the entrance part of the Na^+ channel and inhibits Na^+ channel and Na^+ influx, and generates an active potential, thus, causing the blockade of nerve of muscle function. Narahashi[126] has reviewed the mechanism

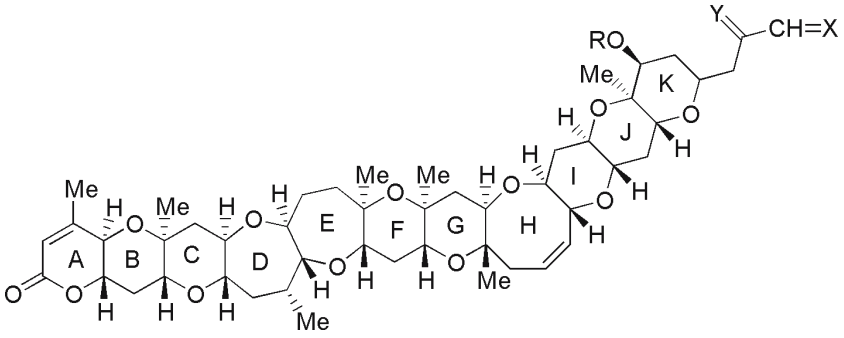
of tetrodotoxin and saxitoxin action. Tetrodotoxin is commercially available and in carefully controlled doses is being used as muscle relaxant and pain killer in neurogenic leprosy and terminal cancer.

3. Neurotoxic Shellfish Poisoning

The dinoflagellate *Gymnodinium breve* (*Ptychodiscus brevis*) often forms blooms along the Florida coast and this leads to mass mortality of fish. Large blooms of this organism (red tide) can kill hundreds of tons of fish a day. Sometimes the blooms cause human irritation in eyes and throat in the coastal areas, and the contamination of shellfish occasionally result in human intoxication. The symptoms of neurotoxic shellfish poisoning (NSP) are mild which generally subside within 36 hrs, and include tingling and numbness in the mouth and digits, ataxias, hot cold reversal of temperature sensation, myadriasis, reduced pulse rate, diarrhea and vomiting.[126] NSP is not a lethal human intoxication.

3.1 Brevetoxins

Several toxins have been isolated from the toxic dinoflagellate, *Gymnodinium breve*[127] and named brevetoxins.[128-131] Brevetoxin-B (GB-2 toxin) (**61**),[132-137] brevetoxin-C (**62**),[138] GB-3 toxin (**63**),[139] GB-5-toxin (**64**),[140] and GB-6 toxin (**65**),[140] GB-7 toxin (**67**)[127] had been isolated from *G. breve*. Of the isolated toxins, brevetoxin-A (GB-l toxin) (**66**)[127] is the most potent toxin in the Florida Red Tide organism *G. breve* (*Ptychodiscus brevis*). Brevetoxins, particularly brevetoxin B is associated with the "red tide" catastrophes that occurs along coastal areas around the world. It is highly poisonous to human life and cases death.[141] The term "red tide" means seawater decoloration by vast blooms of phytoplankton, which constitute the base of the marines food chain. The name "red tide" derives from the red coloration of seawater during this process, although "red tide" may be brown, green or even colorless.



61, X = O, Y = CH_2, R = H (Brevetoxin-B)
62, X = HCl, Y = O, R = H (Brevetoxin-C)
63, X = HOH, Y = CH_2, R = H (GB-3)
64, X = O, Y = CH_2, R = H (GB-4)
65, X = O, Y = CH_2, R = Ac (GB-5)

The story of brevetoxin B may be summarized as for a passage in the bible mentions an event that some believe high have been the first recorded incident of a "red tide."

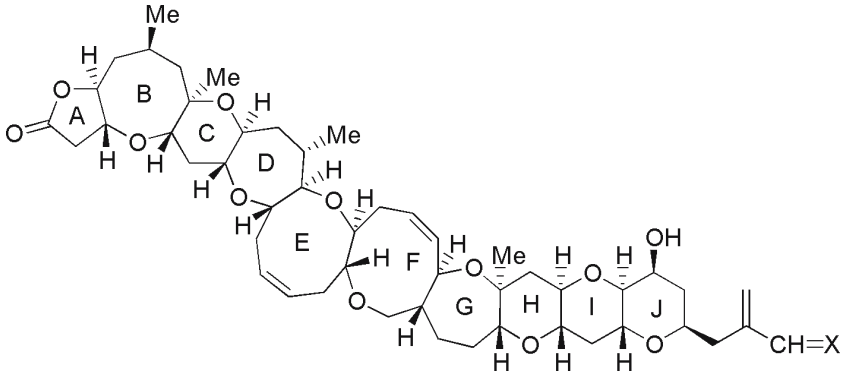
". . . and the waters that were in the river were turned to blood. And the fish that were in the river died, and the river stank and the Egyptians could not drink the water of the river . . ."

[Exodus 7:20-21, 1000 B.C]

The toxin is of particular interest not only because it is the most potent toxin of the family but also because it uniquely binds to sodium channels on excited membrane.

3.1.1 Brevetoxin-A

Brevetoxin-A (**66**)[142,143] was isolated from the cultured cells of *G. breve* by partition and successive chromatographic separation.[127] It forms fine prisms, m.p. 197-199°C; 218-220°C (double-melting point) from acetonitrile. High resolution FAB mass spectrometry gave the molecular formula $C_{49}H_{70}O_{13}$. The ^{1}H and ^{13}C NMR spectra of the toxin showed the presence of two secondary and two tertiary methyl groups, one methylene, one aldehyde, two disubstituted *cis* double bonds, and a carbonyl group. The IR band at 1790 cm^{-1} suggested that nonaldehyde carbonyl group belongs to α-lactone. On the basis of extensive spin-spin decoupling, proton-proton decoupling correlation (COSY), and proton-carbon correlation spectroscopy (hetero COSY) experiments assigned the structure (**66**) to brevetoxin-A which was confirmed by X-ray analysis of a dimethyl acetal derivative (**68**) of the toxin.[127]

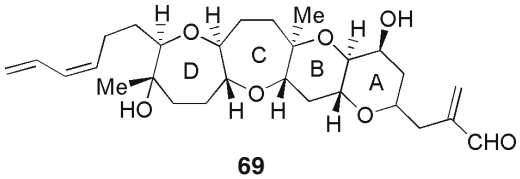


66, X = O (Brevetoxin-A)
67, X = H, OH
68, X = 2 OMe

3.1.2 Brevetoxin-B

Unialgal cultures of *Ptychodiscus brevis* Davis *(Gymnodinium brevis* Davis), isolated during an outbreak at Florida, were grown in an artificial sea-water medium. The medium containing the cells was acidified to pH 5.5, and

extracted with diethyl ether to give 90 mg of crude brevetoxins. Repeated flash chromatography of the crude toxin mixture with 5% methanol in diisopropyl ether (v/v) gave BTX-A (**66**) (0.8 mg), BTX-B (**61**) (5 mg), and BTX-C (**62**) (0.4 mg). Purity of the various toxins was checked by HPLC.[132] The toxins were monitored for ichthyotoxicity with the fresh water "Zebra" fish, *Brachydano rerio*. The lethal doses (LC_{50}) to kill these 0.2-0.6 g fish in 1 hr were as follows: BTX-A, 3 ng/mL; BTX-B, 16 ng/mL; and BTX-C, 30 ng/mL.[132] Brevetoxin-B (**61**) crystallizes from acetonitrile as colourless needles, m. p. 270°C (dec.), mol. for. $C_{50}H_{70}O_{14}$, UV (MeOH) λ_{max}208 nm (ε1600 enal), FT-IR (KBr pellet) 1735 cm^{-1} (lactone), 1691 cm^{-1} (enol). The structure and stereochemistry of the brevitoxin-B (**61**) was determined by X-ray crystallography and NMR studies.[132] Brevetoxin-B[133-137] (**61**) is made up of a single carbon chain locked into a rigid ladder like structure consisting of 11 continuous transfused ether rings. There was no precedent of this extraordinary structure. The remarkable features of the structure of brevetoxin-A (**66**) are the unprecedented presence of all ring systems from five to nine, membered in a single molecule. Another characteristic structural feature is that in the crystal form, the molecule has a 90° twist at ring G. The molecule is essentially composed of two perpendicularly linked polycyclic sheets, rings A-F and rings H-J. It has been found that in solid state, ring G is boat-chair (BC) form. In brevetoxin-B (**61**) and its derivatives, the molecules are essentially planar. It is speculated that brevetoxin-A (**66**) molecule undergoes a rather slow conformational change between BC and crown form in solution.[144] Continued interest on the toxins of *G. breve* led to the isolation of other compounds named hemibrevetoxin-A, B, and C. Hemibrevetoxin-B has been assigned structure (**69**).[145] The structure constitutes essentially the right half of brevetoxin molecules. It was speculated that brevetoxins could be biosynthesized through a cascade of epoxide ring openings triggered by protonation on the carbonyl group at the left terminus of the carbon chain. An alternative mechanism has been suggested in which the cascade is initiated from the right hand side by opening of *cis*-epoxide followed by a hydride ion transfer and consecutive *trans*-epoxide openings.[145] Hemibrevetoxin-B causes the characteristic rounding of cultured mouse neuroblast cells as brevetoxin-A and B. It also showed cytotoxicity at a concentration of 5 mol.[145]



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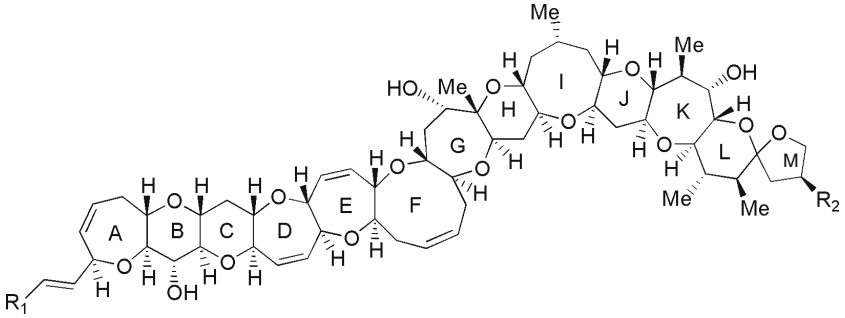
4. Ciguatera (Seafood Poisoning)

The term ciguatera is used to food poisoning caused by ingestion of toxic coral reef fish. Ciguatera not only endangers public health but also hampers

local, fisheries in tropical and subtropical regions of the world. It is estimated that roughly 20,000 people suffer annually from such poisoning. Two groups of compounds implicated in the poisoning are ciguatoxin[147,156] and maitotoxin.[157,164] Both groups of toxins are produced by the epiphytic dinoflagellate *Gambierdiscus toxicus*[165] and transferred to herbivorous fish and subsequently to carnivores through the food chain. Ciguatoxin is regarded as the principal toxin responsible for human illness. The clinical symptoms are diverse.[166] Of these neurologic disturbances are prominent. The most characteristic symptoms of ciguatera are reversal of thermal sensation called "dry ice sensation." Other symptoms are joint pain, miosis, erethism, cyanosis, and prostration. Gastrointestinal disorders are nausea, vomiting and diarrhea. Cardiovascular disturbances are low blood pressure and bradycardia.

4.1 Ciguatoxin and its Congeners

Scheuer's group[167] at the University of Hawaii in 1980 was the first to isolate ciguatoxin (CTX) and characterize it to be a polyether compound. However, an inadequate amount of material prevented this group from determining the structure. Yasumoto' s group in 1989[168] finally elucidated the structure of ciguatoxin. Ciguatoxin (**70**) (0.35 mg) was obtained from the visera of moray eel, *Gymnothorax javanicus* (125 kg). A less polar congener (**71**) (0.74 mg) was obtained from the causative epiphytic dinoflagellate *Gambierdiscus toxicus*, collected in the Gambier island.



70, R_1 = CHOH–CH_2OH, R_2 = OH
71, R_1 = CH_2 = CH; R_2 = H
72, R_1 = CH_2 = CH, R_2 = OH

HR-FAB MS suggested that ciguatoxin had molecular formula $C_{60}H_{86}O_{19}$ (MH^+, *m/z* 1111.3314). In ^{1}H NMR spectra of (**70**) or (**71**) measured at 25°C, signals due to H-22 through H-31 were extremely broadened or missing probably because of slow conformational perturbation of ring F. However, the problem was solved when measurements were taken at low temperatures (−20 or −25 °C) in which missing signals appeared, and broad signals sharpened. Rings F and G were presumed to take a single conformation at the low temperature. The proton connectivities, including hydroxy protons in the

toxin, were mainly established by ^{1}H-^{1}H COSY data obtained under various conditions. The COSY measured at low temperatures clearly revealed connectivities of C_1-C_{32}, C_{34}-C_{38}, C_{40}-C_{51} and C_{53}-C_{55}, but left the skeletal chain in three fragments due to the presence of two quaternary carbons (C_{33} and C_{52} and an unassignable methine C_{39}). Ether linkages were elucidated by NOEs observed on protons or a methyl (C_{56}) attached to ring junction oxycarbons. All the ring fusions are *trans* because coupling constants of angular protons were typical for an antiperiplanar substitution on oxy-carbons. Structural alteration of **(70)** and (**71**) were readily determined on the basis of 2D NMR data, which showed the presence of a *trans*-butadiene moiety and deoxidation at C_{54} in (**71**). The structure and stereochemistry of ciguatoxin (CTX) is shown in (**70**) and its congener tentatively named gambiertoxin-4b (GT4B) (**72**) had been assigned by combined use of ^{1}H NMR, 2D correlation and NOE experiments done with no more than 0.35 mg of CTX and 0.74 mg of the congener. The structure of ciguatoxin was shown to be (**70**) brevetoxin-type, polyether comprising 13 continuous ether rings. The relative stereochemistry except for C_2 of CTX have been clarified.[169] CTX (**70**) was obtained as a white solid from moray eel viscera. The lethal potency of the toxin against mice (ip) was 0.35 g/kg. From *G. toxicus*, GT4b (**72**) was isolated as a white amorphous solid with a potency of ca. 4 g/kg. Physico-chemical properties of these toxins are: CTX: no UV maximum above 210 nm; IR (film) 3400, 1111, and 1042 $cm^{\pm}$. GT4b: UV λ_{max} (CH_3CN) 223 nm (ε 22,000), IR (film) 3400, 1620 and 1040 $cm^{\pm}$. Ciguatoxin (**70**) and its congener (**71**) was extracted in 0.35 mg, and 0.7 mg respectively from moray eels (*Gymnothorax javanicus*) collected in French Polynosian waters.[168-171] Since 1989, chemical studies on ciguatera toxins had made rapid strides. Ciguatoxin congeners had been isolated either from toxic fish (54-deoxy ciguatoxin)[170] or from cultured *G. toxicus*.[172] Furthermore, a dozen of ciguatoxin analogs had been found in fish and in the dinoflagellates.[173] However, only a few of them have been characterized.

The moray eel, which is placed near the top in the coral ecosystem was found to contain more polar (more oxygenated) congeners, whereas the dinoflagellate produces less polar ones. Ciguatoxin (**70**) itself, the more oxygenated member of this class of toxin was absent in the flagellates. These data suggested that less polar congeners produced by *G. toxicus* were probably the precursors of the more polar toxins found in fish. The latter appear to be formed by oxidative enzyme systems in the fish. Interestingly, toxicity of more oxygenated metabolite is often increased, as in the case with ciguatoxin which is 11 times more toxic than its plausible precursor (**72**).

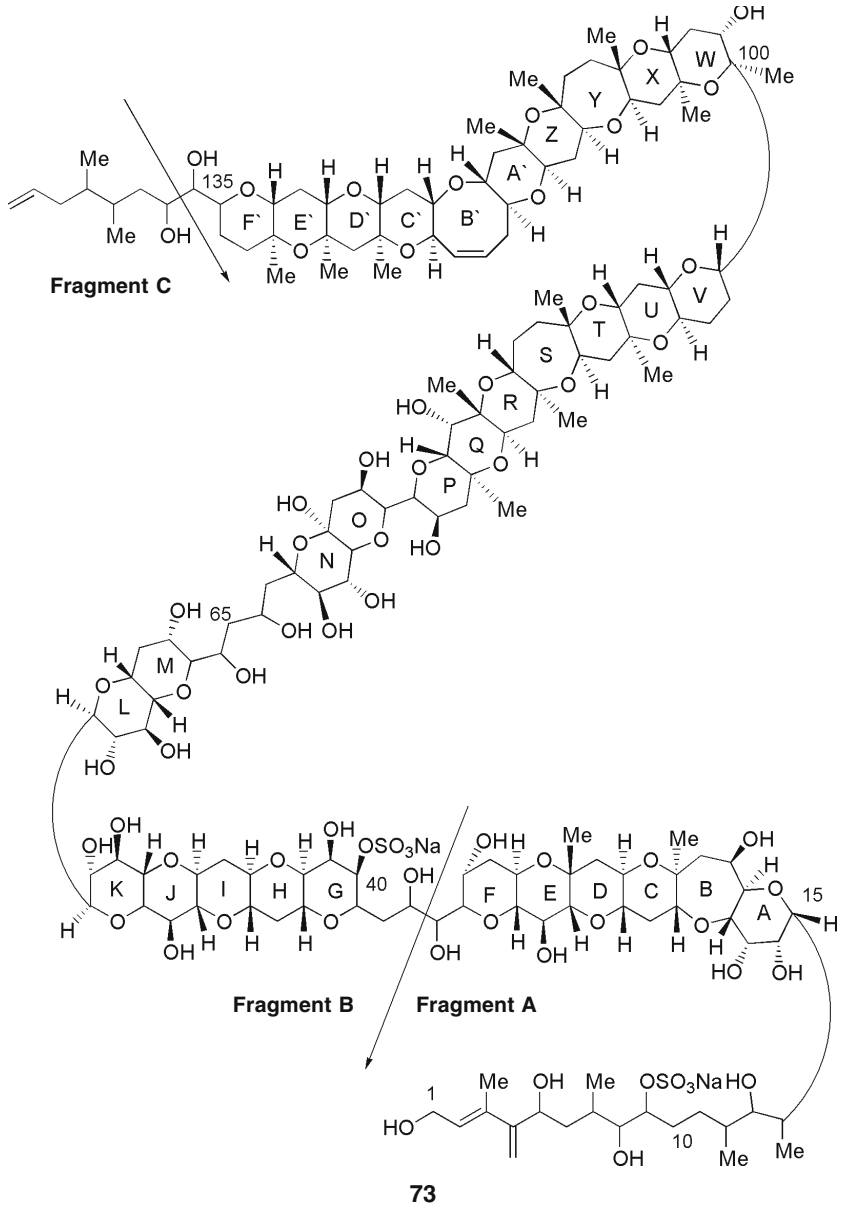
4.2 Mode of Action of Brevetoxins and Ciguatoxins

Pharmacological studies on brevetoxins and ciguatoxins had revealed that the primary site of their action is voltage-sensitive sodium channel (VSSC).[174,175] Brevetoxin-B (**61**) activated VSSC in neuroblastoma cells in

the presence of veratridine. The action was blocked by tetrodotoxin.[174] A binding assay using a radioligand of brevetoxin, tritiated derivative of brevetoxin-B at 42-aldehyde indicated that both toxins shared the same binding site on the VSSC.[176] Several studies have been carried out on ciguatoxin (**70**) to understand its mode of pharmacological and toxicological action because its central role in human illness. Earlier it was thought that CTX is a inhibitor of choline esterase.[177] However, subsequently it was revealed that CTX stimulated sodium ion influx into cells.[178] The polyether toxins of brevetoxins types possess seven-eight, and nine-member ring(s) in the middle of the molecule, which undergo slow conformational changes. Ciguatoxins and gambieric acids possess of 9,7-bicyclic system[179,180] (rings F and G of ciguatoxin), brevetoxin-B has 7,7-bicyclic system. During alteration in conformation, the molecule probably flips around the hinge part. It is speculated that these slow conformational changes have something to do with the binding to VSSC and then lead to alteration of the gating mechanism (the inactivation mechanism) of the channel.[179]

4.3 Maitotoxin

Maitotoxin (**73**)[157,164] had attracted much attention mainly for three reasons: First, it had molecular formula $C_{165}H_{258}Na_2O_{67}S_2$ and molecular weight of 3421.6 Da (as a disodium salt), which exceeded that of any other known natural product,[181] except for biopolymers. Secondly, it presumably plays a role in diversifying ciguatera symptoms, particularly in the poisoning caused by herbivorous fish. Thirdly, it had extremely potent bioactivity. The lethality against mice LD_{50} was ca. 50 ng/kg (ip), which suggested that it might be the most potent, nonproteinous toxin.[181] Thus, the structure determination of maitotoxin was regarded as one of the most exciting challenge in natural product chemistry. Maitotoxin (**73**, MTX) was isolated in 1988 from cultured cells of *Gambierdiscus toxicus*.[181] Approximately 25 mg of MTX was obtained from 5000 L of culture. The structure of MTX has been proposed on the basis of extensive spectroscopic analysis.[182,183] Overlapping as well as poor resolution of both ^{13}C and ^{1}H NMR signals due to the large molecular size prevented from applying routine methods, for structural analysis. In particular the NMR signals arising from the central part of the molecule tended to be broadened because of the predominance of fused cyclic ethers. Several overlapping of both ^{13}C and ^{1}H NMR signals were overcome by repeated measurements in different solvents and by application of new NMR methods. Finally, with the help of 2D NMR a partial structure was initially deduced.[182] The presence of two sulphate esters functions in MTX was deduced by FABMS of the desulfated product (ds-MTX). The position of one sulphate function was assigned to C_9 by comparison of COSY spectra of ds-MTX with MTX. The location of hydroxyl groups was determined mainly by combined use of ^{1}H-^{13}C COSY spectra and deuterium shifts in ^{13}C NMR signals. The six ether rings in the partial structure were assigned on the basis



of NOEs observed as intense cross-peaks in NOESY spectra. NOESY experiments provided essential information not only for connectivities around the ether bonds but also for correlating structural data vicinal to quaternary carbons (C_{22} or C_{28}).

The partial structure was confirmed by periodate degradation. The studies carried out so far demonstrated that MTX had a polyether skeleton similar to brevetoxins, having approximately 32 ether rings, 28 hydroxyl, 21

methyl group and two sulfate esters. The half of the molecule of MTX which included fragment A (**73**), was relatively hydrophilic, while the other half, comprising mostly contiguous fused rings, was hydrophobic, thus accounting for the dual polarity of the toxin.

Treatment of MTX (8.1 mg) with $NaIO_4$ followed by $NaBH_4$ yielded two fragments. Fragment A (1.6 mg) (**73**), and fragment B (5.1 mg) (**73**). The latter fragment was subjected to extensive 2D NMR and FAB MS experiments. Negative FAB MS provided essential information to confirm the structure of fragment B. Since fragment B had a sulphate ester function at C_{40} near one terminus of the molecule, a negative charge was localized at the point, thereby allowing fragments arising from that part of fragment B in the mass spectrum. 2D NMR data enabled to connect fragment B with the rest of the molecule, fragment A and C. Thus, the entire structure (**73**) for maitotoxin was assigned. In maitotoxin the most of the ether rings were probably transfused as in the case with brevetoxin[132] except for ring L/M and N/O, for which NOE data suggested *cis*-fusion.

4.3.1 Mode of Action of Maitotoxin

More than 50 papers are published on the pharmacology and biochemistry of maitotoxin (MTX). The toxin stimulated Ca^{2+} influx across the biomembrane which could be blocked by verapamil, suggesting that MTX acted on a voltage-sensitive Ca^{2+} channel.[184] Lately, diverse actions of MTX have been reported.[185] These are muscle contractions, stimulation of hormones/ neurotransmitter release, activation of phospholipase-C and A_2 and activation of protein kinases some of which do not appear to be directly linked with simple elevation of intracellular Ca^{2+} concentration.[185] Its specificity to tissues or cell lines implies that the primary target of MTX is not a physiological receptors but a ubiquitous membrane component. Recent electrophysiological studies have revealed that the channel activated by MTX has ion selectivity and passes more Ca^{2+} than Na^+, the ratio between Ca^{2+} and Na^+ being about 50:1.[186] It has been suggested that most of MTX actions are probably acted through a receptor-mediated channel.[187] Maitotoxin mode of action appears to be similar to that of dihydropyridine which causes smooth muscle to contract by opening calcium channels.[188]

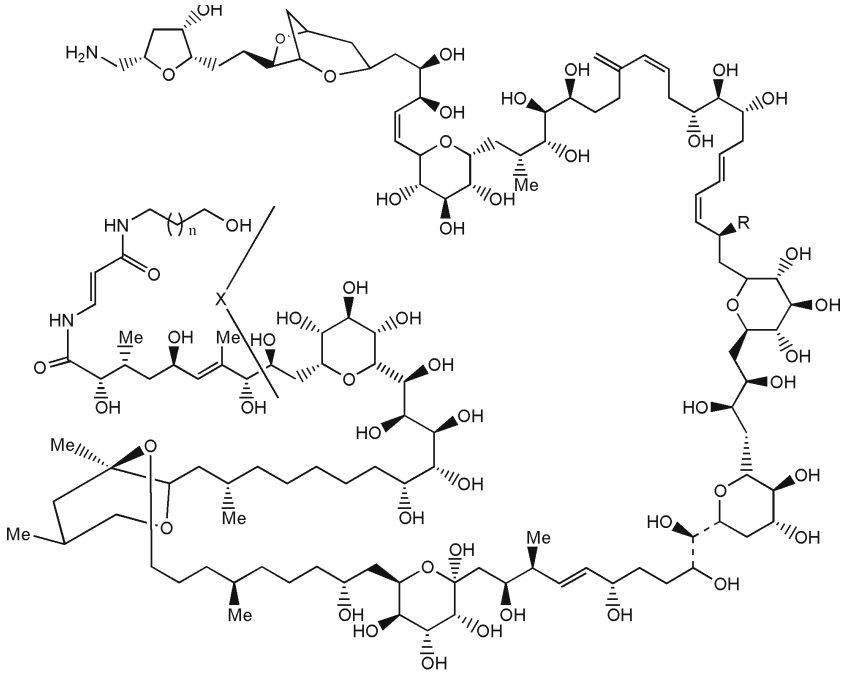
4.4 Palytoxin and Its Congeners

Palytoxin (PTX) (**74**)[189-195] is an extremely poisonous substance found originally in the genus *Palythora* of marine coelenterates.[196] Its intravenous lethality (LD_{50}) was 0.025 g/kg in the rabbit and 0.45 mg/kg in the mouse.[197] The toxicity of *Palythora* was probably noticed for the first time by Hawaiians who used exudates of a rare, but very toxic species, *P. toxica* to poison spear tips for warfare. The Japanese workers became interested in the toxin of *P. tuberculosa* when they traced the dietary origin of a water soluble toxin that had been found in the digestive tract of some toxic filefish *Alutera scripta*.[198,199]

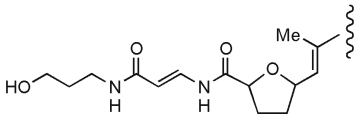
The toxin named aluterin isolated by the Japanese workers was subsequently found identical with palytoxin. The same toxin was found in *P. vestitus,*[200] *P. mamillosa*[201] and *P. caribaeorum*.[201,202] Palytoxin (PTX) or its analogues have not only been found in *Palythora* soft corals but in wide variety of other marine organisms,[203] such as seaweed *Chondria armata,* crabs belonging to the genus *Demaria* and *Lophozozymus,* a trigger fish *Melichthys vidua* and a file-fish *Alutera scripta.* Continuous interest in the toxins of *Palythora* spp. led to the isolation of four minor toxins characterized as homopalytoxin (**75**), bishomopalytoxin (**76**), neopalytoxin (**77**) and dideoxypalytoxin (**78**) from *P. tuberculosa*.[204]

4.4.1 Isolation

Moore and Scheuer[196] were the first to isolate palytoxin from *T. toxica.* The toxin could be completely extracted with 70% ethanol-water from the ungrounded wet animal. Reverse phase chromatography of the defatted extract on powdered polyethylene separated the toxin from inorganic salts and other polar organic materials. Elution of the column with 50% aqueous ethanol gave readily the toxic fraction. Successive ion exchange gel filtration of the



74, n = 1; R = OH
75, n = 2; R = OH
76, n = 3; R = OH
77, n = 1; R = OH, X =



78, n = 1; R = H

toxic material first on DEAE-Sephadex at pH 7 and then on CM-Sephadex at pH 4.5/5, yielded the pure palytoxin in 0.027% yield based on the wet weight of the animal. A similar procedure was used by the Japanese investigators to isolate palytoxin from *P. tuberculosa*.[198] The reverse-phase chromatography was carried out on polystyrene gel instead of powdered polyethylene. Separation of neopalytoxin from palytoxin was not feasible by HPLC, but was possible by HPTLC.[204] Palytoxin was a colorless, water soluble, amorphous, hygroscopic solid. Attempts to crystallize the toxin and various derivatives did not succeed. The toxin had $[\alpha]_D$ + 26° in water and its optical rotatory dispersion (ORD) curve exhibited positive cotton effect with $[\alpha]_{250}$ + 700° and $[\alpha]_{215}$ + 600.[196]

4.4.2 Chemistry

The exact molecular weight 2680.5 dalton and elemental composition, $C_{129}H_{225}N_3O_{54}$, however, were not known unambiguously until after the gross structure had been determined.[205] Hirata et al[206] succeeded in calculating the molecular weight 2681 by ^{252}CF plasma desorption mass spectrometry. A better resolved spectrum and a more precise value 2680.9 dalton were obtained by fast atom bombardment mass spectrometry.[207] The ultraviolet absorption spectrum of the toxin in water had two intense peaks at 233 nm (ε40,500) and 263 nm (ε 23,600). The IR spectrum had a band at 1670 cm$^{\pm}$ for an amide function. Evidence for the weakly basic nitrogen of palytoxin was obtained when the toxin was treated with p-nitrophenyl acetate. Interestingly, the 600 MHz ^{1}H NMR spectrum of N-acetylpalytoxin in dimethyl sulfoxide-d_6 gave well resolved signals for many of the hydroxyl protons. The ^{13}C NMR spectrum showed that there were only three sp^3 quaternary carbon signals. Chemical degradation studies were indispensable for solving the gross structure of palytoxin. Acid and base hydrolysis did not yield much structural information as most of the molecule remained intact. However, ozonolysis and periodate oxidation of N-bromobenzoylpalytoxin provided useful information. The absolute stereochemistry of palytoxin as depicted in structure (**74**) has been unambiguously determined by Hirata's X-ray crystallographic studies[208] and Kishi's synthetic work.[207,209–211]

The structure elucidation of palytoxin (PTX) posed a tremendous challenge to the organic chemists as the toxin had a molecular weight of about 2681 dalton, but lacked repeating units commonly found in biomolecules of this size. With the advent of better separation techniques and analytical methods, in particular high pressure liquid chromatography to separate the complex mixtures that palytoxin produced on chemical degradation and high frequency NMR spectroscopy and field desorption mass spectrometry it became possible to solve the problem. The chemistry of palytoxin had been reviewed.[212]

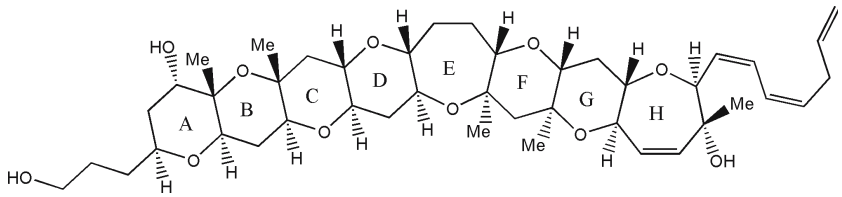
4.4.3 Mode of Action

Extensive pharmacological and biochemical studies have been carried out

on palytoxin.[213] The acute toxicity of PTX in several animal species had been examined in detail. The lethal doses (LD_{50}) of the toxin in rats, mice, guinea pigs, rabbits, dogs, and monkeys ranges between 0.033 and 0.45 g/kg. However, when PTX was administered by the intragastric or intrarectal route, it was found relatively nontoxic. Membrane depolarization, Na^+ or Ca^{2+} influx, stimulation of arachidonic acid release, stimulation of neurotransmitter release, inhibition of Na^+/K^+-ATPase, induction of contraction of smooth muscles and tumor-promoting, studies had been carried out on palytoxin, while it is proposed that palytoxin (PTX) acts through Na^+K^+-ATPase,[213] detailed mechanism of its action is still largely unknown. The primary mode of action of PTX accounting for its variable biological effects is not fully clarified.

4.5 Gambierol

Ciguatoxin congeners and maitotoxin had been isolated from the natural blooms of the marine dinoflagellate *Gambierdiscus toxicus* and assumed that this dinoflagellate is the biogenetic origin of toxins implicated in cigutera fish poisoning, which is prevalent in tropical region. Gambierol (**79**),[214-221] a new toxin reminiscent of ciguatoxin by its ladder-shaped polyether skeleton, was isolated from *G. toxicus* (RGI-strain) cultured cells.[222] *G. toxicus* (RGI-strain) was collected at Rangiroa Atoll, French Polynesia and cultured in seawater. The cultured cells were extracted with MeOH and the extract partitioned between CH_2Cl_2 and $MeOH/H_2O$ (6:4). The toxin was extracted into the organic phase and was further purified by guided mouse bioassay. 1100 L Culture furnished 1.2 mg of gambierol (**79**) as an amorphous solid: FAB-HRMS $[M^+Na]^+$ *m/z* 779.4348. Molecular formula $C_{43}H_{64}O_{11}$ UV λ_{max} (MeOH) 237 nm (ε15,800); LD_{50} in mouse (ip) 50 g/kg. The ^1H NMR spectrum of the toxin showed five singlet-methyl, three hydroxyls, and 4-olefinic (two conjugated) bonds. Analyses of ^1H-^1H COSY and 2D HOHAHA spectra allowed to connect protons H_1-H_6, H_8-H_{10}, H_{12}-H_{18}, H_{19}-H_{20}, H_{24}-H_{29} and H_{31}-H_{38}. Protons $H_{2\text{-}18}$ and $H_{2\text{-}19}$ were difficult to connect by COSY because of the closeness of their chemical shifts. However, a 2D HOHAHA spectrum clarified their connectivity. The number of methylene groups between C_{17} and C_{20} was determined to be two by HSC measurements. NOEs between angular protons or between an angular proton and a singlet methyl, as observed in NOESY and NOE difference spectra, supported the notion that ether ring A-H were *trans*-fused. No NOE between H_{16} and Me-41 was observed at



79

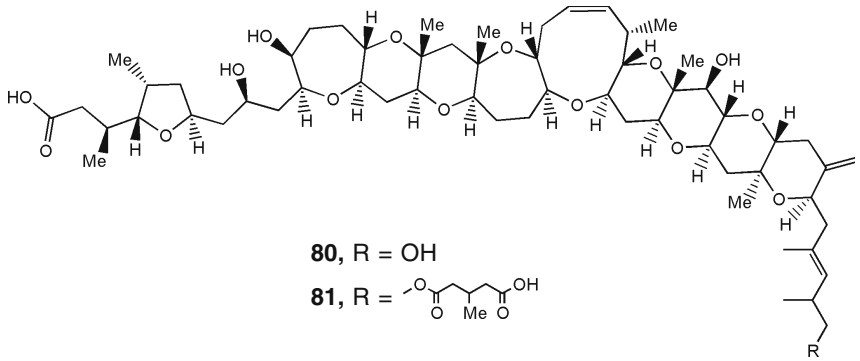
room temperature, probably due to perturbation of ring E, but was clearly detected at ⩾20 °C, as had been the case with ciguatoxin. Coupling constants of angular protons (10 Hz) also supported *trans*-fusion of rings.

The results led to (**79**) as the planar structure of gambierol[222] including relative stereochemistry. The ring system of **79** (6/6/6/6/7/6/6) differs from toxic polyether brevetoxin,[132] and ciguatoxin,[169] thus demonstrating the diversity of ciguatera toxins and the complex biosynthesis of polyether compounds in dinoflagelletes. Production of (**79**) by cultured *G. toxicus* (RGI-strain) and the resemblance between gambierol (**79**) and ciguatoxin (**70**) in molecular size, chromatographic properties and symptoms caused in mice strongly support the hypothesis that *G. toxicus* is the true cause of ciguatera.[222] To date, three total syntheses of gambierol (**79**) have been accomplished.[216-220]

4.6 Gambieric Acids

Marine dinoflagellates produced polyether compounds many of which inhibit the growth of fungi.[223] One strain of *Gambierdiscus toxicus* (GIII strain), an epiphytic species is implicated in ciguatera. During culture the toxins were retained in the algal cells, the antifungal agents were released into the medium. Activity guided purification led to the discovery of four new polyethers designated as gambieric acid, A, B, C and D (GA-A, GA-B, GA-C and GA-D).[224226] Their activity of inhibiting the growth of *Aspergillus niger* was unprecedented potency, exceeding that of amphotericin B by a factor of 2×10^3 [GA-A, GA-B, and a mixture of GA-C and GA-D at 10, 20 and 10 g/disk, respectively] by the paper disk method, while amphotericin B and okadaic acid were inhibiting at doses of 20 and 10 g/disk, respectively. Further, GA-A at a dose of 1 mg/kg showed no toxicity against mice (ip). *G. toxicus* (G III strain), isolated in the Gambier Islands, French Polynesia, was cultured in a seawater medium at 25°C for 38 days. The medium (5000 L) free of alga cells was passed through a column of Amberlite XAD-2. The antifungal agents retained on the column were eluted with MeOH. Purification of the mixture was carried out by solvent partition and column chromatography. Three active constituents were obtained, Ga-A (0.6 mg), Ga-B (0.15 mg), and a mixture of Ga-C and Ga-D (5.8 mg). The major antifungal activity resided in the mixture, but Ga-C and Ga-D were inseparable even by HPLC. Negative FABMS of the mixture suggested molecular weight of 1184 for Ga-C and 1198 for Ga-D. Hydrolysis of the mixture in a methanolic NaOH solution yielded Ga-A (4.1 mg), Ga-B (0.9 mg), and 3-methyl glutaric acid. Thus, Ga-C and Ga-D apparently were 3-methyl glutarate hemiesters of Ga-A and Ga-B, respectively. Ga-A (**80**) was obtained as a white amorphous solid $[\alpha]_D$ + 33° (c, 0.488, MeOH); UV (MeOH) λ_{max} < 210 nm; IR (KBr) 3500, 1735 $cm^{\pm 1}$; HR-FABMS $[M^+Na]^+$ *m/z* 1079.6330 (1079.6280 calcd for $[C_{59}H_{92}O_{16}Na]^+$. IR suggested the presence of a carboxyl function.

Detailed analyses of ^{1}H-^{1}H COSY and 2D-HOHAHA spectra deduced partial structures H_4-H_{18}, H_{22}-H_{34}, and H_{36}-H_{39} and H_{41}-H_{49}. The location of Me-50 was unassignable due to overlapping of the ^{1}H NMR signals and large second order couplings between H_2 and H_3. One-dimensional HOHAHA experiments solved the problem. HMBC spectra clarified the connectivities around the quaternary carbons. The number and location of hydroxyl groups were clarified on the basis of deuterium shifts observed on ^{1}H NMR signals. The results led to (**80**) as the planar structure of Ga-A. The structure of Ga C (**81**) was readily determined by detailed comparison of the 1D and 2D NMR spectra of (**81**) with those of (**80**). Ga-A is unique in possessing an isolated ring in its terminal chain in addition to a continuous chain of fused rings. It is suggested that the extremely potent antifungal metabolites released from the cells may act as repellent against other epiphytic microorganisms.



80, R = OH

81, R =

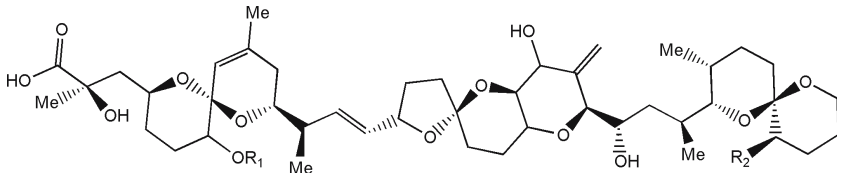
5. Diarrheic Shellfish Poisoning

Diarrheic shellfish poisoning (DSP), a major public health problem even though it is not lethal.[227-230] Epidermiological records indicate 1,300 persons affected between 1976 and 1982 in Japan, 5,000 cases in Spain in 1981, and an everincreasing number of reported cases in the USA. Each year infrequent intoxication is also noticed in the Netherlands and Chile.[231] The toxic symptoms are abdominal cramps (1 hr elapse time), nausea progressing to diarrhea (2-7 hr), and a "raw," "burning" feeling in the stomach. Diarrhea is noted in 92% of all cases, nausea in 80%, vomiting in 79%, abdominal pain in 53%, and chills in 10%.[231] Diarrheic shellfish poisoning was first discovered in 1976[232] when a poisoning due to mussel occurred in North Eastern Japan, although no "red tide" was seen during the infestation period. The causative organisms of the illness have now been identified as several dinoflagellates in the genus *Dinophysis*.[233] Even at a low cell density (200 cells per liter) of dinoflagellates leads to toxification of shellfish. DSP is associated with eating bivalves such as mussels, scallops or clams which have accumulated dinoflagellate toxins. Lack of a proper method of detection of the toxins in the past made it difficult to diagnose the suspected gastroenteritis as the

amount of toxin that can induce illness in man by oral intake is very small, equivalent to 32 g of dinophysistoxin-1 (DTX-1). Causative toxins that have been identified are okadaic acid and its analogs and pectenotoxins.

5.1 Okadaic Acid and its Analogs

Okadaic acid (**82**)[234-238] was first isolated independently from the sponges, *Halichondria okadoi* kadota, a black sponge, commonly found along the Pacific Coast of Japan, and *H. melonodocia* a Caribbean sponge found in Florida keys.[239] Subsequently, it was found in dinoflagellate, *Prorocentrum lima*[240] and *Dinophysis* spp.[241]



82, R_1 = H, R_2 = H (Okadaic acid)
83, R_1 = H, R_2 = CH_3 (Denophysistoxin-1)
84, R_1 = Fatty acid ester; R_2 = CH_3 (Denophysistoxin-3)

5.1.1 Isolation

Methanolic extract of the sponge *H. okadai* by repeated chromatography on polystyrene gel; Sephadex LH-20, SiO_2, followed by crystallization from MeOH and recrystallization from dichloromethane/hexane as crystalline solid (~10^4 % yield), m.p. 171-175°C, $[\alpha]_D$ 21° (c 0.33, $CHCl_3$).[227] The acid was toxic (LC_{50} 192 g/kg) (ip, mice) and inhibited growth of KB cells by more than 30% at 2.5 ng/mL and more than 80% at 5 ng/mL. m.p. 134-135°C, UV (end absorption) and IR (3450, 1740, 1080, 880 cm^{1}) spectra of the toxin were rather uninformative. An electron impact mass spectrum had m/z 804 for composition of $C_{44}H_{68}O_{13}$. ^{13}C NMR spectrum revealed 44 peaks, one carboxyl singlet, six olefinic carbons, three ketal or hemiketal singlet, 12 carbons bearing oxygen, the remaining 22 high field signals included five methyl groups and three methines. A field desorption mass spectrum of p-bromophenacyl okadaiate, subsequently confirmed a molecular formula of $C_{44}H_{68}O_{13}$. The structure (**82**) for okadaic acid with stereochemistry was finally assigned by X-ray diffraction experiments on the O-bromobenzyl ester of okadaic acid.[227] All the tetrahydropyran rings were found in the chair conformation and the tetrahydrofuran ring was in the envelope (C_5) conformation with O_7 as flap. Okadaic acid (**82**) was a complex derivative of a C_{38} fatty acid. Its structural features suggested that it belongs to the class of compounds known as ionophores, which hitherto had been known only from terrestrial micro-organisms. It is suggested that okadaic acid could be a metabolite of an epiphytic microorganism rather than of *Halichondria* spp.[227] The ionophoric features of the toxin, however, were foreshadowed by its toxic properties.

5.2 Dinophysistoxins

Several analogs of okadaic acid had been isolated. Dinophysistoxin-l (DTX_1) isolated from the digestive glands of mussels was found similar in chromatographic properties as okadaic acid (**82**). Comparison of spectral data of DTX_1 with okadaic acid confirmed the structure of DTX, as (35*S*)-methylokadaic acid (**83**). DTX_3 and DTX_4 were indistinguishable from PTX, in UV and mass spectra but were distinguishable by optical rotation suggesting that they are stereoisomer of PTX_1. PTX_5 is a dihydro derivative of either PTX_1, PTX_3 and PTX_4.[242] A series of congeners substituted with various fatty acids, 7-O-acyl-35-(*R*)-methylokadaic acid (DTX_3, **83**) were isolated as the toxic principles of poisonous scallops from Northeastern Japan.[239] 31-Demethyl-35- methyl-okadaic acid (DTX_2) had been isolated from the Irish mussels.[243]

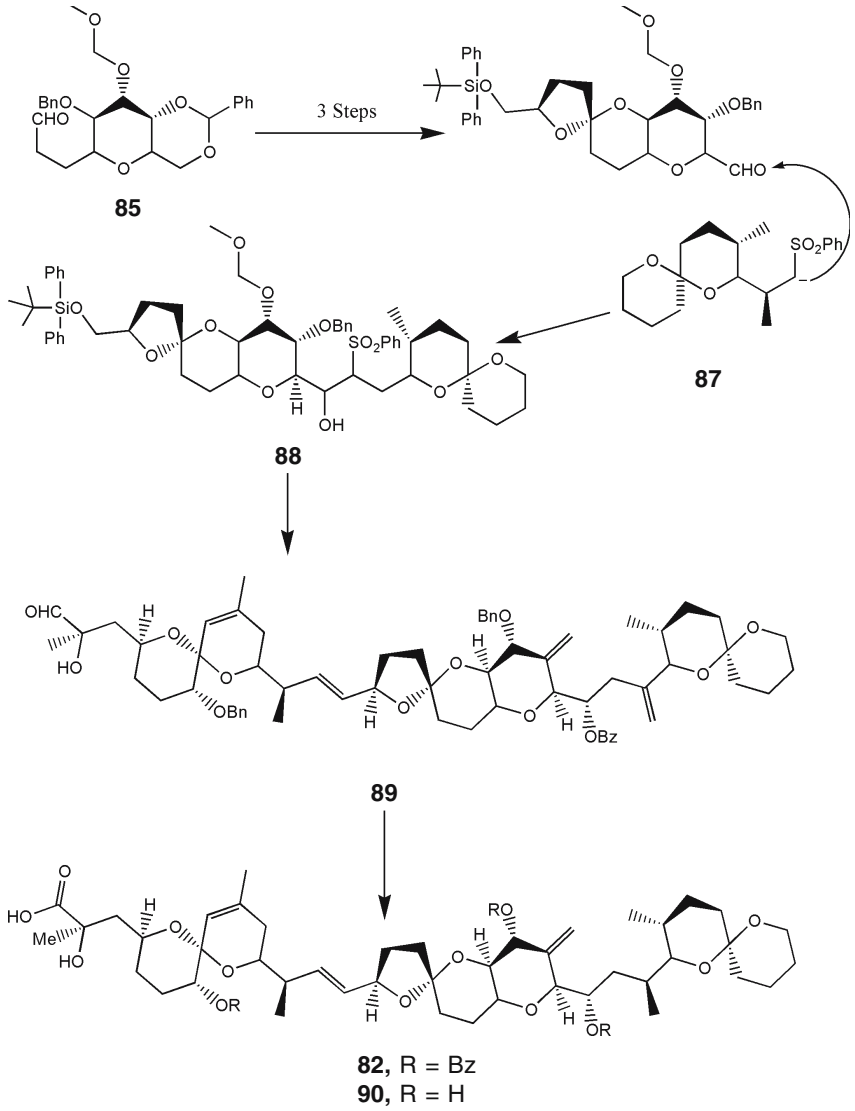
5.3 Total Synthesis of Okadaic Acid

Till now, two total syntheses of okadaic acid (**82**) have been reported.[244] Isobe et al published first total synthesis of (**82**) using D-glucose derived synthon (**85**) through the coupling of three segments A, B, C in 28 steps (**85-90**). The coupling is principally based on the strategy utilizing sulfonyl-carbanion as key reactions. The high acyclic stereoselectivity is attributed to a successful development of switching the *syn*/*anti*-diastereoselectivity in the heterocunjugate addition.

The compound (**82**) was made through a series of steps from (**85**) in good yield. Treatment of (**89**) with $NaClO_2$ at room temperature for 1 hr in aq. t-BuOH (containing $NaHPO_4$ and 2-methyl-2-butene) followed by treatment of (**88**) with lithium metal in liquid ammonia afforded okadaic acid (**82**) in 80% yield.

5.3.1 Mode of Action of Okadaic Acid and its Congeners

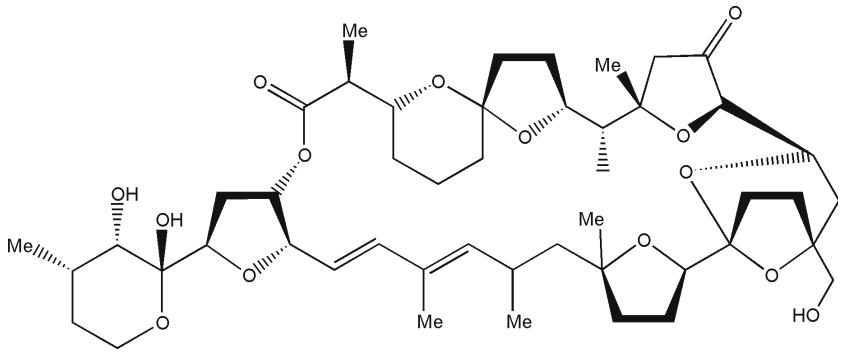
Okadaic acid (**82**) has been found to act as an inhibitor of the protein phosphatases.[245] Numerous biochemical and pharmacological studies have been carried out using (**82**) as a probe. The biological activities of (**82**) are now considered to be due to its inhibitory action against protein phosphatases. Okadaic acid (**82**) inhibited PP2A at the lowest concentration (Ki of 30 pm) PPI inhibited at the next lowest concentration, and PP2B at the highest concentration. Okadaic acid (**82**) showed no effect on PP2C. Okadaic acid and DTX_1 had been reported to be non-phorbol ester type cancer promoter.[246] In contrast to phorbol esters, which activate protein kinase C, okadaic acid inhibited dephosphorylation of proteins, predominantly serine/threonine residues. Okadaic acid and phorbol both cause the accumulation of essentially the same phosphorylated proteins, some of which are involved in tumor promotion.[247] Several groups have studied the structure activity relationship of (**82**). Alteration of the C_1 carboxylic acid or 24-OH greatly reduced the activity. Hydrogenation of C_{14} and C_{15} double bond or deoxidation at C_2



which affect the pseudo cyclic conformation formed by interaction between C_1 carboxylic acid and C_{24} hydroxyl group also reduced the potency.

5.4 Pectenotoxins

Pectenotoxin-l (PTX_1, **91**)[248254] was isolated as one of the diarrheic shellfish toxins from the digestive glands of the scallop, *Patinopecten yessoensis* found Northeastern Japan.[242] The toxins were isolated by repeated chromatography on silicic acid and gel permeation through Sephadex LH-20. DTX_1, DTX_2 and DTX_3 which had bulky fatty acid moiety were separated by this procedure, HPLC on reversed phase column and HPLC on a silicic



91

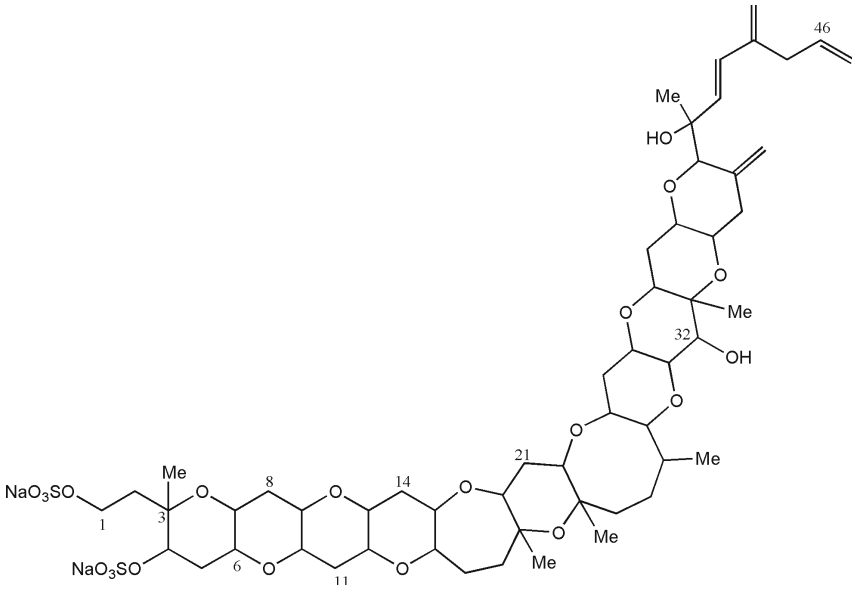
acid column were used to separate PTX_1 from PTX_3 and PTX_2 from PTX_4. 200 kg of digestive glands of scallop yielded pectenotoxin-l (PTX_1) (20 mg) m.p. 208-209°C; $[\alpha]_D$ +17.1° (c, 0.41 MeOH); pectenotoxin-2 (PTX_2) (40 mg) as white amorphous solid, $[\alpha]_D$ +16.2° (c, 0.015 MeOH); pectenotoxin-3 (PTX_3)10 mg; pectenotoxin-4 (PTX_4) (7 mg); and pectenotoxin-5 (PTX_5) (0.5 mg) as colorless solids.

Minimum lethal dose of PTX_1 to mouse was 250 µg/kg (ip); PTX_2 60 µg/kg (ip). Structure (**91**) for pectenotoxin-l (PTX_1) was assigned by X-ray crystallographic analysis.[242] The structures of four pectenotoxin homolog have been elucidated so far.[255] They all have essentially the same structure except at C_{43} where various stages of oxidation from methyl to carboxylic acid are found (PTX_2, CH_3; PTX_1, CH_2OH; PTX_3, CHO, and PTX_6, CO_2H). PTX_1 was also found in dinoflagellate *Dinophysis fortii* along with dinophysistoxin-l.[233]

Histopathological investigations have shown that PTX_1 is hepatotoxic and induces rapid necrosis of hepatocytes. The pathological action of PTX_1 resembles that of phalloidin.[256] PTXs are substantially different from other dinoflagellate toxins, especially in a longer carbon backbone (C_{40}), a C_{33} lactones ring rather than an acyclo structure, and a novel dioxabicyclo moiety. The large oxygen-rich internal cavity is grossly similar to cavities found in the polyether ionophores from terrestrial microorganisms.

5.5 Yessotoxin

Yessotoxin (**92**), a novel polyether toxin, had been isolated from scallops *Patinopecten yessoensis* implicated in diarrheic shellfish poisoning.[242,257–261] Digestive glands of scallops (84 kg) collected at Mutsu Bay, Japan, yielded 60 mg yessotoxin (**92**) as an amorphous solid. The toxin was isolated from the toxin fractions by initial chromatography on an alumina column, and subsequently by successive chromatography on Fujgel (DDS-Q3), Develosil and Toyopearl. It had $[\alpha]_D$ + 3.01 (c, 0.45, MeOH); UV λ_{max} (MeOH) 230 nm (ε 10,600), IR (KBr), 3400, 1240, 1220 cm^{+} ; FABMs (negative), *m/z* 1163 (M^+-Na).



92

The toxin killed mice at a dose of 100 μg/kg (ip), but caused no fluid accumulation in suckling mice intestines even at the fatal dose. Yessotoxin ($C_{55}H_{80}Na_2O_{21}S_2$) in ^{13}C NMR revealed the presence of 55 carbons consisting of six methyls, 18 methylenes, 24 methines and seven quaternary carbons. The connectivities of the protons were established by detailed analysis of ^1H-^1H COSY, ^1H-^1H-RELAY and ^{13}C-^1H COSY. Positions of ether bonds were determined mainly by COLOC (7 Hz) and NOE measurements with use of phase sensitive NOESY and ROESY experiments. The presence of sulphate ester(s) suggested by IR bands at 1240, 1220 and 820 $cm^{\pm}$ was confirmed by elemental analysis for sulphur by ion chromatography of sulfate ions liberated by solvolysis. The position of the esters was determined by comparison between ^1H NMR spectra of desulphated YTX and intact toxin. The data suggested that yessotoxin structure (**92**)[262] partly resembles those of brevetoxins, yet YTX is distinct from brevetoxins in having a longer backbone of 42 carbons or a terminal side chain of nine carbons, two sulphate esters, and in lacking carbonyl groups.

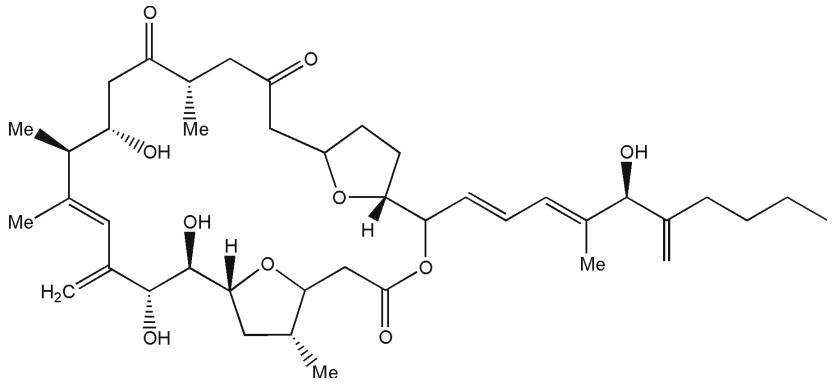
6. Miscellaneous Toxins

Screening of micro algae for toxin production led to the isolation of a wide variety of bioactive metabolites from dinoflagellates. Some of these compounds were possibly implicated in poisonings.

6.1 Amphidinolides

Three groups of macrolides named amphidinolides had been isolated from the dinoflagellate *Amphidinium* spp. symbiotic to flatworm *Amphiscolops*

breviviridis.[263] The cultivated dinoflagellate *Amphidinium* spp. from an Okinawan flatworm *Amphiscolops* spp. furnished amphidinolide-A,[264] and amphidinolide-B[265] and amphidinolide-C (0.0015% wet weight).[263] Amphidinolide-C (**93**), obtained as colorless amorphous solid, $[\alpha]_D$-106° (c, 1.0 $CHCl_3$) had molecular formula $C_{41}H_{62}O_{10}$. ^1H and ^{13}C NMR studies of the toxin revealed the presence of two isolated ketones, an ester carbonyl, five olefins, 12 methines (nine of them bearing oxygen atoms), 10 methylenes, and six methyl groups. Extensive 400 and/or 500 MHz NMR analyses, 2D NMR techniques, in particular, conventional COSY coupled with double relayed coherence transfer (RCT2) experiments were very effective for deducing the partial structures. The assignments of the carbons bearing hydrogen were established by ^1H and ^{13}C COSY via one bond coupling. The phase sensitive 2D COSY facilitated by one-dimensional difference NOE experiments provided useful information to determine the geometries of double bonds. Three segments (A-C) were separated by three carbonyls. The connectivities of three segments (A-C) were clearly established by HMBC spectrum, and finally structure (**93**) was assigned to amphidinolide-C. A number of macrolides with structures similarities, have been isolated from sponges,[266] nudibranchs[267] and cyanophytes.[268,269] The question remains as to whether these macrolides were produced by host animals or symbiotic microorganisms.



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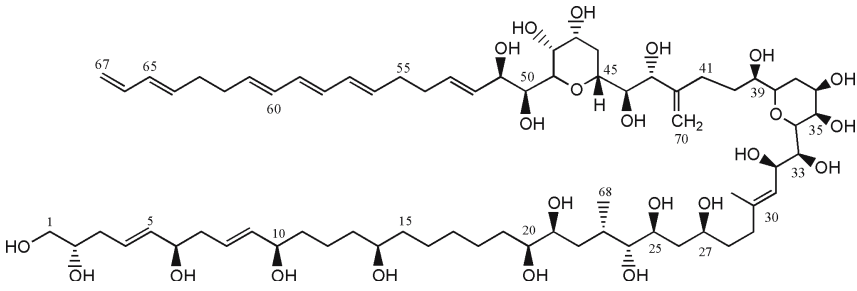
6.2 Amphidinol

Amphidinol 3 (**94**) (12 mg)[270,271] was isolated from cultured cells (440 L) of the dinoflagellate *Amphidinium klebsii,* collected at Ishigaki island, Japan.[272] Recently, Murata et al[273] reported the absolute configuration of amphidinol 3 (**94**) by using newly developed configurational analysis based on carbon-hydrogen spin coupling constant. In order to facilitate measurements of $^{2,3}J_{C,H}$ authors prepared a ^{13}C-enriched sample of (**94**) (8 mg) by making another culture (200 L) in the presence of 12 mM $NaH^{13}CO_3$. Stereochemistry of (**94**) was accomplished as below; (a) the *J*-based method was used for the determination of stereochemistry at the acyclic parts with 1,2- and 1,3-chiral



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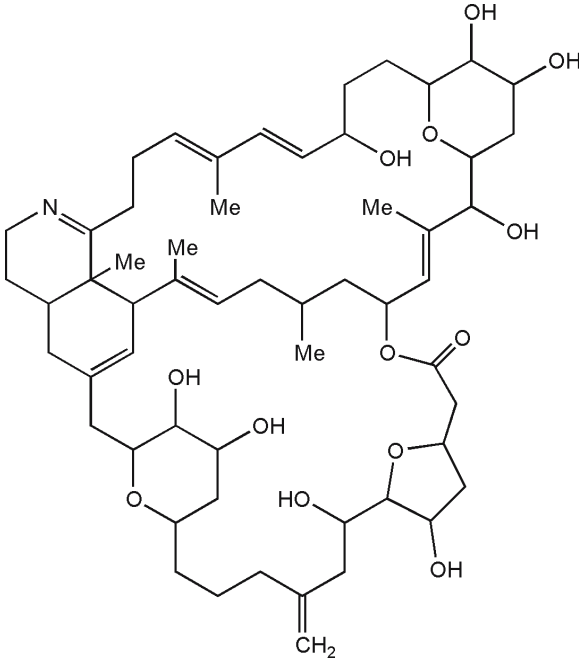
centers, C20-C27, C32-C34, C38-C39, C44-C45, and C50-C51; (b) the NOE analysis combined with *J* analysis was used for two ether cycles and their linkage C39-C44; (c) the modified Mosher method and chromatographic/NMR comparison were used for degradation products to determine the absolute stereochemistry at C2, C6, C10, C14, C23, and C39. $^{3}J_{H,H}$ and $^{2,3}J_{C,H}$ values of intact (**94**) were measured by E. COSY and hetero half-filtered TOCSY (HETLOC), respectively, phase sensitive HMBC was also used for parts where the small magnetization transfer by TOCSY hampered the accurate measurement of $^{2,3}J_{C,H}$ by HETLOC. The values for ^{2}J(C32, H-33) and ^{3}J(C34, H-32) indicate that H-33 is anti to C32-OH[8] and H-32 is gauche to C34, respectively. These interactions unambiguously establish the *threo* configuration for C32-C33. For C38-C39, ^{3}J(H-38, H-39), which is intermediate between anti and gauche, suggests that this bond undergoes a conformational change. The two small values for ^{3}J(C37, H-39) and ^{3}J(C40, H-38) indicate gauche C37/H-39 and gauche C40/H-38 interactions in both conformers. Of the six possible pairs of alternating rotamers arising from *erythro* and *threo* configurations, only one pair in satisfies all of these requirements. The relative configurations of the consecutive stereogenic center in C20-C27 was determined using this method. The diastereomeric relationships of C44-C45 and C50-C51 were assigned in the same manner on the basis of ^{3}JH,H and $^{2,3}J$C,H. The configurations of rings A/B and their linkage (C39-C44) were elucidated using NOEs in combination with ^{3}JH,H and $^{2,3}J$C,H data. These NMR-based analyses using intact (**94**) have revealed the relative configurations of C20-C27 and C32-C51. The absolute configurations of (**94**) was determined by the analysis of the degradation products of Amphidinol 3 (**94**). Treatment of (**94**) with HIO_4/$NaBH_4$, followed by esterification with (*R*)- and (*S*)-MTPA (2-methoxy-2-trifluoromethyl-2-phenylacetic acid) and separation by HPLC, furnished MTPA esters of fragments corresponding to C2-C20, C21-C24 and C33-C50. Based on this elegant study amphidinol 3 (**94**) was assigned the structure with the absolute stereochemistry of 2*S*, 6*R*, 10*R*, 14*R*, 20*S*, 21*S*, 23*S*, 24*R*, 25*S*, 27*S*, 32*R*, 33*S*, 34*R*, 35*R*, 36*R*, 38*R*, 39*R*, 43*R*, 44*R*, 45*R*, 47*R*, 48*R*, 49*R*, 50*S*, and 51*R*. Amphidinol (**94**) is the first representative of a new class of polypeptide metabolites exhibiting potent antifungal and hemolytic activities. Growth inhibiting activity (6 μg/disk) of 94 against

Aspergillus niger was three times that of amphotericin-B; the hemolytic activity was 120 times that of standard saponin.

6.3 Prorocentrolide

Prorocentrolide (**95**)[274,275] has been isolated from the ciguatera associated dinoflagellates *Prorocentrum lima*, the producer of okadaic acid.[276] It is a toxic macro cycle formed from C_{49} fatty acid and incorporating a C_{27} macrolide and a hexahydroisoquinoline moiety in its unique structure. The dinoflagellate was isolated at Sesoko Island Okinawa, and cultured in seawater enriched with ES-1 nutrient at 25°C, 5 weeks. Algal cells (2.7×10^{10}) harvested from 100 L of the culture were extracted with acetone and methanol. Purification of the extract followed by successive chromatography on Toyopearl HW-40 and Develosil ODS-5 columns yielded prorocentrolide (**95**) (70 mg) as an amorphous solid which had mouse lethality of 0.4 mg/kg (ip); $[\alpha]_D$ + 136.5° (c, 0.147 MeOH); UV λ_{max} (MeOH) 235 nm (13,600); IR (KBr) 3400, 1715, 1670, 1640, 1200 and 1060 $cm^{\pm}$. It had molecular formula $C_{56}O_{85}NO_{13}$ (MH^+ *m/z* 980.6168). It gave a positive Dragendorff's test. IR band at 1670 $cm^{\pm}$ suggested the presence of an imines function. ^{13}C NMR analyses revealed the nature of carbons. The proton connectivities were elucidated by detailed analyses of ^{1}H-^{1}H and ^{13}C-^{1}H COSY and long range ^{13}C-^{1}H COSY experiments. The degree of unsaturation derived from the molecular formula and the structural features suggested the presence of three ether rings. The oxycarbons other than those bearing hydroxyl or acetoxy groups were arranged to form one five- and two six-member ether rings. These ether linkages were confirmed



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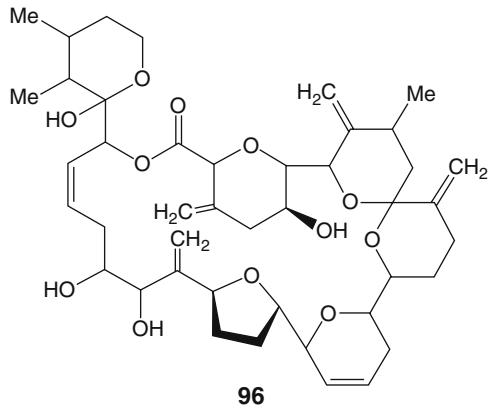
by NOE experiments. Geometry of all double bonds except for C_{19}-C_{20} were determined to be *E* on the basis of phase sensitive NOESY. All these data allowed assigning the planar structure (**95**) for prorocentrolide.[274] The structure is new type of nitrogenous polyether lactones. The co occurrence of (**95**) with okadaic acid (**81**) in *P. limbs*[276] indicated that dinoflagellates are capable of producing polyether of entirely different skeleton.

6.4 Goniodomin-A

The dinoflagellate *Goniodoma pseudogoniaulax* collected in the rock pool at Jogashima, Kanagawa, Japan, had furnished a novel antifungal polyether macrolide goniodomin-A (**96**)[277] (180 mg, 0.05%) by column chromatography on silica gel followed by reverse phase HPLC. Goniodomin-A, $[\alpha]_D$ + 28° (c, 0.13, MeOH) had no UV absorption maximum above 210 nm. Its IR spectrum indicated the presence of hydroxyl (3430 $cm^{\pm}$) and ester (1760 $cm^{\pm}$) groups. ^{13}C NMR revealed 43 carbons which were assigned to one carbonyl, two disubstituted olefins, four exomethylenes, two acetals, 12 oxymethines, one oxymethylene, nine methylenes, three methines, and three methyl groups. Detailed analyses of $^{13}C^{1}H$ COSY and ^{1}H—^{1}H COSY spectra of (**96**) allowed to deduce partial structure A-C. Extensive NMR studies led structure (**96**) for goniodomin-A.[277–281] Goniodomin-A is a unique polyether macrolide and its structure resembles that of pectenotoxin-1 (**91**), obtained from the digestive gland of scallops.[242] It is a novel polyether lactones having antifungal activity.



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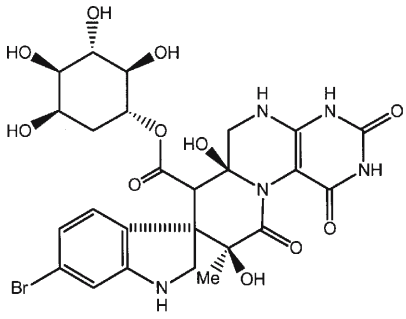
6.5 Surugatoxin

In 1965, intoxication occurred due to ingestion of a carnivorous gastropod, *Babylonia japonica.* The patient complained of visual defects, including amblyopia, mydriasis, with thirst, numbness of lips, speech disorders, constipation and dysuria. Surugatoxin (**97**)[282–285] (10 mg) as colorless prizms, m.p. >300°C, molecular formula $C_{25}H_{26}BrN_2O_{11}$, was isolated from the midget (1 kg) of the gastropod. The toxin was not stable to mineral acids or alkalies, but was fairly stable only in acetic acid. Isolation on column other than Sephadex or CM-Sephadex was not suitable.



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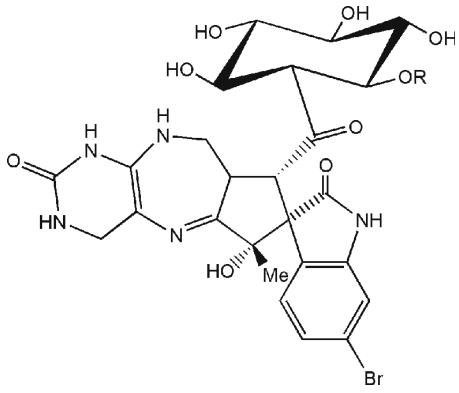
Surugatoxin (**97**), UV 276 nm (15000); IR (KBr) 3200, 1740, 1695 and 1640 $cm^{\pm}$, evoked mydriasis in mice at a minimum dose ca. 0.05 μg/g body weight. It was assigned structure (**97**) on the basis of crystallographic analysis.[286] Surugatoxin was subsequently found to be an artifact produced during purification of toxins.[287] A synthesis of surugatoxin had been reported.[286]

6.6 Neosurugatoxin

Neosurugatoxin (**98**) (4 mg) was isolated from midget glands of the Japanese ivory shell *Babylonia japanica* (20 kg)[178] as a causative agent of intoxication resulting from ingestion of the toxic ivory shell. Midget glands of the shell fish was extracted with 1% AcOH. Gel filtration on Sephadex G25 column then on to a CM-Sephadex ion exchange column and finally by reverse phase HPLC yielded neosurugatoxin (**98**), which evoked mydriasis in mice at a minimum dose of 3 μg/g. It was extremely unstable in alkaline medium and fairly heat labile. Neosurugatoxin has the molecular formula $C_{30}H_{34}BrN_5O_{15}H_2O$ and its structure (**98**) was determined by X-ray crystallographic analysis.[288] The molecule was a glycoside with a pentacyclic aglycone moiety, which was constructed from two-planar parts, 6-bromo-oxindole (D, E ring) and O-α-D-xylopyranosyl (15) myoinositol. Several structural features were common to both neosurugatoxin (**98**) and surugatoxin

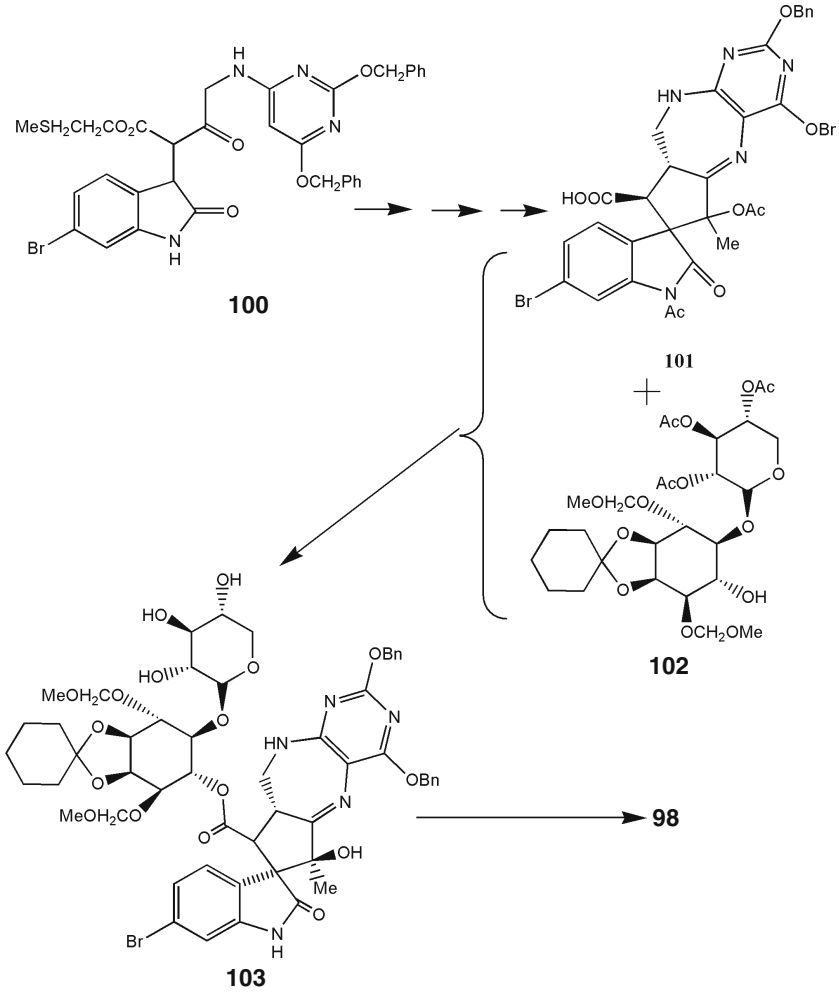


98, R = Xylose
99, R = H

(**97**). Both had an oxindole, a deoxypyrimidine, myoinsositol, and other functional groups. The most striking difference was that neosurugatoxin (**98**) had a ring system with a six-seven heterocyclic system instead of six-six system as in surugatoxin. Neosurugatoxin (**98**) and prosurugatoxin (**99**) have been identified as the causative agents of the poisoning due to ingestion of ivory shell *B. japanica*.[287,289,290] Both (**98**) and (**99**) were over 5,000 times more active as ganglion blocking agents than existing drugs such as mecamylamine or hexamethonium. Since (**98**) and (**99**) specifically block only nicotinic receptors in the ganglion, both toxins are excellent tools for studying the neurosystem or brain. Interestingly, the origin of neosurugatoxin (**98**) and prosurugatoxin (**99**) had been found to be a bacterium belonging to the *Coryneform* group.[290]

6.6.1 Total Synthesis of Neosurugatoxin

A total synthesis of neosurugatoxin (**98**) had been achieved.[291] The synthesis involves the stepwise synthesis of aglycone (**101**) from (**100**), esterification of (**101**) with the optically active xylopyranosyl-myo-inositol derivative (**102**)



and stereo control of the four asymmetric carbons by thermodynamic equilibration of (**102**). Synthone (**100**) was converted through several steps into the aglycone (**101**). Esterification of (**101**) with highly sterically hindered alcohol (**102**) afforded a mixture of four stereo isomeric neosurugatoxin derivatives which was separated to give (**103**).

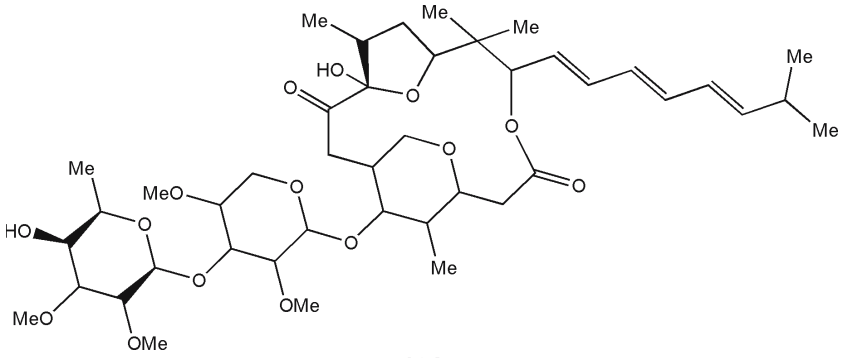
Finally, exposure of (**103**) in 90% TFA (25°C, 1 hr) resulted in removal of all protecting groups to yield the desired neosurugatoxin (**98**) in 77% yield.

6.7 Macroalgal Toxins

Although microalgae are frequently involved in various type of seafood poisoning, however, the involvement of macro algae in seafood poisoning is rare. Human intoxication due to ingestion of the red alga *Polycaverosa tsudai* (formerly *Gracilaria edulis)* occurred in Guam, Japan, in 1991. Thirteen people became ill, three of whom died. As the alga had been eaten widely with no previous record of potential risk, a toxic glycoside named polycavernoside-A (**104**) isolated from the alga has been assumed to be the causative agent of the poisoning.[292]

6.7.1 Polycavernoside-A

P. tsudai (2.6 kg) was collected from Tanguisson Beach, Guam, and toxins were extracted from the alga with acetone, and purified by column chromatography, guided by mouse bioassays. Polycavernoside-A was isolated (400 μg, recovery 14%) as colorless solids, LD 99 in mice (ip) was found to be 200-400 pg/kg . Structure of (**104**) was determined by spectroscopic methods. [**104**, UV, (MeCN) 259, (ε 25 000), 270 (ε 32000), 280 (ε 26000) nm; IR (film) 1630, 1730, 1738 $cm^{\pm}$; HR-FABMS $[M+Na]^+$ *m/z* 847.4483 [calcd for $C_{43}H_{68}O_{15}Na$ *m/z* 847.4455 (M^+)] Partial structures H2-H8, H11-H13, H15-H23,28, Hl′-H5′, and Hl″-H6″ were deduced from detailed analyses of ^1H-^1H COSY and 2D HOHAHA spectra. The conjugated triene (H16-H21) was also supported by the UV maxima; the $^3J_{HH}$ value (15 Hz) determined by the 2D *J* spectrum pointed to *E,E,E* geometry. The ^{13}C NMR spectrum (CD_3CN) confirmed the presence of a ketone (δ 207.4) and an ester (δ 172.1)



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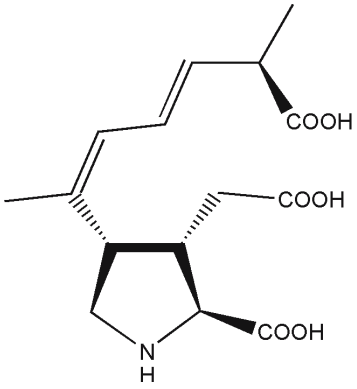
suggested by IR bands. A ^{13}C decoupled HMQC spectrum led to assignments of all ^{1}H and ^{13}C signals except those of two quaternary carbons, C10 (δ 103.9) and C14 (δ 40.5). HMBC spectra clarified the connectivities around quaternary and carbonyl carbons by giving cross peaks due to $^{23}J_{CH}$ between C1/H2a, Cl/H15, C9/H8a, C10/H12a, C10/H12b, C10/Me25, C13/Me26, C13/Me27, C14/H15, C14/Me26, C14/Me27, C15Me26, C15/Me27, C26/Me27, and C27/Me26. The structural features around C14 were supported by NOES (NOESY 270 MHz, ROESY 400 MHz) between H12a/Me26, H13/Me27, H15/Me26, and H16/Me27. The connectivities of C1/C2 and C8/C9 were further supported by the chemical shifts of Hz-2 (δ 2.14, 2.52) and H2-8 (δ 2.07, 2.97) typical for methylenes α to carbonyl. The deuterium exchangeable signals at δ 4.58 and 2.66 in the ^{1}H NMR spectrum (CD_3CN) were assigned to 10-OH and 4″-OH, respectively, on the basis of the cross peaks due to $^{2}J_{CH}$ between C10/10-OH and C4″/4″-OH in HMBC spectra. The connectivities of two remaining quaternary carbons, C9 and C10, were deduced from the NOE between H8b/H11. The adjacent carbonyl (C9) caused a significant downfield shift of 10-OH (δ 4.58) by an anisotropic effect and formation of a hydrogen bond. The ether linkage between C3/C7 was evident from the NOE between H3/H7. The remaining hemiketal carbon (C10) and an oxycarbon (C13) were linked to form a tetrahydrofuran ring; $^{3}J_{HH}$ of H11-H13 agreed with those expected from MM2 energy calculations. Similarly, the structure of the glycosidic residue was confirmed. Recently total synthesis of (**104**) was published.[292] The similarity of observed symptoms in experimental animals and human patients supported the belief that polycavernoside-A and B caused the intoxication (diarrhea, hypersalivation, lacrymation, muscle spasm and cyanosis). The sudden and transient occurrence of the toxins in the alga remained unexplained, but may provide a clue to previous outbreak of fatal poisoning caused by the *Gracilaria chorda* and *G. verrucosa*.[293,294]

6.8 Toxic Substances of *Chondria armata*

The red alga *C. armata* is a folk medicine used as an anthelmintic. However, two palytoxin analogs,[295] and domoic acid (**105**)[296–300] and its seven derivatives were isolated from the alga. Domoic acid (**105**), reported to be produced by the diatoms *Nitzchia pungens, F. multiseries* and *Pseudonitzchia australis* has caused fatal food poisonings, after accumulating in shellfish.[301,302] This poisoning was termed amnesic-shellfish poisoning (ASP). Kainic acid (**106**) a neurotoxic amino acid also occurs in red alga[303,304] but with no intoxication episode.

6.9 Aplysiatoxin and Debromoaplysiatoxin

Accounts of toxicity of sea hares (mollusc-gastropod) without shells date to Roman times. Aplysiatoxin (**108**) and debromoaplysiatoxin (**107**) had been isolated from the ether soluble toxic fraction, LD_{100} 0.3 mg/kg mouse



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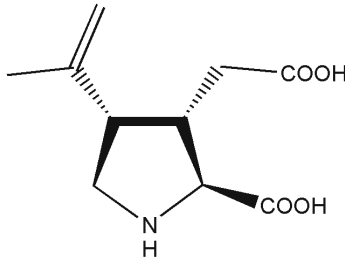

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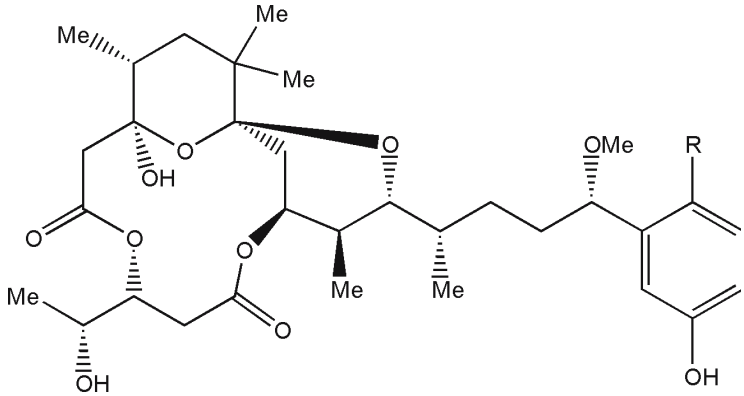
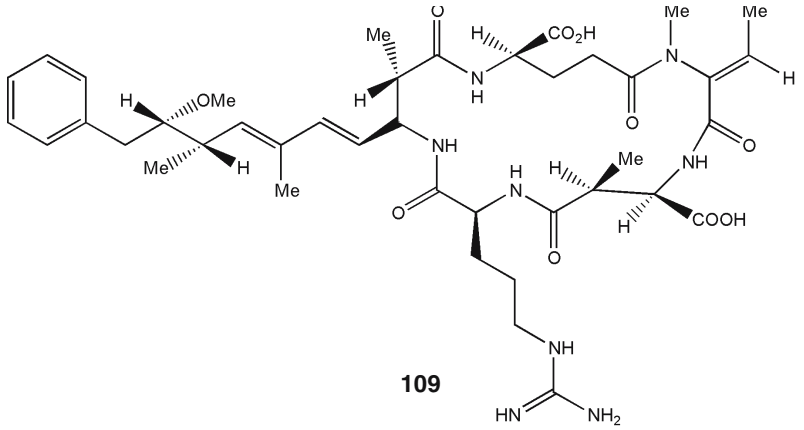
(ip) of the animal as an oily mixture. Aplysiatoxin-A (**108**)[305,306] and debromoaplysiatoxin (**107**)[305,306] are bislactones of 2,4-dihydroxyvaleric acid and 4,6,6,10,12-pentamethyl-3,7,9,11,15-tetraoxy-15-phenyl-cyclododecane. The toxin (**107**) had also been isolated from the blue-green alga *Lyngbya majuscula*[307] as a potent inflammatory agent and responsible for incidents of severe dermatitis among swimmers in Hawaii and Okinawa. Interestingly, (**107**) is reported to have tumor promoting activity similar to shown by phorbol esters.[308]



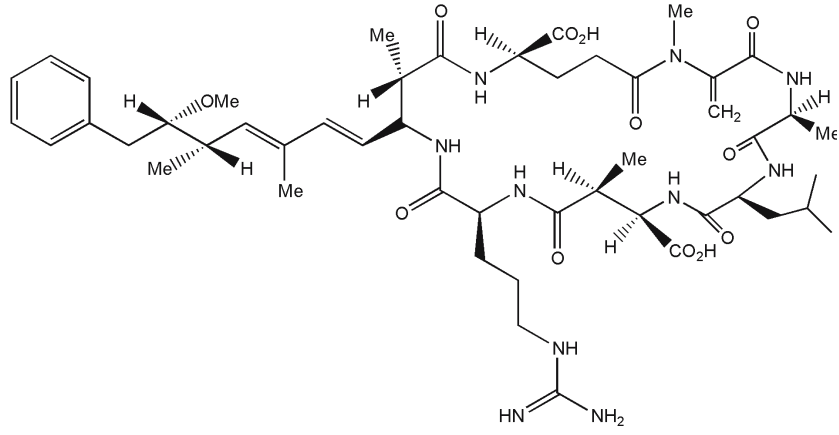
107, R = H
108, R = Br

6.10 Toxic Peptides

The peptides from red, brown green and blue-green algae with toxic properties are known. From the brackish water *Nodularia spumigena* species, which caused problems in Baltic Sea and New Zealand, cyclic pentapeptide, nodularin (**109**) had been isolated.[309] The peptide is closely related to microcystin (**110**),[310] a potent hapatotoxin and protein phosphate 2 and 2A inhibitor isolated from the blue-green alga *Microcystis aeruginosa.* The structures of



109



110

both microcystins (**110**) and nodularin (**109**) are characterized by the presence of two unusual amino acids, 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4,6-decadienoic acid and 3-methylaspartic acid. Moore et al[311] had studied the biosynthesis of these moieties and concluded that the former is a mixed polypeptide made of phenylalanine, acetate, and methyl groups from methionine. It was a very unusual finding and demonstrated that the microalgae sometimes take completely different and unexpected paths to the synthesis of the same structure.

6.10.1 Lyngbyatoxin-A

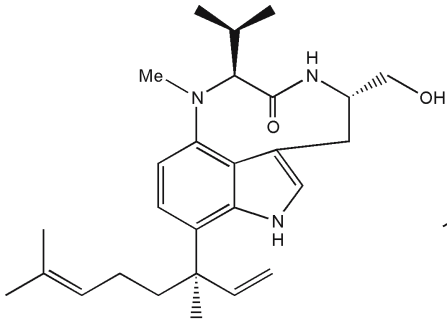
Lyngbyatoxin-A (**111**)[310315] is a marine natural product was first isolated in 0.02% yield from the Hawaiian variant of the marine blue-green alga *Lyngbya majuscula gomont*. This compound was later found to be identical with teleocidin A-1. The structure of teleocidin A-I was determined on the basis of spectral comparison with teleocidin B (**112**), the structure of which had been established by X-ray crystallography.[316,317] The absolute configuration

of (**112**), and therefore of lyngbyatoxin A, was elucidated by comparison of its CD spectrum with that of natural and synthetic indolactam V (**113**) and by chemical degradation. Extraction was carried out on the freeze-dried material by using 50% i-PrOH/CH_2Cl_2 at room temperature. These compounds have attracted much biological interest because they are potent activators of protein kinase C (PKC) which is implicated in the regulation of various cellular responses. Malfunction of PKC can lead *inter alia* to tumor development and diabetic complications, and members of the PKC family are thus promising targets for medicinal chemistry; selective activators and inhibitors of PKC are therefore perceived as useful tools for drug development.

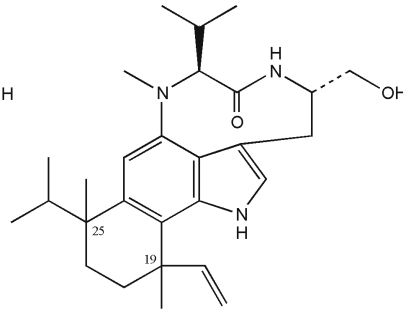
The toxin was found to be a potent tumor promoter in mice similar to that of phorbol ester. The toxin killed baitfish at a concentration of 0.15 μg/mL in seawater.

6.10.2 Isodysiden

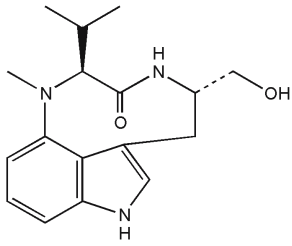
The marine sponge *Dysidea herbacea* had furnished a number of peptides. Of these, only isodysiden (**114**) exhibited ichthyotoxic activity, LD_{50} 5 mg/mL against *Lebistes reticulatus.* It is believed that isodysiden (**114**) is of algal origin.[318]



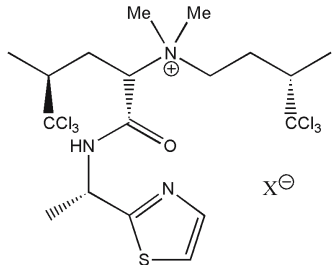
111



112



113



114

7. Concluding Remarks

Seafood poisoning and massive deaths of fish are problems of global concern. They pose serious threats to public health and fisheries. Remarkable progress has been made in recent years in identifying toxigenic sources, as seen in the case of ciguatoxin and tetrodotoxin. It has now been realized that micro algae play an important role in the marine biological system. Many algal metabolites have unique structures and are formed by biosynthetic routes quite different from those followed in the biosynthesis of metabolites from terrestrial sources. A variety of compounds with unique chemical structures and biological activity have been discovered in marine organisms, especially in marine invertebrates. However, there have been persisting questions regarding their true origin. Some of these compounds have been shown not the primary metabolites of the organisms where they are found, but originate elsewhere. The possible primary producers of toxic secondary metabolites of some marine organisms are microalgae, bacteria, and fungi. They are carried through symbiosis, association, food chain, and other forms of nutrient dependency. The uniqueness of chemistry and metabolisms of marine microalgae has been demonstrated by isolation of several types of metabolites. Although immense progress has been made in recent years, many questions remain unanswered such as the trigger mechanisms for the algal blooms and the toxigenicity. The contributions that marine toxins have made to life science as biochemical or pharmacological tools have been invaluable.

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8

Bioactive Marine Nucleosides

Abstract

The chapter deals with the bioactive marine nucleosides. The chemistry and biological activities of spongothymidine, spongouridine, spongosine, isoguanosine, doridosine, 2′-deoxythymidine, 2-deoxycytidine, 3-methyl-2′-deoxycytidine, 3-methyl-2′-deoxyuridine, 2'-deoxyadenosine, 2′-deoxyspongosine, 9-[5-deoxy-5′-(methylthio)-β-D-xylofuranosyl]adenine, 5-iodo-5′-deoxy-tubercidin, mycalisine-A, mycalisine-B and 5-deoxy-5′-dimethylarsinyladenosine, 2′, 3′-didehydro-2′, 3′-dideoxyuridine, aplysidine and phidolopin have been discussed. The biological activities of analogs of spongouridine and spongosine have been reviewed.

1. Introduction

Nucleosides, the nitrogen glycosides of purines and pyrimidines and their phosphate esters known as nucleotides, are vital components of all living cells and are involved in several biological processes. Discoveries made in the area of purine and pyrimidine nucleosides and nucleotide chemistry have contributed substantially to a better understanding of biology at the molecular level. The pioneers of nucleosides chemistry had an interest not only in the naturally occurring nucleosides and their biochemical properties but also in the effects of synthetic nucleosides on living organisms. Abnormal purine metabolism causes a number of human diseases, and specific inhibitors are found effective in the treatment of some human diseases. Allopurinol, a substituted pyrazolo[3,4-d]pyrimidine is used clinically for the treatment of gout and other conditions of rapid purine catabolism. There are several reports which suggest that amino-l-(β-D-ribofuranosyl)-1*H*-pyrazolo[3, 4-d] pyrimidine and related compounds may function as a substrate for anabolic and catabolic enzymes. Microorganisms and marine organisms are capable

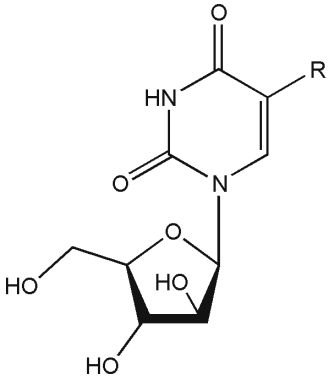
of producing unusual nucleosides. The nucleosides that are produced by microorganisms and inhibit the growth of other microorganisms are called nucleoside antibiotics.[1-5] Recent reviews by Isono et al list more than 200 known naturally occurring nucleoside antibiotics including several highly modified nucleosides isolated from marine invertebrates, such as sponge and algae.[4,5] These nucleosides either inhibit one or several steps of the cellular metabolism in which natural nucleoside participate. For example, a number of nucleoside antibiotics are known to block the incorporation of cell wall and membrane surface components into cell wall. Some nucleosides act as selective inhibitors of protein synthesis in animal virus infected cells, and some interfere with the metabolisms of nucleic acids at different levels. Several nucleosides with unusual structure have been isolated from marine organisms.[6] Biological activities found in the marine nucleosides have been a stimulus for the synthesis of various analogs of these nucleosides for their biological activity evaluation.[7-11] Much of the driving force for the huge amount of work done in nucleoside chemistry[12-14] comes from the possibility that analogs of natural nucleosides might have useful antibiotic, antiviral, antiparasitic and antitumor properties.

2. Pyrimidine and Purine-D-arabinosides

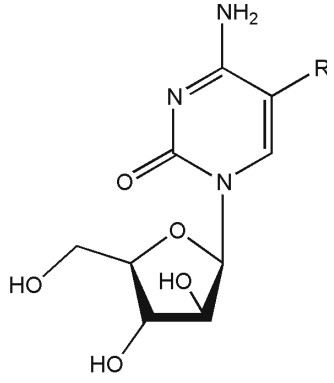
A number of pyrimidine and purine nucleosides having D-arabinose sugar instead of D-ribose have been isolated from marine organisms.

2.1 Spongothymidine (1, Ara-T)

In 1950, Bergmann[15] for the first time isolated an unusual nucleoside named spongothymidine from the sponge *Cryptotethia crypta.* This pioneering work of late Prof. Bergmann stimulated a wide interest in the sponges as a source of novel compounds. Spongothymidine (**1**) was obtained from the sponge *C. crypta* by acetone extraction.[16]



1, R = Me
2, R = H



3, R = Me
4, R = H

The acetone extract during soxhlet extraction of the sponge on cooling yielded a solid mixture of nucleosides. Spongothymidine, as a colorless crystalline compound, was obtained by repeated crystallization of the mixture [m.p. 246-47°C; $[\alpha]_D$ + 80° (c, 1.18 % NaOH)]. Acid hydrolysis of the nucleoside gave thymine[17] hence the compound was named spongothymidine to indicate its origin from sponge and its relationship to thymidine. Degradative method revealed the structure of sugar moiety as D-arabinose.[18-20] Typical acetone extract of a fresh sample of the sponge *C. crypta* was found to contain 2.1 g of spongothymidine per kg of dry sponge.[21] Its 5-phosphate derivative has been prepared[22] and found active against bacteria and viruses.[23] Spongothymidine (**1**) was found effective against HSV-l, HSV-2 and *Varicella zoster* virus (VSV) (ID_{50} 0.25-0.5 μg/mL).[24,25] Its inhibition against HSV-l and HSV-2 is selective and is effective orally.[26] Ara-T is also effective against EMV, but inactive against CMV.[25] It had proved effective in the systemic treatment of HSV-l encephalitis in mice, 5-methylcytosinearabinoside (**3**) can be considered as a prodrug of spongothymidine to the extent that it acts as a substrate for deoxycytidine deaminase.[24]

2.2 Spongouridine (2, Ara-U)

Extensive purification of the mixture of nucleosides obtained by Soxhlet extraction of the sponge *C. crypta* with acetone yielded spongouridine (**2**)[18] as a colorless crystalline compound, m.p. 226±8 °C; $[\alpha]_D$+ 97° (in 8% aqueous NaOH). The positive rotation of the compound indicated it to be a pyrimidine nucleoside. Formic acid hydrolysis gave uracil as the sole heterocyclic moiety. The nucleoside consumed one mole of $NaIO_4$ without formation of formic acid, thus showing it uracil penta furanoside. Further, the fission product of the periodate oxidation of spongouridine and uridine showed the same optical rotation, thereby suggesting that the linkage of sugar to base unit must be similar to uridine. Indication that sugar was D-arabinose came from the study of UV spectrum. The nucleoside showed changes in the UV spectrum at high pH values similar to those shown by spongothymidine, which had been characterized as 1-β-D-arabinofuranoside of thymine. Finally, D-arabinose was isolated after purification of product obtained by the reduction of spongouridine with sodium in alcohol. Almost simultaneously synthetic 1-β β-D-arabinofuranosyluracil[27] became available and spongouridine was found to be identical with it in all respect, thus confirming its chemical structure. Spongouridine (**2**) isolated first from a marine sponge was subsequently obtained from the gorgonian *Eunicella cavolini*.[28] Spongouridine has been used as a starting material for the synthesis of marine nucleoside, spongoadenosine (Ara-A), by a combination of chemical and microbial process.[28] Spongouridine is cleaved reversibly[29] to D-arabinose-l-phosphate and uracil by the enzyme nucleoside phosphorylase. Its phosphate has been prepared for antiviral evaluation. It showed weak antiviral properties[30] and also exhibited very weak activity against *Herpes simplex* virus-l as compared to spongothymidine.

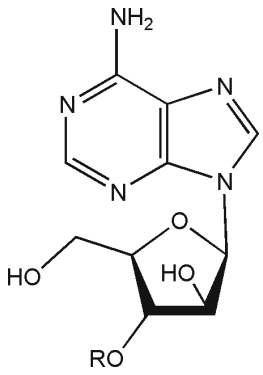
2.3 Analogs of Spongouridine

Several analogs of spongouridine (**2**) have been synthesized where R is substituted by a number of groups. The activity has mainly been evaluated against *Herpes simplex* viruses (HSV). Compounds (**2**, R = CN, NO, CHO, CH_2OCH_3, $CH{=}CHCH_3$, $CH{=}CHCH_2CH_3$, substituted alkynes etc.) are either inactive or weakly active antiviral agents (ID_{50} for HSV >10 g/mL). The compound 1-β-D-arabinofuranosyl-5-fluorouracil (**2**, R = F) is very effective against human cytomegalo virus (HCMV).[29] The compounds [**2**, R = CF_3; CH_2CH_3; $CH{=}CH_2$; CH=CHCl (*E*); and CH=CHBr (*E*)] are generally quite potent against HSV (ID_{50} for HSV-l < 1, g/mL). The most active compounds of this series are (*E*)-5-(2-bromovinyl)-β-D-arabinofuranosyluracil (BV Ara-U) and (*E*)-5-(2-chlorovinyl)-Ara-U with an ID_{50} for HSV-l, 0.1 g/mL. The 5-substituted ara-U as a rule are less active than the corresponding 5-substituted 2-deoxyuridine analogs.[31-38] The antiviral potency of 5-substituted ara-U analogs critically depends on the length of the C-5 side chain. Irrespective of the nature of the C-5 side chain (alkyl, alkenyl or alkynyl), and antiviral activity generally decreases with increasing number of carbon atoms. Uracil nucleosides are predominantly present as *anti*-rotamers,[39] while studies indicate that the binding of uridines to enzyme uridine phosphorylase (Urd-Pase) take place in the *syn*-conformation.[40] Thus ara-U was a very poor inhibitor of Urd-Pase but binds better if a benzyl group is present at the position 5 of pyrimidine ring. This binding is enhanced further if 5-benzyloxybenzyl group is present at position 5 of pyrimidine ring.[40] One special analog of ara-U which must be mentioned here is arabinosyl cytosine (**4**). It is the most active antimetabolite for inducing remission in nonlymphocytic leukemia[41] and in combination with anthracyclines such as daunorubicin, leads to complete remission rates in the range of 60-70 %. The use of ara-C (**4**) with deaminase inhibitors for treatment of leukemia has been discussed.[42] It is also a very potent anti herpes agent. However, it is not selective in its action.[31] Interestingly, ara-C (**4**) also displays immuno suppressive property.[43] The activities of ara-C and its analogs have been reviewed.[44] Several 2-deoxyuridine analogs have been synthesized.[45-49]

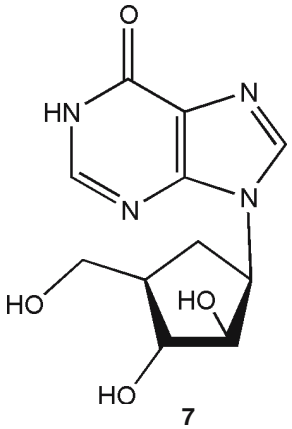
2.4 Spongoadenosine (5, Ara-A)

Spongoadenosine (**5**) and its 3′-O-acetyl derivative (**6**) have been isolated from gorgonian *Eunicella cavolini*.[50] *E. cavolini* (640 g, dry wt.) was extracted (4 times) with acetone. The acetone extract was concentrated and extracted successively with ether and n-butanol. The solvent from n-butanol extract was removed *in vacuo*. The syrup (3.1 g), thus obtained was chromatographed on silica gel column equilibrated with $CHCl_3$: MeOH to give two main fractions. Preparative TLC on silica gel plates (solvent $CHCl_3$: MeOH, 7 : 3) of each fraction afforded pure ara-A (**5**) (50 mg) and its 3′-O-acetyl derivative (**6**). It is interesting to note that ara-A (**5**) has been synthesised[51] prior to its isolation from natural source. Isolation of spongothymidine[18] and

spongouridine[16] from marine source which were resistant to enzymes that repture the base-sugar bond stimulated the synthesis of a series of nucleosides with a fraudulent' sugar arabinose. [52] Among these ŝynthetic analogs', ara-A exhibited significant antiviral activity against DNA virus making it first antiviral drug for the treatment of fatal *Herpes encephalitis*.



5, R = H
6, R = Ac



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Arabinosyladenine (ara-A or vidarabine) was first synthesised in 1960, and the significant biological properties of this compound have ensured continued interest since then.[53-57] It has been found that 9-β-D-arabinofuranosyl-adenine-5′-phosphate, which penetrates the cell without degradation has more sustained toxicity against mouse fibroblasts (L-cells) than does its nucleoside. It has a firmly established role in the management of certain human herpes virus infections[58] and it is also effective in the therapy of herpes keratitis, herpes encephalitis and *Varicella zosten* infections in immnosuppressed patients.[59] β-Anomer of ara-A is also a good antiviral agent.[60] Degradation of ara-A by adenosine deaminase yields 9-β-D-arabinosylhypoxanthine (ara-H, **7**)[61] which is about one-tenth as active as ara-A against HSV culture, but is of comparable activity when assayed *in vivo*.[62] However, low solubility of ara-A and its ready deamination were main restraint to the administration of this drug. To overcome these problems some O-acyl derivatives[63,64] of ara-A were prepared and among these 3-O′-acyl derivative appeared promising, while the best properties were observed with 2′-O-acyl derivative.[63] Carbocyclic ara-A (cyclaradine) has been prepared and found resistant towards deamination by adenosine deaminase.[65] This analog exhibits promising activity *in vivo* against genital herpes and *Herpes encephalitis*. Biosynthesis of ara-A has been studied.[66] It is suggested that ara-A is produced by direct epimerization of C 2′ hydroxy group of adenosine or a derivative thereof. It is also likely that a 2′-keto compound is involved as an intermediate.[67]

3. Pyrimidine-2′-deoxyribosides

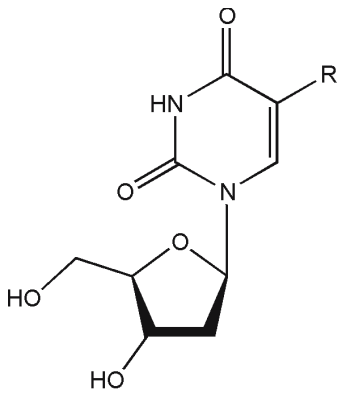
3.1 2′-Deoxyuridine (8)

Marine organisms have furnished pyrimidine 2′-deoxyribosides. 1-(2′-Deoxy-β-D-ribofuranosyl) uracil (**8**) has been isolated from starfish *Acanthaster planci*.[68] Its structure was elucidated with the help of ^{1}H and ^{13}C NMR spectroscopy as well as by EI and FD mass spectrometry.[68]

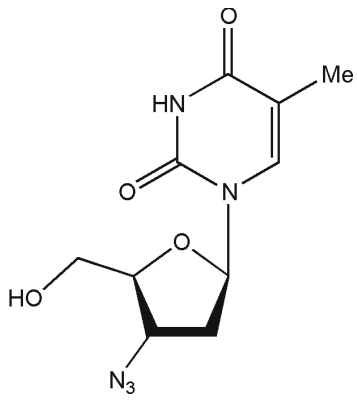
A large number of antiviral and anticancer drugs that are in use or the compounds which have clinical potential can be considered as analogs of 2′-deoxyuridine (**8**).[31] Most of these compounds are directed towards the treatment of herpes virus (HSV-1, HSV-2, and VZV) infections. The most effective compound is: 5-ethyl-2′-deoxyuridine (EDU) (**8**, R = Et) is used in the treatment of *Herpetic keratitis*.[69] It is phosphorylated to a much greater extent by HSV-infected virus cells than by mock-infected cells.[70] Within the infected cells, EDU is preferentially incorporated into viral DNA and is more inhibitory to viral than cellular DNA synthesis.[70] 5-Fluoro-2′-deoxyuridine (**8**, R = F) is an anticancer drug and its congeners[71] are also efficient inhibitors of tumor cell proliferation and the target for their antitumor action appears to be d-TMP synthetase. (*E*)-5-Bromovinyl-2′-deoxyuridine (BVDU) is one of the most potent anti HSV-l agent known.[72] It inhibits the replication of HSV-l, VZV, pseudo rabies virus at a concentration of 0.001-0.01 mg/ml.[31]

3.2 Thymidine (9)

1-(2′-Deoxy-β-D-ribofuranosyl)thymine (**9**) was isolated from the starfish *Acanthastifr plancii*,[68] along with 2′-deoxyuridine (**8**). It binds four to six times less tightly to uridine phosphorylase (Urd Pase) than the corresponding ribonucleoside.[40] One of the close analogs of thymidine (**9**) which got world wide attention of the chemists and biologists in recent times is 3′-azido-2′, 3′-dideoxythymidine (AZT, Zidovudine, Retrovir, **10**) because this compound exhibited potent HIV inhibitory activity.[73-77]

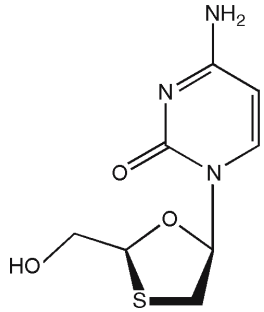


8, R = H
9, R = Me



10

AZT is the first reverse transcriptase (RT) inhibitor approved by the FDA for the treatment of human immunodeficiency virus (HIV) infection. AZT is a synthetic pyrimidine analog that differ from thymidine in having an azido functionality at 3′ position of the deoxyribose in place of hydroxyl group.[78] AZT is a drug of choice for the treatment and management of cognitive impairment in symptomatic HIV infected patients. Other thymidine nucleoside which has been approved by FDA for HIV treatment are 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine, Epivar, **11**)[79] and 2′,3′-didehydro-3′-deoxythymidine (D4T, Stavudine, **12**).[80]



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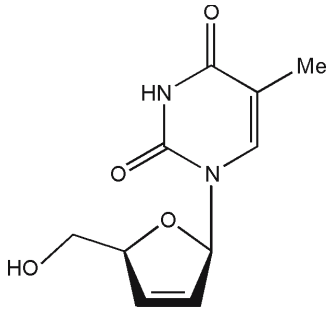
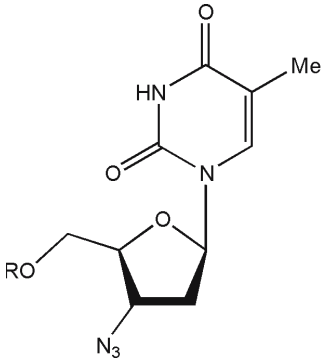


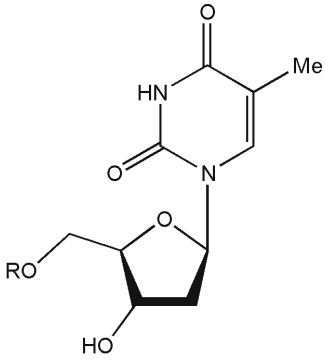
12

It has been shown that cellular enzyme converts AZT to AZT-5′-triphosphate and that inhibit the HIV-reverse transcriptase (HIV-RT). In order to make these nucleosides biologically more active, efforts have been made to improve anti-HIV activity by posphorylation of the AZT.[81] This approach has resulted many nucleotides with potent anti HIV activity (**13-17**).[82-87]

Very recently lipophilic phosphonoformate and phosphonoacetate derivatives of AZT were synthesized, and some of the compounds have shown better



13, R = $(PO_3^-)_2$, AZT-DP
14, R = $(PO_3^-)_3$, AZT-TP



15, R = (PO_3^-), TMP
16, R = $(PO_3^-)_2$, TDP-DP
17, R = $(PO_3^-)_3$, TTP-TP

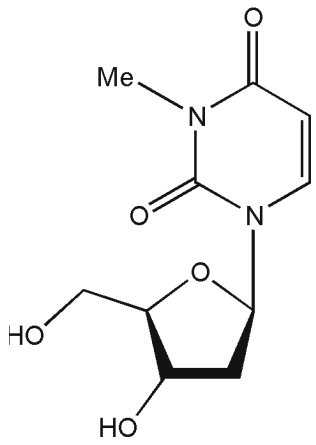
HIV-1 activity than the parent nucleoside.[81] Many modifications in the core structure of the AZT have been carried out,[88] and it was found that substitution at the 4′ or 3′-position by fluorine, cyano, methyl, ethyl, and even an unusual oxetane exhibit interesting antiviral activity. [89-92] AZT delays progression of HIV and improves the survival rate in patient with advanced disease. HIV-1 infection can be reduced in the new born by the use of AZT.[93] It improves the neuropsychological performance in adult patients with HIV-1 infection. Improvements in the neurological functions such as memory, motor function, attention, and general cognitive ability have been observed in the patient taking AZT treatment.[94] Combination drug therapies reduced dramatically the HIV viral loads, and in most of the combination AZT was included. It was found that the combined use of AZT, and another inhibitor (ddC or 3TC) and a protease inhibitor is the most effective.[95,96]

3.3 3-Methyl-2′-deoxyuridine (18)

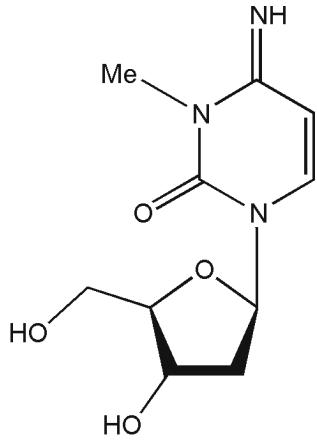
The usual pyrimidine nucleoside, 3-methyl-2′-deoxyuridine (**18**) has been isolated from the marine sponge *Geodia baretti*.[97] Random screenings of the crude extracts of marine organisms *Geodia baretti*, exhibited strong contractile activity in the isolated guinea-pig ileum assay.[97] The aqueous extract was devoid of significant lectinlike activity.[97] Seven fractions were obtained from ethanolic extract of freshly collected material and exhibiting either contractile activity or inhibition of electrically stimulated guinea-pig ileum. *Geodia baretti* was collected in July, 1985 from Swedish west coast and organisms were frozen and freshly thawed material (5.3 kg wet wt) was extracted at room temperature with EtOH (2 X 15 lit). Evaporation of solvent *in vacuo* gave 120 g residue. MeOH extraction and centrifugation led 67 g insoluble material and, after evaporation of the solvent, 50 g soluble material was obtained. The soluble material (25 g) from MeOH extract was chromatographed on a Sephadex LH-20 (400 g) open bed column with MeOH as eluent. Seven fractions were collected. The nature of these fractions were investigated by ^{1}H NMR and TLC. Fraction 1 contained phthalates, presumably artifacts from the isolation procedure. Fractions 2, 3, and 4 lack aromatic protons and uv (254 nm) absorption but have intense signals at δ2.9-3.4 ppm indicating low mol. wt. hydrophilic compounds. Only fractions 5 and 6 were further investigated and resulted in the identification of barettin, adenosine, histamine and inosine. In addition, fraction 5, yielded three N-methylated nucleosides, namely, 3-methyl-2′-deoxyuridine (3 mdUrd) (**18**), 3-methylcytidine (3 mCyd)], and 3-methyl-2′-deoxycytidine (3 mdCyd) (**19**). The structures of these nucleosides were confirmed by comparison of FAB-Mass, ^{1}H-NMR, and ^{13}C-NMR data with values reported for the synthetic compounds.

3.4 3-Methyl-2′–deoxycytidine (19)

3-Methyl-2-deoxycytidine (**19**) has been isolated from marine sponge *Geodia baretti* for the first time as a free natural product.[97] It shows strong contractile activity in the isolated guinea-pig ileum assay.[97]



18



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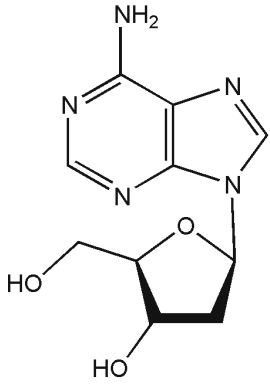
3.5 2′-Deoxyadenosine

2′-Deoxyadenosine (**20**) is a normal component of nucleic acids. However, it has been isolated in free state from the marine sponge *Dasychalina cyathina*.[98] 3′-Azido-2′,3′-dideoxyadenosine (**21**) has been prepared.[99]



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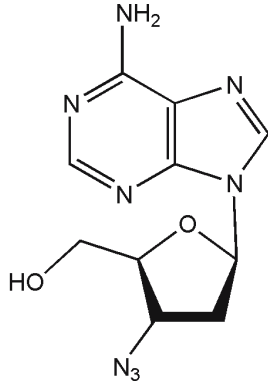
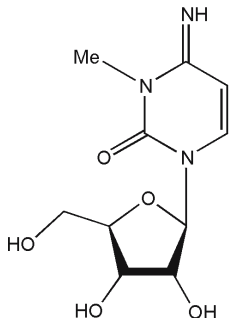


21

4. Pyrimidine and Purine 1-β-D-ribosides

3-Methylcytidine (**22**) is the only pyrimidine riboside isolated from marine sponge *Geodia baretti*[97] along with 3-methyl-2′-deoxycytidine (**19**). It displays contractile activity. Compound (**23**) was found to exhibit potent antileukemic activity, and this compound was commercialized to UpJohn as Arabinoside-C.[100]



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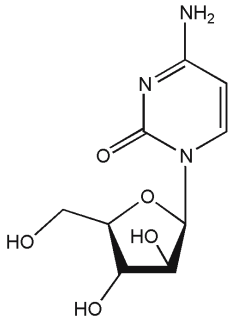

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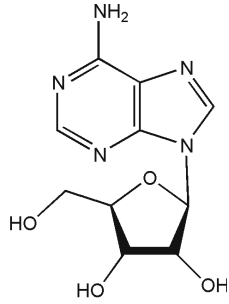


24

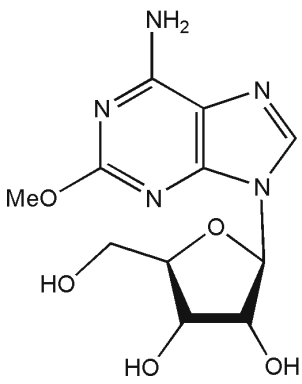
4.1 Adenosine (24)

Adenosine (**24**) is widely distributed in nature. It is one of the principal nucleosides of nucleic acids. Adenosine (**24**) in free state has been isolated from marine sponge *Dasychalina cyanthina*.[98] Its transport,[101] formation, inactivation in different tissues[102] and involvement in the pathophysiology of renal changes observed in various types of renal insufficiency have been discussed. The bronchodilation efficiency of methylxanthine is believed due to the adenosine antagonism. The role of its uptake inhibitors as probe has been established.[103] A number of adenosine analogs having alkylthio groups at position 2 of the heterocyclic moiety has been prepared with a view to modulate the coronary vasodilator activity of adenosine. It has been found that introduction of an alkylthio group at position 2 of adenosine increases the duration of its action and decreases the coronary vasodilation effect as compared to adenosine. Moreover, as the length of alkyl chain is increased, the coronary vasodilator activity becomes more pronounced while branching of the alkyl chain reduced the activity.

4.2 Spongosine (25)

In 1950, Bergmann isolated spongosine (**25**) along with spongouridine (**2**) and spongothymidine (**1**) from the marine sponge *Cryptotethia crypta*.[15,16] The presence of amino group in the nucleoside was established by diazotization to give a dioxy compound. Action of adenosine deaminase confirmed the presence of amino group at position 6 of the heterocyclic moiety, like that of adenosine.[104] By comparing the UV spectra of the base obtained by acid hydrolysis of the nucleoside, with those of other known purines, its structure was inferred to be a methoxy purine which was also in agreement with its elemental analysis.[105] Finally, the structure of spongosine (**25**), as 3-methoxy adenosine was confirmed by its synthesis[104] from 2-chloroadenine. Three syntheses of spongosine (**25**) are reported.[106,107] Recently, an efficient synthesis of spongosine from 2-ethylthio adenine has been reported. Condensation of 2-ethylthioadenine with 1-O-acetyl-2, 3,5-tri-O-benzoyl ribofuranose in dry nitromethane in the presence of $SnCl_4$ gave 2-ethylthio-9(β-D-2′,3′,5′-tri-O-benzoylribofuranosyl)adenine (in 62% yield). Deblocking of the protected

nucleoside with methanolic NH_3 at ambient temperature afforded 2-ethylthioadenosine in 90% yield which on acetylation with acetic anhydride in pyridine afforded triacetyl derivative in good yield. $KMnO_4$ oxidation of the compound in aqueous acetic acid gave 2-ethylsulfonyl-9-(β-D-2′,3′,5′-tri-O-acetylribofuranosyl)adenine which on refluxing with sodium methoxide finally yielded 6-amino-2-methoxy-9-β-D-ribofuranosyl-purine (spongosine) (**25**) in good yield. A new synthesis of spongosine from isoguanosine (**26**) is also reported.[108] Effect of the size of alkoxy groups at position 2 in spongosine has been extensively studied with respect to its coronary vasodilating activity. The result revealed that an n-propoxy group at position 2 instead of a methoxy group resulted in eight fold increase in its activity.



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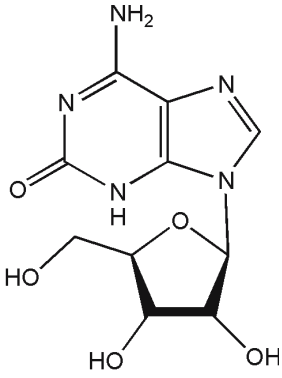

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4.3 Analogs of Spongosine

Several analogs of spongosine have been prepared and evaluated for biological activities. 2-Deoxy analog of spongosine shows moderate antitumour activity.[109] 9-β-D-Arabinosyl-2-methoxy adenine exhibits marginal activity against HSV-1 and found inactive against HSV-2.[110] Its 9-D-arabinosyl analog was found devoid of antiviral activity.[111] 6-Amino-9-[2-hydroxy-1-(hydroxymethyl)-ethoxymethyl]-2-methoxypurine, an acyclic analog of spongosine when evaluated in combination with interferon inducer, mycoviral dsRNA and found protection by 40% against the Semliki Forest Virus (SFV) in Swiss albino mice.[112]

4.4 Isoguanosine (26)

Isoguanosine (**26**) had been isolated from the marine nudibranch mollusc *Diaulula sandiegeness*.[113] It has been isolated earlier from the beans of *Croton tiglium*,[114] and named crotonoside. Isoguanosine (**26**) produced hypotension, bradycardia and relaxation of smooth muscle. It is more potent and much longer acting than adenosine. Like adenosine and its analogs it stimulates accumulation of adenosine 3′,5′-monophosphate (cAMP) in brain

tissue. Although isoguanosine has not shown to be incorporated into the nucleic acids of bacteria, however, it is believed that it might be a constituent of nucleic acids in some animals. Isoguanosine (**21**) has been prepared by selective deamination of 2,6-diamino-9-β-D-ribofuranosylpurine with nitrous acid.[115] However, in the presence of acidic medium the N-C glycoside bond of the purine nucleoside gets rapidly hydrolyzed resulting in a very poor yield of isoguanosine. Recently isoguanosine (**26**) has been synthesed[116] by two methods, in the first method glycosidation of chloro mercuric complex of 6′-acetyl isoguanine with tribenzoyl ribofuranosyl chloride gave 6-acetyl-2′,3′,5′-tri-O-benzoyl-9-β-D-ribofuranosylisoguanonine which on deblocking with methanolic ammonia at 0-5°C yielded isoguanosine (**26**) (yield 25%). The second method consists of nucleophilic transformation reactions at position 6 in xanthosine. 2′,3′,5-Tri-O-benzoylxanthosine on treatment with phosphorus pentasulfide in pyridine affords 6-thio-2-hydroxy-9-β-D-ribofuranosylpurine which on methylation with methyl iodide in the presence of alkali gives 6-methylthio-2-hydroxy-2′,3′,5-tri-O-benzoyl-9-β-D-ribofuranosylpurine. The blocked nucleoside is treated with methanolic ammonia to give finally isoguanosine (**26**) (yield: 20%).

An efficient synthesis of isoguanosine (**26**) in 68% overall yield has been achieved.[117] Silylation of xanthosine with hexamethyl disilizane and catalytic amount of $(NH_4)_2SO_4$ gave the tetrasilylated xanthosine which on aminolysis yielded 6-aminotrisilylated derivative. Removal of the protecting group furnished isoguanosine (**26**). A number of analogs of isoguanosine have been prepared by this procedure in 65 to 73% yields. Of this one compound exhibited 66% inhibition against Ranikhet disease virus.[117] Isoguanosine arabinoside an analog of isoguanosine displayed antiviral activity against *vaccinia* virus and HSV-1 *in vivo*.[118]

4.5 Doridosine (27)

Doridosine (**27**) [119,120] was isolated from marine sponge *Tedania digitala* and *Anisodoris nobilis* almost simultaneously by two groups working independently in different continents (Australia and USA).[121,122] Fuhrman et al[121] in a communication reported that the cardioactive component of the digestive gland of *Anisodoris nobilis* is a new N-methylpurine riboside and named doridosine. Around same time Quinn, Gregson, Cook, and Bartlett at the Roche Institute of Marine Pharmacology, Dee Why, NSW Australia, also reported the isolation of a compound with similar properties from the sponge *Tedania digitata.*[122] These workers have positively established the structure of their product as l-methylisoguanosine by spectroscopic methods, degradation, and synthesis. Authors exchanged the samples, and it was found that these products from the two very different sources the nudibranch from California and the sponge from Australia, are indeed identical. The function and origin of this compound in these marine organisms is not known. Doridosine causes reduced arterial pressure and reduced heart rate in mammalians in a

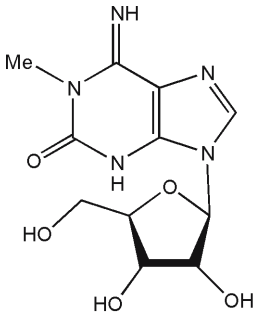
manner that is qualitatively similar to adenosine. It also acts as muscle relaxant and hypothermic activity.

The extracts of active component of *Anisodoris nobilis* was purified by bioassay guided purification on the isolated guinea pig atria. The force of contraction and rate was decreased in both crude and highly purified fraction, and these effects were found to be dependent upon the doses. The negative inotropic action was less variable than negative chronotropic action. Chemical purification of the active component of the lyophilized crude aqueous extracts of *Anisodoris nobilis* was accomplished by repeated gel-permeation chromatography on Bio-Gel P2 followed by chromatography and rechromatography on silica gel with *t*-butyl alcohol, ethyl acetate, water, acetic acid (40:10:2:1) as the eluting solvent. The active component was crystallized from methyl alcohol and water, and washed with small amount of acetic acid, and recrystallization from water, acetic acid, and methanol yielded highly pure compound. The yield of doridosine was about 0.02% based on wet weight of the digestive gland.

The high resolution mass spectrometry indicated the parent ion of doridosine as *m/z* 297.1075 corresponding to a molecular formula of $C_{11}H_{15}N_5O_5$. The 100% intense peak (base peak) was at *m/z* 165.0651 corresponding to a formula for the major fragment of $C_6H_7N_5O$. This latter formula agrees with that of a methylated purine nucleus of guanine or isoguanine ($C_6H_7N_5O$). Thus this mass spectrum can be interpreted as that of a methyl nucleoside in which the major fragmentation is between the methylated purine nucleus and the pentose moiety. Coupled gas chromatography, mass spectrometry of trimethylsilylated doridosine clearly showed the introduction of five trimethylsilyl groups (*m/z* = 657) with two on the purine nucleus *(m/z* = 338) and, therefore, three on the carbohydrate moiety. There was some incomplete trimethylsilylation indicating one difficulty replaceable active hydrogen. Doridosine. therefore, has four readily replaceable hydrogens and a fifth that is more difficult to substitute. The mass spectrum of doridosine has the same parent ion and base peaks as N-methylguanosine. The ultraviolet, long wavelength absorption band of doridosirie at pH 6.5 [λ_{max}, 292 nm (ε 8500)] undergoes a bathochromic shift when the solution is made either acidic [pH 1.5, λ_{max} 282 (ε 9000)] or basic [pH 12, λ_{max}, 286 nm (ε 7500). Similar behavior is observed for isoguanosine; only N^6-methylisoguanosine seems to be reported to show this pattern. A direct comparison of the uv spectra of doridosine and an authentic sample of N^6-methylisoguanosine, showed that they are not the same. Further more, the proton NMR of N^6-methylisoguanosine is not identical to that of doridosine. The decomposition point of doridosine, depending on the rate of heating is 260-265°C that for N^6-methylisoguanosine is 210°C while that of a mixture of the two is 220-230°C. Finally structure of doridosine was determined by ^1H and ^{13}C NMR spectra in both D_2O-CD_3OD and DMSO-d_6 solvents. The ^{13}C NMR spectrum of doridosine is a typical of a purine nucleoside. Resonance at δ139 ppm as a doublet in the

non-decoupled spectrum indicates the presence of a hydrogen at C-8 in the purine ring of the doridosine. The presence of nitrogen, and oxygen at this position was thus ruled out. ^{1}H NMR and ^{13}C NMR data matched well with the known purine nucleosides, and thus confirmed the structure of doridosine. The optical rotation of doridosine was observed to be $[\alpha]_D^{20} -66.2°$ (C = 0.42g/L, MeOH). It was prepared in large quantities and subjected to a number of pharmacological tests. Doridosine has muscle relaxant, hypothermic and cardiovascular effects following oral administration in mice and rats.[120] It interacted directly with adenosine receptors in guinea-pig brain to stimulate adenylate cyclase.[123] Its muscle relaxant activity and other properties have been compared with its various analogs. It has been found that the potency is retained in compounds in which the 1-methylisoguanine moiety is unaltered. Unlike adenosine, doridosine is resistant to deamination by adenosine deaminase. Many pharmacological effects of doridosine are apparently due to its action as long lasting adenosine analog.

1-Methylisoguanosine (**28**) a close derivative of doridosine (**27**) has been reported to occur in the marine animals. Recently Quinn et al[124,125] isolated this compound from the sponge *Tedania digitata* and it has also been reported to occur in the nudibranch *Anisidoris nobilis*[126] and coral *Madracis mirabilis*.[127] This nucleoside shows potent muscle relaxant, blood pressure lowering, cardiovascular, and antiinflammatory activity.[128,129] This compound was originally synthesized by the reaction of 5-amino-4-cyno-1β-(2′,3′,5′-tri-O-acetyl-D-ribofuranosyl)imidazole which upon cyclization and deprotection with methanolic ammonia gave the product in good yield. A series of modification in the 1-methylisoguanosine nucleoside has been carried out and it was observed that these modified nucleosides showed promising biological activities. Recently pyrazolo[3, 4-d]pyrimidine analogues of 1-methylisoguanosine were prepared, and 4-amino-5-N-butyl-1-(3-chloro phenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-15*H*-one was found to be the most active compound of the series with IC_{50} of 19.2×10^{6} M.[130]



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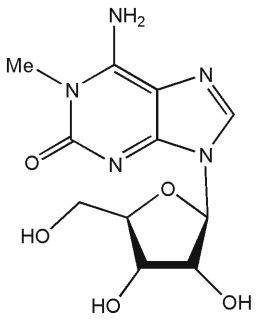

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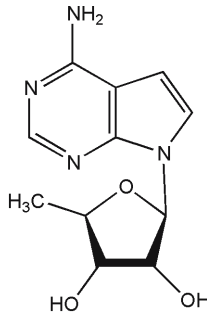

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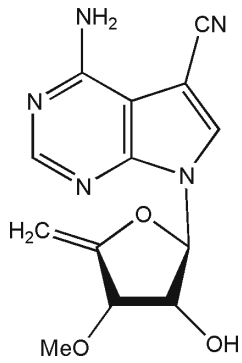
5. Pyrrolo[2, 3-d]Pyrimidine Nucleoside (29)

Marine organisms also elaborate pyrrolo[2, 3-d]pyrimidine nucleoside having a modified D-ribose sugar moiety. Marine alga *Hypnea valendia*[131] has furnished 5-iodo-5-deoxytubercidine (**29**). The sugar moiety in the nucleoside is 5-deoxyribose. The nucleoside displays pronounced muscle relaxant property. It also produces hypothermia in mice.[131] No synthesis of the nucleoside is reported. The marine sponge of the genius *Mycale*[132-135] has yielded pyrrolo [2,3-d]pyrimidine nucleosides name mycalesine-A (**30**) and mycalesine-B (**31**). The sponge is found commonly along the pacific coast of Japan. The ethyl acetate soluble fraction of the ethanol extract of the frozen sponge (9.5 kg) is subjected to SiO_2 flash chromatography. Elution of the column with CH_2Cl_2/EtOAc/MeOH gave an active fraction which on repurification by SiO_2-HPLC and reversed phase HPLC afforded mycalesine-A (**30**, 10 mg) and mycalesine-B (**31**, 48 mg), both as colorless oil.

The mycalesine-A has spectral feature similar to those of nucleoside antibiotic toyocomycin isolated from *Streptomyces* species. It contained 3′-O-methyl-5-deoxy-erythropen-4-eno-furanose sugar moiety. Mycalesine-B was closely related to mycalesine-A. Oxidation of mycalesine-B with alkaline H_2O_2 furnished carboximidine derivative. Both mycalesine-A and mycalesine-B were unstable at room temperature but were stable when stored under nitrogen atmosphere at ⩽0 °C. Mycalesine-A has been synthesized from toyocomycin.[136] Mycalesine-A (**30**) inhibits cell division of fertilized starfish eggs at a concentration of 0.5 μg/M whereas mycalesine-B inhibits the cell division of the egg at a concentration of 200 µg/M.

Isolation of nucleoside antibiotics such as tubercidin,[137-144] toyocamycin,[145-148] sangivamycin,[149] cadeguomycin[150-152] and now of marine nucleoside 5-iodo-5′-deoxytubercydin,[131] mycalesine-B and mycalesine-A[132] has been the main force behind the continued interest of the medicinal chemist in pyrrolo [2,3-d]pyrimidine nucleosides[153-158] Despite the similar chemical structure of these nucleosides they exhibit different biological activities. They are phosphorylated to the triphosphate and then incorporated into nucleic acids.



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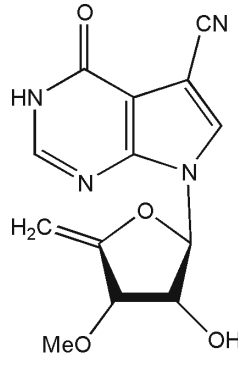

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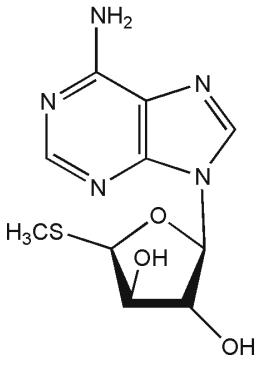
These compounds exhibit potent antitumor activity. They also displayed antiviral activity against RNA and DNA viruses. However, their usefulness as antiviral agents is hampered because of their high toxicity. Analogs of pyrrolo[2, 3-d]pyrimidine nucleosides have been synthesized which exhibit excellent biological activities.[156]

6. 9-[5′-Deoxy-5′-(methylthio)-β-D-xylofuranosyl]Adenine (32)

An unusual nucleoside isolated from marine nudibranch mollusk *Doris verrucosa*[159] has been characterized as 9-[5′-deoxy-5′-(methylthio)-β-D-xylofuranosyl]adenine (**32**). It is the first naturally occurring analog of methylthio-adenosine (MTA). In biological system MTA is formed from S-adenosyl-L-methionine (AdoMet), a ubiquitious enzyme that occurs both in normal and malignant tissues. AdoMet acts as methyl group donor in transmethylation reaction. MAT is known to be involved in several regulatory processes. The nucleoside (**32**) is the first naturally occurring purine nucleoside carrying a substituted xylose sugar moiety. It has been synthesized[160] prior to its isolation from a marine nudibranch. Modification on the core structure of the nucleoside resulted many biologically active MAT derivatives.[161] Nucleoside (**32**) was again synthesized in 1989.[162] Bhakuni et al[163] have recently reported an efficient and convenient synthesis of nucleoside (**32**). Condensation of adenosine with 1,2,3,5-tetra-O-acetyl-D-xylofuranose in the presence of stannic chloride afforded the protected xyloside in very good yield. The β-configuration of xylose moiety at position 9 of adenosine was established with the help of NMR spectral data. Deprotection of the nucleoside with methanolic ammonia afforded 9-β-D-xylofuranosyl adenine. Selective tosylation of hydroxyl function at position 5′ with p-toluene sulfonyl chloride at 0-5°C yielded 9-(5′-deoxy-5′-(tosyl)-β-D-xylofuranosyladenosine which on acetylation with acetic anhydride in pyridine afforded 9-[5′-deoxy-5′-(tosyl)-2′,3′-di-O-acetyl-β-D-xylofuranosyl] adenine. Treatment of this nucleoside with methyl mercaptan in DMF gave 9-[5′-deoxy-5′-(methylthio)-β-D-xylofuranosyl]adenine. Removal of the acetyl protective group finally



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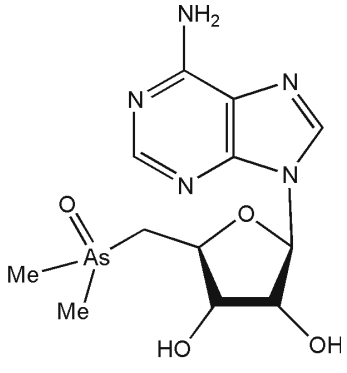

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afforded the desired xyloside (**32**). Number of analogs 9-[5′-deoxy-5′-(methylthio)-β-D-xylofuranosyl]adenine (**32**) have been prepared following above procedure and evaluated their biological activity. Of the compounds tested one displayed 66% inhibition against Ranikhet disease virus.[7]

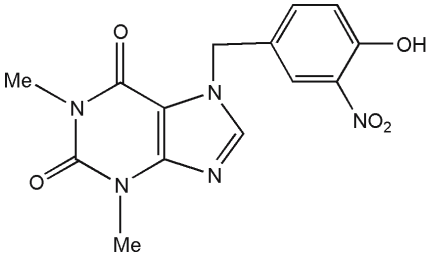
7. 5′-Deoxy-5′-Dimethylarsinyl Adenosine (33)

An arsenic containing the nucleoside characterized as 5′-deoxy-5′-dimethylarsinyladenosine (**33**) has been isolated from the kidney of the giant clam *Tridacna maxima*.[164] Arsenic is generally present in sea water at concentration of 2-3 g/dm^3, chiefly as arsenate. The major form of arsenic in marine algae are dimethylarsinyl ribosides[165] which are probably metabolized to arsenobetaine ($Me_3\ As^+CH_2C\,O_2^-$), the usual form of arsenic in marine animals within food chain. It is believed that algae biosynthesize dimethylarsinylriboside from absorbed oceanic arsenate by the mechanism as described by the Challenger[166] for the biosynthesis of trimethylarsine by microorganism. The giant clam *Tridacha maxima* contain symbiotic algae in its tissues and products of algae metabolism are accumulated in the kidney.[167]

8. Miscellaneous Compounds

8.1 Phidolopin

Marine organism *Phidolopora pacifica* was collected from rocky reefs in Barkley Sound, British Columbia. The freshly collected material was homogenized in methanol. The methanol extract was purified by flash chromatography and preparative TLC to yield phidolopin (**34**) in very low yield (4 mg): mp 226–227 °C; TLC (silica gel) R_f 0.16 (ethyl acetate). UV (CH_3CN) λ_{max} 353 nm (ε 3300), 275 nm (ε 16,800); IR ($CHCl_3$) 3300, 1697, 1626, 1532 cm^{-1} suggested the presence of carbonyl, and hydroxyl functionalities. HRMS-EI, *m/z* 331.0917 (M^+) (calcd for $C_{14}H_{13}N_5O_5$ 331.0917), 180 ($C_7H_8N_4O_2$), 152 ($C_7H_6NO_3$, base peak). Benzylic cleavage was observed in the mass spectrum of phidolopin (**34**), which results in the nitrophenol residue giving rise to the observed base peak at *m/z* 152. The remaining of phidolopin molecule contain fragment $C_7H_7N_4O_2$ which have six degree of unsaturation. ^1H NMR (270 MHz, $CDCl_3$) δ 3.39 (*s*, 3 H), 3.59



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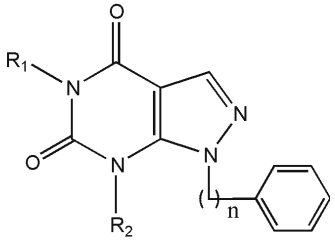

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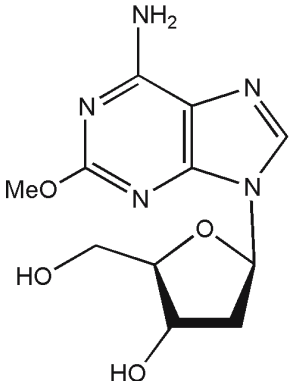
(*s*, 3 H), 5.46 (*s*, 2 H), 7.16 (d, J = 8.6 Hz, 1 H), 7.61 (dd, *J* = 2.2, 8.6 Hz, 1 H), 7.63 (s, 1 H), 8.08 (d, *J* = 2.2 Hz, 1 H), matches well with the proposed molecular formula. Resonance at δ 10.56 (1 H, exchanges with D_2O) confirmed the presence of phenolic OH group. ^{1}H NMR resonances at δ 5.46, 7.16, 7.61, 8.08, and 10.56 were assigned to 4-hydroxy-3-nitrobenzyl residue by comparing these chemical shifts to the literature values for 4-hydroxy-3-nitro toluene. ^{1}H NMR resonances at δ 3.39 (*s*, 3 H) and 3.59 (3 H) indicated presence of two methyl groups attached to either oxygen or nitrogen atoms and the IR spectrum suggested the presence of at least one amide carbonyl. A purine nucleus containing oxygen, methyl, and 4-hydroxy-3-nitrobenzyl substituents could account for all the structural requirements of phidolopin. Finally structure of phidolopin (**34**) was solved via a single-crystal X-ray diffraction analysis on its p-bromophenacyl derivative. Phidolopin (**34**) is a new addition to a very small but important group of naturally occurring xanthine derivatives which include caffeine, theophylline, and theobromine. It is of special interest because it is of animal rather than of plant origin. Further, it contains a nitro functionality which is relatively rare in natural products. It displays high order of antifungal and antialgal activities *in vitro*.[168] Phidolopin had been synthesized by Hirata et al.[169] Recently, Avasthi et al[170] has reported a convenient synthesis of phidolopin. Reaction of 4-methyl-2-nitrophenol with methyl iodide in the presence of anhyd. K_2CO_3, in dry DMF gave 4-methoxy-3-nitrotoluene, which on bromination with N-bromosuccinimide (NBS) gave 4-methoxy-3-nitrobenzyl bromide in good yield. Stirring a mixture of theophylline and 4-methoxy-3- nitrobenzyl bromide in dry DMF in presence of anhyd. K_2CO_3 gave a single product which on alkaline hydrolysis yielded phidolopin (**34**). A number of analogs of phidolopin has been prepared following this procedure and evaluated for antibacterial (*in vitro*), antifungal (*in vitro*) and antiallergic (% PCA inhibition, *in vivo*) activities. The screened compounds showed weak antibacterial and antifungal activities and moderate antiallergic activity.

Avasthi et al synthesized large no of monomeric (**35**), and dimeric pyrazolo [3,4-d]pyrimidine analogs of phidolopin, and observed that the these compounds shows moderate antibacterial, antifungal, and antiallergic activities.[171-173]

Searle and Molinski[174] have examined a sponge collected from Western Australia, and isolated two nucleosides, spongosine (**17**), isolated by Bergmann[104,105] from *Cryptotetbya crypta*, and 2′-deoxy-spongosine (**36**). Prior the isolation of this natural product, compound (**36**) has been obtained as a synthetic product.[175] Searle et al[174] elucidated the structure of (**17**) and (**36**) by spectroscopic methods. The orange sponge was collected from Exmouth Gulf, Western Australia in January 1993. The freeze-dried animals were extracted with MeOH and the extract was partitioned. The $CHCl_3$, and n-BuOH fractions exhibited distinctive signals in their ^{1}H-NMR spectra and TLC indicated only three polar spots in uv light. All of the spots were



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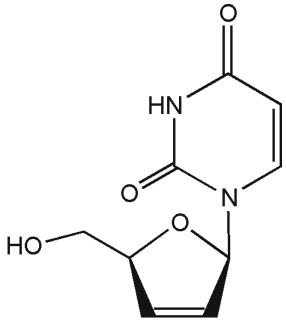

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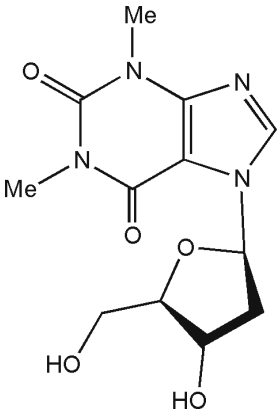
separated by flash chromatography and compound (**17**) was obtained in 0.43% and 2′-deoxy-spongosine (**36**) in 0.39% yield. Crystallization of compound (**36**) from H_2O afforded microcystalline white solid with mp 174–175 °C, $[\alpha]_D$ –22.5 ° (c = 1.0, DMSO). Molecular ion peak was obtained at m/z 282.1178 (MH^+) in the mass spectrum indicating the molecular formula as $C_{11}H_{16}N_5O_4$. Examination of the ^{1}H-NMR and COSY spectra in DMSO-d_6 revealed two substructures. Presence of 2-deoxyribose sugar moiety was confirmed by analysis of the COSY spectrum which indicated a contiguously coupled spin system from H-1′ through to H-5′, including most notably an upfield methylene group H- 2′, δ 2.22 (1H, ddd, J = 13.4, 7.8, and 2.7 Hz) and δ 2.73 (1 H, ddd, J = 13.2, 6.2, and 5.8 Hz). The remainder of the ^{1}H NMR spectrum consist of a methoxy signal at δ 3.80 (*s*, 3H), a sharp downfield one proton signal at δ 8.12 (*s*, H-8) and a broad NH signal at δ 7.30 (NH-6), suggestive of a 2-methoxyadenosine. Complete structure was determined by 2D HETCOR and COLOC experiments. The NH protons and H-8 showed long-range correlations to C-5, δ 115.7(s) in the COLOC spectrum. The methoxy protons at δ 3.80 (s) showed a three-bond correlation to C-2 at δ 161.7 (s) and confirmed the presence of 2-methoxyadenine base. FAB-mass also supported the presence of 2-methoxyadenine nucleoside which showed the expected fragmentation pattern and the base peak at *m/z* 166 (MH^+, 2-methoxyadenine). Finally The structure of (**36**) was confirmed by comparison of spectral data with an authentic sample of the synthetic material.[175]

Recently, synthetically known antiviral agent 2′,3′-didehydro-2′,3′-dideoxyuridine (**37**) and aplysidine (**38**) have been obtained from Okinawan marine sponge *Aplysia* sp.[176] which was collected off Kerama Islands, Okinawa. The residue obtained in methanol extract was partitioned between ethyl acetate and water. The aqueous portion was subsequently extracted with n-butanol. The n-butanol fraction was purified on a silica gel column. The product thus obtained was repurified by reversed-phase HPLC on ODS (H_2O/CH_3CN, 90:10) to give aplysidine (**38**) in 0.0003% (wet weight) yield together

with 2′,3′-didehydro-2′,3′-dideoxy uridine (**37**) in 0.001% yield. Molecular formula of (**38**) was determined to be $C_{12}H_{16}N_4O_5$ (m/z 296.1118) by EI-HRMS. Presence of 12 carbon was further confirmed by ^{13}C NMR spectrum. Nucleoside nature of (**38**) was confirmed by the fragment ion peak at *m/z* 181, 180, 117 in the EI mass spectrum which correspond to $[aglycon + 2H]^+$, $[aglycon + H]^+$ and [deoxysugar], respectively.[177] The IR spectrum of (**38**) indicated the presence of hydroxyl group (3360 $cm^{\pm}$) and amide carbonyl group (1660 $cm^{\pm}$). The UV absorption [λ_{max} (H_2O) 274 nm (ε 8500],[178] and resonances at δ 154.2 (s), 150.9 (s), 147.7 (s), 139.9 (d), 105.5 (s), 29.4 (q), and 27.6 (q) in the ^{13}C NMR spectrum indicated the presence of 1,3-dimethylxanthine moiety in aplysidine (**38**).[179] The ^1H NMR spectrum showed a singlet at δ 8.38 due to H-8 of the xanthine ring. N-1 and N-3 methyl proton of 1,3-dimethylxanthine appears as a singlet at δ 3.23, and 3.43, and these values matches well with the literature data. Presence of deoxy sugar unit was confirmed by the decoupling experiments. Pseudotriplet signal of H-1′ indicated the β configuration at the anomeric carbon.[180] Finally, the structure (**38**) was confirmed by its synthesis. Treatement of sodium salt of theophylline with 1-chloro-2-deoxy-3,5-di-O-p-toluoyl-α-D-erythro-pentofuranose in acetonitrile gave 7-(2-deoxy-3,5-di-O-p-toluyl-β-D-erythro-pentofuranosyl)theophylline, which on treatment with sodium methoxide yielded a compound the spectral data of which were found to be identical with aplysidine (**38**). Based on this structure of aplysidine (**38**) was determined as 7-(2-deoxy-β-D-*erythro*-pentofuranosyl)theophylline.

This is the first example of the nucleoside with theophylline ring as a base from marine source. Aplysidine (**38**) found to have antagonistic activity to adenosine A1 receptor comparable with xanthine N-7-ribosides.[181]

Recently, 2,3′-didehydro-3′-deoxythymidine and the carbocyclic 2-amino-6-cyclopropylaminopurine, abacavir (**39**) have been approved by FDA for the treatment of patient infected by HIV.[182-185]



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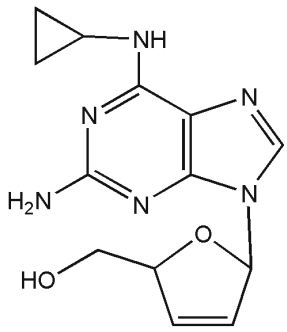

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9. Concluding Remarks

Several bioactive marine nucleosides have been isolated from marine organisms. So far marine sponge has been the best source of these nucleosides. The heterocyclic moiety in bioactive marine nucleoside is either a substituted pyrimidine, purine or pyrrolo[2,3-d]pyrimidine moiety. The sugar moiety is either D-arabinose, D-ribose, 2′-deoxyribose, 2′,3′-didehydro,2′,3′-dideoxyribose or a substituted xylose sugar. In mycalesine-A and mycalesine-B the sugar moiety is 3′-O-methyl-5-deoxyerythropen-4-enofuranose. In the nucleoside isolated from the kidney of the giant clam *Tridacna maxima*, the hydroxy group at position 5 of D-ribose is substituted with 5′-dimethylarsinyl function. Several of these nucleosides have been synthesized. In some cases the compounds have been synthesized prior to the isolation from marine source. Marine nucleosides display antiviral, anticancer, vasodilator, muscle relaxant, and hypertensive activities. Some of them produced bradycardia, and relax smooth muscles. The biological activity of the arabinosides is most prominent. The efficacy of the ara-A in the management of certain human *Herpes virus* infection is firmly established. Ara-A has been found effective in the therapy of *Herpes keratitis*, *Herpes encephalitis* and *Varicella zoster* infections in immuno suppressed patients. Ara-A is one of the best antiviral drug. Marine nucleosides have provided new "Lead compounds"for drug design particularly in the area of viral and parasitic infections. Several analogs of bioactive marine nucleosides have been synthesized and evaluated for biological activities. The structure-activity relationship studies have furnished very useful information for optimization of the activity. The studies carried out have shown that the analog of marine nucleosides display high order of antiviral, anticancer, antilesishmanial and antiallergic activities.

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9

Bioactive Marine Alkaloids

Abstract

The chapter deals with bioactive marine alkaloids. The chemistry and biological activities of pyridoacridines, pyrroloacridines, indoles, β-carbolines, pyrroles, isoquinolines, and tyrosine derived alkaloids have been discussed and reviewed.

1. Introduction

The alkaloids are generally defined as naturally occurring basic nitrogenous compounds. Majority of this class of compounds display biological activity. The reviewers[1-4] of "marine alkaloids" have, therefore, included amino acids, purines, primidines and their nucleosides, peptides, nitrogenous marine toxins, guanidine etc. under "marine alkaloids." The chemistry and biological activities of marine toxins, nucleosides and peptides have been dealt separately in Chapters 7, 8 and 10, respectively. The chemistry and biological activities of the bioactive marine alkaloids for convenience have been discussed and reviewed.

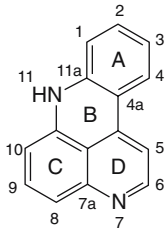
2. Pyridoacridine Alkaloids

Marine pyridoacridine alkaloids have been the subject of intense study due to their significant biological activities.[4-13] Over 75 pyridoacridine alkaloids have been isolated and characterized from marine source and it is expected that more of these alkaloids will be isolated in future. Almost all known pyridoacridine alkaloids are reported to have significant cytotoxicity. The compounds of this group also display several specific biological properties, such as inhibition to topoisomerase II,[12-14] antiHIV activity,[15] Ca^{2+} release activity,[16] metal chelating properties[17] and intercalation of DNA[17] property.

Pyridoacridines have a common tetracyclic heteroaromatic parent-pyrido [4,3,2-m,n]acridine (**1**) system. They are distributed across several phyla of marine invertebrates which are an intriguing fact, and it needs further investigation. One possibility is that in the biosynthesis of these alkaloids probably symbiotic microbes are involved, but it has not yet been tested. Marine pyridoacridine alkaloids have been reviewed extensively.[18-23]

2.1 Occurrence and Chemical Properties

Pyridoacridines have been isolated from marine sponges, tunicates, anemone and molluscs which are often ornately decorated with bright colors and patterns. Tropical tunicates (ascidians) in particular are generally richly pigmented in colors which vary from yellow to deep red, orange, blue and purple. It is often found that pyridoacridines isolated from such tunicates are the pigments (zoochromes) responsible for their coloration. Pyridoacridines act as a pH indicator. The indicator properties is correlated with the presence of at least two basic pyridine like nitrogen and is probably associated with electronic perturbations of an extended chromophore with charge-transfer properties. Simple indicator properties are absent in the less basic iminoquinones, such as cytodytin-A (**2**) and diplamine (**3**). Alkaline solution of the free base generally appears orange or red, while in acidic solution they are green-blue to purple. Some quaternary ammonium alkaloids, like petrosamine (**4**), are deep blue or purple salts.



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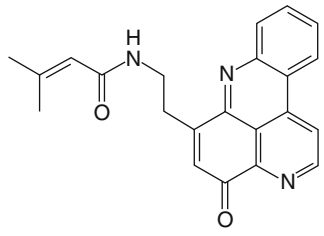

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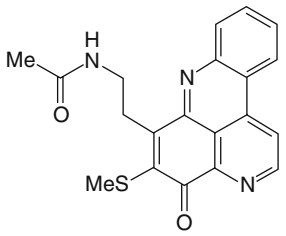

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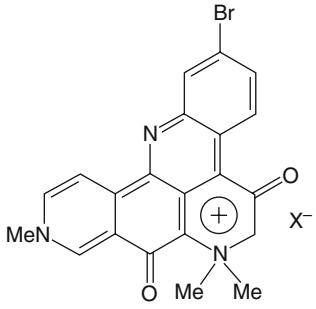
Pyridoacridines are generally obtained as microcrystalline solids with melting points above 300°C. They have also been isolated as hydrochloride salts. Few pyridoacridines are found to be optically active. The optical activity of these compounds is due to the presence of additional asymmetric side chain. The majority of pyridoacridine alkaloids have planar heterocyclic system.

Because of variability in oxidation states of the heterocyclic nucleus, pyridoacridines exhibit facile redox reactions. For example, the iminoquinone substructure (**5**) in many alkaloids is easily reduced by $NaBH_4$. Partially saturated nitrogen containing rings in pyridoacridines are easily aromatized by air oxidation (auto oxidation) upon storage or heating in solution. Although

several pentacyclic pyridoacridines have a 1,10-phenanthroline subunit, they do not react with Fe(II) salts to form red complexes. This lack of reactivity must be interpreted cautiously while assigning possible structures to new alkaloids as it does not provide evidence for lack of 1,10-phenanthroline substructure.



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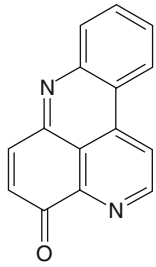

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2.2 Assignment of Structure

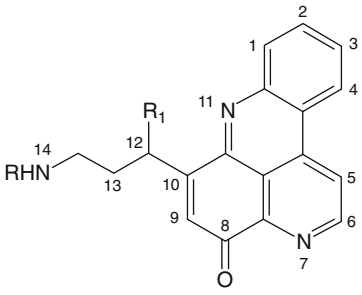
The assignment of structure in general by NMR in highly condensed heterocyclic aromatic compounds is complicated because of the difficulty in defining the correct regioisomer from among many possibilities. However, these problems can be solved by employing new powerful multipulse NMR techniques like HMQC, HMBC, INADEQUATE, INAPT. J_{CH} Coupling constant analysis has been helpful in the resolution of ambiguous structural assignments. When suitable crystals of the compound are available, single crystal X-ray diffraction analysis has given definitive structures. Because the ring system (**1**) is highly conserved, some general features in the appearance of the ^{1}H NMR spectra are common to most of these alkaloids and useful in identifying a member of this class of compounds. The disubstituted benzo ring A gives rise to a distinctive linear four proton coupled spin network (H_1-H_4, 7.0-9.0 ppm, J = 8-9 Hz) with H_1 resonating at lowest field due to the deshielding acridine nitrogen. A second AB spin system (8.5, 9.0 ppm, J = 5.6 Hz) is assignable to H_5-H_6, the protons of a trisubstituted pyridine ring. A strong NOE (ca. 20%) is seen between the two 'bay region' protons, H_4-H_5, thus, linking these two nonscalar-coupled substructures.

2.3 Structural Subtypes

Pyridoacridines vary in structure by appendage of different side chains or fusion of rings to ring C, and occasionally to the acridine nitrogen. Halogen substitution in the pyridoacridines are rarely seen, and when present, this is always bromine at C_2 in ring A. Oxidation states of the rings vary, and in some cases ring D is partially saturated. Additional rings are more commonly appended to ring C.

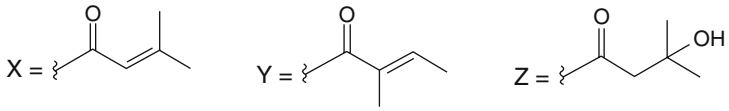
Tetracyclic Alkaloid

The yellow tunicate *Cystodytes dellechiajei* from Okinawa has yielded nine cytotoxic tetracyclic pyridoacridine alkaloids named cystodytins A-I (**2**, **6-11**).[16,24] The cystodytins A-C are the first tetracyclic pyridoacridine alkaloids isolated from a marine tunicate. Colored tunicate (900 g, wet weight) was collected and kept frozen until used. The methanol-toluene (3:1) extract of *C. dellechiajei* was partitioned with toluene and water. It was observed that toluene soluble portion exhibited potent cytotoxicity against L1210 murine leukemia cells. The toluene soluble portion was chromatographed by using $CHCl_3/CH_3OH$ (98.5:1.5) as an eluant followed by a Sephadex LH-20 column ($CHCl_3/CH_3OH$, 1:l) to yielded yellow crystals of cystodytins A (**2**) and B (**6**) in 0.022% yield. Separation of (**2**), and (**6**) was very difficult as both of the compounds had the same retention times on HPLC, silica gel or ODs under different solvent systems. The aqueous layer also exhibited modest cytotoxicity against L1210 and was purified by the same procedure and afforded cystodytin C (**7**) in 0.0003% yield as yellow crystals in addition to 38 mg of mixture of cystodytin A and B. Both of the compounds were separated, and EIMS of free base (**2**) showed the molecular ion at *m/z* 357 (M^+). Molecular formula for compound (**2**) was determined to be $C_{22}H_{19}N_3O_2$ by FAB-HRMS *(m/z* 357.1707). The UV spectrum of (**2**) exhibited absorptions at 225 (ε 35000), 272 (ε 25000), and 380 (ε 14300) nm. Absorption at 1640 and 1660 $cm^{\pm}$ in IR and the resonances at δ 167.8-170.3 and 183.2 in the ^{13}C NMR indicated the presence of an amide and a conjugated ketone carbonyl group, respectively in compound (**2**). The RCT-COSY spectrum of (**2**) revealed cross peaks of H-5 to H-6, H-9 to H2-12 and H2-13, H2-12 to H2-13, H-16 to H3-18 and H3-19, H3-18 to H3-19, and among H-1-H-4, respectively. The final structure of compound (**2**) was determined with the help of ^{l}H-^{13}C COSY experiments.

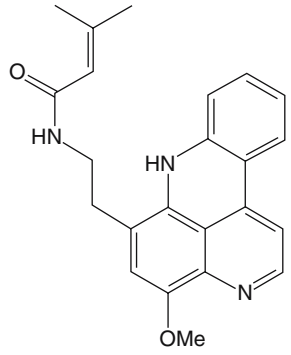


6, R = Y, R_1 = H
7, R = Z, R_1 = H
8, R = X, R_1 = OH
9, R = Y, R_1 = OH
10, R = X, R_1 = OMe
11, R = Y, R_1 = OMe

12, R = X, R_1 = $OCO(CH_2)_7CH{=}CH(CH_2)_7CH_3$
13, R = Y, R_1 = $OCO(CH_2)_7CH{=}CH(CH_2)_7CH_3$



Cystodytins D-I (**8-13**) are chiral levorotatory compounds, due to the presence of 2-amido-1-hydroxyethyl side chain, N-substituted with one of the above mentioned C_5 carboxylic acids. Hydration of Cystodytin-A (**2**, 6% aqueous HCl l00°C, 3 h) gives cystodytin C (**7**). When treated with diazomethane, (**2**) formed a methylether (**14**) (23%). This transformation is unusual as it constitutes a reductive methylation. The iminoquinone system in (**2**) is readily reduced in the ionization stage of a mass spectrometer, (M+2 ion in EIMS, MH+2 for FABMS), a typical for quinones. The vivid purple-colored ascidian *Lissoclinum vareau*[25] from Fiji has furnished two bright crimson pigments varamine-A (**16**) and varamine-B (**17**) which occurs with antitumor alkaloid, varacin (**15**).[26] Varamine-A (**16**) and varamine-B (**17**) have a parent tetracyclic aromatic ring system at the same oxidation level as the systodytin-A methylation product (**14**). However, the varamines also contain a methyl thioether substituted at C_9.



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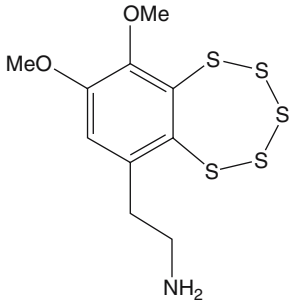
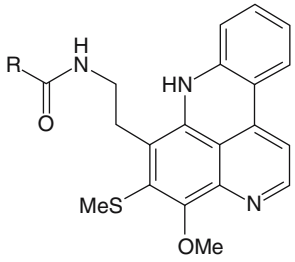


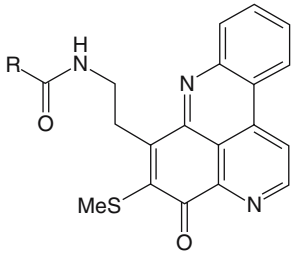
15

The bright red tunicate *Lissoclinum vareau* (Monniot and Monniot, 1987), was collected from Yasawa Island chain, in the Fiji Island. Crude methanol extract of the tunicate exhibited potent antifungal and cytotoxicity against the L1210 murine leukemia cell line. The chloroform soluble fraction of the extract yielded two bright red pigments, varamine A (**16**) and varamine B (**17**). The spectral data of (**16**) and (**17**) revealed that the two compounds (**16, 17**) were related as homologues, and structure elucidation was carried out primarily on the trifluoroacetate salt of **16**. Molecular formula of varamine A (**16**) was determined to be $C_{22}H_{23}N_3O_2S$ by FABMS data (*m/z* 394.1589 M^+). The ultraviolet spectrum of the free base of (**16**) revealed strong bands from 232 to 494 nm. In particular, the striking color change from yellow to intense red upon acidification of the freebase of (**16**) was correlated with a reversible bathochromic shift 464 nm (ε 5170) to 527 nm (ε 5670 nm). In the ^{1}H NMR of TFA salt of (**16**) six proton signals found between δ 7.20 and 8.30 ppm were assigned to deshielded protons of the heteroaromatic ring. The resonance at δ 7.52 (*d*, *J* = 6.5 Hz) and 8.21 (d, *J* = 6.5 Hz) were assigned to H-5 and H-6, respectively, in a trisubstituted pyridine ring.

A strong nuclear overhauser effect from H-5 to H-4 indicates the close proximity of the respective ring residues. Stretches around 1650 and at 3200, 3280, and 3450 $cm^{\perp}$ confirmed the presence of amide, and NH functionalities. A thiomethyl group (SCH_3) appeared as singlet of three proton at δ 2.66. Additional evidence comes from $^1J_{CH}$ values for the methyl groups; these were most easily obtained by measuring the separation of the inner peaks of the methyl ^{13}C satellites in the proton spectrum of (**16**) or (**17**). The moderately large one bond coupling constant ($^1J_{CH}$ = 141 Hz) for the methyl carbon at 18.7 ppm is similar to that measured for the electronegative methoxy group. Finally structure (**16**), and (**17**) were confirmed by 2D NMR spectroscopy. Ring C of varamine-A (**16**) isoelectronic with hydroquinone, was readily oxidized by aqueous ceric ammonium nitrate to the iminoquinone (**18**) in quantitative yield.[25] The corresponding oxidation product (**3**) of varamine-B (**17**) was found identical with diplamine from the Fijian tunicate, *Diplosoma* sp.[27] Another homologue in this series, isobutyramide (**19**),[18,27] has been characterized from an unidentified Australian tunicate.



16, R = CH_2CH_3
17, R = CH_3

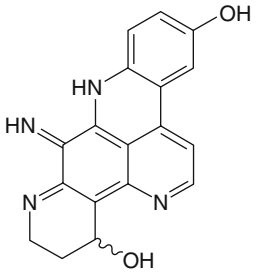


18, R = CH_2CH_3
19, R = $CH(CH_3)_2$

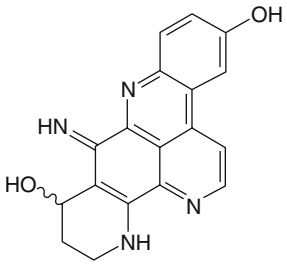
Pentacyclic Alkaloids

The pentacyclic pyridoacridine alkaloids could be classified into two groups: (a) those having one additional angular-fused ring at C9, 10 of the acridine system at C8, C9 of ring C and (b) those having linear-ring fusion at C8, C9 of ring C. Typical ring appendages include pyridine, tetrahydropyridine, pyridone, thiazine, or even a thiazole heterocycle. In some cases, a substituted 2-ethylamino side chain is also attached to the acridine C ring. The bright yellow zoochrome calliactine isolated from the mediterranean anemone, *Calliactis parasitica,* has a long history and is probably the first marine pyridoacridine alkaloid isolated from marine organisms. Calliactine attracted the attention of Lederer et al[28] in 1940 and later by Barbier[29] however, the structure determination work was hampered by low solubility of the compound and the difficulties in purification. In 1987, Cimino et al[30] reported their studies on the degradation and NMR spectroscopy of calliactine and its derivatives. Calliactine is readily aromatized (presumably autoxidation elimination with concomitant hydrolytic loss of ammonia) by boiling with dilute HCl to give "chlorocalliactine" or with water to give "neocalliactine"

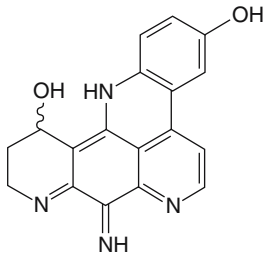
which in turn yielded neocallicatine acetate when treated with acetic anhydride in pyridine. The molecular formula $C_{18}H_{21}N_4O$ was established for calliactine, and several possible structures (**20a-20d**) were advanced. Four possible structures (**21a-21d**) for neocalliactine acetate were also proposed. However, definitive assignments have not yet been reported on either of these compounds. The amphimedine (**22**) is the first pyridoacridine alkaloid to be fully characterized.[31]Schmitz et al[31] isolated amphimedine (**22**), a sparingly soluble yellow solid (m.p. 360°C) sponge *Amphimedon* sp. was collected from Guam Island. Ambient temperature extraction with CH_2Cl_2, $CHCl_3$-MeOH, MeOH followed by hot Soxhlet extraction with $CHCl_3$ and finally purification by coloumn chromatography yielded pure compound. Molecular formula of (**22**) was established as $C_{19}H_{11}N_3O_2$ by high-resolution mass spectral analysis (*m/z* 313.08547, M^+). In the mass spectrum few fragments were observed which indicated that the compound is highly stable. The electronic absorption



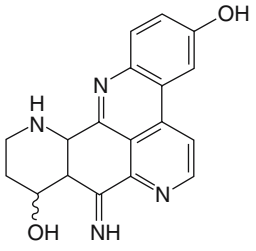
20a



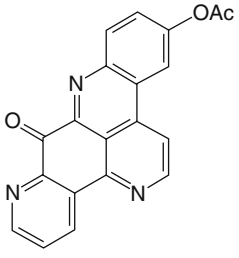
20b



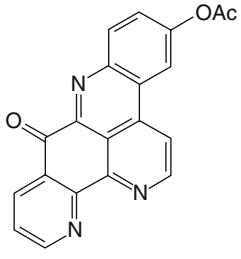
20c



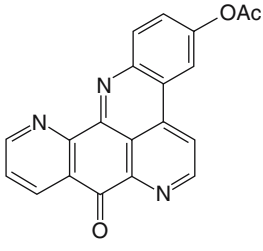
20d



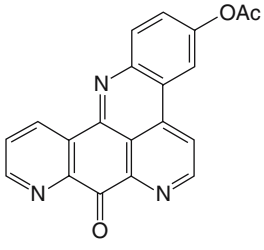
21a



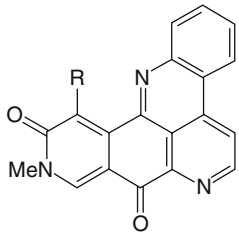
21b



21c



21d



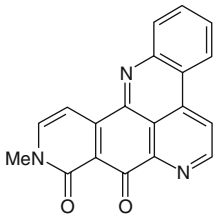
22, R = H
23, R = Br

spectra of **22** showed absorption at λ_{max} 210 (ε 19690), 233 (ε 39393), 281 (ε 9099), 341(ε 6060). Significant changes were observed in the absorption with $NaBH_4$ [λ_{max} nm 235 (ε 12879), 280 (ε 9090)], suggesting the presence of conjugated carbonyl functionality. Two strong absorptions 1690 and 1640 $cm^{\pm}$ in the IR spectrum confirmed the presence of two carbonyl groups. Further since the absorption was observed at lower frequency so these peaks were attributed to α,β-unsaturated ketone and amide functionalities, respectively. The ^{13}C NMR data reconfirmed the presence of an amide carbonyl (C-11 , δ 165.9) and a cross-conjugated ketone (C-8, δ 175.0). The 2D NMR ^{13}C-^{13}C INADEQUATE NMR techniques were used for the structural elucidation of amphimedine (**22**).[32,34]

Amphimedine (**22**) is selectively brominated (Br_2, acetic acid) to give the mono bromo derivative (**23**). Neoamphimedine (**24**),[23] along with amphimedine (**22**) and debromopetrosamine (**25a**), have been isolated from the Micronesian sponge *Xestospongia carbonaria*.[9] Neoamphimedine (**24**) is a regioisomer of amphimedine (**22**). Kobayashi et al[35] have isolated ascididemin (**26**)[6,17,36] from a species of *Didemnum* collected in Okinawa. The structural proof relied on extensive use of long-range ^{1}H-^{13}C correlation (COLOC) data and comparison, with the properties of amphimedine (**22**). It is noteworthy that ascididemin (**26**), like related pyridoacridine alkaloids which have a 1,10-phenanthroline ring system, does not form a bright red complex with Fe^{2+} that is characteristically observed with 1,10-phenanthroline itself.

Schmitz et al[37] have isolated 2-bromoleptoclinidone from *Leptoclinides* sp. collected in Truk Lagoon. 2-Bromo-leptoclinidone was assigned structure (**28**) based on interpretation of long range 2D ^{1}H-^{13}C NMR correlation data and the absence of a color reaction with Fe^{2+}. However, this structure was later revised[38] and correct structure (**27**) was shown to have a alternate pyrido ring orientation.[38] This was confirmed by selective long range 1D ^{1}H-^{13}C INAPT experiments[39] and debromination of bromoleptoclinidone to ascididemin (**26**).

Pyridoacridine alkaloids had not only been obtained from marine invertebrates of tropical waters, the pentacyclic phenolic alkaloid is also obtained from a South Australian temperate water tunicate *Amphicarpa meridiana*,[14] and more recently from a Caribbean sponge *Corticum* sp.[40] The structure of meridine (**29**)[40] is determined by single-crystal X-ray diffraction analysis.[14] An isomer of meridine was also isolated from *A. meridiana* and assigned structure as (**30**). The regiochemistry of (**30**) was assigned on the basis of NOE studies. Rapid tautomerism of pyridoacridine alkaloids has been observed. On standing, in $CDCl_3$ compound (**30**) undergo isomerization to (**29**) at room temperature. The Caribbean sponge *Petrosia* sp. when viewed under water looked jet black due to its deep dark pigmentation. Samples were immersed in methanol imparted a deep green-blue color to the solvent. This extract when diluted with water, the color changed to purple. The brominated pigment petrosamine (**31a** and **31b**) along with tryptamine



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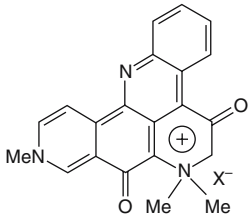
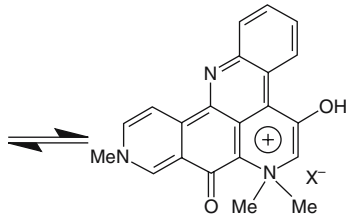
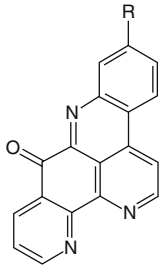


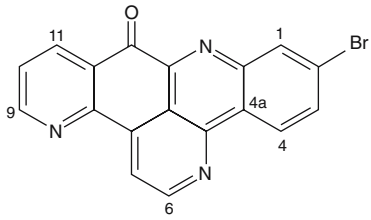
25a



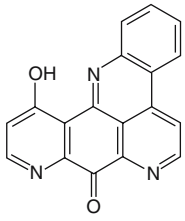
25b



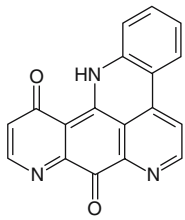
26, R = H
27, R = Br



28



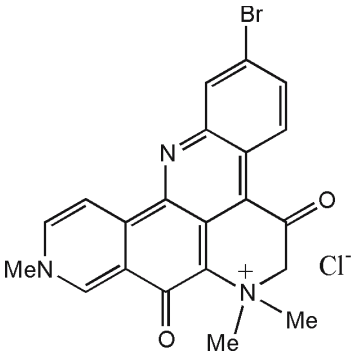
29



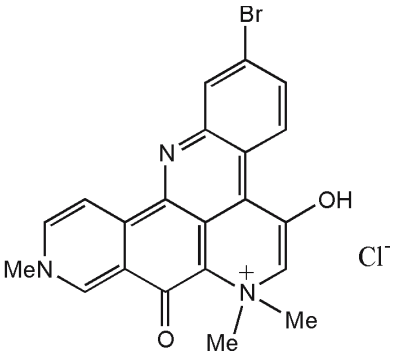
30

have been (**29**) isolated from this extract.[41] Faulkner et al[41] isolated petrosamine (**31**), from marine sponge *Petrosia* sp. while attempting to purify an antimicrobial constituent amphimedine (**22**). Petrosamine is remarkable compound since color of the solution containing (**31**) changes by the addition of dilute organic or aqueous solutions. It was observed that blue colored methanol extracts of *Petrosia* sp. exhibited antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*. The marine sponge *Petrosia* sp. was collected from Carrie Bow Cay, Belize and methanol soluble material portion was partitioned to ethyl acetate, n-butanol, and aqueous extracts. A blue band and pale yellow band from n-butanol soluble material was separated on Sephadex LH-20 (MeOH) column. The blue compound was repurified on Sephadex LH-20 column and yielded petrosamine (0.1% dry weight) as dark green crystals, m.p. >330°C. Molecular formula of petrosamine was established as $C_{21}H_{17}BrN_3O_2$ by high resolution mass spectrum. Both the ^{1}H and ^{13}C NMR spectra indicated the presence of three N-methyl signals, two of which were equivalent. The remaining signals were all in the aromatic region of the

spectra, except for a ^{13}C NMR signal at δ 187.4 (s) that could be assigned to a single quinone-type carbonyl group. X-ray crystallography in the solid state of petrosamine revealed that the pigment exists as the chloride salt of a quaternized pyridone-acridine ring system (**31a**). Correlation of the solvent-dependent changes in the UV and NMR spectra suggested that the remarkable color change observed by varying solvent polarity, was associated with shifts in the position of a keto enol equilibrium, favoring the enol form (**31b**).



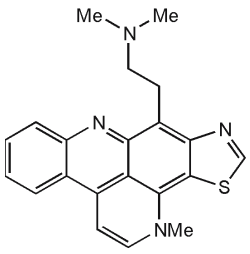
31a



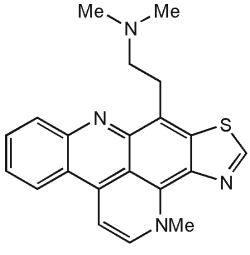
31b

The deep-water sponge *Dercitus* sp. has yielded the dark violet pigment named dercitin (**32**) together with dimethylindolinium chloride.[42] The structure of (**32**) was assigned on the basis of spectroscopic data including 2D INADEQUATE. This structure was subsequently revised to (**33**)[17] by interpretation of the magnitudes of long-range carbon-proton coupling constants.

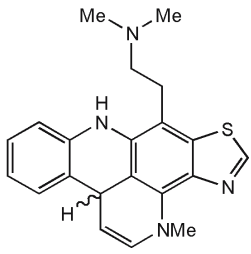
The earlier assignment error arose from inherent difficulties in the interpretation of 2D ^{1}H-^{13}C NMR spectra in highly condensed heteroaromatic compounds. In the revised structure, the nitrogen and sulphur of the thiazole ring are in correct position relative to other acridine substituents. The thiazole ring proton exhibits different J_{CH} to each of the two ring junction quaternary carbons, thus providing unambiguous assignment of the respective ^{13}C signals.[43] The ease with which a partially reduced pyridoacridine system can be aromatized was demonstrated when the dihydropyridoacridine (**34**), obtained



32



33

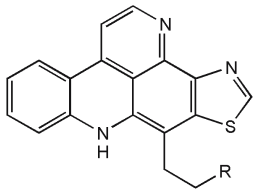


34

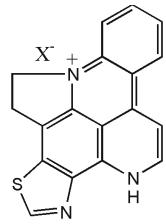
by reduction of dercitin (**33**)[44,45] with sodium borohydride rapidly autoxidized back to dercitin during workup.

Gunawardana et al[44] have reported the isolation of four minor congeners from *Dercitus* sp. Of these, nordercitin (**35**) and dercitamine (**36**) are related to dercitin (**33**). Reductive methylation of dercitamine (HCO_2H, HCHO) gave nordercitin (**35**). Dercitamicle (**37**) contains a propionamide side chain and cyclodercitin (**38**) is a hexacyclic quaternary salt.

The purple colored colonial tunicate *Trididemnum* sp.[46-48] has furnished two related bases, shermilamine-A (**39**)[47] and shermilamine-B (**40**).[47] Kuanoniamines A-D (**41-44**), along with shermilamine-B (**40**), are found in the lamellarid mollusc *Chelynotus semperi* and its prey, an unidentified tunicate, both collected in Pohnepei.[43] Kuanoniamines-B (**42**) and D (**44**) are homologues of kuanoniamine C having isovaleramide and acetamide side chains, respectively. Kuanoniamine A (**41**) differs from the other three alkaloids in lacking the 2-amidoethyl side chain and contains an iminoquinone structure analogous to those found in 2-bromoleptoclinidone and ascididemin.



35, R = NMe_2
36, R = NHMe
37, R = $NHCOCH_2Me_2$



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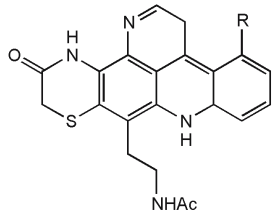
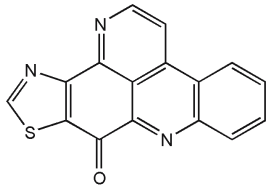


39, R = Br
40, R = H



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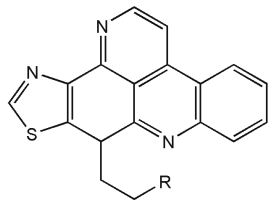
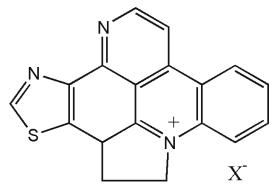


42, R = $NHCOCHMe_2$
43, R = $NHCOCH_2CH_3$
44, R = $NHCOCH_3$



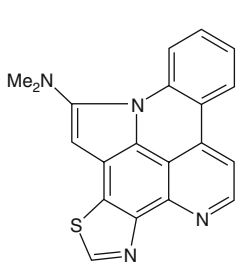
45

Hexacyclic and Heptacyclic Alkaloids

Two hexacyclic alkaloids have been reported from the deep-water sponges *Dercitus* sp. and *Stelleta* sp. Cyclodercitin (**38**) is found along with dercitin (**33**) and other related compounds in *Stelleta* sp.[43] The sixth ring in cyclodercitin is formally derived by cyclization of the 2-aminoethyl side chain to the acridine nitrogen, while the pyridine ring is substituted with an N-methyl group. When dissolved in TFA-d_4 cyclodercitin spontaneously autoxidizes

to the hexacyclic pyrrolo compound (**45**). Recently, the hexacylic pyrrolo compound (**46**) is obtained as a minor compound from *Stelleta* sp. and its structure has been determined by X-ray analysis.

The red sea tunicate *Eudistoma* sp. has yielded six alkaloids, including the known pyricloacridine alkaloid shermilamine B (**40**).[49] In addition to these segoline-A (**47**) and isoegoline-A (**48**) are regioisomeric hexacyclic pyridoacridine alkaloids.



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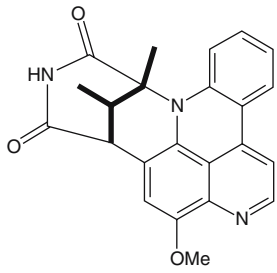

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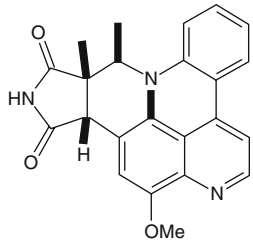
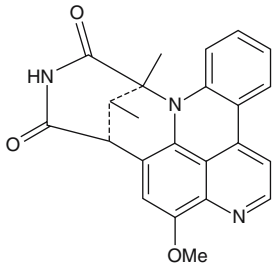


48

Segoline-A (**47**) and isosegoline-A (**48**) are the first optically active pyridoacridines isolated from the marine source.[50,51] Segoline A (**47**) was isolated from *Eudistoma* sp. in 0.4% (dry weight) yield. Molecular formula of segoline A (**47**) was established as $C_{23}H_{19}N_3O_3$. Intensive 1D and 2D NMR experiments such as COSY, NOE, short- and long-range CH correlations, COLOC, and HETCOSY studies were conducted on (**47**). The proton NMR data revealed a trisubstituted benzo-3,6-diazaphenanthroline ring system for segoline A. The pyridine ring of the diazaphenanthroline ring was characterized by the H-2 and H-3 signals. The pyridine moiety was hydrogenated easly to the 1,2,3,3a-tetrahydro derivative indicated that it was part of a quinoline ring. The heterocyclic ring system was confirmed by the NOEs between H-2 and H-3, H-3 and H-4, H-5 and H-6, and H-6 and H-7. Unequivocal structure of (**47**) was determined by single X-ray analysis. The structure of isosegoline A (**48**) is determined by NMR spectroscopic techniques. Segoline B (**49**) is a diastereoisomer of segoline A (**47**). In segoline B (**49**), the bridge across the cyclic imide ring is inverted. This is supported by the strongly bisignated CD curves for (**48**) and (**49**), which are almost exactly opposite in sign. The structure of eilatin (**50**) and unusual pyridoacridine "pseudo-dimer," has been solved by X-ray diffraction. Eilatin (**50**)[51] is the only known heptacyclic pyridoacridine alkaloid.

Octacyclic Alkaloids

Chiral pyridoacridine alkaloids are rare. Eudistone A (**51**) and eudistone B (**52**), the two optically active octacyclic alkaloids obtained from the Seychelles tunicate *Eudiste* sp.,[52] differ from other members of the class by having additional dihydroisoquinolone bicyclic ring system fused to a quaternary



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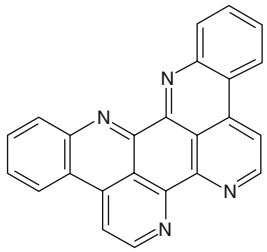

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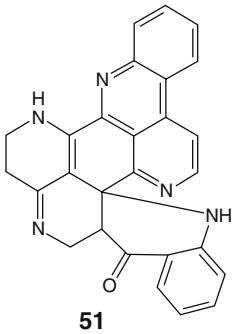
carbon of the acridine system. The relative stereochemistry of the carbon skeleton was determined by comparison of NMR coupling constants with values predicted from molecular modeling. The two compounds were correlated by autoxidation. Eudistone (**51**) aromatized to (**52**) when air is bubbled in a solution of (**51**) in DMSO.[52] The circular dichroism spectrum of eudistone-B (**52**) exhibited a strong bisignate cotton effect. However, the absolute configuration of the two compounds remains unknown.



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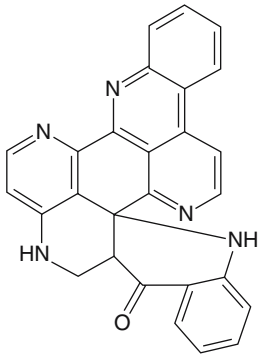

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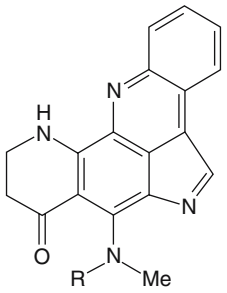
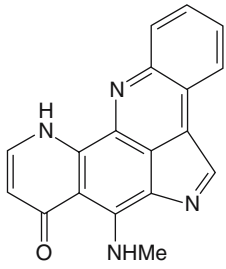
3. Pyrroloacridine and Related Alkaloids

The alkaloids of this group are mostly cytotoxic. Pyrroloacridines have similar ring construction to the pyridoacridine alkaloids. However, they contain a fused pyrrole ring rather than the familiar trisubstituted pyridine ring. The first example of this class are plakinidine[53–55] A (**53**) and B (**54**) which have been isolated from the sponge *Plakortis* sp.[53] Ireland et al[56] reported the isolation of plakinidine-A (**53**), B (**54**) and C (**55**) from *Plakortis* sp. collected in Fiji.

Bioassay-guided isolation of red sponge *Plakortis* has afforded two novel alkaloids named plakinidine-A (**53**) and B (**54**). Both of the compounds showed *in vitro* activity against the parasite *Nippostrongylus brasiliensis* (at 50 pg/mL). A pyrrolo[2,3,4-kl]acridine fused-ring skeleton in (**53**) and (**54**), represents a new structural variation within polycyclic aromatic alkaloids



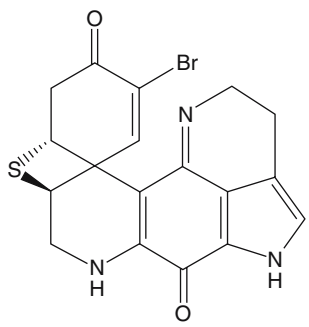
53, R = H
54, R = Me



55

from marine organisms. The organism was collected by scuba at 10 m off Hideaway island, Port Vila, Vanuatu. Red viscous oil (3.87 g) was obtained from aqueous methanol extracts of the preserved organism (0.2 kg, wet). The crude oil after partitioned between aqueous MeOH and the solvent series of hexanes, CCl_4, CH_2Cl_2 were separated by column chromatography (reversed phase and Sephadex LH-20/methanol). Plakinidine A (**53**) was isolated as deep purple solid from methanol (52 mg, 0.026% wet weight, mp. 248-250°C) along with 24 mg (0.012%) of a purple oil identified as plakinidine B (**54**). The molecular formula of (**53**) was determined as $C_{l8}H_{14}N_4O$, from EI-HRMS (*m/z* 302.1169, M^+) and an APT ^{13}C NMR spectrum. Four separate proton spin systems were confirmed by COSY experiments in DMSO-d_6 and $CDC1_3$-TFA-d_4 (1:1). Protons on N-8 to C-10 were assigned to a $CH_2CH_2N(H)$ group, and protons on C-3 to C-6 were confirmed to be a part of ortho-disubstituted benzene ring. Other protons H-2, was a low-field singlet (δ 8.84) with a large $^1J_{CH}$ coupling constant (200.4 Hz in $CDCl_3$-TFA-d_4), indicating a nitrogen was adjacent to C-2.[57] Based on the ^{13}C chemical shifts remaining fragment consisting C_9N atoms were assumed to have five double bonds. Long-range ^1H-^{13}C COSY experiments were done to confirm the structure of the compounds. Three bond correlations to H-4 and H-5 revealed the location of quaternary carbons in the molecule. The existence of a six-membered ring β-enamino ketone was confirmed by the IR (1624 $cm^{\perp}$) and COSY NMR correlations from H_2-9 to C-7b and from H_2-10 to C-11 and C-1la. Finally, two dimensional ^{13}C-^{13}C INADEQUATE was used to verify most of the structural elements of plakinidine A.[57] Bioassay guided purification of the methanol-toluene extract of the tropical green sponge *Prianos melanos* from Okinawa gave a cytotoxic pigment, prianosin A (**56**), prianosin B (**57**), prianosin C (**58**) and prianosin D (**59**).[58,59]

Several independent reports described the identification of discorhabdins, compounds similar to prianosins.[6064] The first report described the isolation and structure elucidation of discorhabdin-C (**60**) an achiral spiro-alkaloid. Discorhabdin-A identical to prianosin-A, (**56**) was isolated together with discorhabdin-B (**61**) from three species of *Latrunculia* sponge collected the



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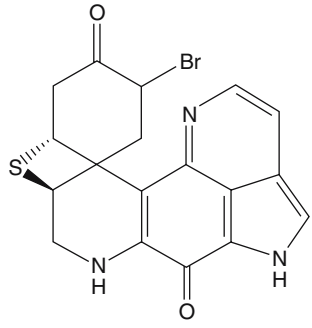

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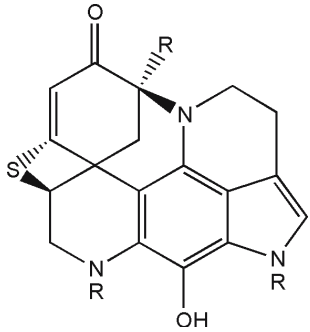
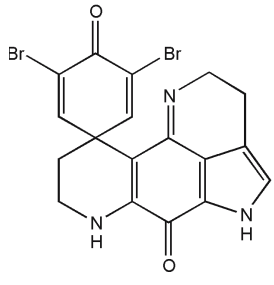


58, R = OH
59, R = H

temperate waters around New Zealand. The quaternary iminium salt discorhabdin D (**62**) is isolated from both *Latrunculia brevis,* from New Zealand and *Prianos* sp. from Okinawa.[65] Each discorhabdin or prianosin contains an unsaturated cyclohexenone and spiro-fused to a tetracyclic ring system. Each ring system, with exception of discorhabdin C (**60**) is also bridged with tetrahydrothiophene ring. A deep-water collection of *Batzella* sp. from Bahamas has furnished three pyrroloquinolines, batzellines A-C (**63-65**).[66] Each alkaloid has tetrahydroquinoline nucleus further, bridged across both rings by a trisubstituted pyrrole ring. An unusual chlorine atom is present in all the alkaloids.



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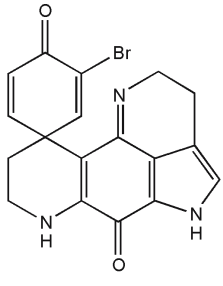

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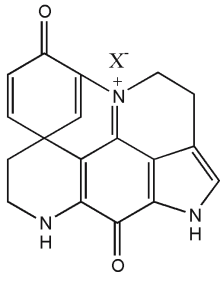

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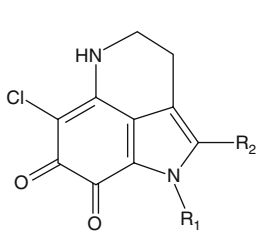
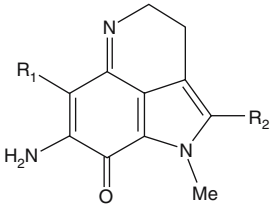
The sponge *Bratzella* sp. has also furnished four additional pyrroloacridine alkaloids named isobatzellines A-D (**66-69**).[67]



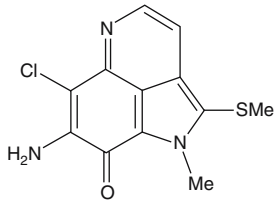
63, R_1 = Me, R_2 = SMe
64, R_1 = H, R_2 = SMe
65, R_1 = Me, R_2 = H



66, R_1 = Cl, R_2 = SMe
67, R_1 = H, R_2 = SMe
68, R_1 = H, R_2 = Cl

Isobatzellines are not strictly isomeric with batzellines, but differ in replacement of one of the quinone carbonyl group with an amino group. Autoxidation of isobatzellin A (**66**) to D (**69**) is facile and could be observed within a few hours during thin layer chromatography or treatment with DDQ.

Isobatzelline A (**66**) can be converted into bratzelline A (**63**) by diazotization substitution in aqueous nitrous acid. The Fijian tunicate *Clavelina* sp.[68,69] has yielded wakayin (**70**) a unique alkaloid. Ireland et al[68,69] isolated a new pyrroloiminoquinone based alkaloid, wakayin (**70**) from the ascidian *Clavelina* sponge. Wakayin (**70**) exhibited potent *in vitro* cytotoxicity against the human colon tumor cell line (HCT116 IC_{50} 0.5 μg/mL). Wakayin was found to inhibit topoisomerase II enzyme (250 μM) and based on related biological data it was concluded that wakayin exhibit its biological activity by damaging the DNA. This compound also exhibited antimicrobial activity against *Bacillus subtilis* (MIC = 0.3 μg/mL). The crude methanol-chloroform extract was partitioned by reversed phase flash chromatography using methanol-aqueous trifluoroacetic acid solvent systems. Purification of combined biologically active fractions by repeated elution through Sephadex LH-20 yielded 15 mg (0.005% wet weight) of wakayin (**70**) as a triflouroacetate salt. Molecular formula of this compound was established as $C_{20}H_{15}N_4O$ by the use of FAB-HRMS (MH^+ 327.1262, calculated 327.1246). Several features of the ^1H and ^{13}C NMR spectra of (**70**) were matches with discorhabdins and isobatzellines,



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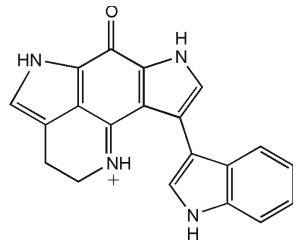


70

suggesting the presence of a pyrroloiminoquinone moiety. The spin system comprising δ 13.04 (br *s*, NH1), 7.11 (*d*, *J* = 2.5 Hz, H2), 2.93 (*t*, *J* = 8.0 Hz, H24), 3.78 (br *t*, *J* = 8.0 Hz, H25), and 10.32 (br s, NH6) was established by DQCOSYD and 1-D lH difference NOE[70] NMR experiments. This spin system was assigned to part of a pyrrolodihydropyridine moiety by HMQC[71] and HMBC[72] NMR experiments. IR absorption at 1662 and 1447 $cm^{\perp}$ indicated the presence of an iminoquinone ring which was reconfirmed by ^{13}C NMR signals at δ 166.28 (CO) and 156.72 (C7). The connectivities between the pyrrolodihydropyridine system and C7, C8, and C9 of the iminoquinone ring was established by long range ^{1}H-^{13}C NMR correlations observed for H2 (δ 120.74). The ^{1}H NMR signals at δ 13.41 (br *s*), 7.28 (*d*, *J* = 2.5 Hz) and ^{13}C NMR signals at δ 134.25 (s), 120.44 (s), 114.15 (s), 125.14 (*d*, $^{1}J_{CH}$ = 190 Hz) confirmed the presence of another 2,3,4 trisubstituted pyrrole ring. Long range ^{1}H-^{13}C NMR correlations experiment also confirmed this and proved that the pyrrole ring was bound to C11 and C15 of the iminoquinone ring. ^{1}H and ^{13}C NMR data were also suggested the presence of a 3-substituted indole moiety [δ 11.54 (br *d*, *J* = 2.0 Hz), 7.73 (*d*, *J* = 2.0 Hz), 7.52 (*d*, *J* = 8.0 Hz), 7.49 (*d*, *J* = 8.0 Hz), 7.19 (dd, *J* = 8.0, 8.0 Hz), 7.09 (dd, *J* = 8.0, 8.0 Hz); ^{13}C [δ 105.90 (s), 112.07 (d, *J* = 161 Hz), 118.86 (*d*, *J* = 161 Hz), 119.66 (*d*, *J* = 161 Hz), 121.85 (*d*, *J* = 161 Hz), 124.94 (*d*, *J* = 183 Hz), 125.49 (s), 136.76 (s)]. This was further confirmed by HMQC and HMBC NMR experiments and by comparison with the ^{13}C NMR data reported for the indole-imidazole moiety of topsentin.[73] Long-range ^{1}H-^{13}C correlations by HMBC and selective INEPT NMR experiments between H13 and C16 established connectivity between the bipyrroloiminoquinone moiety and the 3-substituted indole, and confirmed the structure wakayin.

Sources and cytotoxities of many marine pyridoacridines that have been reviewed recently[74] are summarized in Table 1.

Table 1 Cytotoxic marine pyridoacridines and their sources

S. No.	Pyridoacridine	Source	References
1	Labuanine-A	*Biemna fortis* sponge (Indonesia)	75
2	Lissoclin-A	*Lissoclinum* sp. ascidian (Australia)	76
3	Lissoclin-B	*Lissoclinum* sp. ascidian (Australia)	76
4	Lissoclin-C	*Lissoclinum* sp. ascidian (Australia)	76
5	Lissoclinidine	*Lissoclinum notti* ascidian (New Zealand)	77
6	9-Aminobenzo [*b*]pyrido[4,3, 2-de]-[1,1 0]-phenanthrolin-8(8H)-one	*Biemnafortis* sponge (Indonesia)	75
7	Amoamine-A	*Cystodytes* sp. ascidian (Arno Atoll, Rep. Marshall Is.)	76, 77
8	Amoamine-B	*Cystodytes* sp. ascidian (Arno Atoll, Rep. Marshall Is.)	77, 78

(Cond.)

S. No.	Pyridoacridine	Source	References
9	Pantherinine	*Aplidium pantherinum* ascidian (S. Australia)	79
10	Sagitol	*Oceanapia sagittaria* sponge (Palau)	80
11	Sebastianines-A	*Cystodytes dellechiajei* ascidian (Brazil)	81
12	Sebastianines-B	*Cystodytes dellechiajei* ascidian (Brazil)	70, 81
13	Biemnadin	*Biemna fortis* sponge (Indonesia)	75
14	2-Bromoleptocl inidinone	*Leptoclinides* sp. ascidian (Truk Lagoon)	82, 83
15	Meridine	*Amphicarpa meridiana* ascidian (S. Australia), *Leptoctinides* sp. sponge (Truk Lagoon), *Cortidum* sp. sponge (Bahamas)	84, 85
16	Meridine regioisomer	*Biemna fortis* sponge (Indonesia)	75
17	5-Methoxyneo-amphimedine	*Xestospongia carbonaria,* X. cf. *exigua* (Indopacific)	86
18	Neoamphimedine	*Xestospongia* sp. sponge (Philippines) *Xestospongia* cf. *carbonaria* (Micronesia) *Xestospongia* c *carbonaria,* X. cf. *exigua* (Indopacific)	86, 87, 88, 89
19	Neoamphimedine-Y	*Xestospongia* c *carbonaria,* X. cf. *exigua* (Indopacific)	86
20	Neoamphimedine-Z	*Xestospongia* cf. *carbonaria,* X. cf. *exigua* (Indopacific)	86
21	Nordercitin	*Stelletta* sp. sponge *Derdtus* sp. sponge (Bahamas)	90, 91
22	Stellettamine	*Stelletta* sp. sponge	90
23	Styelsamine-A	*Eusynstyela lateridus* ascidian (Indonesia)	92
24	Styelsamine-B	*Eusynstyela lateridus* ascidian (Indonesia)	92, 93, 94
25	Styelsamine-C	*Eusynstyela lateridus* ascidian (Indonesia)	92
26	Styelsamine-D	*Eusynstyela lateridus* ascidian (Indonesia)	92
27	Varamine-A	*Lissoclinum vareau* ascidian (Australia)	76, 95
28	Varamine-B	*Lissoclinum vareau* ascidian (Australia)	96, 95
29	Cystodytin-D	*Cystodytes delleehiajei* ascidian (Okinawa)	96, 97
30	Cystodytin-E	*Cystodytes dellechiajei* ascidian (Okinawa)	96, 97
31	Cystodytin-F	*Cystodytes delleehiajei* ascidian (Okinawa)	96, 97
32	Cystodytin-G	*Cystodytes delleehiajei* ascidian (Okinawa)	96, 97
33	Cystodytin-H	*Cystodytes delleehiajei* ascidian (Okinawa)	96, 97
34	Cystodytin-I	*Cystodytes delleehiajei* ascidian (Okinawa)	96, 97
35	Cystodytin-J	*Cystodytes* sp. ascidian (Fiji), *Lissoclinum nolli* ascidian (New Zealand)	13, 77, 96, 98
36	Cystodytin-K	*Lissoclinum nolli* ascidian (New Zealand)	77
37	Dercitamine	*Stelleta* sp. sponge, *Dercitus* sp. sponge (Bahamas)	90, 99
38	Diplamine	*Diplosoma* sp. ascidian (Fiji), *Cystodytes* sp. ascidian (Fiji), *Lissoclinum nolli* ascidian (New Zealand)	13, 93, 98, 100
39	Eilatin	*Cystodytes* sp. ascidian (Fijian), *Eudistoma* sp. ascidian (Eilat)	13, 93, 101, 102
40	Isodiplamine	*Lissoclinum nolli* ascidian (New Zealand)	77

Total synthesis of many of the alkaloids have been achieved. Synthesis of cystodytin-A and B have been published by Cinfolini et al[103] using an efficient intramolecular photochemical nitrene insertion into an aryl substituted dihydroisoquinoline. Three total synthesis of amphimedine have been reported.[104406] Synthesis of bromoleptoclinidone and ascidemin have also been published.[107,108] Total synthesis of most of the pyridoacridines given in Table 1 have also been achieved. New pyridoacridine structures provide fertile area for the design and execution of biologically active heterocyclic molecules.[109] Newer synthetic strategies are emerging for efficient assembly of tetracyclic and pentacyclic ring system.

Biological Activity of Pyridoacridines

Mostly all of the pyridoacridines discovered so far have shown strong *in vitro* cytotoxic activity. The activity has been related to the ability of pyridoacridines to intercalate DNA, so inhibit DNA metabolizing enzymes. Pyridoacridines also exhibited antiviral, antimicrobial, insecticidal, fungicidal, and other activities. Some pyridoacridines have also shown excellent antitumor activity in various models, while others have proven too toxic to be useful for clinical purposes. Pyridoacridine alkaloids show *in vitro* cytotoxicity against cultured tumor cells (L 1210 murine leukemia, P_{388}, etc.) or antineoplastic activity in whole animal experiments. Dercitin (**33**) inhibits a variety of cultured cell clones at nanomolar concentrations and shows antitumor activity in mice and modest antiviral activity against *Herpes simplex* and A-58 murine corona virus at micromolar concentration.[42] Dercitin (**33**) exhibited strong inhibition of HSV-1 at 5 δg mL^{4} with moderate cytotoxicity. It also completely inhibited murine A59 coronavirus at 1 μg mL^{4} with no cytotoxicity.[17] A thorough study by Burres et al[110] has shown that dercitin (**33**) inhibits both DNA and RNA synthesis upto 83% at 400 nM, but protein synthesis is effected to a lesser extent. Dercitin also binds to calf thymus DNA and relaxed supercoiled $\phi \times 174$ DNA at 36 nM and inhibited DNA polymerase and DNase nick translation at 1 nM. Collectively these results suggest that inhibition of enzyme activity by dercitin is of secondary importance, and its activity is entirely consistent with potent intercalation of nucleic acids. The activities of dercitin (**33**) and its analogues have been compared, and the nature of pharmacophores in dercitin responsible for antiviral and antitumor activities has been identified. In contrast to dercitin (**33**), neoamphimedine (**24**) is found to be a potent inhibitor of purified mammalian topoisomerase II (IC_{50} 1.3 μM), but not of topoisomerase I. Neoamphimedine (**24**) is also shown to intercalate DNA with a K_m of 2.8×10^5 M^{4} and a binding site size of 1.8 base pairs per molecule of 24. Interestingly, the isomeric base amphimedine (**22**), petrosamine (**4**) and debromopetrosamine (**25**), had little effect on topoisomerase I or II activity, despite displaying comparable cytoxicity. It is postulated that the cytotoxicity of (**24**) towards mammalian cells can be explained by DNA damage resulting from native

topoisomerase II inhibition. However, the other pyridoacridin alkaloids may elicit cytotoxicity through as yet unidentified mechanisms associated with DNA processing. Ascididemin (**26**) and shermilamine B (**40**) also inhibited topoisomerase II, albeit at higher concentration (75 and 30 μM, respectively), while shermilamine A (**39**) and meridine (**29**) are found inactive.[14]

Benzo[4,5]sampangin and ascididemin possess potent antiviral activity while kuanoniamine A appeared inactive. Benzo[4,5]sampangin showed complete inhibition of HSV-1 at 80 μg mL^{4} with no host cell toxicity (BSC-1 green monkey cells). It also displayed activity against polio virus type 1 (partial inhibition at 80 μg mL^{4} with no detectable cytotoxicity to the Pfizer vaccine strain) and HIV-1 (46% protection at 0.7 μM with host cell toxicity at 10 μM).[111] Hollow fiber assay and tumor implant assays have been developed for antitumor activity assays to assess the antitumor activity of pyridoacridines.[112415] Mainly two of types of the tumor models have been used to assess pyridoacridines. In the first, mouse leukemia cells (P338 cells) are injected into the peritoneum of DBA/2 mice. These mice live for approximately 10 days. The tumor bearing mice are then treated with the test drug, which is administered i.p. and response is measured as increased life span (%ILS). 2-Bromoleptoclinidinone had an excellent *in vitro* cytotoxicity, but when tested in xenograft models, proved too toxic to yield significant antitumor responses.[116] Ascididemin was also tested *in vivo* at the National Cancer Institute, USA against twelve human tumor cell lines by hollow fiber assay and was shown to exhibited significant activity (%T/C < 50) against six of the cell lines.[117] *In vitro* activity of several pyridoacridines on markers of leukemia and tumor growth have been assessed. Dercitin (**33**) and Kuanoniamine have been evaluated for the cytotoxic acitvity and observed that they are approximately equally potent against a solid human lung cancer line and a mouse leukemia line.[118]

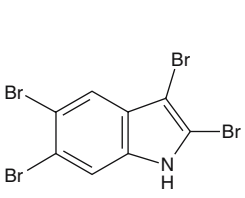
Although wakayin (**70**) exhibits marginal inhibitory activity toward topoisomerase II (250 μM), it showed an interestingly differential cytotoxicity against mammalian cell clones that is indicative of DNA damage or interference with DNA procesisng.[68] It has been demonstrated that the fluorescence spectrum of kuanoniamine D (**44**) is quenched upon addition of calf thymus DNA, and the emission wavelength of 534 nm (excitation at 350 nm) changed to 593 nm. Both of these findings are suggestive of alkaloid binding to DNA. The two 'bay region' nitrogen's in 2-bromoleptoclinidone (**27**) is ideally disposed to present two donor nitrogen atoms of a bidentate ligand to metals. Alkaloid (**27**) forms an octahedral complex with Ru (II) salts that induces photo activated single strand cleavage of supercoiled PBR 322 DNA under visible light irradiation.[119] It is interesting to note that cytotoxic cystodytins-A (**2**) and B (**6**) are found to be potent Ca^{2+} release agents, and stimulated calcium release from the sarcoplasmic reticulum (SR) at 36 and 13 times the potency of caffeine, respectively.[16]

Ascididemin, benzo[4,5]sampangine, deazaascididemin were found activity against *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, and *Cladisporium resinae*, with an MIC of 0.39 μg mL^{-1} against *C. albicans*.[120] Cystodytin was found active against *E. coli* and *Staphylococcus aureus*.[110] Sampangine and several analogues exhibited potent antimicrobial activity against the opportunistic infection organisms *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Mycobacterium intracellulare*.[121] Limited information is available in the literature regarding the insecticidal activity of pyridoacridine.[122] Kuanoniamines were studied extensively using neonatal *Spodoptera littoralis* larvae and the 50% lethal concentration (LC_{50}) was determined.

4. Indole Alkaloids

The majority of marine indole alkaloids are rather simple compounds. However, some of the indole alkaloids carry unique structural features. Bacteria and algae have yielded halogenated simple indoles, while more complicated structures have been isolated from marine snails, sponges, bryozoans, etc.[123–126] Strong antibacterial and antiyeast activities were associated with the red alga *Laurencia brongniartii*.[123] The alga yielded the antibacterial agent (**71**). The compound has been characterized by its ^{1}H and ^{13}C NMR spectra. The proton at C7 in (**71**) exhibits substantial solvent-dependent downfield shift.

Six unique bisindole alkaloids have been isolated from the blue-green alga R*ivularia firma* exhibiting antiinflammatory activity. The major alkaloid (**72**) (m.p. 239–240 °C) is responsible for the antiinflammatory effect.[127] Sponges have yielded brominated tryptamines, 5,6-dibromotryptamine (**73**) and N_b-methyl-5,6-dibrmotryptamine (**74**) are obtained from the sponge *Polyfibro spongia australis*. The bromoindole derivatives showed *in vitro* Gram-negative, Gram-positive antibacterial activity, and platelet aggregation inhibitory activity.[128]



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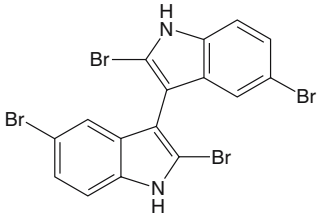

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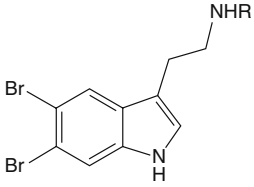
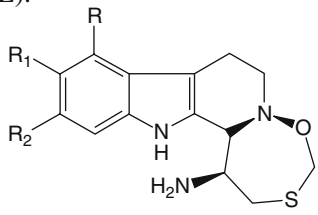


73, R = H
74, R = Me

Carboline Alkaloids

Rinehart and others have searched systematically pharmaceutically useful compounds from marine species.[129–133] Initial efforts have been involved

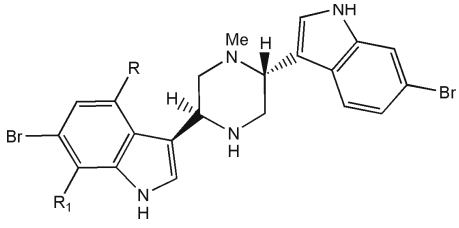
surveying of antibacterial and antifungal organisms, with bioassays carried out on shipboard. Later on, focus was shifted to antiviral and cytotoxic compounds. The most active antiviral extract in shipboard testing was obtained from a tunicate subsequently identified as *Eudistoma olivaceum,* but the extract was surprisingly found inactive in the secondary assay. When the tunicate was recollected, its extracts proved very active in both primary and secondary assay against *Herpes simplex* and other viruses. The *Eudistoma* extract yielded eudistomins variously substituted β-carbolines. All the compounds isolated had antimicrobial or antiviral activity, but the most potent are the tetracyclic eudistomins with the unique oxathiazepine ring (eudistomins C, E, K, L).[130-133] Their structures are assigned by spectroscopic techniques (high resolution, FABMS, high resolution EIMS, ^{13}C NMR, UV) and by partial synthesis. Eudistomins C, E, K, and L have been assigned the structure (**75**), (**76**), (**77**) and (**78**), respectively.[129,134] The stereochemistry of eudistomins (**76-78**) has been determined by NMR,[135] and the absolute stereochemistry of eudistomin K (**77**) was determined by single crystal X-ray structural, analysis of its 4-bromobenzyl derivative.[136,137] A new manzamine related tetrahydro-β-carboline alkaloid with a methylene bridge between N-2 and N-27 and named eghedin-A was isolated from an Okinawan marine sponge *Amphimedon* sp. Its structure was determined on the basis of spectroscopic data. The compound exhibited antibacterial activity against *Sarcina lutea* (MIC = 2.8 g/mL), and *Corynobacterium xerosis* (5.7 g/mL) and showed cytotoxicity against murine leukemia L 1210 cells (IC_{50} = 4.4 g/mL).[136]



75, R = H, R_1 = OH, R_2 = Br
76, R = Br, R_1 = OH, R_2 = H
77, R = H, R_1 = H, R_2 = Br
78, R = H, R_1 = Br, R_2 = H

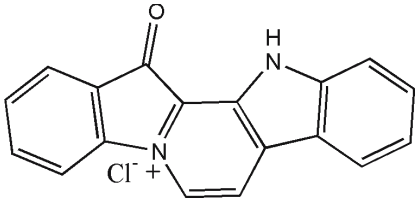
Complex Indole Alkaloids

A deep water Caribbean sponge *Dragmacidon* sp. has yielded an alkaloid named dragmacidin (**79**) which inhibits the growth of several cancer cell lines.[138] Dragmacidin (**79**) contains two tryptamine units and an unoxidized piperizene ring which had not been found before in marine natural products. The pacific sponge *Hexadella* sp.[139] collected from the coast of British Columbia afforded dragmacidon A (**80**) another member of the group, exhibits potent cytotoxicity.



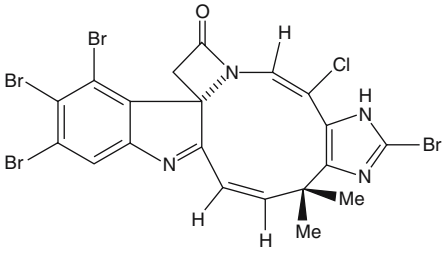
79, R = OH, R_1 = Br
80, R = R_1 = H

Fascaplysin (**81**),[140–143] a blood red pigment obtained from a Fijian sponge *Fascaplysinopsis* sp. Fascaplysin (**81**), exhibits antimicrobial and cytotoxic properties.[144] Chartelline A (**82**) a unique β-lactam indole alkaloid, which exhibits cytotoxicity *in vitro* against KB, and PS cell lines, has been isolated from a marine bryozoan *Chartella papyracea* collected in North Britanny waters.[145] The biosynthesis of this new class of alkaloid appears quite interesting. It will be worth to know whether chartelline-A (**82**) is a true bryozoan metabolite or produced by some microorganism.



81

Five flustramine derivatives,[146–149] dihydroflustramine-C (**83**), and its N-oxide flustramine-D (**84**) and its N-oxide and isoflustramine have been isolated from bryozoan *Flustra foliaceae*.[146] Flustramines are tricyclic indole alkaloids responsible for the antimicrobial activity of the methylene chloride soluble fraction of aqueous methanolic extract of the bryozoan. Oxidation of dihyro flustramine-C (**83**) and D (**84**) with m-chloroperbenzoic acid yielded the corresponding N-oxide, (**85**) and (**86**), respectively.



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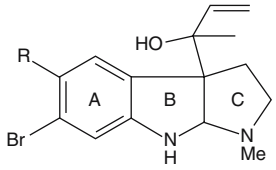
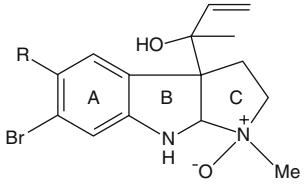


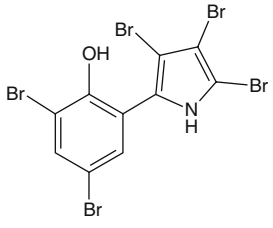
83, R = H

84, R = 



85, R = H

86, R = 



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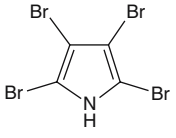

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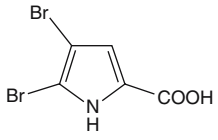
5. Pyrrole Alkaloids

Several simple brominated pyrroles have been isolated from marine bacteria and sponge. A highly brominated pyrrole derivative (**87**) and low molecular weight biologically active compounds have been obtained from a bacterial source.[150,151] The compound (**87**) is composed of more than 70% bromine by weight and is characterized by X-ray crystallographic analysis.[152] It displays impressive *in vitro* antibiotic properties against Grampositive bacteria. However, it was found inactive *in vivo.* It also displayed antitumor activity. The alkaloid (**87**) has been synthesized.[153] The purple colored bacterium of the genus *Alteromonas*[154] yielded a tetrabromo pyrrole (**88**) along with other metabolites. The compound (**88**) displays moderate antimicrobial activity *in vitro* against *Stayphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Number of brominated pyrrole alkaloids have been isolated from sponge *Agelas sventres*.[155-157] The dichloromethane-methanol extract of this sponge resulted in the isolation of the known alkaloids, oroidin (328 mg), 4,5-dibromopyrrole-2-carboxylic acid (35 mg), hymenidin (417 mg) and new bromopyrrole-derived alkaloid named sventrin (75 mg). The known brominated alkaloids hymenidin, oroidin, and 4,5-dibromopyrrole-2-carboxylic acid (**89**) were characterized by comparison of their spectroscopic data with those reported in the literature.[158-160] Isotopic cluster of sventrin at *m/z* 400, 402, and 404 $[M+H]^+$ in the ratio 1:2:1 in ESI mass spectrum provedd the presence of two bromine atoms. The molecular formula of was determined as $C_{12}H_{14}Br_2N_5O$ by (*m/z* 401.9565, $[MH]^+$), by high resolution FABMS. The ^1H and ^{13}C NMR data were in agreement with the proposed molecular formula. The NMR spectra of the base was compared with a oroidin and it was found that it has one less NH signal than oroidin and an additional methyl signal at 3.89 ppm which was assigned as an *N*-methyl group. The presence of HMBC correlations from the *N*-methyl group to C-2 and C-5, and absence of the pyrrole NH established the structure of sventrin as N(pyrrole)-methyl oroidin = sventrin). Dibromo pyrrole carboxylic acid showed *in vitro* immunosuppressive activity.[161] The Caribbean colonial tunicate *Eudistoma olivaceum* has afforded a number of β-carboline alkaloids. Of these, eudistomin-A (**90**) has a pyrrole-β-carboline and eudistomin-G (**91**) has pyrrolinyl-β-carboline structures.



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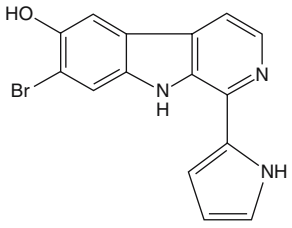

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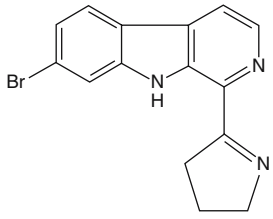
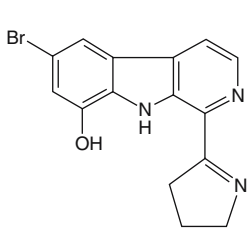


91

Several other eudistomins have also been isolated from this source.[162,163] An Okinawan tunicate *Eudistoma glaucus* has yielded eudistomidin A derivative (**92**).[164] The compound (**92**) exhibits strong calmodulin antagonistic activity, and is the first calmodulin antagonist of marine origin. It is about 15 times more potent than W-7, a well-known calmodulin antagonist.

Eudistomidins-B, C and D have also been obtained from the same tunicate.[165,166] The absolute configuration of eudistomidin B (**93**) is determined by NMR and CD data, whereas of eudistomidin C (**94**) is determined by synthesis of 6-methyl-1-10(*R*)-eudistomidin C. These new β-carbolines display antileukemic activity. Eudistomidin-B (**93**) inhibits Na^+, K^+ ATPase, but activates octomyosin ATPase, whereas eudistomidin C (**94**) shows calmodulin antagonistic activity. Eudistomidin-D (**95**) induces Ca^{2+} release from SR. The New Zealand ascidian *Ritterella sigillinoides* has afforded, eudistomin K (**96**) sulfoxide and shows antiviral activity.



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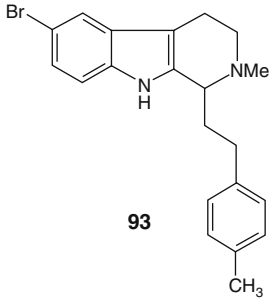

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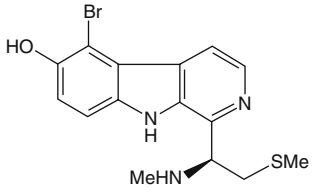
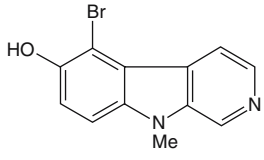


94

More than sixty bioactive bromopyrroles have been isolated from marine sponges of the genus *Agelas*.[167] Most of them are either C11 or C12 oroindine derivatives with exception to latondulin-A[168] and 4,5-dibromopyrrole-2-cabonitirle.[169] Very recently a fluorescent alkaloid ageladine-A (**97**) was isolated from marine sponge *Agelas nakamurai*.[170] Ageladine-A (**97**) is the first example of this family which contains 2-amino-imidazolopyridine.[170] Ageladine-A (**97**) was isolated from the combined extract of the sponge, and purified by ODS flash chromatography, gel filtration, and ODS HPLC. Repetitive reversed phase HPLC of active fractions afforded ageladine-A



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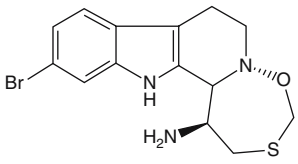

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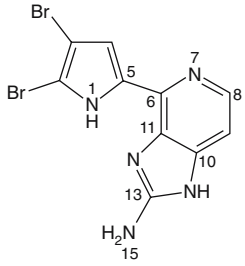


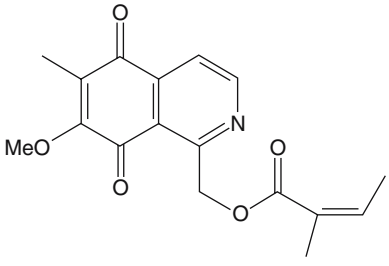
97

(**97**) as yellow powder. The structure of this novel marine natural product was determined by the use of spectral techniques such as LR, HR-FABMASS, 1D/2D-NMR, ^{13}C NMR. The FABMS spectra showed $(MH)^+$ clusture at *m/z* 356/358/360 in the 1:2:1 ratio. Presence of clusture of peaks in the ratio of 1:2:1 is typical for the presence of two bromine atoms. On the basis of FAB-HRMS and ^{13}C NMR spectral data molecular formula of ageladine-A (**97**) was established as $C_{10}H_7N_5Br_2$ indicating the presence of nine double bonds in the compound. The final structure of this compound was established by using 2D NMR and ^{13}C NMR spectral data. Ageladine-A (**97**) showed antiangiogenic activity.[170]

6. Isoquinoline Alkaloid

Arai et al[171] have published a review on isoquinoline alkaloids of actinomycetes and sponges. The bright blue sponge *Reniera* sp. has yielded a variety of isoquinoline alkaloids. Of these, renierone (**98**) exhibits high order of antibacterial activity.[172,173] Since the sponge contained relatively small quantities of the alkaloids, the possibility of a symbiotic microorganism origin cannot be ruled out. All the alkaloids isolated from the sponge *Reniera* sp. exhibit antimicrobial activity against selected terrestrial and marine microorganisms. Renierone (**98**) and N-formyl-1,2-dihydrorenierones (**99** and **100**) both inhibit cell division in the fertilized sea urchin egg assay.[174]

New dimeric isoquinoline alkaloid Juromycin has been isolated from the skin and mucus of the pacific nudibranch *Jorunna funebris*. The compound exhibited potent cytotoxicity against various cancer cell lines.[175] An usual isoquinoline alkaloid imbricatine (**101**) has been isolated from the starfish *Dermasterias imbricata*.[176] The alkaloid (**101**) displays significant anti-neoplastic activity.



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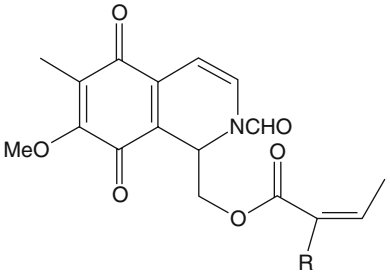
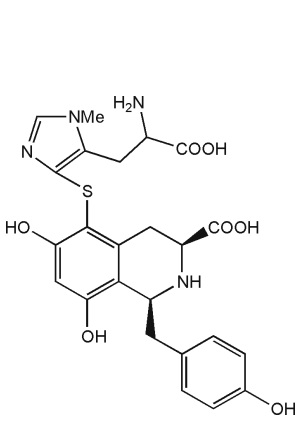


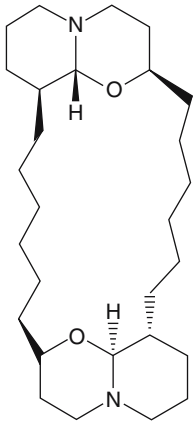
99, R = H
100, R = Me

7. Miscellaneous Alkaloids

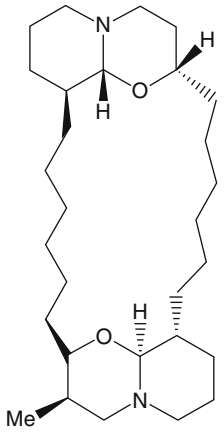
Halichlorine (**102**)[177-181] and Pinnaic acids (**103**)[182-185] two closely related alkaloids were isolated by from the Okinawan bivalve *Pinna muricata* and the sponge *Halichondria okadai* Kadota, respectively. Both compounds exhibit antiinflammatory activity, by different mechanisms. Pinnaic acid inhibits cPLA2 *in vitro* (IC_{50} = 0.2 mM) whereas halichlorine is a vascular cell-



101



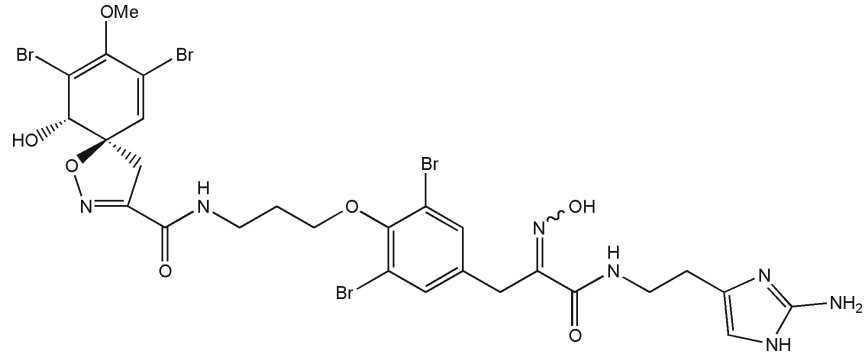
102



103

adhesionmolecule-1 (VCAM-1) antagonist. Although pinnaic acid and halichlorine inhibit different target proteins, but close examination of these compounds reveals that these alkaloids share a common azaspiro unit. The structure of (**102**) and (**103**) was elucidated by detailed spectroscopic analysis. Halichlorine consists of a sterically hindered 15-membered lactone, an azabicyclo [4.4.0] ring, and a [5.6]-spiro ring moiety. Asymmetric syntheses of these novel alkaloids have been achieved recently.

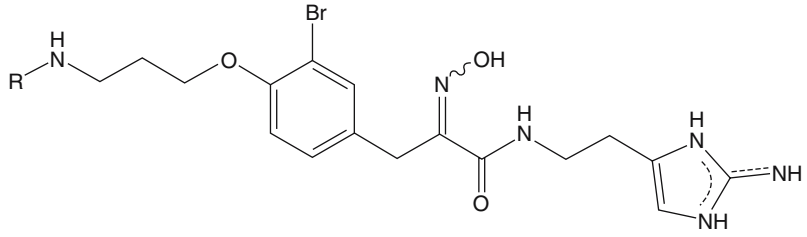
Several bromotyrosine derived alkaloids have been isolated from sponges. Purealin (**104**) and lipopurealins A-C (**105-107**) have been obtained from an Okinawan sponge *Psammaplysilla purea*.[186]



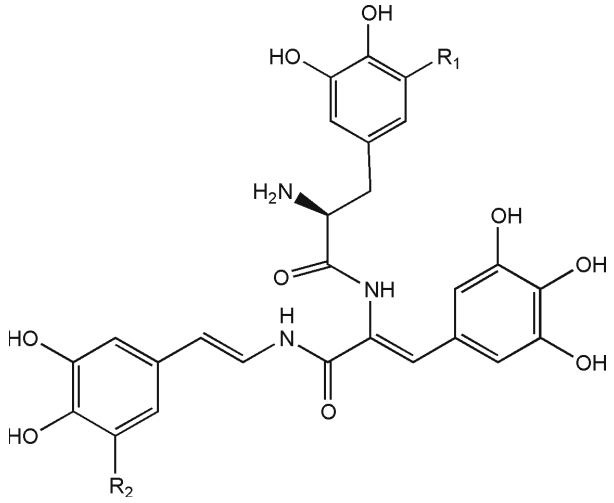
104

Purealin (**104**) and lipopurealin A and C (**105** and **107**) exhibit inhibitory activity on Na^+, K^+-ATPase. Purealin (**104**) is the first natural product that was found to activate myosin K. EDTA-ATPase whereas this enzyme activity is inhibited by lipopurealin B (**106**).

Tunicates selectively accumulate vanadium (or iron) in their specialized blood cells. The tunicate *Molgula manhattensis* has yielded two tunichromes (**108** and **109**) which are reported to accumulate the metal.[187]

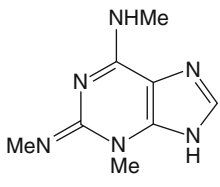


105, R = $CO(CH_2)_{12}CH_3$
106, R = $CO(CH_2)_{11}CH_3)_2$
107, R = $CO(CH_2)_{14}CH_3$

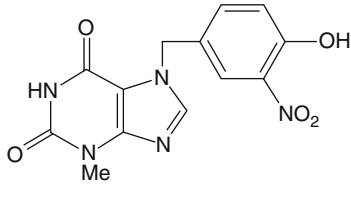


108, $R_1 = R_2 = H$
109, $R_1 = H$, $R_2 = OH$

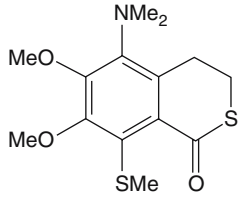
The sea anemone *Sagartia troglodytes*[188] has afforded 1-iminomethyl-3-methyl-6-aminomethyl-9*H*-purine (**110**) which inhibits the growth of tumors viruses of plant or bacteria. However, its activity is less than 1,2-diaminopurine. The North East Pacific bryozoan *Phidolopora pacifica*[189-192] contained desmethylphidolopin (**111**) which exhibits antimicrobial activity. The *Astroides calucularis*[193] collected in the Bay of Naples yielded 2-amino-6-(1*R*, 2*S*) [1′,2′-dihydroxypropyl]-3-methylpterin-4-one (**112**). The compound (**112**), exhibits cell growth inhibitory activity.[193]



110

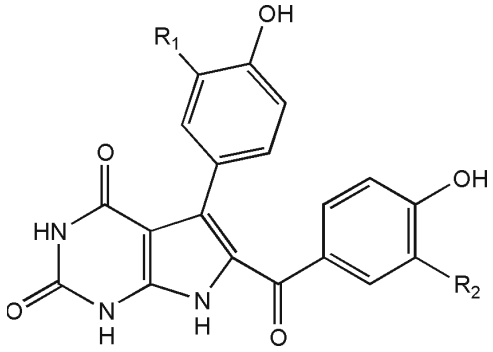


111



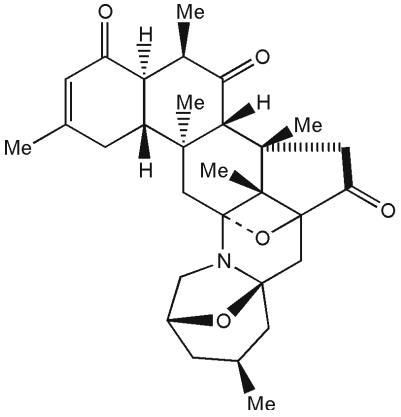
112

A Pyrrolopyrimidine alkaloid rigidin (**113**)[194,195] has been obtained from the Okinawan tunicate *Eudistoma* cf. rigida.[194] Rigidin (**113**) displays calmodulin antagonistic activity.[194] The tunicate *Cystodytes* sp. (1.6 kg, wet weight) was collected from Ie Island, Okinawa, and was extracted with MeOH which gave EtOAc soluble materials. Ethyl acetate soluable portion was purified on silica gel coloumn and C18 column chromatography and C18 HPLC to afford known compounds rigidin (**113**, 12 mg, 0.00075%), iejimalides A-D[196,197] and dytesinins A and B[198] along with unknown alkaloids rigidins B (**114**, 4.9 mg, 0.00031%, wet weight), C (**115**, 1.3 mg, 0.00008%), and D (**115**, $R_1 = R_2 = OMe$, 0.6 mg, 0.00004%). Molecular formula of rigidin B (**114**) was established as $C_{20}H_{16}N_3O_6$ by HR-FABMS [*m/z* 394.1029 $(M+H)^+$] which was larger than that of rigidin (**113**) by a OCH_2 unit. Absorption bands at 3420 and 1666 $cm^{\pm}$ in the IR spectrum indicated the presence of OH, NH and carbonyl group(s), respectively. Presence of conjugated phenol chromophore was confirmed by the UV absorptions at 364 (ε 9400), 319 (ε 9600), 304 (sh), 278 (ε 13200), 236 (sh), and 206 nm (ε 31400). Out of 13 proton signals in the ^1H NMR spectrum of (**114**), five of were D_2O-exchangeable, and the others were due to seven methines and one methoxy group. In the ^{13}C NMR spectrum, all 19 carbon resonances except that of a methoxy carbon (δ 55.19) were observed between δ 95 and 190. ^1H-^1H COSY experiments confirmed presence of 1,4-di and 1,3,4-trisubstituted benzene rings. ^1H-^{13}C HMBC correlations for H-9 and H-13 to C-11, H-10 and H-12 to C-8, and OH-11 to C-10, C-11, and C-12 indicated the presence of an 11-hydroxyphenyl group (C-8-C-13). On the other hand, the existence of an 18-hydroxy-17-methoxybenzoyl moiety (C14-C20) was inferred by ^1H-^{13}C HMBC correlations for H-16 to C-14, C-18, and C-20, H-19 to C-15 and C-17, H-20 to C-18, 17-OCH3 to C-17, and 18-OH to C-17, C-18, and C-19. A pyrrolo[2,3-d]pyrimidine-2,4-dione core of (**114**) was assigned by detailed analysis of the ^1H-^{13}C HMBC and ^1H-^{15}N HSQC spectra and NOE experiments. The molecular formula of rigidin C (**115**) was established as $C_{20}H_{16}N_3O_6$ and rigidin D as $C_{20}H_{18}N_3O_7$ by HRFABMS data.[199]



113, $R_1 = R_2 = H$
114, $R_1 = H$, $R_2 = OMe$
115, $R_1 = OMe$, $R_2 = H$

Zoanthamine (**116**),[200–203] zoanthenamine (**117**),[204] zoanthamide (**118**),[204] deoxyzoanthanamine (**119**)[205] and zoanthaminone[206] a new class of alkaloids have been isolated from a new species of colonial zoanthial of the genus *Zoanthus* collected from the Bay of Bengal. The structure of zoanthamine (**116**) has been determined by X-ray crystallographic analysis.[200]



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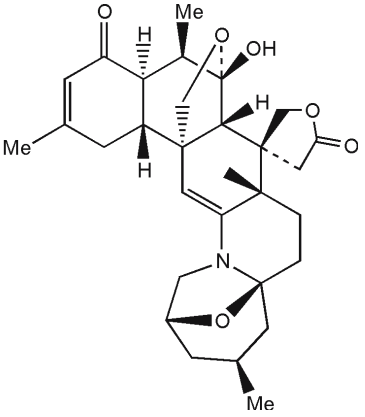

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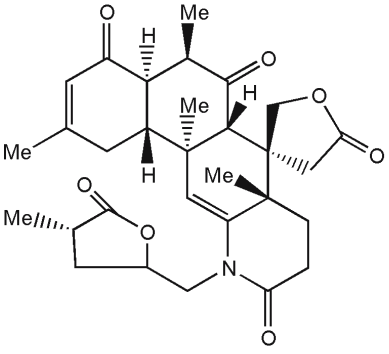

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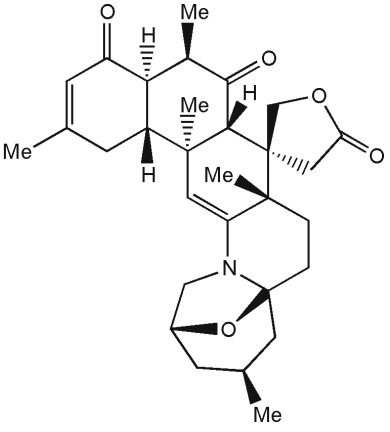
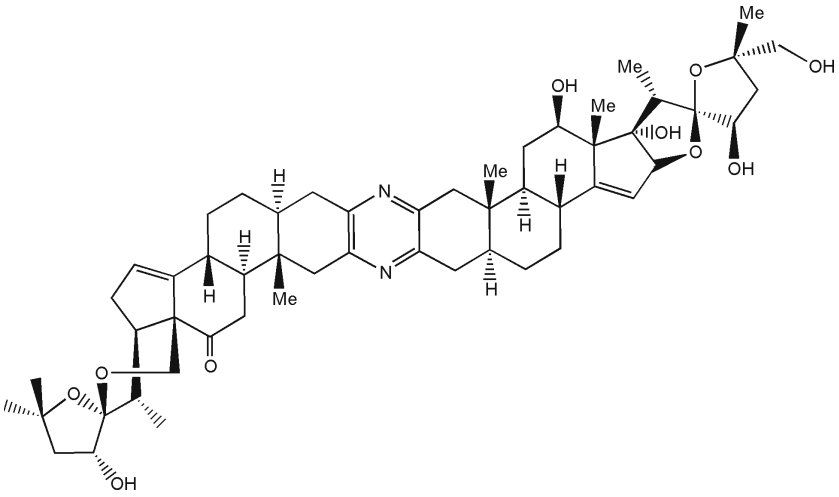


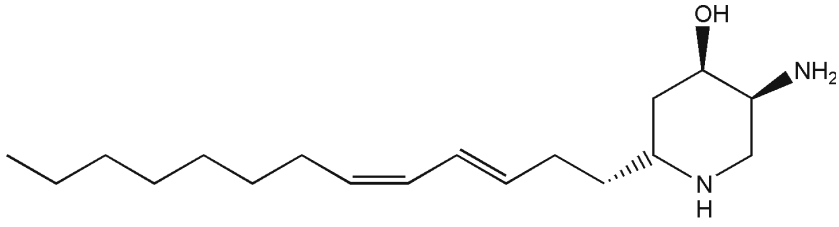
119

The alkaloids (**116-119**) display inhibitory activity in the phorbol acetate (PMA)-induced mouse ear inflammatory assay as well as analgesic activity. The biosynthesis of these alkaloid is very fascinating and the studies will be rewarding. The marine worm *Cephalodiscus gilchristi* collected in the Indian Ocean has afforded cephalostatins which were powerful cell growth inhibitory substances against PS cell lines.[207–213] The structure of cephalostatin I (**120**) has been determined by X-ray analysis. It is suggested that cephalostatins could be formed in nature by condensation of 2-amino-3-oxo-steroid units. The two steroidal units are connected by a pyrazine ring.



120

Extract of the Okinawan tunicate *Pseudodistoma kanoko* yielded the anti neoplastic piperine alkaloids pseudodistomin A (**121**) and B (**122**) which displayed calmodulin antagonistic activity.[214] Pseudodistomins are the first piperidine alkaloids found in marine organisms. The Fijian sponge *Niphates* sp. has afforded the pyridine alkaloids, niphatynes A and B.[215]



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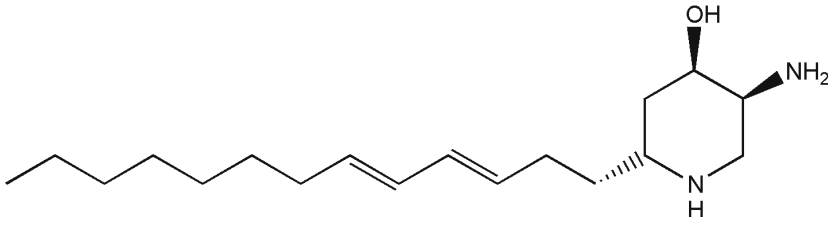
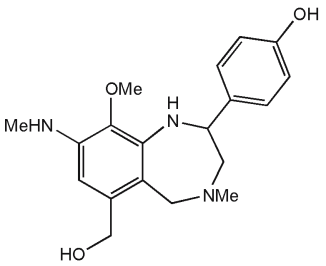


122

Aplysepine (**123**)[216] the first 1,4-benzo-diazepine alkaloid of marine origin was isolated from the sea hare *Aplysia kurodi*. The structure of aplysepine was elucidated on the basis of the spectral data. The new Guinea shell-less mollusk *Dolabella auricularia* was found to contain a series of green-blue

chlorins. One of these compounds was found to be the nickel chelat tunichlorin (**124**) which was isolated previously only from the caribbean tunicate *Tridemnum solidum*. Discovery of tunichlorin (**124**) in sea hare suggested that its occurrence is algae consuming marine animals may be more common than earlier realized. It is suggested that it may have a role in electron transfer or other metabolic process in mullusc.[217] Three imidazole alkaloids, leucettamines A and B, and leucettramidine were isolated from the Palawan sponge *Leucetta microraphis* and their structures were elucidated on the basis of extensive spectral analyses. Leucettamine A showed potent leukotriene B4 receptor binding activity (K_i = 1.3 M).[218]



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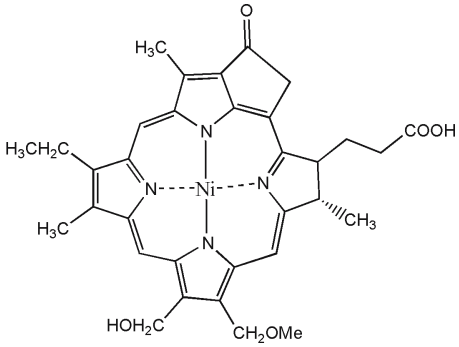
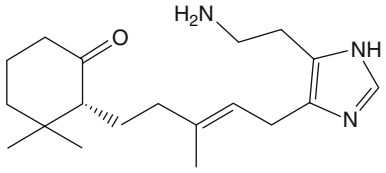


124

Hamacanthin was isolated from a new deep water sponge, *Hamacantha* sp. The hamacanthins were found to be the growth inhibitors of *Candida albicans* and *Cryptococcus neobormans*.[219222] The major metabolite of the Philippine sponge *Oceanapia* sp. was the antimicrobial alkaloid oceanapamine (**125**) which was isolated a a trifluoroacetate salt. The structure and absolute configuration of oceanapamine were determined by the spectroscopic data. The compound showed antimicrobial activity, it inhibited *B. subtitis* and *E. coli* at 25 g/disk, *S. aureus* and *C. albicans* at 50 g/disk and *P. aeruginosa* at 100 g/disk.[223] Pyrrolo[4,3,2-de]quinolines and pyrido[4,3,2-mn]acridine were of major interest as metabolites in sponge and ascidians. Many of these compounds had generated interest both as challenging problems for structural elucidation and synthesis as well as for their cytotoxicity. The principal structural feature of these alkaloids was the core of a planar iminoquinone moiety which tercalate into DNA and cleave the DNA double helix or inhibit

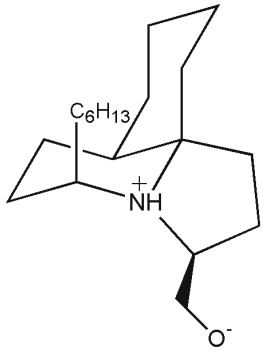


125

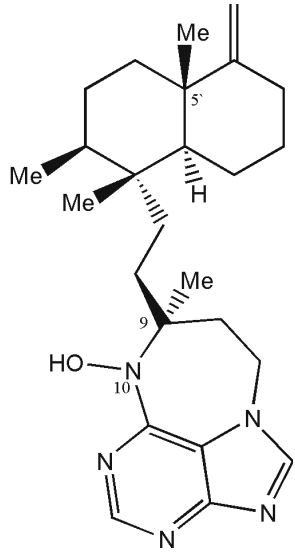
the action of topoisomerase II. Of the makaluvamines, makaluvamines A and F were the most cytotoxic to the HCT 116 cell line. They were also the most potent inhibitors of topoisomerase II. Both makaluvamines A and C decreased tumor size in a solid human tumor model. The new member of the pyrrolo[4,3,2-de]quinolines, and pyrido[4,3,2-mn]acridine such as veitamine, discorhabdin G, and epinartins were reported.[12]

Biard et al[224] reported the isolation and structure elucidation of a marine alkaloid lepadiformine (**126**) from the marine tunicate *Clavelina lepadiformis*[224] in 1994 and later from *C. moluccensis*.[225] Lepadiformine exhibited cytotoxic activity against the various tumor cell lines. Recently, it was found that lepadiformine is very active *in vivo* and *in vitro* in the cardiovascular system.[225] Structure of lepadiformine was determined on the basis of extensive spectral analysis. Recently, Kibayashi et al[226,227] reported the total synthesis of (-)-lepadiformine.

Asmarines A (**127**) and B (**128**) were isolated from the ethyl acetate extract of marine sponge *Raspailia* sp. collected from Red Sea marine invertebrates.[228] These alkaoilds exhibited potent cytotoxic activity against four human cancer cell lines.[229] The structure of asmarine A was ascertained by an X-ray diffraction analysis.[229] Recently total synthesis of these alkaloids have been achieved.[230]

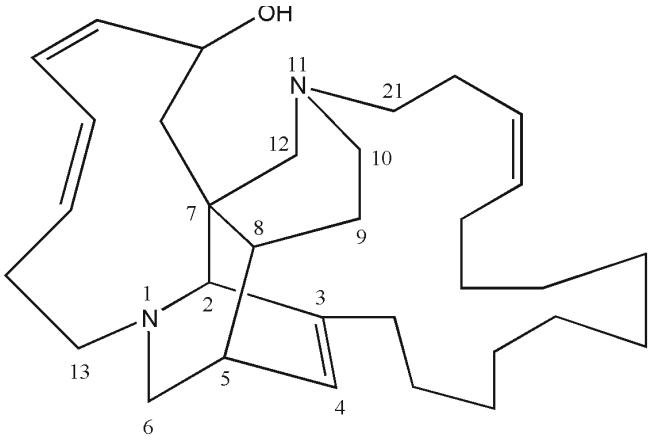


126

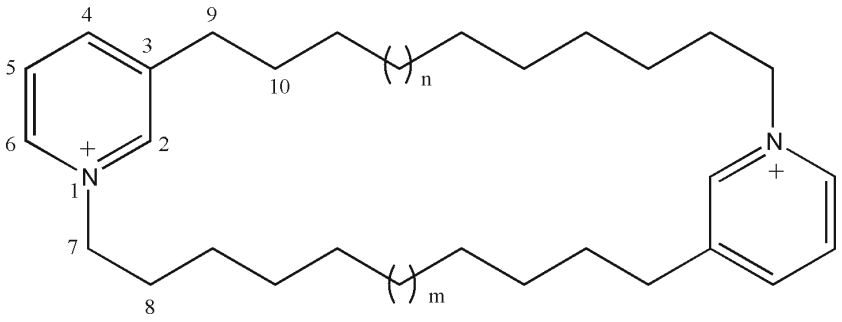


127
128 (5′ Epi)

Recently Berlinck et al[231] had reported the isolation and structural elucidation of marine alkaloid ingenamine G (**129**), and mixture of new cyclostellettamines G, H, I, K and L (**130–134**) from marine sponge *Pachychalina* sp. The methanolic extract of the ingenamine G displayed cytotoxic activity against



129



130, m = 2, n = 3
131, m = 1, n = 3
132, m = 1, n = 4
133, m = 1, n = 5
134, m = 2, n = 5

HCT-8 (colon), B16 (leukemia), and MCF-7 (breast) cancer cell lines, antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and four oxacilin-resistant *S. aureus* strains, and antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv. The sponge, (2.0 kg) was extracted with EtOH. Both EtOH and MeOH extracts were subjected to solvent partition with EtOAc and with n-BuOH. EtOAc extract after evaporation was dissolved in MeOH-H_2O (9:1) and partitioned with hexane. The 90% of the solvent was evaporated, and the residue thus obtained was dissolved in CH_2Cl_2-0.5 N HCl. Compound (**129**) was isolated as an optically active glassy solid after repeated chromatographic separations. Molecular formula of the compound was determined as $C_{32}H_{51}N_2O$ by its HR-FABMS (*m*/*z* 479.40007, calcd 479.40014). The molecular formula indicated the presence of nine degrees of unsaturation. By the use of ^1H

NMR, and ^{13}C NMR data it was concluded that this compound must have five rings. Additionally, NMR analysis indicated the presence of three sp3 methines (δ 62.1, 40.4, and 37.6), one sp3 (δ 44.6) and one sp2 (δ 144.5) quaternary carbons, and five typical nitrogen-substituted methylene resonances (δ 50.6, 51.2, 56.3, 58.0, 59.4), which suggested an ingenamine-type skeleton for (**129**). Comparison of the ^{13}C NMR data of (**129**) with related alkaloids indicated the presence of a tricyclic central core. Extensive analysis of NMR spectra including HSQC, HMBC, ^{1}H-^{1}H COSY, HSQCTOCSY, and NOESY confirmed the structure of these mairne alkaloids. Similarly, structure of other alkaloids (**130-134**) have been confirmed.

8. Concluding Remarks

The field of marine alkaloids is firmly established and rapidly expanding. Over 75 pyridoacridine alkaloids have been isolated from marine sponges, tunicates and molluscs and characterized. There is little doubt that their number will increase in future. Marine alkaloids, particularly brominated or chlorinated ones, do have distinct features as compared to alkaloids from terrestrial sources. There are ample circumstantial evidences which suggest that some of the marine alkaloids are metabolites of an associated flora. Since the origin of a number of alkaloids is uncertain, the attempts of construction of chemotaxonomical system based on secondary metabolites analysis must be viewed with caution. Although quite a number of marine alkaloids display high order of antineoplastic and antiviral activity, a drug from this source is still very far. However, studies conducted on marine alkaloids have enriched basic organic chemistry and the biological activities have widened the vision of chemists and biologists. The biological effects of bioactive marine alkaloids have expanded greatly our knowledge of several basic phenomena in biology. The new structures have provided fertile area for design and synthesis. The biosynthesis of bioactive marine alkaloids is fascinating and remains unexplored.

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10

Bioactive Marine Peptides

Abstract

The chapter deals with the structure, conformation and biological activities of bioactive marine peptides. The chemistry and biological activities of bioactive peptides and modified peptides of marine algae, sponges, tunicates, ascidians, coelenterates, and molluscs have been discussed and reviewed. The chemical nature and biological properties of cone snail venoms, sperm activating peptides of sea urchin egg jelly, peptides of marine worms and marine vertebrates have also been reviewed and analyzed.

1. Introduction

Despite excitement generated by the isolation of arabino nucleosides from sponge,[1] the early literature was dominated by reports of non-nitrogenous metabolites which included halogenated terpenes and fatty acid derivatives from red algae, sea hares, cembranes and eicosanoids from coelenterate, sesquiterpenes from sponges, and nudibranches, and diterpenes from brown algae.[2–4] Only few reports on isolation of nitrogenenous metabolites from marine source led to the belief that it is due to the fact that nitrogen is a limiting nutrient in the ocean. However, subsequent researches revealed that it is not true. A diverse array of nitrogenous metabolites, including nucleosides,[5] alkaloids,[6,7] isonitriles,[8–11] guanidines,[12] amino acids[13] and peptides[14–16] were isolated from marine organisms.

The field of marine natural products is now becoming more sophisticated. Instead of simply searching for new metabolites, the search is now on for compounds which exhibit pharmacologically useful biological activities. Assay methods are now available to detect a diverse array of biomedically relevant compounds, such as CNS membrane active toxins, ion channel effectors,

anticancer agents, tumor promoters, antiviral, antiinflammatory agents, and metabolites which affect microfilament mediated processes.[17] There has been considerable activity in the area of marine natural products during the last two decades. To date approximately 16,000 marine natural products have been isolated from marine organisms and reported in about 6,800 publications. In addition to these publications there are approximately another 9,000 publications which covers syntheses, reviews, biological activity studies, ecological studies etc. on the subject of marine natural products. In the year 2003, over 656 marine natural products were isolated from marine organisms and reported in 243 research papers. Chemical investigation of different phyla of marine organisms had yielded different class of compounds.[18] For example, during the years 1977 to 1985, 85% of the metabolites isolated from coelenterates were terpenoids; sponges yielded 37% terpenoids and 41% nitrogenous metabolites, and ascidians showed a specialized ability to biosynthesize amino acid derivatives, producing up to 89% nitrogenous compounds. Although some of these reports may not be reflecting the actual chemical make up of the organisms since isolation of a particular class of compound is influenced by the expertise of the chemists involved and also techniques used in the isolation, however, it is true that sponges and ascidians are rich sources of nitrogenous metabolites. Of the several class of nitrogenous compounds isolated from marine organisms, the peptides are of considerable interest both from the point of view of structural types and type of biological activities.

2. Peptides Conformation

For many years keen interest has been evinced in the structure of bioactive small peptides, but in recent years much work has been done on the conformation of these peptides in solution. The general concept regarding the structure and conformation of small peptides is that these compounds exist as a large number of rapidly interconverting conformation in solution at ordinary temperature. More recent evidence suggests that although this general picture is true for many peptides, however, in several cases the smaller peptides have a fairly well-defined conformation in solution. Many of the smaller peptides display a wide variety of biological activities including compounds that function as hormones, antibiotics, toxins, antidote, ionophores etc. A relationship between the conformation of these peptides in solution and their biological activities does exist, hence considerable time has been spent to understand their conformation in solution. The principal methods used to study the conformation of peptides in solution are nuclear magnetic resonance (NMR) spectroscopy, optical rotatory dispersion (ORD), circular dichroism (CD) and infrared (IR) spectroscopy. These studies are often supplemented and complimented by X-ray diffraction studies or both, and by theoretical calculations. For study in solution spectroscopic studies are

also often supplemented by various other physical studies. The most prominent of these are: (a) hydrogen exchange studies to provide information on hydrogen bonding and shielding of exchangeable hydrogen; (b) thin film dialysis studies for information regarding the relative sizes and shapes of peptides; (c) dipole moment measurement for geometrical information, and (d) partition chromatography to indicate relative shape and size of the peptide. Energy calculations often used in conjunction with spectral and other physical evidence have also been extremely useful in many conformational studies. Intramolecular hydrogen bonds in peptides are important because they can be expected to be one of the major non covalent interactions providing conformational stability to a small peptide in solution. Furthermore, the presence of intramolecular hydrogen bonds severely restricts the conformations possible.

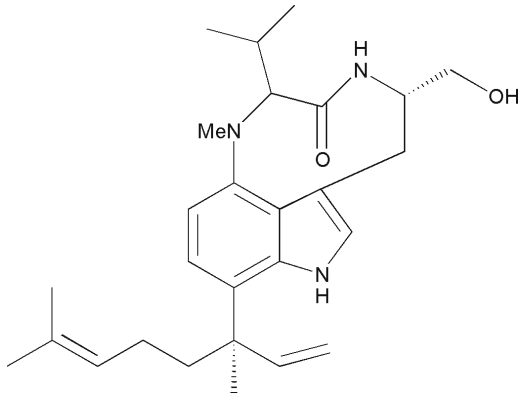
Significant difference in chemical shifts of amide or other protons in the NMR spectrum of the peptides give clues about certain conformational features. This is particularly useful if the shifts are quite large, since they usually reflect the magnetic anisotropy of neighboring groups (in a spatial sense). Group which produce substantial effects are the aromatics found on the side chains of individual amino acid residues including phenyl, hydroxyphenyl, indole, and imidazole systems. Infrared (IR) spectroscopy has been widely used for studying dipeptide conformations. The principal information can be obtained by this method and the cis-trans nature of peptide bond can be ascertained. Cyclization of peptides greatly restricts the number of possible conformations that a peptide can assume. A study of the conformations of the cyclic dipeptides in solution provides considerable information regarding the relationship between the various spectral probes and the conformational characteristics associated with these spectral properties. This is because the degrees of rotational freedom are quite restricted in cyclic peptides and thus the number of conformations are quite limited. It is intriguing that small linear peptides, which are devoid of covalent crosslink such as disulfide, amide, or ester bonds can have a unique or highly restricted conformation(s) in solution which is of considerable importance to both biology and chemistry. A number of bioactive marine peptides have D-amino acids and some unusual amino acids. All the 20 amino acids that occur in proteins have L-configuration. However, a large numbers of "non-protein" amino acids have very diverse structures. The majority of these amino acids have been found in plants, microorganisms and marine organisms. The peptide antibiotics and some marine peptides are good source of D-amino acids and abnormal amino acids.

3. Bioactive Marine Peptides

3.1 Marine Algae

Certain varieties of *Lyngbya majuscula,*[19-27] a toxic marine blue-green alga

found in warm water have been implicated as the causative agents of Swimmer's itch, a form of contact dermatitis. Lyngbyatoxin-A (**1**)[28,35] was isolated from *L. majuscula* and the gross structure was determined as the prenylated cyclic dipeptide. The configuration at the C-14 quaternary carbon in (**1**) was subsequently shown to be (*R*) by degradation of (**1**) to dimethyl 2(*S*)-2-ethyl-2-methylglutarate.[36] Lyngbyatoxin-A (**1**) represents a new structural class of potent tumor promoters that exhibits tumor promoting activity in mice similar to that induced by phorbol esters.[37] Lyngbyatoxin-A was also found to kill baitfish (*Poecilia vittata*) at a concentration of 0.15 µg/mL in seawater suggesting *L. majuscula* perhaps produce lyngbyatoxin-A as an ichthyotoxic chemical for defense. Lyngbyatoxin-A has been synthesized.[38]



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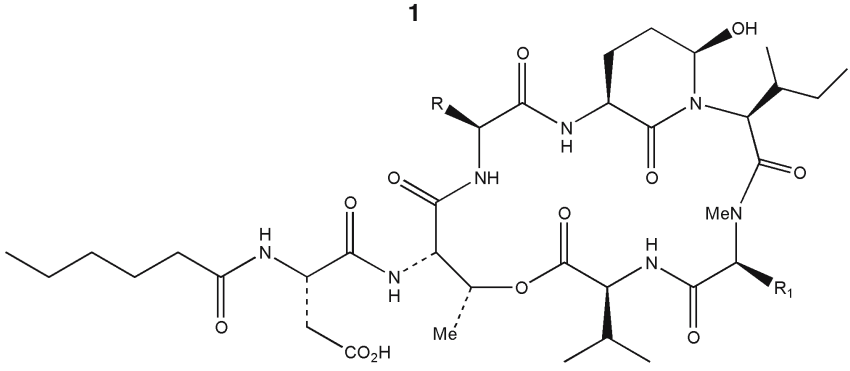

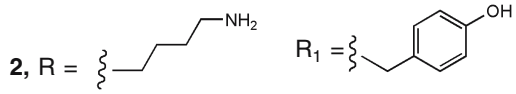

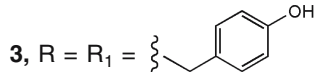

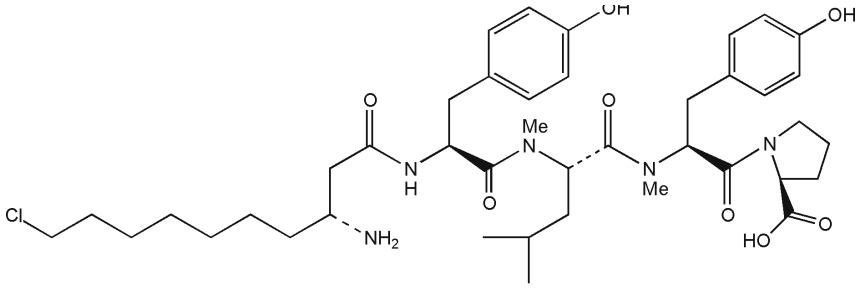

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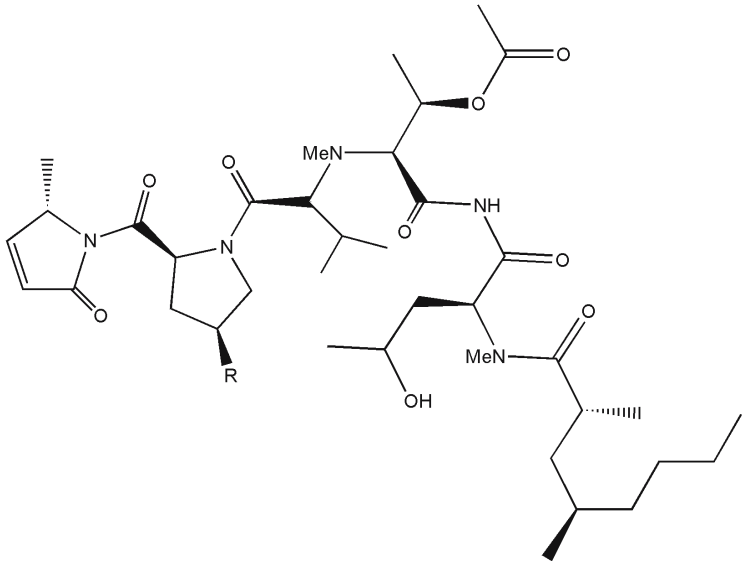


5, R = OH
6, R = H

Blue-green alga *Microcystis aeruginosa* has yielded four new protease inhibitors, micropeptins SD944 (**2**), SD979 (**3**), SD999 and SD1002.[39,40] Micropeptins SD944 (**2**) and SD999 inhibited trypsin with IC_{50} at 8.0 and 4.0 μg mL^{-1}, respectively, but both compounds did not inhibit chymotrypsin at 45 μg mL^{-1}. Micropeptin SD979 and SD1002 inhibited chymotrypsin at 2.4 and 3.2 μg mL^{-1}, respectively, but not trypsin at 18.0 μg mL^{-1}. The structures of these peptides were elucidated on the basis of 2D NMR data and chemical degradation. Microginin (4)[41] has been isolated from *M. aeruginosa* and it inhibited angiotensin converting enzyme (IC_{50}, 7.0 μg/mL), but did not inhibit pepsin, trypsin, chymotrypsin and elastase at 100 μg/mL. A Venezuelan sample of the blue-green alga *Lyngbya majuscula* had recently furnished two new immunosuppressive peptides microcolin-A (**5**) and microcolin-B (**6**).[4245] The microcolins are potent inhibitors of the murine mixed lymphocyte response and murine P-388 leukemia *in vitro.*

3.2 Sponges

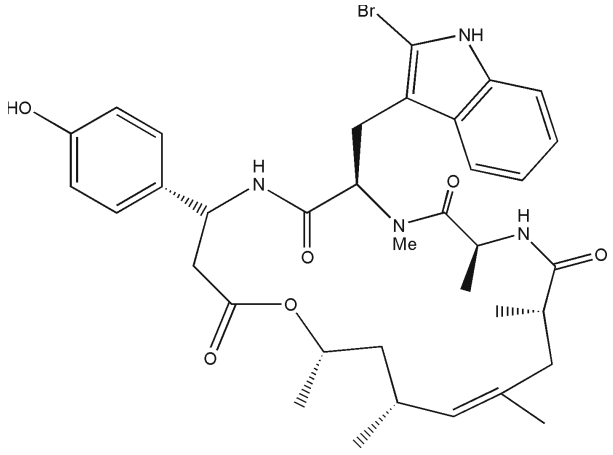
Marine sponges continue to be source of secondary metabolites with unusual chemical diversity and remarkable biological activity. Large number of peptides have been isolated from marine sponges. They have attracted considerable attention because of their unique structural framework, rich physiochemical properties, and thus potential as important drug candidates.[4649] The sponges so far had furnished only a few "true" peptides. Most sponge peptides are highly modified whereas some contains unusual amino acids. Sponges provide lodging to many organisms, such as brittle stars, bivalve, gastropods, crustaceans, and annelid worms. Sponge peptides are suspected to be of microbial origin because some of them contain D-amino acids and unusual amino acids. Fusetani et al[50] were the first to isolate bioactive peptide discodermin A from the marine sponge *Discodermia kiiensis* in 1985. The peptide contained the rare tert leucine, cysteic acid and several D-amino acids. Since this report many peptides had been isolated from marine sponges, some of which not only exhibit interesting biological activities but also contain new amino acids.[14] Several reasons have been given for the rapid progress in the chemistry of sponge peptides; development of reversed-phase HPLC enabled the isolation of peptides from a mixture of related metabolites. Advances in spectroscopy, especially 2D NMR and FAB mass spectrometry for structural determination because sequence analysis of unusual peptides cannot be accomplished by Edman degradation due to the presence of blocked N-termini and/or amino acid residues. Progress in chiral chromatography allowed the assignment of absolute configuration of amino acids with small amount of material. Lastly, the marine natural product chemists happen to investigate sponges of the order Lithistida, rich in bioactive peptides. Sponge peptides have been reviewed.[14,16,46] The species that have yielded bioactive peptides are: *Jaspis, Pseudaxinyssa, Geodia, Discodermia, Theonella, Cliona, Axinella, Pseudoaxinella, Malaysistin, Leucophloeus* and *Hymeniacidon*.

Jaspamide (**7**)[5157] was the first bioactive peptide isolated from a sponge of the genus *Jaspis* from Fiji and Palan. MeOH extract of *Jaspis* was obtained by soaking 73 g of pulverized freeze-dried tissue. The methanol extract was subjected to a solvent partition to give 500 mg material which was found solubale in CCl_4 and $CHCl_3$. Filtration of this material through a silica gel 60 column (2.4 cm × 10 cm, EtOAc) followed by HPLC (Partisil 10, 4.6 mm × 25 cm; EtOAc/Hexane, 8:2) gave jaspamide (**7**) as a colorless oil. Molecular formula of (**7**) was established as $C_{36}H_{45}BrN_4O_6$ by HR-FABMS $C_{36}H_{45}BrN_4O_6$ (MH^+ 709.2596). The depsipeptide nature of jaspamide was evident from IR bands at 1715, 1684, 1674 and 1638 cm^{-1} and ^{13}C NMR signals at δ175.1, 174.4, 170.5, and 168.9 ppm indicating the presence of four carbonyl functionality. Finally, the structure (**7**) was confirmed by extensive 2D NMR spectroscopic data.

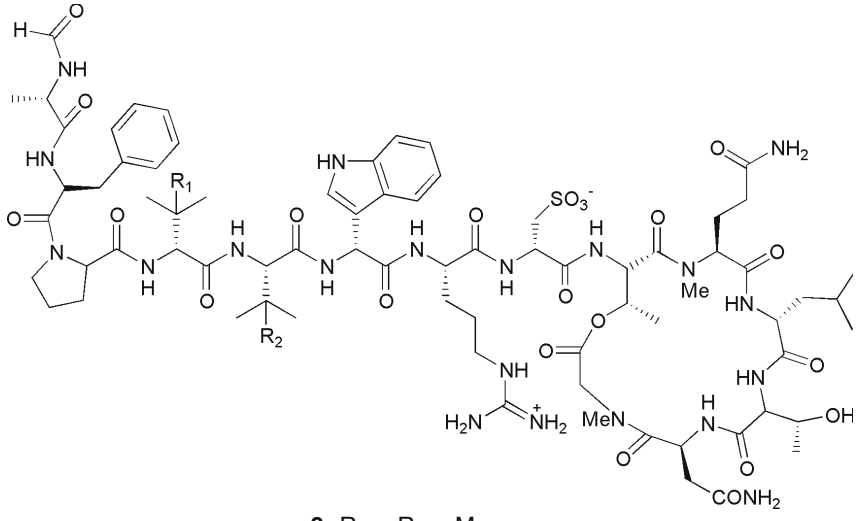
Total synthesis of jaspamide (**7**) has been accomplished. Jaspamide exhibited insecticidal activity against *Heliothis verescens* with an LC_{50} of 4 ppm. It was

toxic to nematode [*Nippostrongylus brasiliensis* (LD_{50} < 1 μg/mL)]. Jaspamide showed *in vivo* topical activity against a vaginal *Candida* infection in mice and was cytotoxic against a larynx epithelial carcinoma cell line (IC_{50} 0.32 μg/mL) and a human embryonic lung cell line (IC_{100} 0.01 μg/mL).

Sponges of the genera *Discodermia* and *Theonella* had proved to be a rich source of bioactive peptides. Discodermins were the first bioactive peptides isolated from marine sponge.[58,59] Discodermins A-D (**8-11**) were isolated from *D. kiiensis*. They are tetradecapeptides with the N- terminus blocked by



7

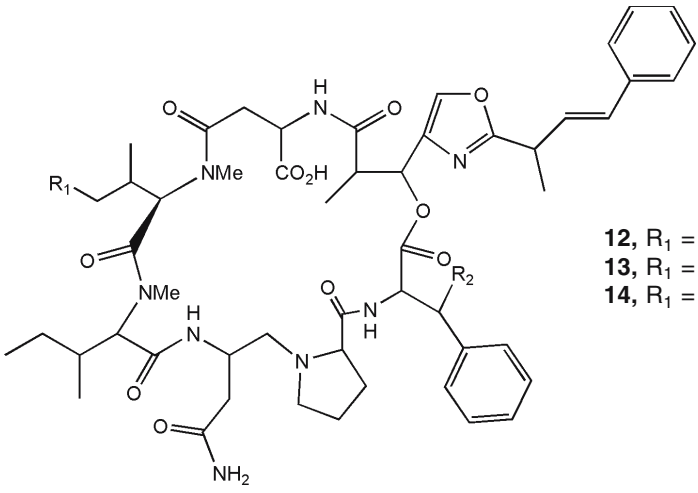


8, $R_1 = R_2 = Me$
9, $R_1 = H$, $R_2 = Me$
10, $R_1 = Me$, $R_2 = H$
11, $R_1 = R_2 = H$

a formyl group and the C-terminus lactonized with the ninth (Thr.) residue from the N-terminus.

The structure of discodermins B-D (**9-11**) differed in the fourth and fifth amino acid residues, which were Val-t-Leu in discodermin B, t-Leu-Val in discodermin C, and Val-Val in discodermin D. The most unusual component in the discodermins is the t-Leu residue, which had only been reported as a constituent of actinomycete peptides, bottromycins.[60,63] These facts along with the presence of several D-amino acids suggest the microbial origin of discodermins. Discodermin A inhibited *Bacillus subtilis* and *Proteus mirabilis* at 3 and 1.6 μg/mL concentrations respectively. Discodermins also inhibited the development of starfish embryos at concentrations 2-20 μg/mL. They were found to be potent inhibitors against phospholipase A_2 (IC_{50}, 3.5-7.0 × 10^{-7} M).[64] Discodermin A exhibited antiinflammatory activity in the mouse ear pretreated with okadaic acid. Discodermin A also inhibited tumor promotion by okadaic acid. Treatment with 500 μg of discodermin A before application of okadaic acid (1 μg) reduced the percentage of tumor bearing mice from 86.7% to 46.7% and the average number of tumors per mouse from 4.7 to 1.1.[14]

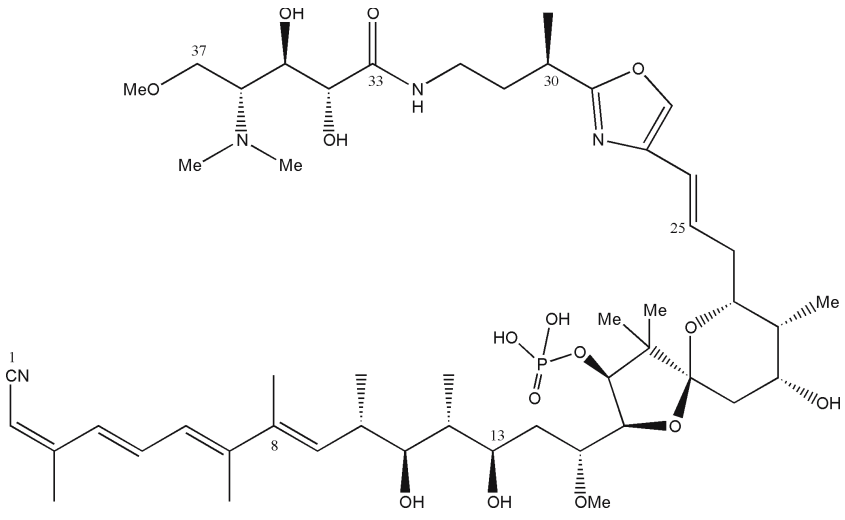
Caribbean sponge *Discodermin* sp. collected at a depth of 274 m off the coast of St. Lucia, Lesser Antilles furnished a bioactive peptide, polydiscamide A[65] which had common structural features as discodermins. Polydiscamide A was cytotoxic against the cultured human lung cancer A_{549} cell line with an IC_{50} of 0.7 μg/mL and inhibited *B. subtilis* with an MIC of 3 μg/mL. *Discodermia kiiensis* had yielded unrelated cyclic depsipeptides, namely discokiolide A-C (**12-14**).[66] These peptides had unusual β-hydroxy acids as well as β-methoxyphenylalanine residues. The mixture of peptides was separated by reversed-phase HPLC after conversion to the methyl esters.



12, R_1 = H, R_2 = OMe
13, R_1 = Me, R_2 = OMe
14, R_1 = Me, R_2 = H

Discokiolides exhibited marginal cytotoxic activity; IC_{50} μg/mL. P_{388}, 2.6, P_{388}/ADM, 7.2, B16-BL6; 1.6, Lewis, 1.2; Lu-99, 0.7; HT-29, 1:2; CCD-19Lu 0.5. Eight bioactive peptides calyculins A-H containing an octamethyl polyhydroxylated C_{28} fatty acid linked to two amino acids were isolated from the sponge *Discodermia calyx*.[67,68] Absolute stereochemistry of the calyculins was determined on the basis of CD spectrum of an amino acid fragment obtained by acid hydrolysis,[69] which was later confirmed by a synthesis of the fragment[70] and total synthesis of an enantiomer of calyculin A.[71] Calyculin A (**15**) exhibited *in vivo* antitumor, cytotoxic and antifungal activities. Calyculin A showed potent tumor promoter activity.[72] It inhibited protein phosphatases 1A and 2A.[73] An Okinawan *Theonella* sp. had yielded cyclic peptides, named keramamides B-D (**16-18**).[74] Another related peptide keramamide F (**19**) was also obtained from the same sponge.[75] Keramamide F (**19**) was found moderately cytotoxic, whereas keramamides B-D (**16-18**) inhibited the generation of super oxide anion in human neutrophils treated with formyl-Met-Leu-Phe at concentrations of 5×10^{5} M.

Konbamide (**20**)[76] and keramamide-A (**21**),[77] were isolated from Okinawan *Theonella* sponge and found to be moderate inhibitors of calmodulin-activated phosphodiesterase and Ca^{2+}-ATPase, respectively. Both the peptides had common pentapeptide feature, the side-chain amino group in the N-terminal Lys residue formed an amide bond with the C-terminal carboxyl group and the amino group of the Lys residue formed as urea bond with an amino acid. Hymenamide[78-80] A and B (**22**, **23**) the two cyclic heptapeptides having a prolylproline segment have been isolated from the Okinawan marine sponge *Hymeniacidon* sp. Their structures have been elucidated on the basis of 2D NMR and FAB-MS data.



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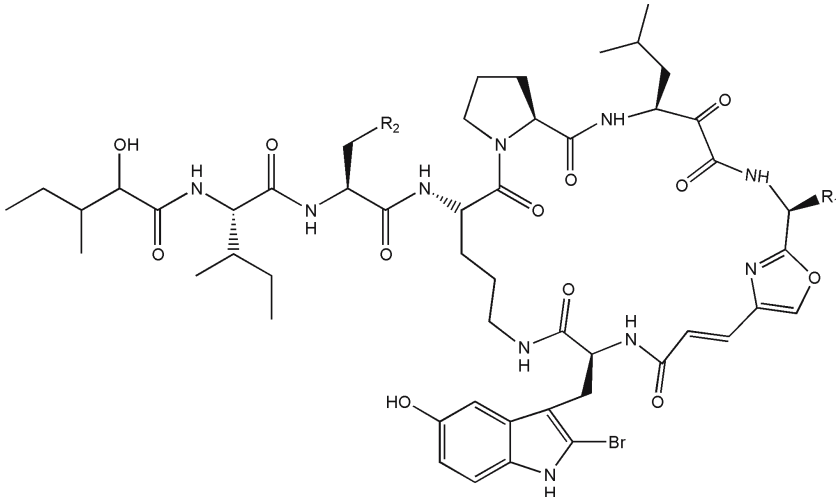
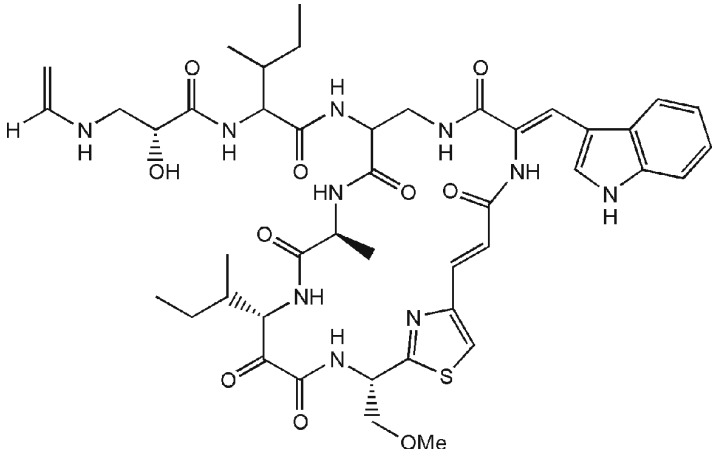


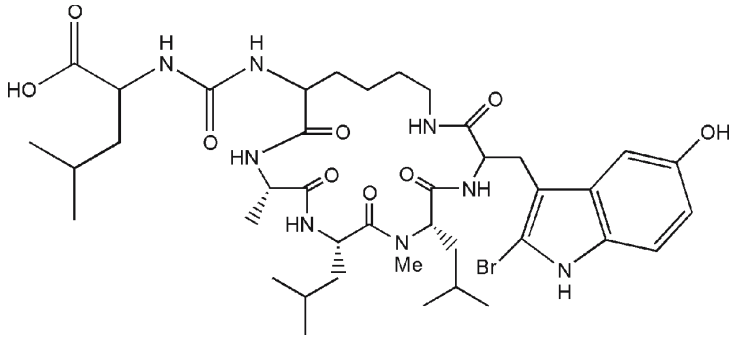
16, $R_1 = R_2 = CH_2CH_3$
17, $R_1 = CH_2CH_3$, $R_2 = CH_3$
18, $R_1 = R_2 = CH_3$



19

Hymenamides A, B (**22**, **23**, respectively) showed antifungal activity against *Candida albicans* (MIC 33 and 66 µg/mL respectively) and *Cryptococcus neoformans* (<133 and 33 µg/mL, respectively). Hemanamide B (**23**) also showed cytotoxicity against murine lymphoma-L 1210 cells and human epidermoid carcinoma KB cells with IC_{50} value of 3.2 and 6.0 µg/ml *in vitro*, respectively.

Theonella sp. have been a source of structurally diverse biologically active peptides.[46] Recently, phakellistatins (phakellistatins 1-11 and isophakellistatin



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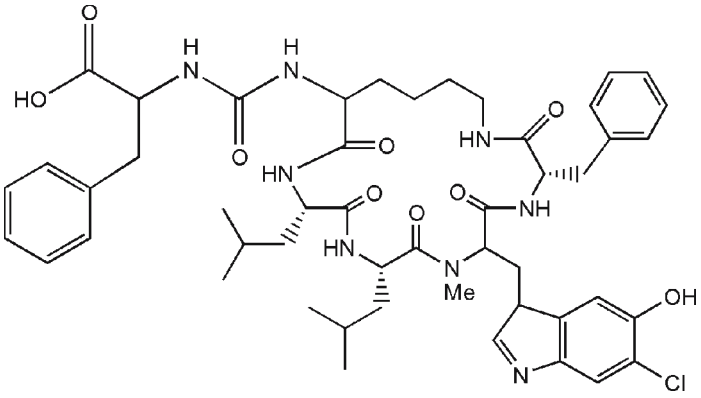

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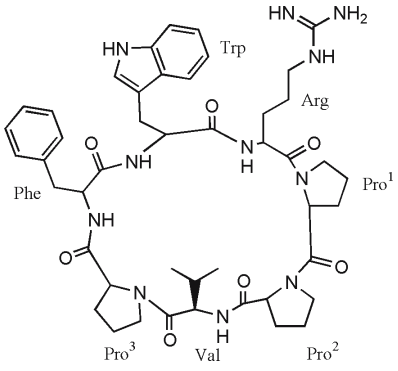

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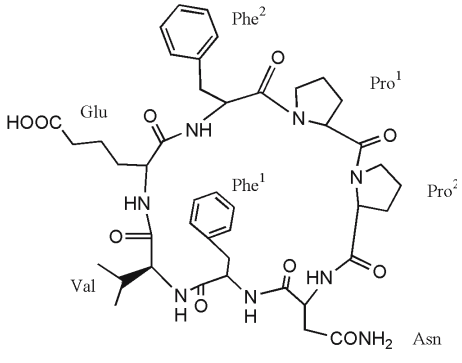
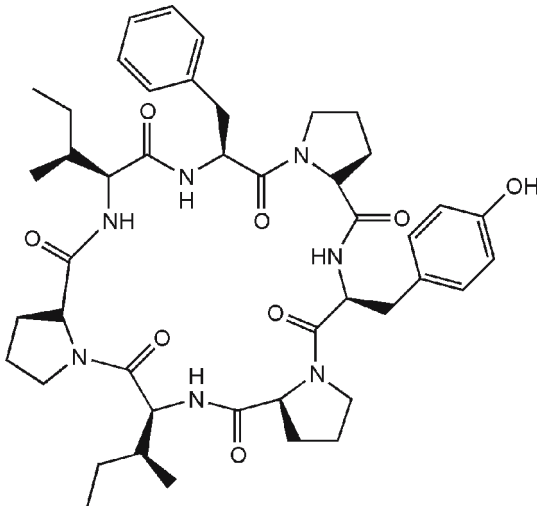


23

13), a series of cyclicpeptides, have been isolated from *Phakellia* sp. (class Demospongiae, order Axinellida).[81-89] All of the phakellistatins have common

proline unit. Phakellistatins exhibit moderate cytotoxicity.[81] Phakellistatin 1 (**24**),[81] a cell growth inhibitory (P-388 murine leukemia, (ED_{50} 7.5 μg/mL) cycloheptapeptide was isolated from two Indo Pacific sponges, *Phakellia costata* and *Stylotella aurantium*. Its structure was accomplished using high field NMR, amino acid analyses and mass spectral techniques (FAB, Tandem MS) followed by chiral gas chromatographic procedures for absolute configuration assignments (all *S*-amino acid units). The structure (**24**) was finally confirmed by X-ray analysis.



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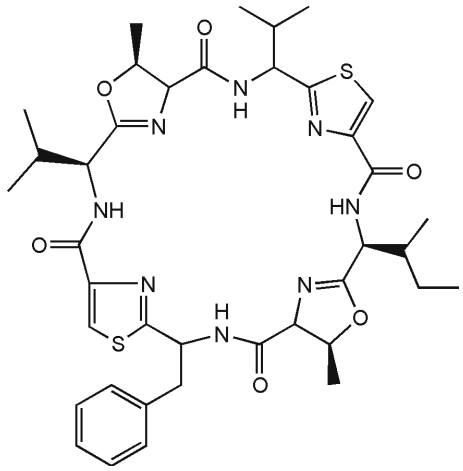
The ascidians *Lissoclium patella* collected from Pulan Salu, Singapore yielded a cyclic peptide patellamide E (**25**)[90] which exhibited mild cytotoxicity against human colon tumor cells *in vitro* (IC_{50} 125 μg/mL). Nazumamide-A (**26**),[91] a thrombin inhibitory linear tetra peptide of marine origin, was efficiently constructed using diethyl phosphorocyanidate (DEPC) as coupling reagent and *tert*-butyloxycarbonyl as the temporary N-protecting group. The protecting groups were finally removed by catalytic hydrogenation.

Ethanol extraction of the frozen sponge *Discodermia* sp. collected from Bahamas at a depth of 180 m, yielded an extract which was partitioned between EtOAc and H_2O. The EtOAc soluble fraction on bioassay guided silica-gel chromatography followed by reversed-phase HPLC purification furnished two antifungal peptides~~di~~scobahamin-A (**27**) and discobahamin-B (**28**).

Discobahamins are related structurally to cyclic peptides orbiculamide A[92] and keramamides B-D isolated from *Theonella* sp.[93] Both discobahamin A and B exhibited weak antifungal activity against the yeast form of *Candida*



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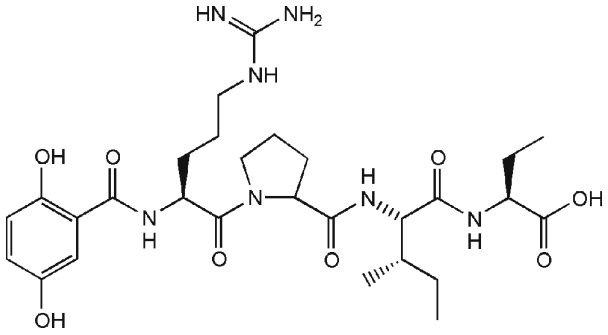

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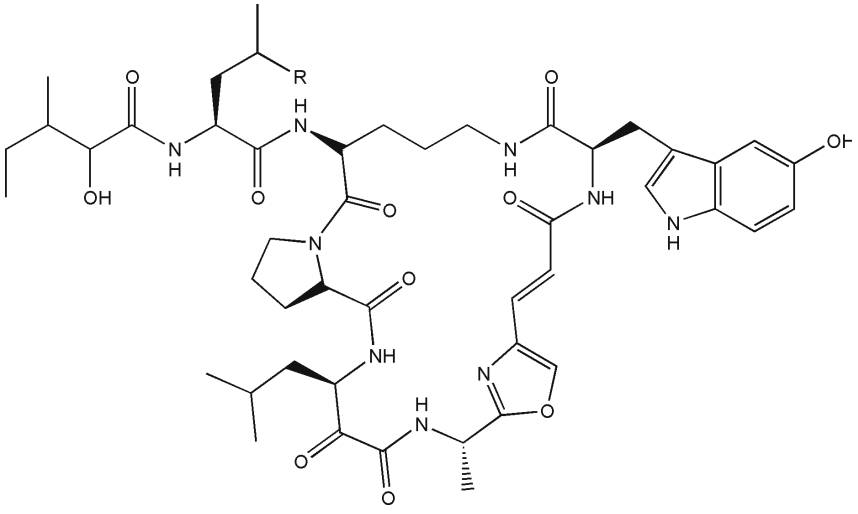
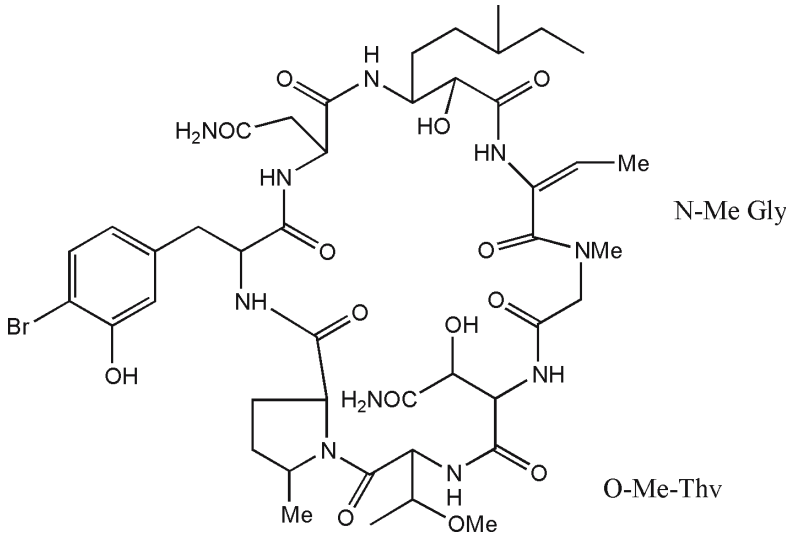


27, R = H
28, R = Me

albicans. A sponge of the genus *Theonella* collected at a depth of 15 m by SCUBA near Perth, Western Australia contained a cyclic octapeptide perthamide B,[94] Its structure was elucidated by spectroscopic methods.

Perthamide B (**29**) weakly inhibited the binding of [^{125}I] IL-1 to intact EL 4.6.1 cells with IC_{50} 27.6 μM. Methanolic extract of the frozen sponge was partitioned between n-butanol and water. The residue of the active organic layer was chromatographed on an LH-20 stationary phase and further purified by HPLC to give perthamide-B, m.p. 228-231°C.

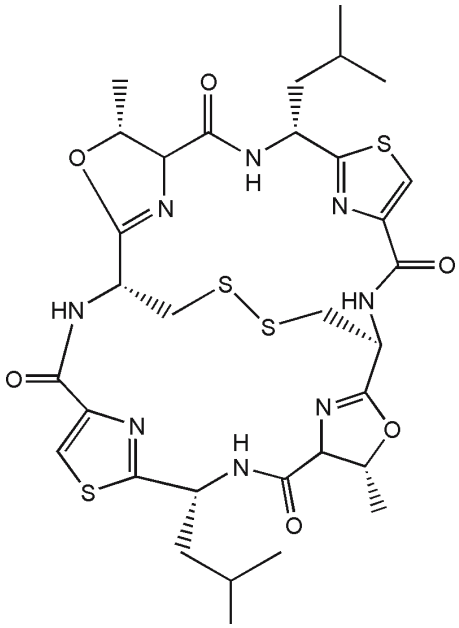


29

Most of the bioactive peptides isolated from marine sponges are cyclic and lipophilic. The chemists involved in the isolation of these bioactive peptides were using a specific bioassay method. It is likely that due to this reason the linear or more polar peptides are being missed during isolation. The most significant feature of sponge peptides is the presence of unusual amino acids, such as keto amino acids, and vinylogous amino acids in the molecules. Some peptides of blue-green algae and sponges share some common features in constituent amino acids. There remain some unanswered questions. For example, blue-green algae participate in the synthesis of peptides in sponges. Why are some peptides found in large quantities, while some are present in trace amounts? The answers to some of the questions may be arrived at when it will be possible to culture sponge cells or symbiotic microbes. Sponge peptides appear to have drug potential. Cyclotheonamides may serve as "Lead compounds"for development of antithrombin drugs. Discodermins are potential antitumor promoting agents and calyculins are useful biochemical reagents.

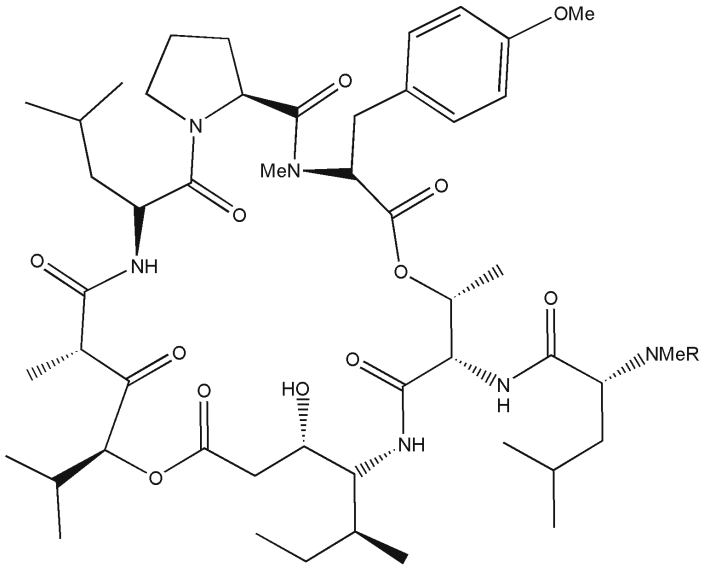
3.3 Tunicates

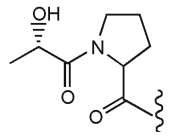
There are roughly 2,000 living species of tunicates[95-100] of which ascidians are the most abundant. Adult ascidians are sessile filter feeders, either solitary or colonial, and live preferentially in regions where there is freely flowing sea water. Tunicates (sea squirts) received relatively little attention from marine chemists. Over 50 secondary metabolites isolated so far from tunicates a number of them are peptides with significant biological activities. Peptides were first isolated in 1980 from *Lissoclinum patella*.[101] The peptides of tunicates have been reviewed.[16,102] Of the several peptides isolated from *L. patella* ulithiacyclamide[103-107] (**30**) were found to be most active. It exhibited *in vitro* anticancer activity against L 1210 (0.1 µg/mL), HeLa (0.1 µg/mL), and CEM (0.01 µg/mL) cell lines[103] and *in vivo* activity against the P 1534J murine leukaemia (TIC 188 at 1 mg/kg, repetitive doses). There are now 12 members of this cyclic peptides, all containing atleast one thiazole and usually an oxazoline amino acid. The oxazoline ring apparently plays an important role in the biological activity of ulithiacyclamide.

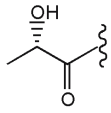


30

Tridemnum sp., a Caribbean tunicate, furnished didemnins A-C (**32**, **31**, **33**)[108-117] a new class of cyclic depsipeptides. Didemnin-B had been synthesized.[118] Didemnins exhibited impressive *in vitro* and *in vivo* anti-viral activity.[119] Didemnin-A and B inhibited *Herpes simplex* I and II at 1.0 µM and 0.05 µM concentrations, Rift Valley Fever virus at 1.37 and 0.04 µg/mL, Venezuelan *Equine encephalomyelitis* at 0.43 and 0.08 µg/mL, and yellow fever virus at 0.4 and 0.08 µg/mL, respectively. Mice infected



31, R = 

33, R = 

32, R = H

with Rift Valley Fever virus showed 90% survival when treated with didemnin-B (**31**) at 0.25 mg/kg. There were, however, some drug related deaths at this dose.[120] Didemin-B had *in vivo* anticancer activity against P 388 murine leukemia (TIC 199 at 1.0 mg/kg).[119] It was subsequently evaluated *in vitro* against human tumors in a stem cell assay.[121] Tumor cells from 8 of 17 patients showed sensitivity to didemnin-B with the median IC_{50} 4.2×10^{-3} μg/mL. Didemnin-B (**31**) has completed phase II human clinical trials against advanced adenocarcinoma of the kidney,[122] advanced epithelial ovarian cancer,[123] and metastatic breast cancer.[124] Unfortunately, it failed to show significant anticancer activity, but demonstrated significant toxicity. Didemnin-B had shown immuno-suppressant activity. In a Simonsen parental-to-F_1 graft versus-hot assay, didemnin showed 71% inhibition of splenomegally with repetitive doses of 0.3 mg/kg.

3.4 Ascidians

Peptides continued to be one of the major classes of the secondary metabolites from ascidians. Several new didemnins have been reported since the structures of didemnin-A (**32**), B (**31**) and C (**33**) were determined.[108,118] Several structures which were originally proposed on the basis of mass spectral data but were not confirmed. The structure of nordidemnin-B originally proposed on the

basis of GC-MS analysis of hydrolysate products was confirmed by synthesis and NMR spectral data of the compound completely assigned.[125,126] Didemnins Sand Y, which were new addition to the didemnin family of depsipeptides, contained three and four L-glutamine residues respectively, capped with a 3-hydroxydecanoyl unit as a new N-terminal blocking group. Conformational studies on didemnin-B and related analogs had been carried out.[127–129] Biological activities of didemnins varied considerably on acy substitution pattern.[130] For example, the cytotoxic activity was enhanced when the side chain was acetyl or longer. Acylating both the isostatin hydroxyl and the free N-methyl leucine amino group, or by bridging the amino groups of the N-methylleucine and the threonine moieties with methylene group, the cytotoxicity was reduced often without reduction of antiviral activity.[130]

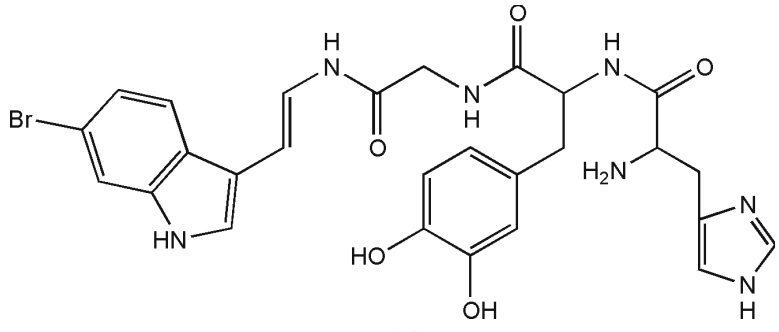
The genus *Lissoclinum* is a rich source of cyclic peptides. Two classes of cyclic peptides, the heptapeptide lissoclinamides and the octapeptide patellamides, ulithiacyclamides have been isolated from the tunicate *L. patella*.[131] Each of these classes of peptides have been characterized by the presence of thiazole and oxazoline amino acids. Recently, *L. patella* collected from different localities has furnished five new octapeptides lissoclinamides,[132–134] three new heptapeptides patellamides D and E[135] and ulithiacyclamide (**30**).[136] The amino acid sequence of all the lissoclinamides is the same. The difference lies in the oxidation states of the two sulphur containing rings and in the absolute stereochemistry of the amino acids. Most of the lissoclinamides exhibited only mild cytotoxicity. The exceptions are lissoclinamide which was cytotoxic against MRC54CVI and T24 cell lines with IC_{50} values of 0.04 μg/mL and lissoclinamide which was found active against the same cell lines with an IC_{50} values of 0.8 μg/mL. The other cyclic peptides isolated from collection of *Lissoclinum patella* were ulithiacyclamide-B and patellamides-D and E. Ulithiacyclamide-B incorporates a phenylalanine in place of the alanine found in ulithiacyclamide.[137] The highly cytotoxic ulithiacyclamide B showed no selective activity against solid tumor cell lines. An X-ray analysis showed that patellamide-D adopts a severely folded conformation with the two thiazole rings nearly parallel to each other.[29] This conformation which deviates drastically from the nearly square shape reported for the related peptide ascidiacyclamide,[138] is stabilized by four transannular N-H-O hydrogen bonds.

Recently *L. patella* collected in the Southern Philippines had yielded two new cyclic octapeptides tawicyclamide-A and B.[139] Tawicyclamides possess one thiazoline and two thiazole amino acids, but lacks oxazoline rings which were characteristic of all previously isolated *Lissoclinum* peptides. Twicyclamides and their dehydro analogs exhibited only weak cytotoxicity against human colon tumor cells (IC_{50} 30 μg/mL). *L. patella* collected in the Fiji islands has given a new class of cyclic peptides called patellins. The patellins include both hexapeptides and octapeptides, and are unique since they lack thiazole and oxazoline rings, instead contain a thiazoline ring and

two novel threonine amino acids which are modified as their dimethylallyl ethers.[140] Each peptide exits in solution as a mixture of conformers, complicating the interpretation of NMR spectral data. The structure determinations of patellins therefore, relied heavily on the evaluation of tandem mass spectral data.

Three new cyclic hexapeptides have been reported from the related ascidian *Lissoclinum bistratum*,[141–144] collected from the Great Barrier Reef, Australia. Bistratamide-A contains oxazoline and thiazoline rings. Bistratamide-B also contains a second thiazole ring. Bistratamides A and B showed only marginal cytotoxicity against the human MRC5CVI fibroblasts and T 24 bladder carcinoma cells with IC_{50} in the range of 50 and 100 μg/mL respectively. The ascidian *Cystodytes dellechiajei* was the source of three cyclic peptides[145] each of which was a symmetrical dimer of either L-Leu-L-Proc, L-Leu-L-Val, or L-Pro-L-Phe. The structure of these peptides had been determined by spectroscopic methods and confirmed by synthesis. These peptides were cytotoxic to L1210 leukemia with IC_{50} 0.5 μg/mL. Diazonamides-A and B[146–149] are perhaps the most unusual peptides isolated from ascidians. They were isolated from *Diazona chinensis* collected in the Philippines. These chlorinated peptides are highly unsaturated, and derived from 3,4,5-tri-substituted L-tyrosine, tryptophan substituted at 2 and 4 positions of the indole moiety and L-valine. Diazonamide-A was cytotoxic *in vitro* against the human colon cancer HCT 116 and B 16 murine melanoma cell lines with IC_{50} values less than 15 ng/mL.

The majority of ascidian secondary metabolites have been isolated by extraction of whole body. However, some investigators have concentrated on specific tissues or physiological fluids. Two novel linear tetrapeptides, halocyamines A (**34**) and B (**35**), were isolated from the morula blood cells and from the blood of the solitary ascidian *Halocynthia oretz*.[150,151] These peptides are composed of several amino acids which are unusual to ascidian secondary metabolites, including histidine-3,4-dihydroxyphenylalanine, and 6-bromo-8,9-didehydrotryptamine. The remaining amino acids of halocyamines-A (**34**) and B (**35**) were glycine and threonine, respectively.



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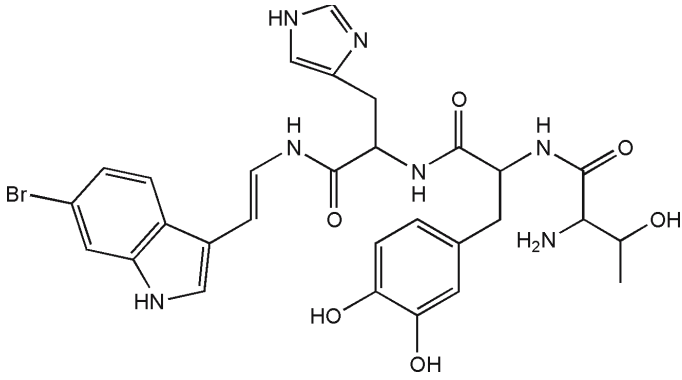


35

Halocyamines exhibited antiviral activity against fish RNA viruses in RTG2 cells. They also showed antimicrobial activity against several Gram-positive bacteria and yeasts. Halocyamines were also cytotoxic to some cultured mammalian cells. Ascidian morula cells have been implicated in immunological responses such as phagocytosis and lysis of foreign substances. It is suggested that halocyamines which are found only in the morula cells of *H. roretzi* might be playing a role in the defense of this organism.

Ascidians remain unique among the marine invertebrates as they overwhelmingly produce nitrogen containing metabolites. Although investigations on ascidians as potential source of drugs were initiated more recently than on some other marine invertebrates, it is significant that the first marine natural product to enter human clinical trials, is didemnin-B (**31**), an ascidian secondary metabolite. A survey of the biological activities of the secondary asidian metabolites reveals that cytotoxicity is the most frequently observed activity against a variety of tumor cell lines. Next is antimicrobial activity followed by antiviral and antiinflammatory activities. These results however, are highly biased, reflecting the selection of assay system by individual researchers. A more reliable data on bioactivity would require new metabolites to be tested for a wider array of biomedically important activities. Ascidians, like many of the other marine invertebrates, are known to exist in obligate and non-obligate symbiosis with microorganisms. The unicellular prokaryotic alga *Prochloron*[152,153] as well as other *Cyanophytes*[154] are commonly associated with ascidians. The exact nature of the symbiosis is unclear, the increased incidence of algal-tunicate symbiosis in low nutrient tropical waters tends to suggest that the algae may play a nutritional role in the host's survival.[155] The most interesting problem for natural product chemists is to know whether the symbionts contribute to the production of secondary metabolites. The isolation of biosynthetically unrelated secondary metabolites from a single ascidian organism may suggest different origins of each class of compounds. For example the lissoclinamides, patellamides and the patellazoles were obtained from *Lissoclinum patella,* an ascidian known to

harbor *Prochloron*. Another example is that of *Lissoclinum bistratum* which was the source of the cyclic peptide bistratamides and the polyether bistratenes (bistramides). The findings of Hawakins et al[141] reveal that bistratamides were localized in algal cells, while bistratenes were concentrated in tunicate tissue. These facts are consistent with different origins of these metabolites. The contribution of symbiotic alga to the biosynthesis of secondary metabolites of ascidian has not yet been elucidated because the symbiotic algae have been resistant to culture. However, there has been some success in the mariculture of ascidians. Evidence has been obtained that some secondary metabolites produced by ascidians provide a chemical defense.[156] Didemnin-B (**31**), nordidemnin-B and patellamide-C have been demonstrated to have feeding deterrent activities towards generalist fishes in the field. Experimental results are beginning to supplant the long standing speculation about the ecological functions of marine secondary metabolites. However, much research remains to be done. Ascidians will undoubtedly, continue to be the source of novel secondary metabolites. However, the lack of a secure source of large quantities of material will make the development of these compounds difficult as pharmaceutical products. A potential solution to this problem involves the study and culture and symbiotic microorganisms.

3.5 Coelenterates

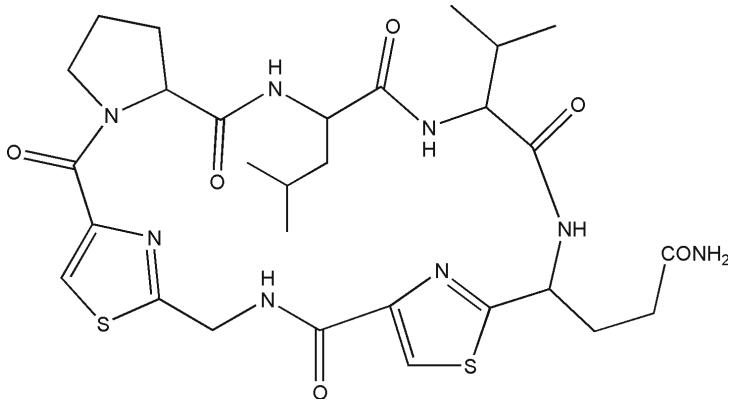
Soft corals, gorgonians, sea anemones, Portuguese man-of-war and jellyfish all come under coelenterates. Peptides most of which are venoms, have been isolated from these marine organisms. Coelenterate toxins can be divided into neuropeptides and neurotoxins. The first of these neuropeptides, a morphogenetic head activator, was isolated from a fresh water hydroid (*Hydra attenuata*). Subsequently, the sea anemone *Anthopleura elegantissima* was found to contain a substance which appeared identical by biological and chemical methods to the *Hydra* peptide. Twenty micrograms of the peptide was isolated from 200 kg of anemone and it was sequenced.[157] Structurally, the peptide was unusual as it lacked a free amino terminus since the terminal glutamate was cyclized to a pyroglutamyl ring. This sequence was confirmed by synthesis. The head activator is required to initiate head specific growth and to affect the corresponding cellular differentiation.

The second groups of neuropeptides from coelenterates were related to the molluscan neuropeptide RMF amide. These peptides are believed to be ubiquitous in coelenterates. The largest groups of peptides from coelenterates are the neurotoxins from sea anemones. These neurotoxins could be divided into two groups, viz long and short. The long group of neurotoxins has 45-50 amino acids residues. These are further subdivided into type I and II. Type I was isolated from *Anemonia sulcata*[158–160] and *Anthopleura xanthogrammica*.[161,162] These peptides, show a great deal of sequence homology. All long toxins of type-II were isolated from anemones of the Stichodactylid family.[161–166] These peptides also show a large degree of

sequence homology. However, between the two types of long peptides, there is only a small amount of sequence homology, primarily in the N-terminal region. The second category of sea anemone neurotoxins is the "short" peptides having about 30 amino acids residues. Despite the fact that there are three types of sea anemone toxins which share little sequence homology, all have the same mechanism of action. All alter the function of sodium channels in excitable membranes of muscles and nerves primarily by delaying the inactivation process. Because of this, the toxins are considered important probes of Na^+ channels and related physiological functions. These peptides have been studied extensively for their physiological function and mechanism of action.[167-172]

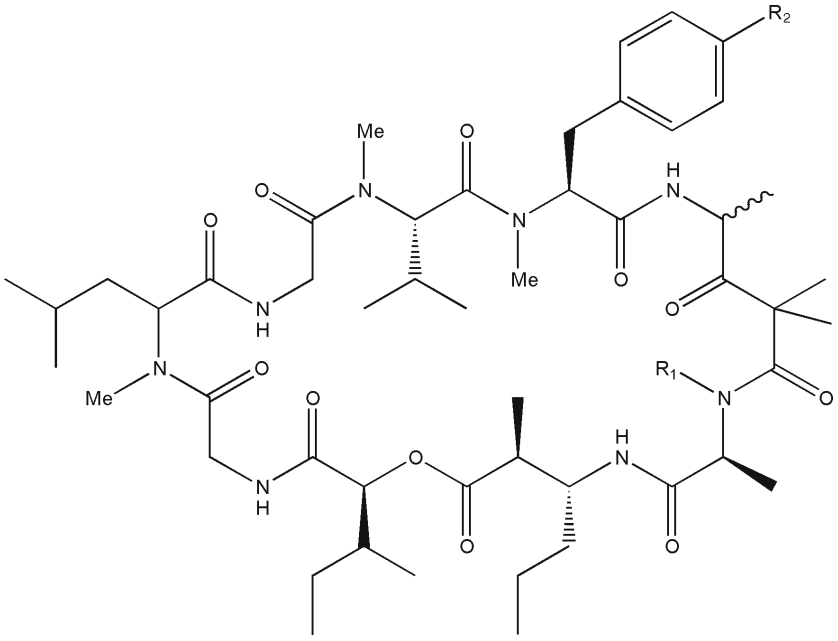
3.6 Molluscs

A large number of secondary metabolites derived from amino acids have been isolated from marine molluscs. Many of these are venoms,[173] while others have been implicated in the regulation of sexual and digestive functions.[174,175] With a few exceptions, these can be classified as unmodified linear peptides comprised of (*S*)-amino acids. The Indian Ocean sea hare *Dolabella auricularia* has yielded the cyclic pepides dolastatins.[176-184] Dolastatins are powerful cell growth inhibitors of the P388 lymphocytic leukemia cell line with dolastatin-3 (**36**)[185] having ED_{50} 1×10^4 to 1×10^7. The structure for dolastatin-3 was proposed largely from NMR studies and acid hydrolysis. Electron impact mass spectrometry determined the amino acid sequence. However, this structure was not confirmed by synthesis.[185] Dolastatin-10, which contained a number of unusual amino acids including a modified proline and O-methylisostatine, was claimed be the most potent antineoplastic agent known (176%). Curative response to NCI human melanoma xenograph in mouse at 3.25 μg/kg; T/C 238 at 11 μg/kg against B16 melanoma and 202 at 4 μg/kg against P388.[186]



36

Janolusimide[187] isolated from *Janolus cristatus* is the only peptide known from a nudibranch. The peptide appears to be derived by condensation of N-methyl-(*S*)-alanine, 4-amino-3-hydroxy-2-methyl valeic acid, valine and isobutyric acid. Janolusimide was toxic to mice at 5 mg/kg (ip.) apparently acting as a cholinergic agent. At lower doses its action was antagonized by atropine. The Japanese sea hare, *Dolabella auriculata* has yielded a new depsipeptide, dolastatin 11 (**37**), and dolastatin 12 (**38**)[188,189] exhibiting weak cytotoxicity. The absolute stereochemistry of (**38**) was elucidated by spectroscopic analysis and chemical degradation and confirmed by synthesis.



37, R_1 = H, R_2 = OMe
38, R_1 = Me, R_2 = H

Opioid type peptides have been reported from molluscs. Met and Leu-enkephalin and Met-enkephalin-Arg-Phe were isolated from neural tissue of the mussel *Mytilus edulis*.[190] FMRF amide (Phe-Met-Arg-Phe-$NH_{2)}$ and a number of related peptides were originally isolated from cerebral, pedal and visceral ganglia of the venus clam *Macrocallista nimbosa*.[191] Subsequently, several analogs of MRF amide including a family of heptapeptides had been reported from molluscs.[192] A wide range of activities on cardiac and noncardiac muscle tissue from several mollusc species had been reported.[193] Much of the work in this area concerns the pharmacology rather than the chemistry.

4. Cone Snail Venoms

Cone snails are venomous predators. Their venoms contain a large number of small conformationally constrained peptides each with highly potent and

specific biological activity. Most of the peptides have 10-30 amino acid residues. The number of peptides in each venom is unprecedented both in the great variety of sequence and pharmacological spectrum. The *Conus* peptides are being used for a wide variety of physiological and pharmacological investigation in both vertebrate and invertebrate nervous systems. Some *Conus* peptides, such as the conotoxin have become well established neurobiological tools. *Conus* peptides have been used in several studies as probes for their receptor targets since a number of features make them attractive as probes. The chemistry and biochemistry[15,194] and mechanisms of pharmacological action[195] of *Conus* peptides have been reviewed.

5. Sea Urchins

Several peptides have been isolated from sea urchins egg jelly. Their structures and activities were specific for sea urchin spermatozoa at the ordinal level. These peptides have been given a general name, sperm-activating peptides (SAP). The chemistry and function of SAP have been reviewed.[196201] Speract (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Val-Gly) and ten of its derivatives obtained from the egg jelly of sea urchins, order Echinoida, stimulated the respiration and motility of spermatozoa of sea urchins belonging to the same order. Resact (Cys- Val- Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu NH_2) and mosact (Asp-Ser-Asp-Ser-Ala-Glu-Asn-Leu-Iie-Gly) and two of its derivatives obtained from *Arbacia punctulata* eggs, and *Cypeaster japonica* eggs, stimulated the metabolism and motility of respective sea urchin spermatozoa. Two other peptides (Gly-(X)-Pro-(X)-Gly-Gl-Als-Val- and Gly-Cys-Pro-Trp-Gly-Gly-Ala-Val-Cys) were isolated from the egg jelly of the sea urchin *Diadema setosum.* These are specific for *Diadema setosum* spermatozoa. Biochemical responses of the spermatozoa to the egg jelly peptide include at a net hydrogen ion efflux and elevation of cAMP and cGMP concentation.

The first sperm-activating peptides (SAP) were isolated from the egg jelly of the sea urchin *Hemicentrotus pulcherrimus* by sequential chromatography on Sephadex G-25, DEAE-Sephadex A-25, Sephadex G-15 and Avicel thin layer plate.[202] The peptides isolated were (H-1: Gly-Phe-Asp-eu-Thr-Gly-GlyGly-Val-Gly and H-2: Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly). Both the peptides were quite potent in stimulating *H. pulcherrimus* sperm respiration that had been lowered under acidic condition (pH 6.8). The higher concentration of the peptides did not increase the rate of respiration. About 20 liters of soluble egg jelly from 5,000 female sea urchins gave 15 mg of pure peptides (7 mg H-1 and 8 mg H-2). The peptides H-1 and H-2 were named (Thr^5)-speract and speract respectively.[203] The isolation procedure was subsequently modified and sequential HPLC on reverse-phase column was used to purify the peptides.[204] Using the modified purification method eleven sperm activating peptides from four other species of sea urchins (*Lytechinus pictus, Pseudocentrous depressus, Strongylocentrotus purpuratus* and *Anthocidaris*

crassipina) of the order Echinoida were isolated.[205-207] Although speract and its derivatives are effective in stimulating the respiration of *Hemicentrotus pulcherrimus* spermatozoa as well as sperm respiration of sea urchins in the order Echinoida, they did not stimulate sperm respiration of sea urchins in different taxonomic order.[208] This suggested that sperm activating peptides were species specific at the ordinal level in the respiratory stimulation of sea urchin spermatozoa. Resact[209,210] respiration activating peptide was isolated from the egg jelly of *Arbacia punctulata*.[209] From the egg of fifty female *Glyptocidaris crenularis,*[211] six sperm-activating peptides were isolated. The peptide, Lys-Leu-Cys-Pro-Gly-Gly-Asn-Cys-Val, was obtained in the largest amount (240 nM) from the egg jelly and named alloresact. The other five peptides were named as alloresact derivatives.[211] Three structurally similar sperm-activating peptides were obtained from the egg jelly of the sea urchin *Clypeaster japonicus*,[212] order Clypeasteroida. These peptides stimulated *C. japonicus* sperm respiration at one half of the maximum rate at about 0.5 nM. The peptides, speract, resact and alloresact did not cross react. The peptide Asp-Ser-Asp-Ser-Ala-Gln-Asn-Leu-Ile-Glyu from *C. japonicus* was named mosact which stands for motility activating peptide and the two other peptides were called mosact derivatives.[204]

Sperm-activating peptides and their analogs had been synthesized by the liquid phase method.[212] Although decreased respiration rates of sea urchin spermatozoa caused by acidification of sea water can be reversed by the addition of sperm-activating peptides, the stimulated respiration rate did not exceed that of spermatozoa in normal sea water. The respiratory stimulation induced by the peptide[213] was dependent on the concentration of external Na^+. Further, the peptide, caused substantial increase in both cAMP and cGMP within a few seconds.[213] Chemotaxis of animal spermatozoa to eggs or secretions of the female reproductive system is a wide spread phenomenon. It has been suggested that the sperm chemo attractants of certain echinoderms and hydroids might be peptides.[214] When 10 nM of the peptide resact was delivered into the *Arbacia punctulata* sperm suspension, many spermatozoa appeared to be forming a cluster in the area of the injected material.[215] The effects of resact on the clustering of spermatozoa were dose dependant. The clustering required millimolar concentrations of Ca^{2+}. Further, the chemo attracting property of resact was species specific. Resact is the first egg derived molecule of known structure shown to be a chemo attractant of animal spermatozoa. Sea urchin egg jelly consists of two major macromolecular components and several oligopeptides. These components have important roles for sperm-egg interaction during fertilization. The experiments conducted suggested that the peptides in the egg jelly play important roles in the process of normal fertilization of sea urchins. Most animal eggs have more or less an extra cellular matrix, like the egg jelly of sea urchins. It is suggested[196] that the matrix of other animal eggs, including that of mammalian eggs, may have similar peptides or substances whose function is similar to that of these

peptides. Over 20 peptides have been isolated from nine species of sea urchins distributed over four taxonomic orders. Their structures and activities were specific for sea urchin spermatozoa at ordinal level. It is expected that more species specific peptides will be isolated from the egg jelly of sea urchins belonging to different orders.

6. Marine Worms

A few peptides have been isolated from marine worms. Two classes of polypeptides were obtained from the mucus secretion of the skin of the heteronemertine worm *Cerebratulus lacteus*.[216,217] Of these, toxin A-III was a basic polypeptide composed of 95 amino acids residues with disulfide cross linkages between residues 17 and 38, 23 and 48 and 48 and 61. The toxin caused rapid lysis of human erythrocytes, Ehrlich Ascites cells and cardiac muscle cells at concentration 1.1 to 3.1 g/mL. The smaller toxins B-II and B-IV were neurotoxins which act specifically on the axons of crustaceans by delaying inactivation of Na^+ channels.[218-220] There was a high degree of homology between toxin B-II and B-IV. However, neither was significantly homologous with scorpion or sea anemone neurotoxins which displayed similar activity.

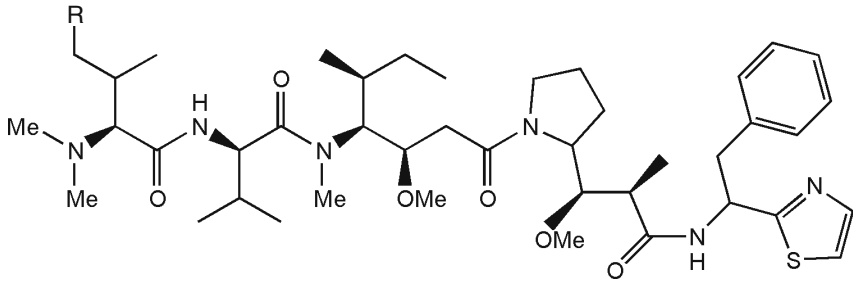
7. Marine Vertebrates

Soles (flat fish) of the genus *Pardachirus* are known to secrete an ichthyotoxic material from glands lining the dorsal and fins.[221] This secretion from *P. marmoratus* (Moses sole) was shown to have shark-repellant activity.[222] Three toxic peptides pardaxins P-1, P-2 and P-3[223226] had been isolated from *P. pavoninus.* Both pardaxin P-1 and P-2 caused death in *Oryzias latipes* (killifish) in 30 min at a concentration of 25 µg/mL. Pardaxins also exhibited pharmacological activities similar to the bee Venom melittin.[227] Tropical marine fish from several families secrete toxic peptides from specialized glands in the skin. The peptides known as gramistins appear to be universal in the secretion of the soap fishes.[228,229] Similar compounds had also been isolated from species of *Gobiodon* and the clingfish *Diademichthys lineatus*.[230,231] All these substances had shown both ichthyotoxic and hemolytic activities and impart a bitter taste to the mucus of the skin.

8. Marine Peptides and Related Compounds in Clinical Trials

8.1 Dolastatin-10 (39)

The tubulin interactive agent dolastatins-10 (**39**) entered into Phase I clinical trials in early 1990s.[232235] Since the natural abundance of dolastatin was very low, a total synthesis of this compound was accomplished. Later this compound entered in to Phase II clinical trials and tolerated at the doses



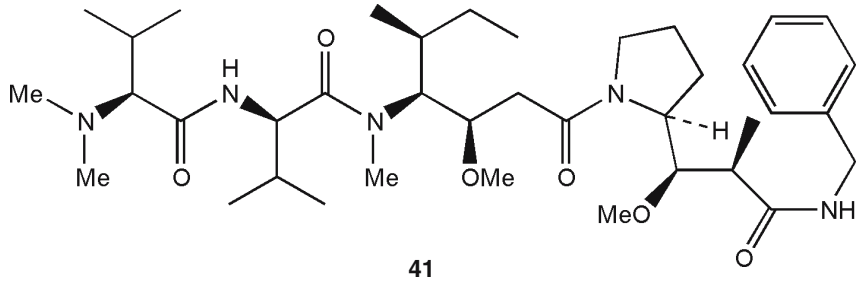
39, R = H
40, R = Me

used. Unfortunately it did not show significant antitumor activity against prostate cancer and metastatic melanoma in man[236,237] in the Phase II clinical trial. Very recently dolastatin-10 was isolated directly from another marine cyanobacterium that was known to be grazed on by *D. auriicularia*.[238] Some of dolastatins derivatives are still in clinical and preclinical trials.

Simplostatin-1 (**40**) which differs from dolastatin-10 by the addition of a methyl group on the first N-dimethylated amino acid was isolated from the marine cyanobacterium *Simploca hynoides*.[239] Biological activities of symplostatin-1 was found to be similar to dolastatin-10 but to be somewhat more toxic to mice at comparable doses.[239]

8.2 Soblidotin (TZT-1027, Auristatin PE)

Due to its potent anticancer activity a large number of dolastatins derivatives have been synthesized. One of the compound TZT-1027 (Auristatin PE or Soblidotin, **41**) is in Phase I clinical trials in Europe, Japan, and the United States.[240,241] Recent study indicated that in nude mice the transfected vascular endothelial growth factor (VEGF)-secreting human lung cell line SBC-3/VEGF and the mock transfected cell line were totally inhibited at early or advanced stage xenografts at levels of 1 or 2 $mg.kg^{\pm}$. The interesting part apart from these findings was that TZT-1027 also exhibited a potent antivascular effect at these concentrations.[242] Phase II trial is currently recruiting patients. In this study effect of soblidotin in patients having advanced or metastatic soft tissue sarcoma, including rhabdomyosarcoma is being studied. Patients receive soblidotin IV over 1 hour on days 1 and 8. Treatment repeats every



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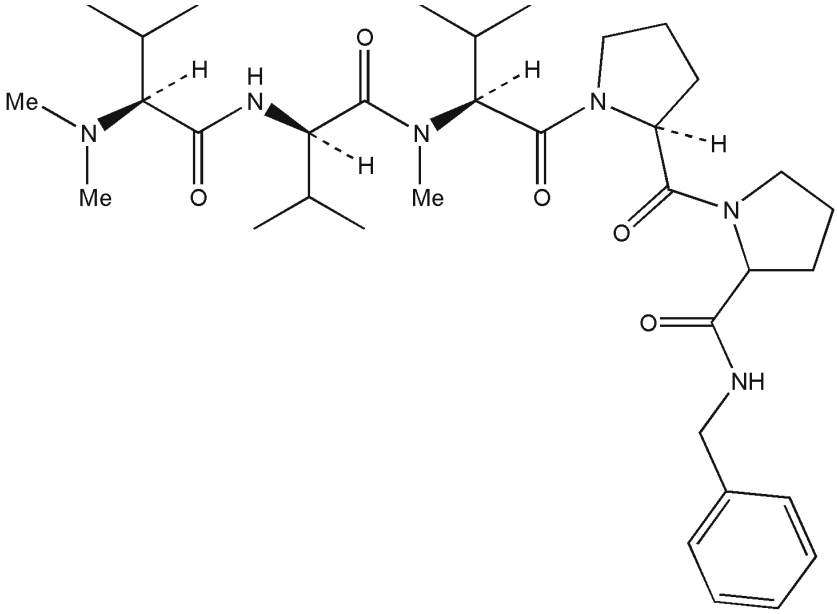
3 weeks for at least two courses in the absence of disease progression or unacceptable toxicity. A total of 27 patients will be accrued for this study. Patients 15 years of age and above are eligible. This study is being conducted at centers in Alabama, New York, and Texas.[242]

8.3 Cematodin (LU-103793)

Cematodin (**42**, LU-103793) another derivative of dolastatin 15 entered into Phase I clinical trials for the treatment of breast and other cancers. The Phase I level data were found to be encouraging, and this compound have been recommended for Phase II studies against malignant melanoma, metastatic breast cancer, and non-small-cell lung cancer.[243250] Amador et al[251] report Phase II trials still ongoing as in 2003 in breast, ovarian, lung, prostate, and colon carcinomas, but currently (01/2004) it is listed as discontinued in the Prous Ensemble database.



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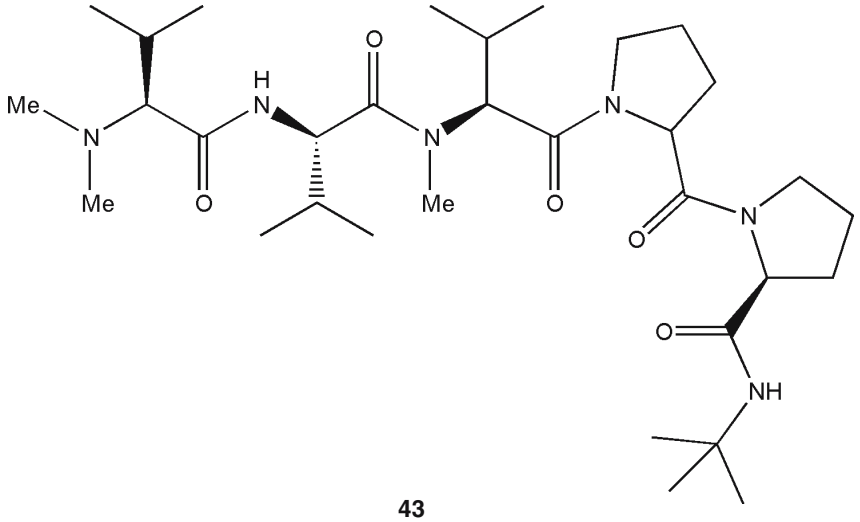
8.4 Synthadotin

Synthadotin [ILEX 651, (**43**)] is an orally active synthetic pentapeptide analog of the natural substance dolastatin 15 and has a unique mechanism of action targeting tubulin.[252255] ILEX-651 has acquired exclusive worldwide rights to ILEX-651 in 2000 from BASF Pharma in Germany.[256] Ilex Oncology has initiated a Phase II study on the effect its anticancer compound ILX-651 has on metastatic melanoma. ILEX-651 has been chemically modified to provide improved pharmacologic properties and is orally bioavailable with a potentially enhanced therapeutic window. The Phase II study are going on to examine the efficacy and tolerability of ILEX-651 in patients with recurrent or metastatic



43

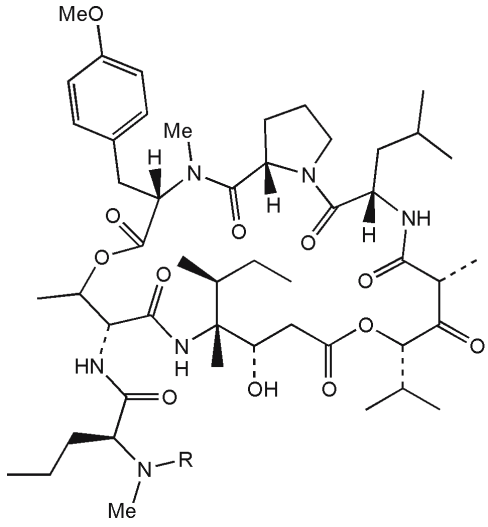
melanoma. Preliminary results from an earlier study show that the agent was active in a range of solid tumors.[257]

8.5 Applidine (44)

Dehydrodidemnin-B (aplidine, **44**) was first reported in 1990 in a patent application.[258] The antitumor property of aplidine was first reported in 1996 by Pharma Mar.[259,260] Total synthesis of this compound was achieved in 2000.[261] The compound with generic name "aplidine or dehydrodidemnin-B" and trade name of Aplidin, was placed into Phase I clinical trials in 1999 for the treatment of solid tumors and non-Hodgkin's lymphoma. Recently this compound entered into Phase II clinical trials. Phase II trials are going on in Europe and Canada covering renal, head and neck, and medullary thyroid. The mode of action of this novel molecule is not yet known, but it appears to block VEGF secretion and blocks the corresponding VEGF-VEGF-Receptor-1 autocrine loop in leukemic cells.[262] Didemnin-B (**45**) which is a close analogue of (**44**), was isolated from *Tridemnum solidum*, and this compound exhibited potent antiviral activity, and cytotoxicity against P388 and L1210 murine leukemia cell lines. Didemnin-B was advanced into preclinical and clinical trials. This compound turns out to be very toxic, and trials were terminated recently.[263]

8.6 Kahalalide F (46)

The cyclic depsipeptide kahalalide-F (**46**) was isolated from the Sacoglossan mollusk *Elysia rufescens.* After isolation and characterization, it was found that this depsipeptide also occurs in the alga. From 216 g of the animal, 2.1 g of kahalalide-F (**46**) was isolated, while 5 mg was isolated from 3 kg of the alga collected at the same site.[264266] University of Hawaii licensed this compound to Pharma Mar in the 1990s, and later it entered into preclinical



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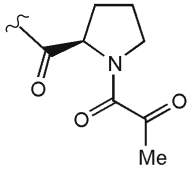
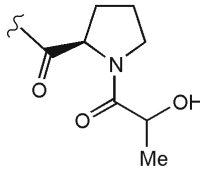
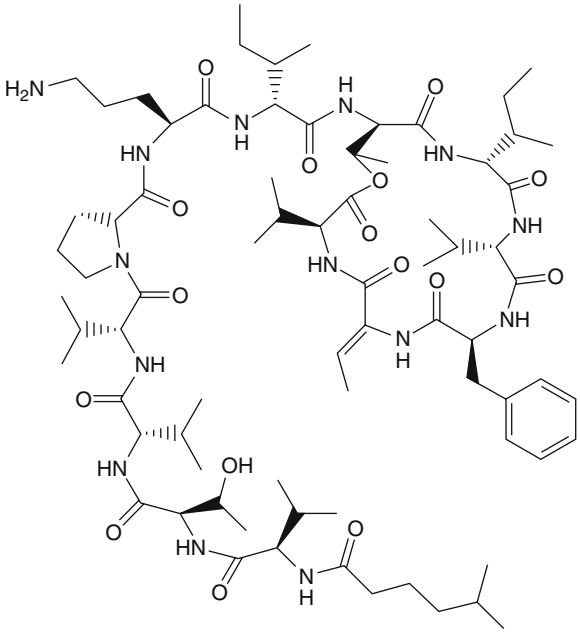


45, R =



trials. Actual mode of action of this compound had not yet been fully determined, but it was known to target lysosomes[267] thus it was suggested that it may have selectivity for tumor cells such as prostate tumors which have high lysosomal activity. The compound was synthesized by solid phase peptide techniques[268] and found to be active for the treatment of androgen-

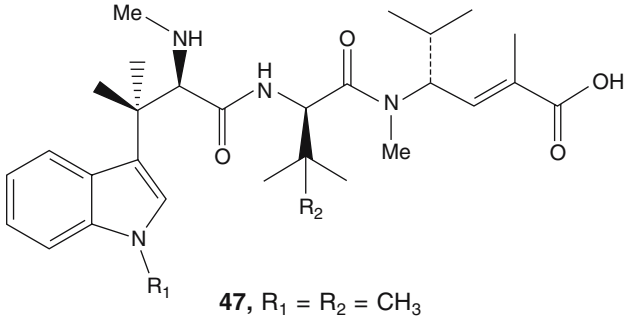


46

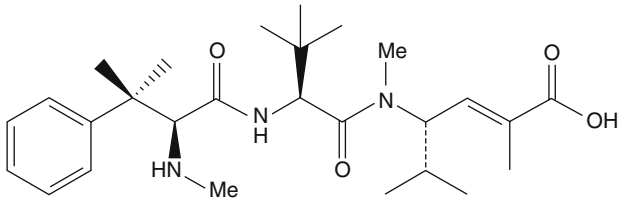
independent prostate cancer. Scheuer et al in 1999,[269] raised the questions about the stereochemistries given in the original structure by Hamann. A later stereochemical assignment of the natural product was reinvestigated and it was observed that Valine 3 should be D-Val and Valine 4 should be L-Val, rather than the reverse.[270] Currently this compound is in the Phase II clinical trials for the treatment of prostate cancers.[271] Recently, Munoz et al[272] demonstrated that kahalalide-F induces cell death via ŏncosis'þossibly initiated by lysosomal membrane depolarization in both prostate and breast cancer cell lines.

8.7 Hemiasterlin (HTI-286)

Hemiasterlin (**47**) was originally isolated from the South African sponge *Hemiasterella minor*,[273] an organism that has been a source of geodiamolide TA and jaspamide. Later a group of cytotoxic peptides were isolated from a Papua New Guinea sponge. This sponge produced a large number of peptides, including geodiamolides A-F, hemiasterlins-A (**48**) and B (**49**), and other criamides and geodiamolides.[274] Hemiasterlin and the A and B derivatives were found to interact with tubulin and gives microtubule depolymerization in a identical manner to that reported for vinblastine and nocodazole. Recently hemiasterlin, and its derivatives have been synthesized.[275–277] Andersen's group[276] licensed the hemiasterlins and analogues including HTI-286 (**50**) to Wyeth for development as part of the NCNPDDG. This compound is currently in Phase I clinical trials and scheduled to enter Phase II shortly. The *in vitro*



47, $R_1 = R_2 = CH_3$
48, $R_1 = H$, $R_2 = CH_3$
49, $R_1 = R_2 = H$



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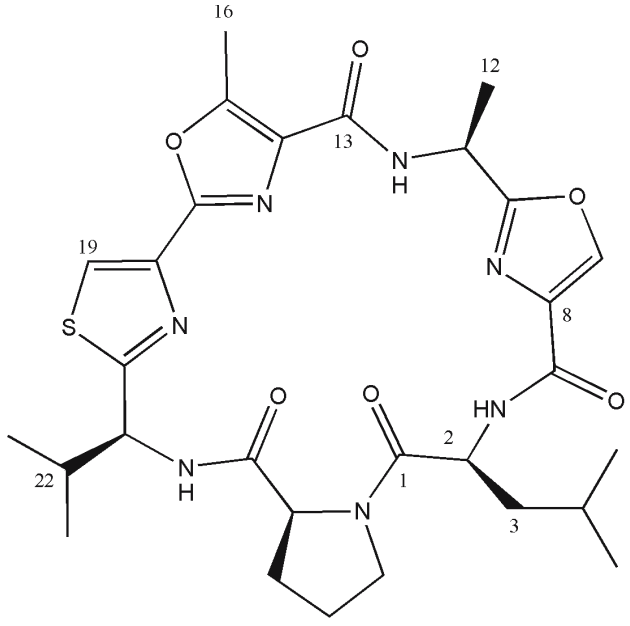
and *in vivo* animal data were recently published.[278] Very recently interesting data on HTI286-dolastatin-10 hybrids[279,280] have been published and the hybrids were found to be much more active than dolastatin-10 in cells that express the P-glycoprotein efflux pump.

9. Miscellenous Peptides

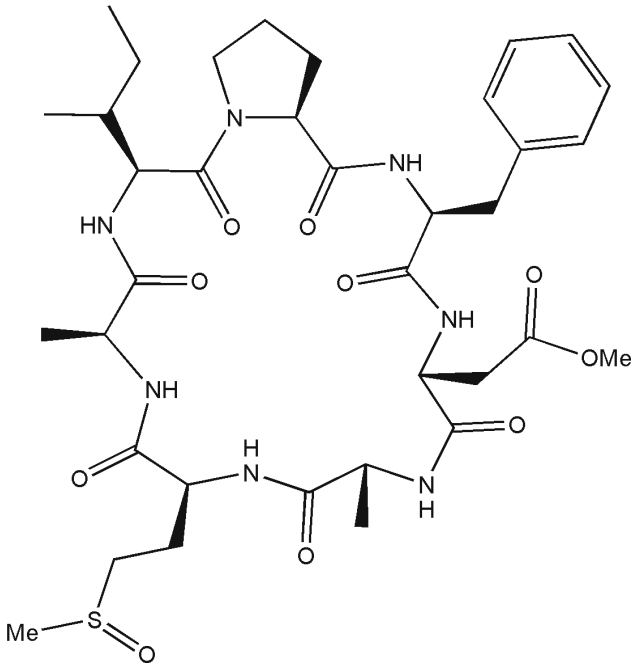
A bioactive cyclic heptapeptide, leucamide-A (**51**),[281] was isolated together with the known compound BRS1,[282] from the dichloromethane extract of the Australian marine sponge *Leucetta microraphis*. The compound was unique in the sense that it contains mixed 4,2-bisheterocycle tandem pair of a methyloxazole and thiazole subunit. The CH_2Cl_2 extract of the freeze-dried sponge tissue (67.7 g) gave 0.9 g (1.33%) of green extract, which on fractionation by vacuum liquid chromatography (VLC) over silica gel using gradient elution with nonpolar to polar solvents yielded five fractions. Fraction four was further fractionated by VLC over Polygoprep 60–50, C18 and subsquent reversed-phase (RP) HPLC separation of the second fraction (column: Knauer C_8 Eurospher-100, 250 × 8 mm, 5 µm; MeOH/H_2O (8:2), 2.0 mL/min) yielded semipure compound (**51**). Final purification was achieved by RP HPLC (column: Phenomenex Max C_{12}, 250 × 4.6 mm, 5 µm; MeOH/H_2O (8:2), 0.8 mL/min) to afford 7.2 mg of (**51**) as a white amorphous solid. The planar structure of leucamide-A (**51**) was determined by spectroscopic techniques such as NMR, MS, UV, and IR. The absolute stereochemistry of leucamide-A (**51**) was established by chemical degradation, derivatization,



51

and chiral GC-MS analysis. Leucamide-A (**51**) was found to be moderately cytotoxic toward several tumor cell lines. Total synthesis of leucamide-A (**51**) was achieved recently.[283]

Marine porifera genus *Phakellia* was found to be a rich source of anticancer cyclic peptides. The investigations employing *Phakellia carteri* from Republic of the Comoros and *Phakellia* sp. from Chuuk in the Federated States of Micronesia led to the isolation and structural elucidation of several bioactive cyclopeptides commonly known as phakellistatins 1-12.[284291] Very recently a new compound phakellistatin-14 (**52**) is isolated from *Phakellia* sp. in 8.8×10^{-7} % yield. The structure of phakellistatin-14 (**52**), cyclo-Phe-β-OMe-Asp-Ala-Met-(SO)-Ala-Ile-Pro, was determined by 1D and 2D NMR spectral data supported by HRFABMS. The chirality of each amino acid unit was determined to be *S* using chiral HPLC methods. Phakellistatin-14 (**52**) was found to be very active against murine P388 lymphocytic leukemia (ED_{50} 0.0042 μg/mL).[292]



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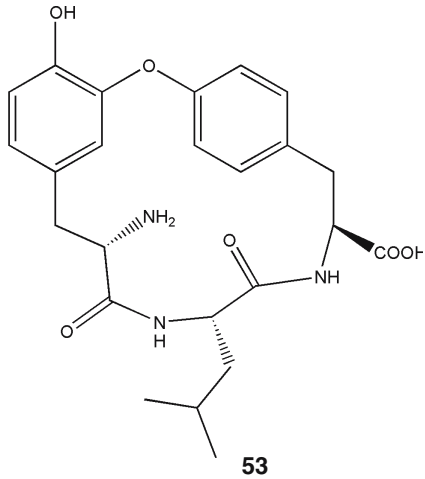
Cyclic tripeptide renieramide (**53**)[293294] was isolated from the polar extract of the Vanuatu sponge *Reniera* sp. The preliminary biological tests showed immunomodulating activity in this marine cyclic tripeptide. This compound has not been reported previously as natural product, but was found to be identical to a synthetic cyclic peptides of the OF4949 family of anticancer

agents.[295] Renieramide (**53**) possesses a 17-membered cyclic side chain linked biphenyl ether skeleton, typical of the class that includes the natural products OF4949 I-IV, K13, and eurypamides. The complete structural elucidation of renieramide (**53**) was acheived by 1D and 2D NMR, ESIMS, and MS data, along with chemical analysis. Stereoselective synthesis of renieramide (**53**) has been reported.[296] New cyclic depsipeptide ulongapeptin (**54**),[297,298] was isolated from a Palauan marine cyanobacterium *Lyngbya* sponge in 0.10% yield. The structure was elucidated through one-dimensional TOCSY experiments and other spectroscopic techniques. The absolute and relative stereochemistry of the β-amino acid, 3-amino-2-methyl-7-octynoic acid (AMO), in (**54**) was determined by synthesis of the saturated R-alkyl-β-amino acid. Ulongapeptin (**54**) was cytotoxic against KB cells at an IC50 value of 0.63 μM.



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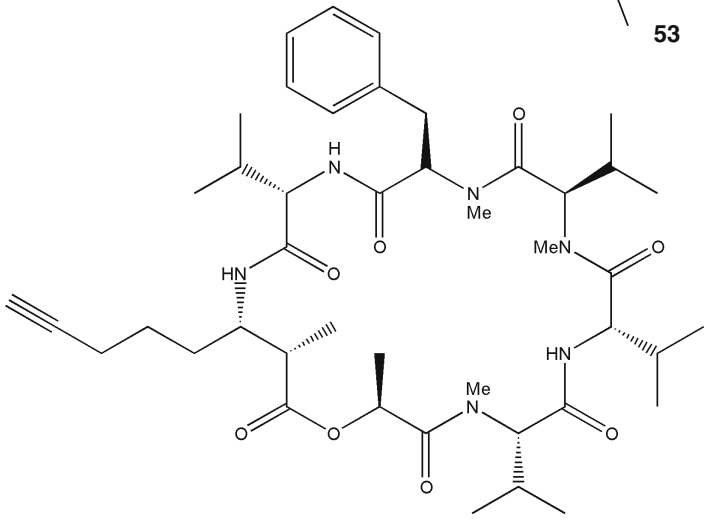
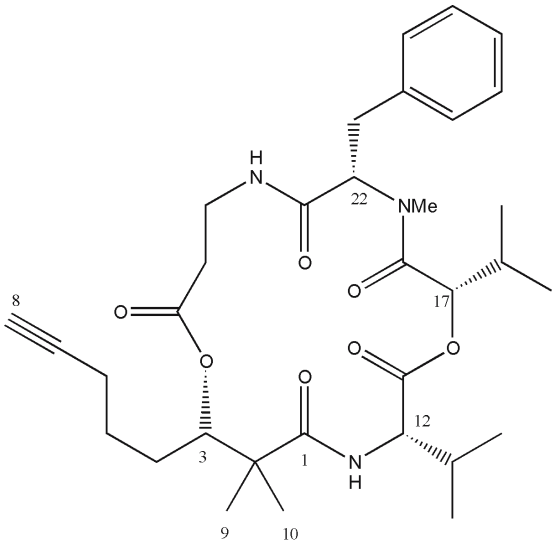
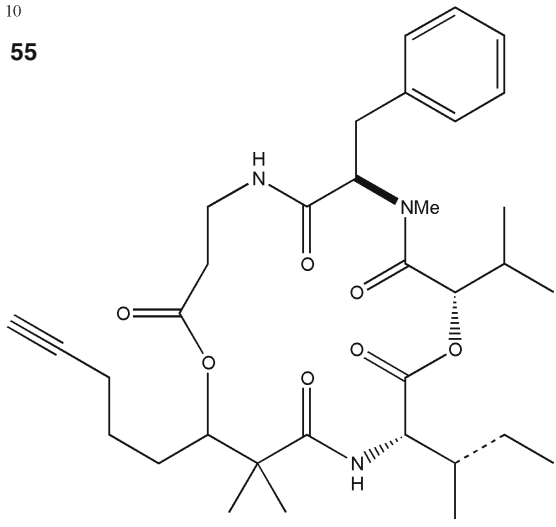


54

Yanucamides-A (**55**) and B (**56**) were isolated from the lipid extract of a *Lyngbya majuscula* and *Schizothrix* sp. collected at Yanuca island, Fiji.[299] The structures of these compounds (**55**) and (**56**) were established by detailed spectroscopic experiments. Common feature of these compounds is the presence of 2,2-dimethyl-3-hydroxy-7-octynoic acid unit. This unit is present only in kulolide-1 and kulokainalide-1, metabolites from the marine mollusk *Philinopsis speciosa*.[300,301] Isolation of the yanucamides proves the fact that the kulolides and related metabolites are of cyanobacterial origin.[302] Both yanucamides-A (**55**) and B (**56**) exhibited strong brine shrimp toxicity (LD50, 5 ppm). The stereochemistry at C-3 in yanucamides-A (**55**) was determined configuration (*S*) of 2,2-dimethyl-3-hydroxyoctynoic acid (Dhoya) group,

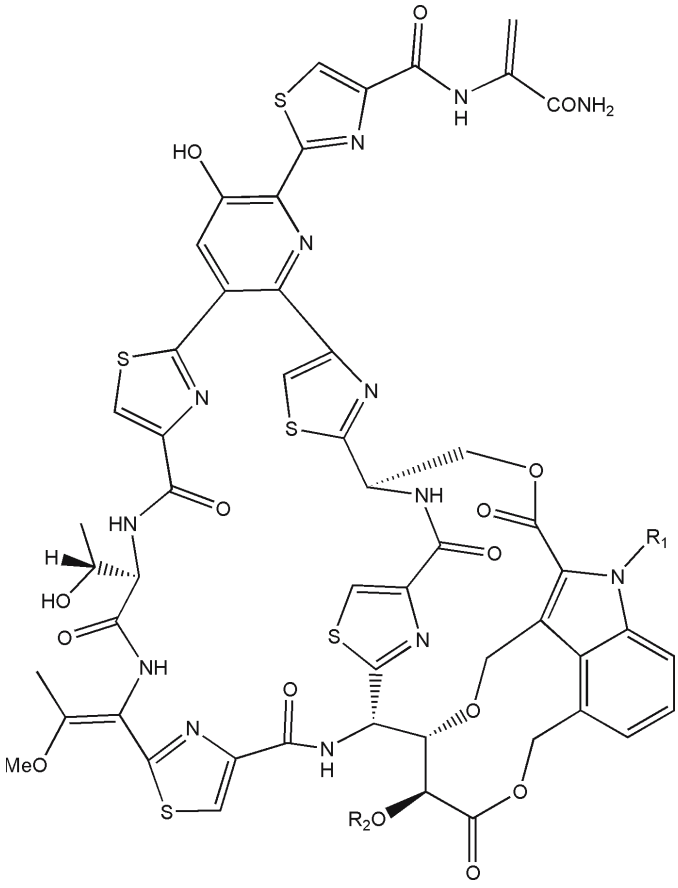


55



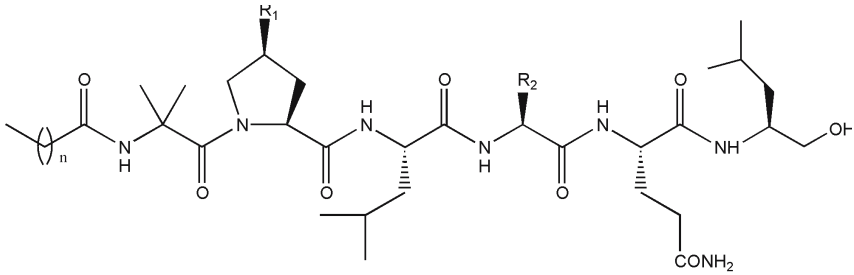
56

which is present in the natural products kulolide-1 and kulokainalide-1. The stereochemistry of yanucamides-A (**55**) has been recently revised, and total synthesis of this novel peptide was achieved.[303] The stereochemistry at positions 3, 12, 17 and 22 was revised, and finally, positions 3*S*, 12*S*, 17*S*, 22*S* were assigned for the natural product.[303] The thiazolyl peptide antibiotics, nocathiacins I–III (**57**-**59**), have been isolated from the culture broth of *Nocardia* sp.[304–309] The nocathiacins exhibit potent *in vitro* activity against

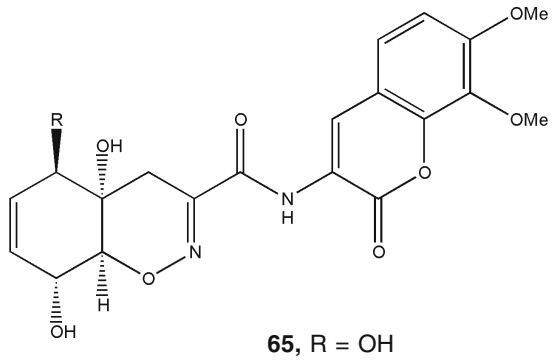


a wide range of bacteria, including several multiple-drug resistant pathogens and also exhibit excellent *in vivo* efficacy in a systemic *Staphylococcus aureus* infection mouse model.[307] Total synthesis of nocathiacins I (**57**) has been reported recently.[310]

The halovirs AE (**60**-**64**), lipophilic linear peptides, have been isolated from a *Scytalidium* sp. sourced from the Caribbean sea grass *Halodule wrightii.*[311] These compounds exhibit potent *in vitro* inhibitory activity against *Herpes simplex viruses* 1 and 2. The structures of these new peptides were determined by a combination of chemical and spectral techniques. Peculiar structural features of the halovir include the presence of a nitrogen terminus acylated by myristic (C14) or lauric (C12) acid, an unusual Aib-Hyp dipeptide segment, and a carboxyl terminus reduced to a primary alcohol. Synthesis of halovirs have been achieved recently.[312] Modified dipeptides, trichodermamides-A (**65**) and B (**66**), were isolated from cultures of *Trichoderma virens* isolated from the ascidian *Didemnum molle* and from the surface of a green alga of the genus *Halimeda*, both collected in Papua New Guinea. Trichodermamide-A (**65**) with traces of trichodermamide-B (**66**) was isolated from the ascidian-derived culture while a greater quantity



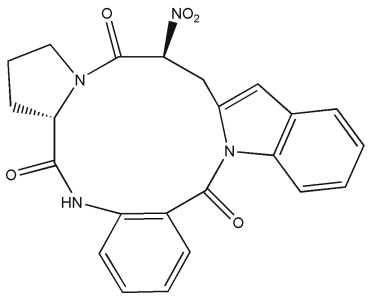
60, R_1 = OH, R_2 = $CHMe_2$, n = 12
61, R_1 = OH, R_2 = Me, n = 12
62, R_1 = H, R_2 = $CHMe_2$, n = 12
63, R_1 = OH, R_2 = $CHMe_2$, n = 10
64, R_1 = H, R_2 = $CHMe_2$, n = 10



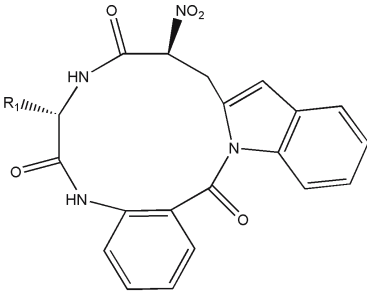
65, R = OH
66, R = Cl

of trichodermamide-B (**66**) was isolated from the algal derived strain. The trichodermamides possess a rare cyclic O-alkyl-oxime functionality incorporated into a six-membered ring. The structure of (**65**) was assigned by X-ray diffraction analysis while the absolute stereochemistry was determined using the modified Mosher method. Trichodermamide B exhibited potent *in vitro* cytotoxicity against HCT-116 cell lines, moderate antimicrobial activity against amphoterocin-resistant *C. albicans*, MRSA and vancomycin-resistant *E. faecium*.[313] Trichodermamide A is closely related to a natural product penicillazine, isolated from a marine-derived *Penicillium* sp.[314]

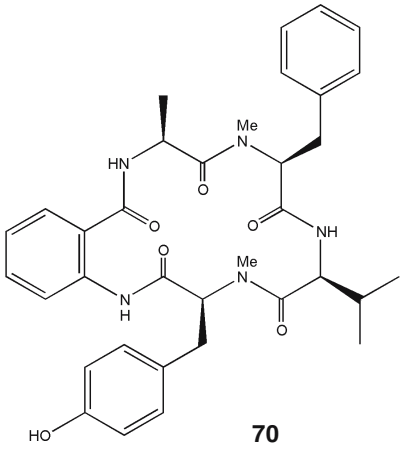
Psychrophilin A-C (**67-69**) and cycloaspeptide-D (**70**),[315,316] fungal metabolites, along with the known cycloaspeptide-A[317] were isolated from the psychrotolerant fungus *Penicillium ribeum*. These compounds were isolated by using high-speed countercurrent chromatography (HSCCC)[318] and preparative HPLC. The structures were determined by 1D and 2D NMR techniques, HREIMS, tandem mass spectrometry (ESMS/MS), and X-ray crystallography. The L configuration of all chiral centers in psychrophilin-A (**67**) and cycloaspeptide-D (**70**) were confirmed by Marfey's method. Psychrophilin-A (**67**) is the first natural cyclic peptide containing a nitro group instead of an amino group.



67

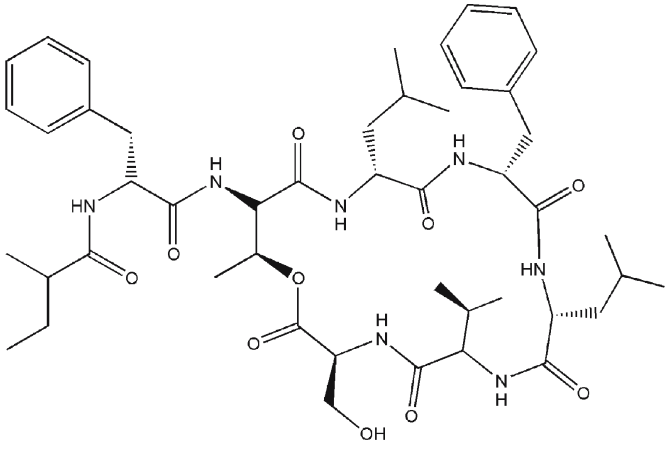


68, R = $CH(CH_3)_2$
69, R = CH_3

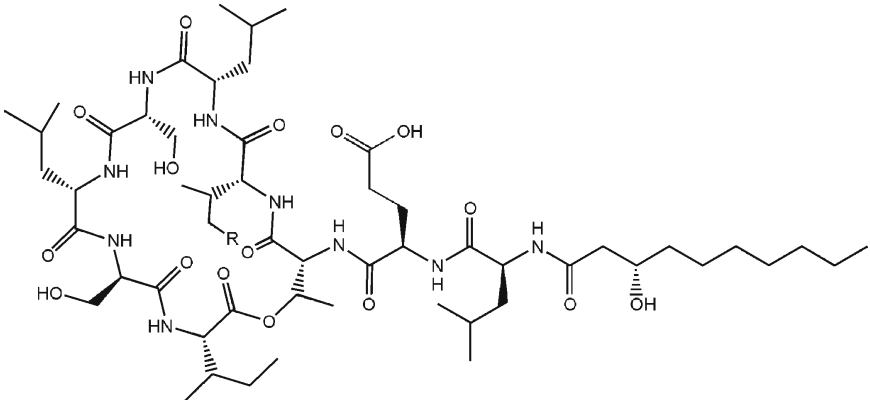


70

In the year 2000, structurally diverse marine natural product and the semisynthetic compounds (total 48 compounds) were screened[319] for *in vitro* activity against *M. tuberculosis*. Kahalalide-A (**71**) was found to be most active within this set, inhibiting 83% of the growth of *M. tuberculosis* at (H37Rv) 12.5 μg/mL and emerged as a promising new lead. Kahalalide-A (**71**) is one of the member of natural peptides isolated[320323] from the marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. Kahalalide-F (**46**) among these, has attracted the most attention[324326] and is currently in phase II clinical trials as an anticancer and antipsoriatic agent, while kahalalide-B has been the subject[327] of a total synthesis. Kahalalide-A does not have significant homology to these other antimycobacterial cyclic peptides. Furthermore, it is devoid of obviously reactive functional groups, and it is not cytotoxic to various tumor cell lines, suggesting a selective antibacterial target. Solid phase total synthesis of kahalalide-A (**71**) has been achieved.[328] Kahalalide-A (**71**) is the third example of marine derived peptide having antimicrobacterial tuberculosis activity after massetolide-A (**72**) and viscosin (**73**). Massetolide-A (**72**) and viscosin (**73**) are cyclic depsipeptides isolated



71



72, R = CH_3
73, R = H

from cultures of two *Pseudomonas* species isolated from marine alga and tube worm respectively.[329,330] When tested against *M. tuberculosis*, massetolide-A (**72**) and viscosin (**73**) displayed MIC values of 5-10 and 10-20 μg/mL respectively.[329,330]

10. Concluding Remarks

The field of marine peptides is firmly established. Instead of simply searching for new peptides, search is now for peptides of biomedical importance. Assay methods are available to detect diverse array of biomedically relevant peptides. Blue green algae (Cyanophyta) are found rich source of peptides with diverse structure and biological activity. Among the marine animals, sponges and ascidians are good source of peptides. The sponge peptides are generally highly modified. It is believed that some sponge peptides are produced by interaction with symbiotic micro-organisms. Many peptides had been isolated during the last two decades from marine sponges. Several reasons have been given for rapid progress in the chemistry of sponge peptides, such as development of reveres phase HPLC technique for isolation, advances in spectroscopy especially 2D NMR and FAB mass spectrometry, chiral chromatography had allowed the assignment of absolute configuration of amino acids with small amounts of material. A number of bioactive peptides had been synthesized. Nazumamide A, a thrombin inhibiting linear tetrapeptide, was efficiently constructed using diethyl phosphorocyanidate as coupling reagent and tert-butyloxycarbonyl as N-protecting group. Most of the bioactive peptides isolated from marine sponges are cyclic and lipophilic. The chemists involved in the isolation of these peptides were using a specific bioassay method. Hence, it is likely that the linear or more polar peptides are being missed. However, some questions remain unanswered. Do blue-green algae which live symbiotically with sponges, participate in the synthesis of peptides in sponges? Why are some peptides found in large quantities, while some are present in trace amounts? The answers to some of these questions may be arrived at when it will become possible to culture sponge cells or symbiotic microbes. Sponge peptides appear to have drug potential. Cyclotheonamides may serve as "Lead compounds" for developments of antithrombin drugs. Discodermins are potential antitumor promoting agents and calyculins are found useful as biochemical reagents.

Ascidians remain unique among marine invertebrates in that they over whelmingly produce nitrogen containing metabolites, almost all being derived from amino acids. Although investigations on ascidians as potential source of drugs were initiated more recently than some other marine invertebrates, it is significant that the first marine natural product to enter human clinical trials is didemnin B, an ascidian secondary metabolite. Ascidians, like many of the other marine invertebrates, are known to exist in obligate and non-obligate symbiosis with micro-organisms. The contribution of symbiotic algae to the biosynthesis of secondary metabolites of ascidian remains

unanswered because the symbiotic algae have been resistant to culture. However, there has been some success in the mariculture of ascidians. Most of the bioactive peptide of coelenterates is neurotoxins. These peptides show a large degree of sequence homology. Since all alter the function of Na^+ channels in excitable membranes of muscles and nerves. These toxins are considered important probes of Na^+ channels and related physiological functions. The *Conus* peptides are being used for a wide variety of physiological and pharmacological investigations in both vertebrates and invertebrate nervous systems. Several sperm activating peptides have been isolated from sea urchins egg jelly. These peptides are specific at the ordinal level in the respiratory stimulation of sea urchins spermatozoa. The peptide named resact is the first egg derived molecule of known structure, shown to be a chemo attractant of animal spermatozoa. Over twenty peptides have been isolated from nine species of sea urchins distributed over four taxonomic orders. It is expected that more species-specific peptides will be isolated from the egg jelly of sea urchins belonging to different orders.

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11

Marine Prostaglandins

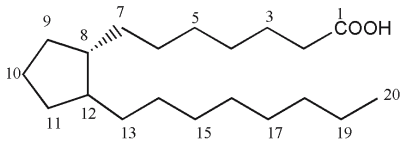
Abstract

The chapter deals with the prostanoids of marine organisms. The isolation and characterization of (15*R*)-PGA_2, methyl ester acetate of (15*R*)-PGA_2; PGE_2; 13-14-*cis*-PGA_2-15-acetate; 13,14-dihydro-PGA_2; PGF_2; 5-*trans* PGA_2; PGF_2 -9-O-acetate methyl ester from Caribbean soft coral *Plexaura homomalla* (Esper); chlorovolones; clavulones; claviridenones and C-20 acetoxy clavulones from stolonifer *Clavularia viridis* Quoy and Gaimara, a soft coral from Okinawa, Japan; methyl-11-acetoxy, 9,15-(*S*)-hydroxy-5-*cis*-12-*trans*-prostadienoate; methyl-11,18-diacetoxy-9,15-(*S*)-hydroxy-5-*cis*,13-*trans* prostadienoate, and the corresponding acetate and diacetate of free acids from *Lobophyton depressum,* a soft coral collected in the Gulf of Eilat (The Red Sea); unusual prostanoids, punaglandins from the octocoral *Telesto riisei* and PGF_2 and PGE_2 from the marine red alga *Gracilaria lichermids* from West Head, Victoria, Australia have been discussed.

1. Introduction

Prostaglandins (PGs) are physiologically active substances occurring primarily in human and sheep seminal plasma. They are also present in lesser amounts in uterus, lungs, brain, iris, thymus, pancreas and kidney tissues. Prostaglandins are biosynthesized from C_{20} polyunsaturated fatty acids and, widely distributed in mammalian tissues. In contrast to hormones, PGs neither circulate in the body nor stored in tissues. Rather they are synthesized locally on demand, perform a tissue-specific function and then rapidly inactivated by metabolic enzymes. They act in nanogram quantities on reproductive, gastrointestinal, respiratory and cardiovascular systems to produce physiological effects. Their importance in therapeutic abortion and regulating menstruation, fertility and

conception is well established. Their use in prevention and treatment of peptic ulcers, thrombosis and control of blood pressure is envisaged. Due to their pivotal role in the biological system and application in pharmaceuticals use, extensive work has been done in this area.[1-25] Chemically prostaglandins are C_{20} carboxylic acids consisting of a five member ring with two side chains. One of them is seven carbon atoms and the other of eight carbon atoms. All prostaglandins may be regarded as derivative of prostanoic acid (**1**), a trivial name. The stereochemistry of substituents on 5-member cyclopentane ring could be α or β. The configuration of C-15 hydroxyl function is 15(*S*). Naturally occurring prostaglandins (**2-23**) could be divided into nine groups (A-I) based on the substitution pattern on the cyclopentane ring. Individual member of each group is distinguished by the number of double bonds in the side chain which are denoted by the subscript 1, 2, 3. In the F-series, a further subscript is added to define the stereochemistry of the C_9 hydroxy group.



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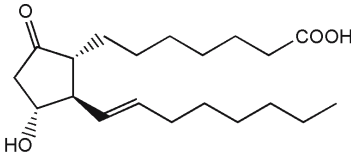

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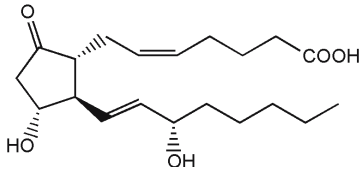

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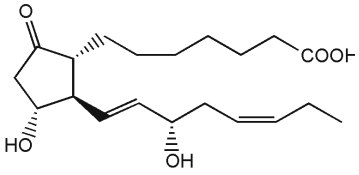

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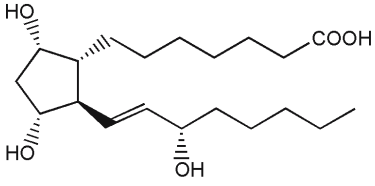

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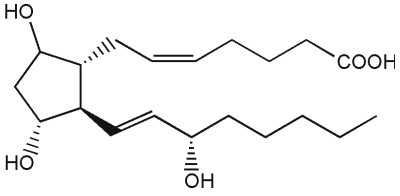

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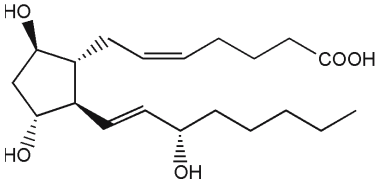

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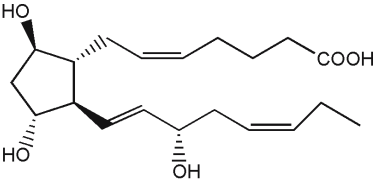

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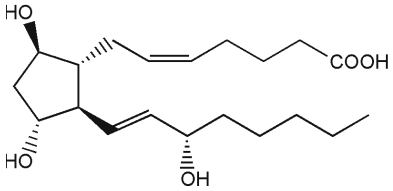

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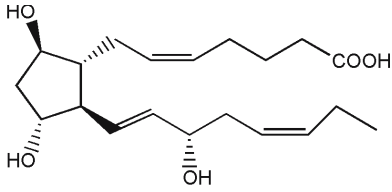

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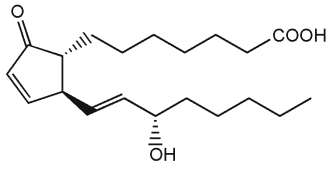
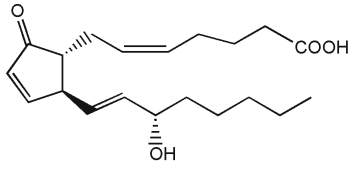
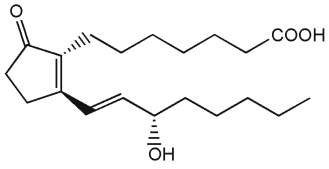


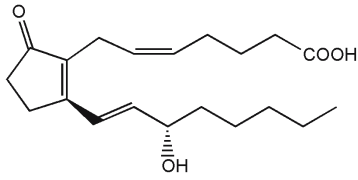
11, PGA_2



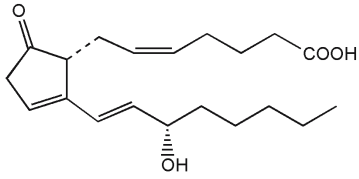
12, PGA_2



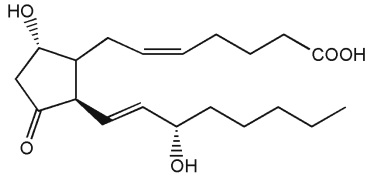
13, PGB_1



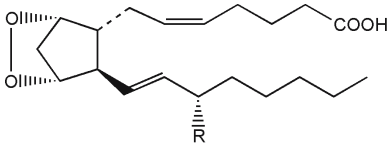
14, PGB_2



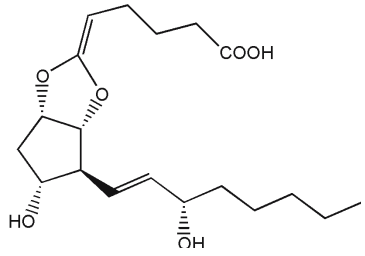
15, PGC_2



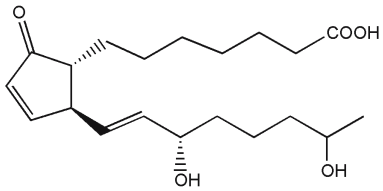
16, PGD_2



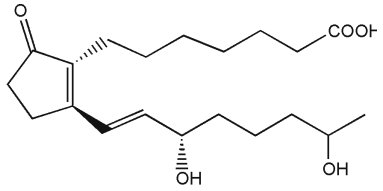
17, R = OOH, PGG_2
18, R = OH



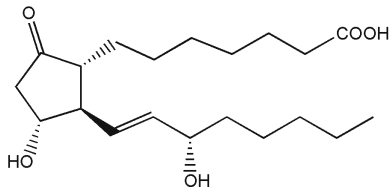
19, PGI_2



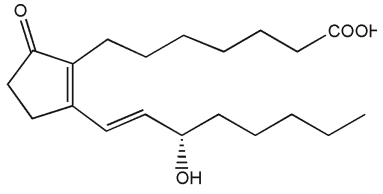
20, 19-Hydroxy-PGA_1



21, 19-Hydroxy-PGB_1



22, Dihydro-PGE_1



23, 19-Hydroxy-PGB_1

Prostaglandins are biosynthesized from the essential polyunsaturated fatty acids (Figure 1), such as 8,11,14-eicosatrienoic acid (dihomolinolenic acid (**24**); 5,8,11,14-eicosatetraenoic acid [arachidonic acid (**25**)] and 5,8,11,14,17-eicosapentaenoic acid (**26**). Van Drop et al[26,27] have demonstrated that the fatty acids (**24**), (**25**) and (**26**) are precursors of the prostaglandins PGE_1, PGE_2 and PGE_3, respectively. Arachidonic acid (**25**) in mammalian system is converted into the prostaglandins (PG_2 series, Figure 2) by various enzymes via endoperoxide route. The enzyme phospholipase-A releases the precursor such as arachidonic acid (**25**) which exists in tissues as phospholipids. It is then acts upon by other prostaglandin synthesizing enzymes.

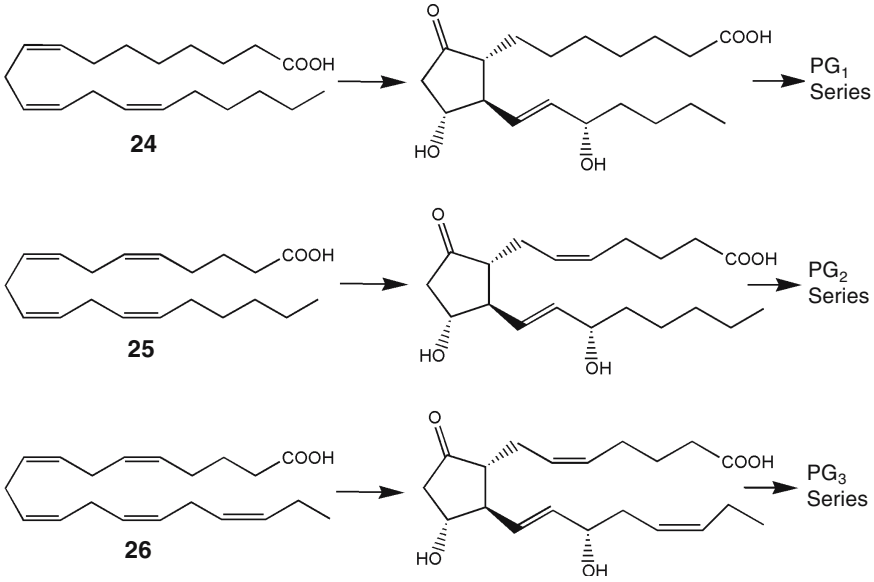


Figure 1 Biosynthesis of mammalian prostaglandins.

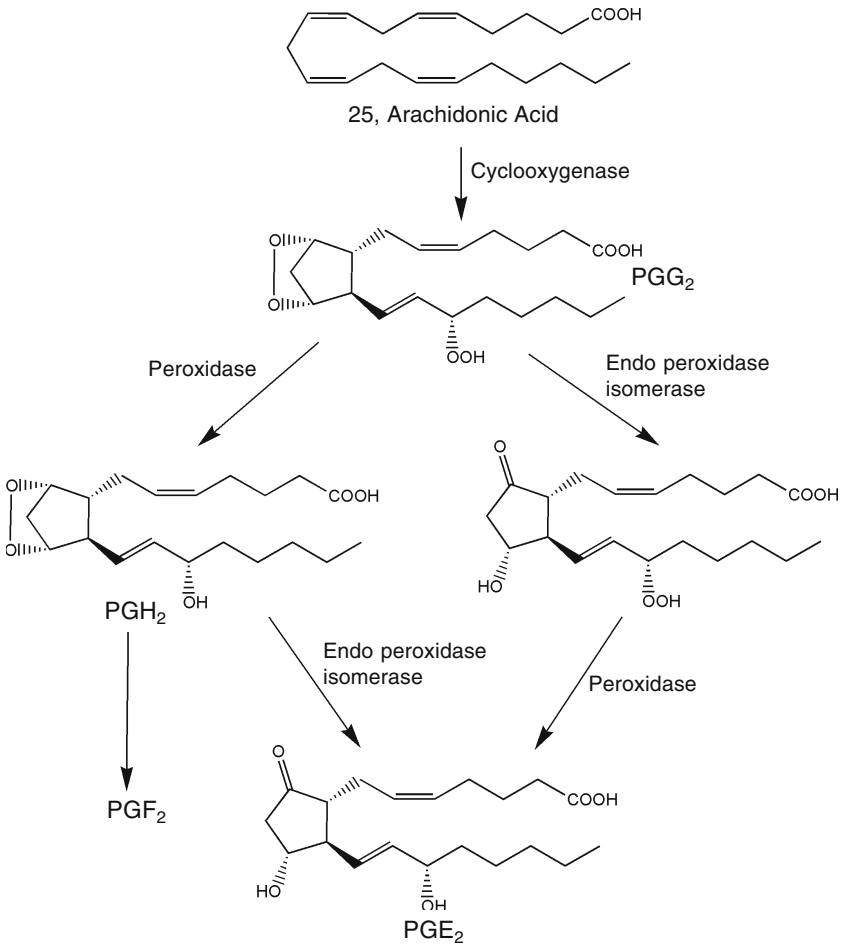
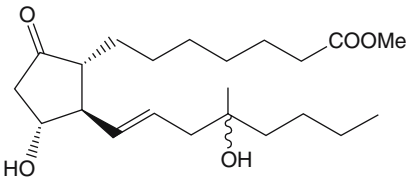


Figure 2 Biosynthesis of the prostaglandins.

The biosynthesis of prostaglandins consists of many steps that involve a variety of enzymes and co-factors. Inhibitions of prostaglandin synthesis may occur by blocking any of the steps involved in the biosynthesis. Prostaglandins display a wide range of biological effects in concentrations as low as 10^9 . They all are biologically active compounds. The action of E, F and A series are often dissimilar and sometime opposite. The biological activities of prostaglandins were of academic interest till 1971 when Vane and his co-workers demonstrated that prostaglandins play a crucial role in causing inflammation. It was demonstrated that prostaglandins have the ability to suppress the enzyme cyclo-oxygenase and thereby arrest the production of prostaglandins. This finding drew the attention of several scientists and prostaglandins study snowballed at a very fast pace. Among the prostaglandins the PGE_2, PGD_2 and PGF_2 are mainly involved in the manifestation of inflammation, pain and swelling. These prostaglandins are also involved in

causing fever. Although PGs have been found to be chemical indicator in several abnormalities the predominant use of E type PGs has been found in the treatment of peptic ulcer and cardiovascular diseases. The F type PGs are useful in the treatment of gynaecological disorders or fertility regulation. To date, the only widely marketed PGE analogue is misoprostol (**27**) used to prevent gastroduodenal ulcers caused by non-steroidal antiinflammatory drugs. Prostaglandin E_1 is involved in protecting the lining of the stomach. Misoprostol mimicks the action of prostaglandin E_1 in the body. It is used in the treatment of gastro-intestinal ulcers including those occurring in people who are taking one of a group of medicines known as non-steroidal antiinflammatory drugs (NSAIDs) to manage their arthritis. One of the adverse effects of NSAIDs is stomach and duodenal ulcers (gastrointestinal ulceration). Unfortunately misoprostol also mimicks the labour-inducing effects of prostaglandin E_1. Therefore, women who are pregnant or who may become pregnant should avoid taking misoprostol as it may cause contraction of the uterus.[28] PGF compounds have been found of limited utility in human reproductive regulations. However, they are more extensively used for farm animal estrus synchronization.

Prostaglandins are susceptible to air oxidation like any other polyunsaturated fatty acid derivatives. The F type prostaglandins are the most stable ones. The E type prostaglandins are very labile towards bases, and also get dehydrated under acidic conditions. Under basic conditions all E to A prostaglandins undergo for dehydration and also undergo for the isomerisation to the B-type prostaglandins. Mammalian prostaglandins occur in minute quantities. In 1969, Weinheimer and Spraggins[29] reported the first high yield isolation of nonmammalian type, (15*R*)-PGA_2 (**28**) and its methyl ester acetate (**29**) from the Caribbean gorgonea *Plexaura homomalla* (Esper). This report stimulated a worldwide search of PGs in marine life. Subsequently it was found that some forms of *P. homomalla* contained various mammalian-type PGs, such as (15*S*)-PGA_2 (**32**), PGE_2 (**3**), 13-14-*cis*-PGA_2, 15-acetate, 13,14-dihydro-PGA_2. Later on by monitoring the activity towards isolated guinea-pig ileum, PGF_2 (**6**) was isolated from the Japanese coastal gorgonean, *Euplexaura erecta*.[30] The occurrence of PGs in marine life other than gorgoneans was also reported, PGE_2 (**3**) and PGF_2 (**6**) are even isolated from the Australian red alga *Gracilaria lichienoides.*[31] This was the first report of



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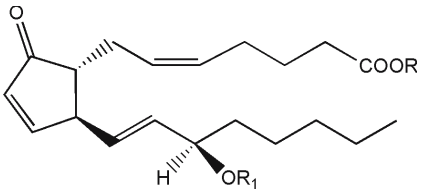


28, R = R_1 = H
29, R = Me, R_1 = Ac

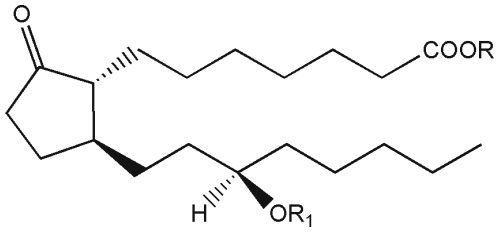
the occurrence of PGs in plant. The red soft coral *Lobophyton depressum*[32] was found to contain PGF_2 (**6**) 11-acetate methyl ester and its 18-acetoxy derivative as well as their two corresponding free carboxylic acid. The Hawaiian octocoral *Telesto riisei*,[33] which lacks symbiotic photosynthetic algae, yielded a series of highly functionized halogenated prostaglandins named punaglandins. The Okinawan soft coral (Stolonifer) *Clavularia viridis* Guoy and Gaimard (*Stolonifera clavulariidae*)[34] afforded a number of prostaglandins name claviridenones.

2. Marine Organisms

2.1 *Plexaura homomalla* (Esper)

P. homomalla[35-39] coffee brown in color, highly branched bush like the Caribbean soft coral, found in abundance is extraordinarily rich in PGA_2, methyl ester (**29**) (up to 2% of dry weight) or the 15-epimer depending on subspecies.[29] 15-*epi* PGA_2 (**28**) and its diester (**29**) isolated from the air dried cortex in the yield 0.2% and 1.3% respectively,[29] were epimeric with potent mammalian hormone at allylic hydroxyl centre C-15. They were, however, devoid of the dramatic blood pressure lowering (dog) effect of PGA_2 (**12**) itself. Their function like that of other compounds occurring in the gorgonian remains speculative. The diester (**29**) was isolated by chromatography of the crude hexane extract in benzene/ethyl acetate on silicic acid. High resolution mass spectrometry established its composition as $C_{23}H_{34}O_5$ and demonstrated the loss of acetic acid and the even-mass fragment, $C_6H_{10}CO_2Me$. The IR spectrum of (**29**) showed carbonyl absorptions at 1735 $cm^{\perp}$, typical of simple ester, and at 1710 $cm^{\perp}$ compatible with a conjugated cyclopentenone moiety. Hydrogenation of the diester (**29**) and the acid (**28**) over Palladium gave the hexahydro-diester (**30**) and the hexahydro acid (**31**), respectively. The compound (**30**) showed no hydroxyl absorption in the infrared and carbonyl absorption only at 1735 $cm^{\perp}$. The ultra violet absorption maximum at 300 nm confirmed the presence of cyclopentanone group in (**30**).

The mass spectrum of (**30**) showed the loss of the even mass fragment. $C_6H_{10}CO_2Me$, suggesting the presence of this side chain in the α position of the cyclopentanone, as well as the loss of acetic acid and fragment C_8H_{15} suggesting the presence of an acetate bearing side chain. The NMR spectrum

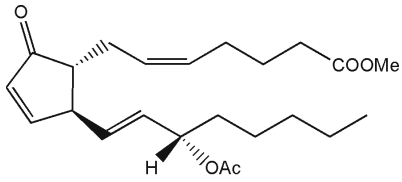


30, R = Me, R_1 = Ac
31, R = R_1 = H

of (**29**) showed two sharp methyl singlet at δ1.98 (acetate) and 3.61 (methoxy) and a third methyl group as a perturbed triplet characteristic of an alkyl chain terminus at δ0.89. A pair of double doublets at δ6.12 and 7.42 (J = 2 Hz, 6.2 Hz) indicated the presence of conjugated double bond (C-10, 11) and their multiplicity clearly indicated a single substituent at C-12. By decoupling experiments it was shown that the H-10 and H-11 protons are coupled to the one proton singlet at δ3.22 (H-12) which was also coupled with H-13 and H-14 protons. Thus, the prostaglandins (**28**) and (**29**) isolated from gorgonian were found epimeric with C-15 in PGA_2 (**12**).

All known natural mammalian prostaglandins have the *S*-configuration at the C-15 asymmetric centre.[35] Weinheimer and Spraggins[29] had reported the "non mammalian" prostaglandins, 15- *epi*- PGA_2 (**28**) and its acetate methyl ester (**29**) isolated from the Caribbean soft coral *Plexaura homomalla* which had *R*-configuration at C-15. Schneider et al[40] reinvestigated the samples of *P. homomalla* and found that some forms of soft coral contained instead of the (*R*)-prostaglandins, esterified derivative of (15*S*)-PGA_2 (**32**) and (15*S*)-PGE_2 (**33**) identical with the prostaglandins derived from mammalian sources.

In some single specimen of this gorgonian, both (15*R*) and (15*S*), prostaglandins were also found. The (15*R*) and (15*S*) forms of these two prostaglandins, differ slightly in silica gel TLC, using the AIX polarity system, the (15*R*)-isomer being less polar, so that preliminary identification in coral extraction can be made. The origin of primary prostaglandins in marine organisms raises many intriguing biochemical questions. Bundy et al[41] have reported the conversion of both (15*S*)-(**32**) and (15*R*)-(**29**) PGA_2 into biologically important primary prostaglandins, PGE_2 (**3**) and PGF_2 (**6**). (15*R*)-PGA_2 methyl ester (**29**) was treated with methane sulphonyl chloride in pyridine and the resulting crude 15-mesylate was solubilized in acetone-water to give modest yields of the C15 inverted product, (15*S*)-PGA_2 methyl ester. Acetylation of the product in acetic anhydride/pyridine gave (**32**) and thus, ultimately to PGE_2 (**3**) and PGF_2 (**6**). *Plexaura homomalla* var. (*R*) and var. (*S*) are thus both suitable sources of (coral) prostaglandins useful in the synthesis of PGE_2 and PGF_2. From the (*S*) variety PGE_2 can be obtained in three steps and PGF_2 in four steps.



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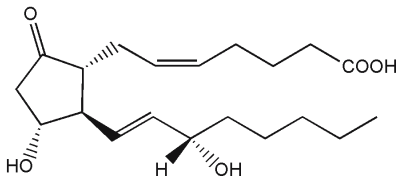
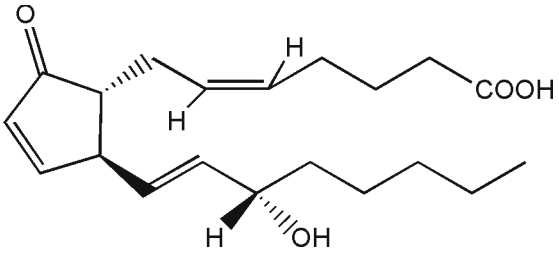


33

5-Trans-PGA₂

During the chromatographic purification of (15*S*)-PGA_2 from the gorgonian *P. homomalla* var. (*S*) a new natural prostaglandin identified as 5-*trans*-

PGA_2, was isolated.[42] This compound was less polar than PGA_2 on silver nitrate impregnated silica gel. Column chromatography of crude (15*S*)-PGA_2 on Amberlite-15 Ag^+ form or on silver nitrate impregnated silica gel gave a minor component to which the structure (15*S*)-15-hydroxy-9-oxo-5-*trans*-10,13-*trans*-protatrienoic acid C_5-*trans*-PGA_2 (**34**) was assigned. Content of the *trans*-isomer usually ranged between 5 and 15% of the PGA_2. 5-*trans*-PGA_2 was an oil, λ_{max} 217 nm (ε 9050); $[\alpha]_D$ 128° ($CHCl_3$); molecular ion peak at 478.2998 for TMS derivative (calcd for $C_{26}H_{46}O_4Si_2$ 478.2932) and mass spectrum was found to be identical with PGA_2.



34

Irradiation of prostaglandin E_2 (**3**) in oxygen free benzene-methanol solution with 3500 Ålight for 24 hr in a Rayonet photochemical reactor in the presence of diphenyl sulphide gave, after careful chromatography on acid-washed silica gel a 22% yield of 5-*trans*-PGE_2, m.p. 75–77 °C (which was identical with the material derived from *P. homomalla*). In a similar fashion and in similar yield, crystalline 5-*trans*-$PGF_{2\beta}$ and 5-*trans*-PGF_2 were prepared from the corresponding 5-*cis*-prostaglandins and were also found identical with the coral derived compounds. Re-examination of the extracts of *P. homomalla* var. (*S*) prior to hydrolysis showed that while small amount of the free acids are present however, the 5-*trans* isomer was predominantly in the form of its 15-acetate methyl ester. It is not yet clear whether the presence of *trans*-isomer represents biosynthetic formation from 5-*trans*-arachidonic acid endogenous to *P. homomalla* or a subsequent transformation product of 5-*cis*-PGA_2.

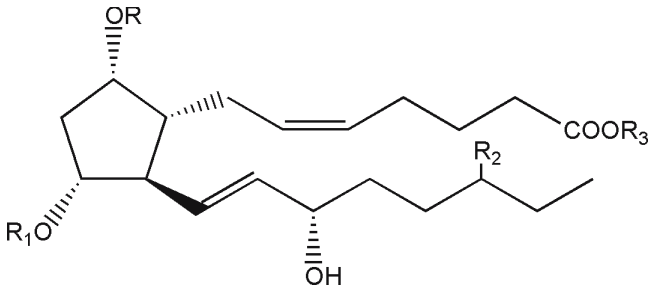
PGF_2-9-O-acetate methyl ester

Weinheimer and Spraggins[29] had discovered that the Caribbean gorgonian *Plexaura homomalla* (Esper, Plexauridae) contained remarkable quantities (in excess of 2% dry weight) of 15-*epi* and 15-normal prostaglandin PGA_2 (**12**). Smaller quantities of PGB_2 (**14**) and PGE_2 (**3**) were also found in the gorgolean. Groweiss and Fenical[43] investigated the extracts of *P. homomalla* collected in the Bahama Islands and isolated a simple prostaglandin derivative characterized as $PGF_{2\alpha}$-9-O-acetate methyl ester (**35**). $PGF_{2\alpha}$-11-O-acetate methyl ester (**36**), an acetate positional isomer of **35** had been isolated as a natural product from the Red Sea soft coral *Labophytom depressum.*[44]

Prostaglandin (**35**) was isolated as viscous oil that was analyzed for $C_{23}H_{38}O_6$. The compound showed spectral characteristic for multiple ester linkage (1750 $cm^{\perp}$ br) and hydroxyl functionalities (3400 $cm^{\perp}$ br). Consideration of ^1H and ^{13}C NMR data, and specifically the results of COSY analysis allowed the complete structure (**35**) to be assigned. The 15*S* configuration in (**35**) was assigned based upon the similar ^{13}C NMR shift of C-13, C-17 in comparison with those reported for compound (**37**). A careful literature survey revealed that (**35**) had been prepared as a synthetic intermediate.[45] However, this is the first report of the ester occurring as a natural product.

13,14-Dihydro and 13,14-cis-unsaturated prostaglandins

Enzymatic hydrolysis of prostaglandin esters in the coral *P. homomalla* obtained from Cayman islands, gave a mixture from which PGA_2 (**12**); 5,6-*trans*-PGA_2, PGE_2 (**3**) and traces of PGE_3 had been isolated chromatographically. Further separation by silica gel chromatography of fractions less polar than PGA_2 had disclosed the presence of other prostaglandins of which, 13,14-*cis*-PGA_2; 15-acetate (**38**) and 13,14-dihydro-PGA_2 acetate methyl ester (**39**) were characterized.[46]



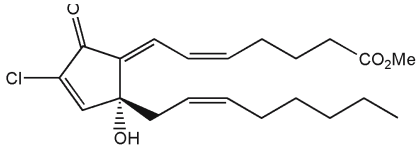
35, R = Ac, R_1 = R_2 = H, R_3 = Me
36, R = H, R_1 = Ac, R_2 = H, R_3 = Me
37, R = H, R_1 = Ac, R_2 = H, R_3 = Me
38, R = H, R_1 = R_2 = Ac, R_3 = Me
39, R = H, R_1 = Ac, R_2 = H, R_3 = H

2.2 *Clavularia viridis* QUOY and GAIMARA

Chlorovulones

The marine prostanoids named clavulones isolated from *Stolonifer viridis*[47-50] had received much attention because of their unique structural features and remarkable anticancer activities.[51-60] The freeze dried *C. viridis* (210 g) collected at the coral reef of Ishigaki Island (Okinawa, Japan) were extracted with ether. The ether extract (7.88 g) was chromatographed on a silica gel column using n-hexane-ethyl acetate (5:1) as an eluent to give a mixtures of chlorovulones (100 mg). Chlorovulones were eluted prior to the elution of clavulones. Repeated HPLC [silica gel, n-hexane-ether (2:1)] of

the mixture gave chlorovulone-I (**40**) (colorless oil, 20 mg, $C_{21}H_{29}ClO_4$) and chlorovulone-II (**41**) (colorless oil, 4 mg, $C_{21}H_{29}ClO_4$) in a ratio of 10:1.2 in order of increasing polarity. Although chlorovulone-IV (**42**) was not isolated from the chlorovulones mixture in a pure state, its presence was suggested by the isolation of the corresponding acetate **43**, when the mixture was subjected to acetylation with acetic anhydride/pyridine to give the easily separable acetates mixture (**43**, **44**, **45** and **46**) of chlorovulones which was easily separated by silica gel column and HPLC.



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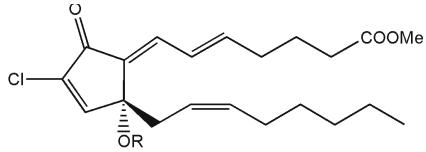
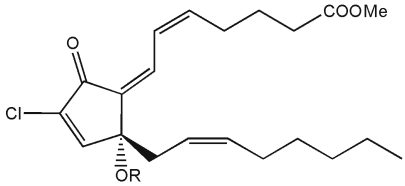
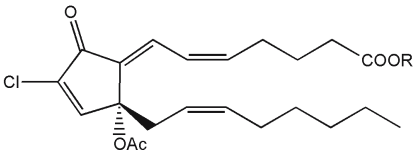


41, R = H
45, R = Ac



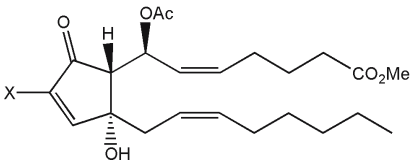
42, R = H
43, R = Ac



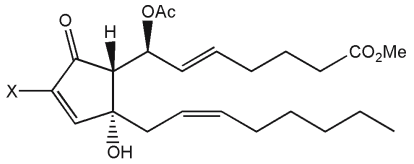
44, R = Me
46, R = H

Chlorovulones (I-IV) had the stereochemistry of the carbon-carbon double bonds (5*Z*, 7*E*, 14*Z*), (5*E*, 7*E*, 14*Z*), (5*E*, 7*Z*, 14*Z*) and (5*Z*, 7*Z*, 14*Z*) which corresponds to those of clavulone I, II, III and IV, respectively. The structures of chlorovulones had been assigned by extensive use of mass spectrometry, ^{1}H NMR and ^{13}C NMR spectroscopy. Chlorovulone-I (**40**) displayed strong antiproliferation and cytotoxic activities in human *Promyelocytic leukemia* (HL-60) cells *in vitro*. The IC_{50} value of (**40**) in the HL-60 cells was 0.03 M (0.01 g/mL) which is about 13 times stronger than clavulone-I.

Iguchi et al[51,60,61] reported the isolation of new halogenated prostanoids (**47-51**) from the Okinawan soft coral *Clavularia viridis*. Mainly NMR spectral data were used in order to elucidate the structure of (**47**) and the relative and absolute configurations of the compound was determined by analysis of NOESY, CD data, chemical conversion, and the modified Mosher's method. The structures of (**48–51**) were deduced by comparison of their spectral data with those of (**47**). Hexane extract of 6.83 g crude isolate (out of 14.5 g, freeze-dried soft coral 470 g) was chromatographed on a silica gel column eluted with hexane : AcOEt and AcOEt : MeOH (3 : 1 and 1 : 1) to obtain five fractions. The second fraction (eluted with hexane:AcOEt, 3 : 1) was further subjected for separation and purification by MPLC and HPLC on normal- and reversed-phase columns to obtain compounds (**47**) (29.8 mg), (**48**) (2.6 mg), (**49**) (1.1 mg), (**50**) (0.3 mg), (**51**) (0.6 mg) and (**40**) (0.1 mg).



47, X = I
48, X = Br



49, X = I
50, X = Br
51, X = Cl

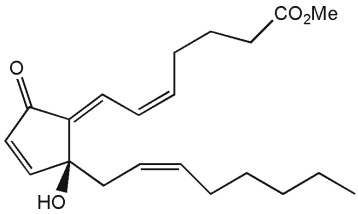
Analytical data such as HR-EIMS and ^{13}C NMR indicated that compound (**47**) possess an iodine-containing molecular formula of $C_{23}H_{33}IO_6$ [472.1109 (M^+ -CH_3CO_2H), (calcd for $C_{21}H_{29}IO_4$, 472.1111]. In the UV and IR spectra, the presence of an α, β-unsaturated carbonyl group [λ_{max} 251 mn], an acetate ester (IR 1732, 1240 $cm^{\pm}$), and a hydroxyl group (3470 $cm^{\pm}$) was suggested. Compound (**47**) showed potent cytotoxic activity.

Clavulones

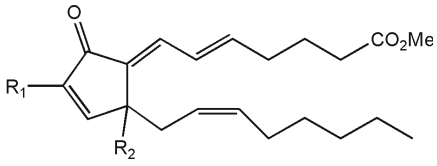
Iguchi et al[62,63] have isolated three new prostanoids named clavulones (I-III) from the Japanese coelenterate, Stolonifer *Clavularia viridis* QUOY and GAIMAR. Clavulones are the first examples of the prostanoids having oxygen functions at C-4 and C-12 positions and olefins at C-7 and C-14 positions. The methanol extracts of *C. viridis* (5 kg, wet weight), collected at the coral reefs of Okinawa Japan, was suspended in water and extracted with ethyl acetate. The ethyl acetate extract (30 g) was chromatographed on silica gel column using benzene : ethyl acetate (10 : 1) as eluant to give four fractions. The fraction four was decolorized by passing through a polystyrene gel column using benzene : ethyl acetate (10 : 1) as an eluant to give clavulone-I (870 mg, $C_{25}H_{34}O_7$; $[\alpha]_D$ -28.9 ° as a pale yellow oil. Similar separation procedure of the fraction 3 and 2 gave clavulone-II (pale yellow oil, $C_{25}H_{34}O_7$; $[\alpha]_D$ +10.9° and clavulone-III (pale yellow oil, 253 mg, $C_{25}H_{34}O_7$; $[\alpha]_D$ -45.5 ° respectively. Structure of clavulones-I, III had been determined by UV, IR, ^1H NMR, ^{13}C NMR and chemical reaction. The absolute configurations at the C-4 and C-12 positions of clavulones had been determined.[64] The clavulones showed a significant antiinflammatory effects at 30 g/ml in the fertile egg test.[62]

More recently Shen et al[65] reported the isolation of seven new clavulone and its derivatives from the CH_2Cl_2:MeOH extract of *Clavularia virdis* collect in Tiwan, and designated as 4-deacetoxyl-12-O-deacetylclavulone I (**52**), 4-deacetoxyl-12-O-deacetylclavulone II (**53**), bromovulone II (**54**), iodovulone II (**55**), 4-deacetoxyl-12-O-deacetylclavulone III (**56**), bromovulone III (**57**), and iodovulone III (**58**).

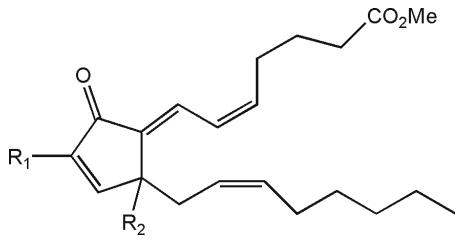
The dichloromethane and methanol extract of *C. viridis* was fractionalized between ethyl acetate and water. Silica gel column chromatography and HPLC chromatographic purification yielded seven new compounds. These



52



53, R_1 = H, R_2 = β-OH
54, R_1 = Br, R_2 = α-OH
55, R_1 = I, R_2 = α-OH



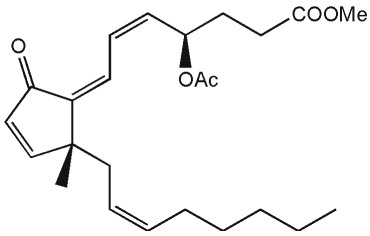
56, R_1 = H, R_2 = β–OH
57, R_1 = Br, R_2 = α-OH
58, R_1 = I, R_2 = α-OH

compounds were designated as 4-deacetoxyl-12-O-deacetylclavulone I (**52**, 1.1 mg), 4-deacetoxyl-12-O-deacetylclavulone II (**53**, 2.6 mg), bromovulone II (**54**, 3.1 mg), iodovulone II (**55**, 9.1 mg), 4-deacetoxyl-12-O-deacetylclavulone III (**56**, 2.2 mg), bromovulone III (**57**, 25 mg), and iodovulone III (**58**, 4.4 mg), in addition to seven known prostanoids from the EtOAc soluble fraction. The known compounds were identified as clavulones I (15 mg), II (6 mg), and III (8 mg), 7-acetoxy-7,8-dihydroiodovulone (6 mg), chlorovulones II (17 mg) and III (0.9 mg), by comparison of their spectral data (^{1}H, ^{13}C NMR, MS, and optical rotation) with reported values. Structure of these unknown compounds was determined by the use of ^{1}H NMR, ^{13}C NMR, HRFABMS, and 2D NMR. Pharmacological study revealed that bromoclavulone (**57**) exhibited potent cytotoxicity against human prostate (PC-3) and colon (HT 29) cancer cells.

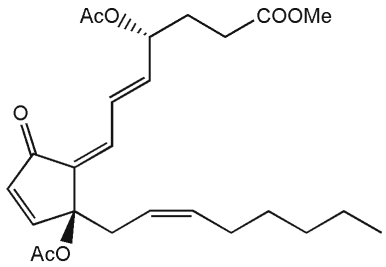
Claviridenones

Four new antitumor prostanoids named claviridenone[61] (a-d, **59-62**) have been isolated from the Okinawan soft coral *Clavularia viridis*. The acetone extract of the fresh soft coral from Okinawa Japan was partitioned by a EtOAc-water solvent system. Purification of the EtOAc soluble portion by repeated SiO_2 column and HPLC (PORASIL) provided claviridenone-a (**59**), $C_{24}H_{34}O_5$; $[\alpha]_D$-82.2° ($CHCl_3$); claviridenone-b (**60**), $C_{25}H_{34}O_7$, $[\alpha]_D$ +26.7° ($CHCl_3$); claviridenone-c (**61**) and claviridenone-d (**62**). The absolute stereo structures of these prostanoids had been elucidated on the basis of chemical and physicochemical evidences which including the application of the CD exciton chirality method to their various benzoyl derivatives having benzoate and conjugated diene chromophores.[66] Iguchi et al[62] had reported the isolation of three prostanoids, named clavulones-I-III from *C. viridis*. Subsequent

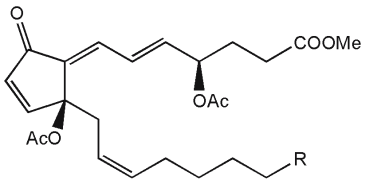
studies by Kitagawa et al[66] established that clavulone-I, II and III were identical with those of claviridenone-d (**66**), claviridenone-c (**61**) and claviridenone-b (**60**), respectively.



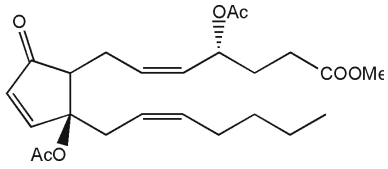
59, Claviridedone-a



60, Claviridedone-b



61, R = H, Claviridedone-c
63, R = CH_2OAc, 20-acetoxy claviridenone

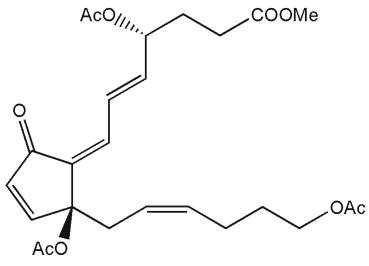


62, Claviridedone-d

C-20 Acetoxy clavulones

Three novel C-20 oxygenated prostanoids named as C-20 acetoxy clavulones had been isolated from *C. viridis*.[67] Silica gel chromatography (benzene-ethyl acetate, 10:1) of the ethyl acetate extract of *C. viridis* (210 g, wet weight) gave fractions (1-3).

Further, repeated silica gel chromatography and preparative TLC of the fractions gave 20-acetoxy claviridenone-d (**62**) (13 mg, $C_{25}H_{34}O_7$; $[\alpha]_D$-31.1°, c 0.9, $CHCl_3$); 20-acetoxy claviridenone-c (**63**) [215 mg, $C_{27}H_{36}O_9$, $[\alpha]_D$ 3.7 ° (c, 0.054, $CHCl_3$) and 20-acetoxy claviridenone-b (**64**) [16 mg,

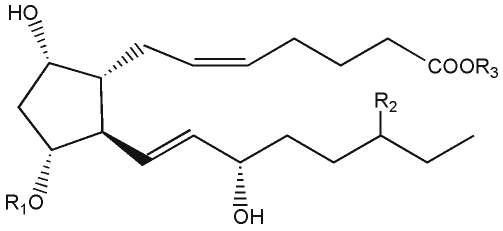


64, Acetoxy claviridedone-b

$C_{25}H_{32}O_9$, $[\alpha]_D$ +26.40° (c, 0.86, $CHCl_3$)] as pale yellow oils. The absolute stereostructures of these prostanoids had been elucidated on the basis of chemical and physiochemical evidences.[66,67]

Labophyton depressum

L. depressum (Alcyonacea, Alcyoniiudae) a soft coral collected in the Gulf of Eilat (the Red Sea) had afforded four PGF derivatives, (15*S*) $PGF_{2\alpha}$-11-acetate methyl ester (**65**); 18-acetoxy derivatives (**66**) of compound (**65**) as well as their two corresponding free carboxy acids (**67**) and (**68**). The soft coral *L. depressum* was collected in the Gulf of Eilat and extracted with CH_2Cl_2. A crystalline compound ($C_{23}H_{38}O_6$) m.p. 55°C; IR (KBr): 3700, 3610, 3510 (OH) 1740, 1730 $cm^{\perp}$ (OC=O) obtained from the CH_2Cl_2 was characterized mainly on the basis of its spectral data as methyl-11-acetoxy-9,15 (*S*)-hydroxy-5 *cis*-13-*trans*-prostadienoate (**65**).



65, R_1 = Ac, R_2 = H, R_3 = Me
66, R_1 = Ac, R_2 = H, R_3 = Me
67, R_1 = Ac, R_2 = H, R_3 = H
68, R_1 = R_2 = Ac, R_3 = H

The second compound was obtained as an oil ($C_{25}H_{40}O_8$). The ^1H and ^{13}C NMR spectra of the compound were similar to (**65**), but it had 2-hydroxyl and 2-acetate functionalities. It was characterized as methyl 11,18-diacetoxy-9, 15 (*S*)-hydroxy *5-cis,* 13-*trans* prostadienoate (**67**) (the 11,18-diacetate 18-hydroxy PGF_2 : methyl ester). The more polar compounds isolated from the crude ethyl acetate extract of *L. depressum* turned out to the corresponding acetate and diacetate of free acids (**67**) and (**68**). Esterification of the acids with CH_2N_2 gave the corresponding esters (**65**) and (**66**), respectively.

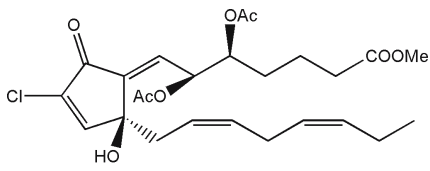
Telesto riisei

Scheuer et al[68] have isolated unusual halogenated antitumor eicosanoids named punaglandins from the octocoral *Telesto riisei.* Punaglandins are characterized by C-12 oxygen and unprecedented C-10 chlorine functions. Punaglandin-3 (**69**) inhibits L1210 leukemia cell proliferation, with an IC_{50} value of 0.02 g/mL, which represents 15 fold greater activity than displayed by the corresponding clavulone.

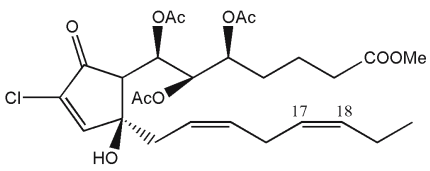
It is interesting to note that *Telesto riisei* a source of punaglandins, is an octocoral that lacks symbiotic algae. The freeze-dried animal (760 g) was refluxed with hexane to yield a (9.5 g) residue. Of which 1.9 g partitioned into 80% MeOH:H_2O containing the punaglandins. Column chromatography on Biosil-A (hexane:EtOAc, 7:3) monitored at 254 nm yielded four fractions, punaglandin-IV (**70**, 0.08% of the freeze-dried animal), punaglandin-III

(**69**, 1%), punaglandin-II (**71**, 0.2%) punaglandin-I (**72**, 0.8%) as colorless oil, each further purified by HPLC (Lichrosorb magnum RP-18, 80% MeOH/H_2O). Desorption chemical ionization MS revealed molecular ions and the presence of chlorine, which was confirmed by combustion analysis. The 17,18-dihydro relationship of (**72**) and (**71**) and of (**69**) and (**70**) was evident from ^1H and ^{13}C NMR data and from their formula each pair differing by two mass units. The structure of punaglandin-I (**72**), $C_{27}H_{37}ClO_{10}$ was deduced as follows as: The ^1H NMR and ^{13}C NMR showed the presence of three acetates as three quaternary carbonyl carbons were observed at δ171.3, 170.5, 170.4; and three singlet (for three protons each) at δ2.11, 2.8, 2.00 ppm and presence of one OMe group was supported by the resesonence at 3.63 ppm in the ^1H NMR spectrum (δ173.8). An α,β-enone system was supported by the UV absorption at 228 nm. Presence of 3° OH was confirmed by the IR spectroscopy. Extensive decoupling experiments at 500 MHz fully documented all protons from C-2 to C-8 and C-13 to C-20 and, hence, both side chain. A 3° hydroxy at C-12 was supported by the non-equivalence of the C-13 ^1H NMR signals (δ 2.53, 2.45) and their coupling to H-14, δ 5.30 ppm (J = 7.0 Hz), thereby unequivocally placing chlorine at C-l0. Punaglandin-I (**72**) loses AcOH when treated with pyridine affording a 3:1 mixture of (*Z*)-7,8-punaglandin-3 (**73** and **70**) under the same reaction conditions **71** was transformed into (*Z*)-7,8-punaglandin-4 (**74**) and its E isomer, also in a ratio of 3:1. These reactions showed that all four punaglandins belong to the same stereochemical series. The relative stereochemistry of the five centers was deduced by NOE experiments. If one assumes that the punaglandins had the same stereochemistry as other marine eicosanoids, the structures proposed represent the correct absolute stereochemistry.

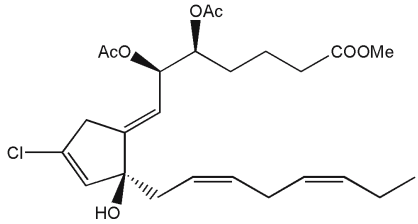
Recently, Ireland et al[69] isolated punaglandins from *Telesto riisei*. The freeze-dried organism *Telesto riisei* collected from Hawaii was macerated and subjected to a hexane Soxhlet extraction. The hexane-soluble material



69
70, 17,18-Dihydro of 69



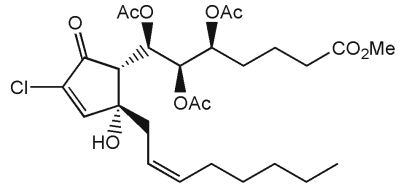
71, 17,18-Dihydro of 72
72



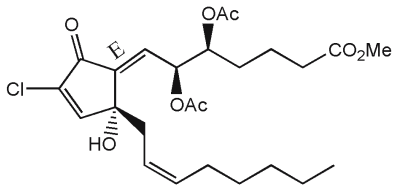
73
74, 17,18-Dihydro of 73

was extracted with 70% $CH_3OH : H_2O$ (70 : 30) resulting in hexane and aqueous CH_3OH fractions. The ^1H NMR spectrum confirmed the presence of punaglandins in the aqueous fraction. The water soluble material was purified by silica gel flash chromatography, yielding three fractions containing mixtures of punaglandins as determined by ^1H NMR. Each fraction was further purified by reversed-phase C-18 HPLC leading to the following punaglandins: PNG_2 (**75**), PNG_4 (**76**), *Z*-PNG_4 (**77**), PNG_6 (**78**), PNG_3 (**79**). The structures of the punaglandins were confirmed by ^1H and ^{13}C NMR and EIMS. Additionally, HMQC, HMBC, and DEPT data were collected for PNG_2 (**75**), *Z*-PNG_4 (**77**), and PNG_6 (**78**). All data were consistent with literature values. These punaglandins were tested for the ubiquitin-isopeptidase activity. It was anticipated that these punaglandins would inhibit ubiquitin-isopeptidase activity of the proteasome pathway with greater potency due to chlorination at C10. The activity was determined by the use of simple isopeptidase substrate, ubiquitin-PEST (Ub-PEST), a full-length ubiquitin molecule fused with an 18-amino acid C-terminal peptide extension.[70,72] The polypeptide extension is rich in Glu (E), Ser (S), Pro (P), and Thr (T) residues called PEST motifs that are responsible for rapid degradation of these and other unstable proteins.[71] Ub isopeptidases enzyme specifically cleaves this peptide extension, yielding free, full-length Ub. PNG_2 (**75**), PNG_3 (**79**), and *Z*-PNG_4 (**77**) were analyzed for isopeptidase activity *in vitro* in HCT 116 lysates. Isopeptidase activity buffer was used to lyse the cells. and the protein concentration was adjusted to 0.3 mg/mL per sample.

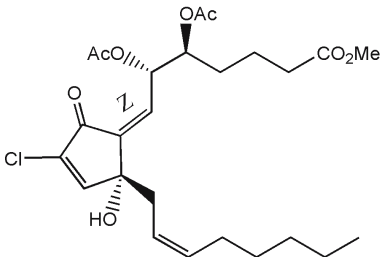
Each sample was subsequently incubated with 2, 6, 20, 60, or 200 μM of punaglandins (**75**), (**78**) and (**79**) in addition to 50 μg/mL of Ub- PEST. The



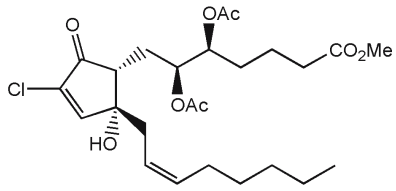
75



76

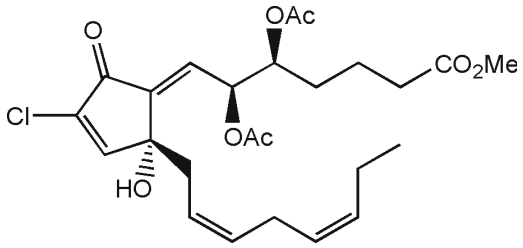


77



78

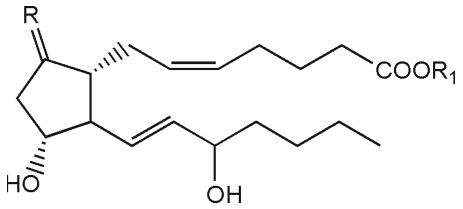
negative control cells were treated with 0.5% DMSO (vehicle) and equivalent Ub-PEST. The reaction was terminated after 45 min. The amount of fused Ub-PEST and free Ub product was determined by SDSPAGE fractionation. The vehicle control cells revealed a 1:1 ratio of fused Ub-PEST (10.5 kDa) to free Ub (8.5 kDa) as predicted to occur at a 45 min time interval. The punaglandins were found to inhibit the Ub-PEST degradation. Inhibition was initially observed at 20 μM with complete inhibition occurring at 60 μM, indicating that PNG_2 (**75**), PNG_3 (**79**), and *Z*-PNG_4 (**77**) are more potent inhibitors of isopeptidase activity *in vitro* than PGJ_2. All of these compounds exhibited potent *in vivo* isopeptidase activity, and they represent a new chemical class of cancer therapeutics.



79

Gracilaria lichenoides

All the naturally occurring marine prostaglandins so far had been isolated from marine invertebrates particularly from soft corals and gorgonian. Quinn et al[73] reported for the first time the occurrence of prostaglandins PGE_2 and $PGF_{2\alpha}$ in the Red alga *Gracilaria lichenoides.* The red alga (8.3 kg wet weight, 945 g dry weight) was collected from West Head, Victoria, Australia. It was frozen, stored at ⁄20 °C then cryogenically ground in liquid nitrogen. Extraction of the ground material twice with water followed by lyophilization of the combined extract afforded powder-A (456.5 g) which displayed potent antihypertensive activity when given intravenously to pentabarbitone, anaesthetized hypotensive rats. Isolation of the antihypertensive agent from *G. lichenoids* was achieved by a sequence of chromatographic separations monitered by the hypertensive rat assay. Aqueous extract of power-A (260 g) was adsorbed onto Amberiite XAD-2 in water and the active fraction-B (1.99 g, 0.3%) was eluted with the methanol. Fraction-B (1.95 g) was chromatographed on Sephadex G-25 in water and the antihypertensive activity of the eluate was confirmed to a fraction C (780 mg, 0.15 %). Preparative HPLC of C (750 mg) on octadecyl silica with methanol water stepwise gradient resulted in the elution of an active fraction D (200 mg) in water-methanol (6 : 4). Further HPLC of D (200 mg) run isocritically in water-methanol (6 : 4), afforded the antihypertensive agent (**80**) (27 mg) and a chromatographically similar, inactive constituent (**81**) (43 mg).



80, R_1 = O, R_1 = H
81, R = α-OH, R_1 = H
82, R = O, R_1 = H
83, R = β-OH, R_1 = Me

To facilitate complete characterization and the attainment of optical purity, (**80**) and (**81**) were esterified with diazomethane and the esters (**82**) and (**83**), respectively, purified by preparative TLC (benzene-dioxane, 5:4). It was unequivocally proved that (**82**) and (**83**) were methyl esters of PGE_2 (**3**) and $PGF_{2\alpha}$ (**6**) by comparing them with the ^{13}C NMR, ^{1}H NMR, TLC and mass and optical rotation data. $PGF_{2\alpha}$ (**6**) was present 0.07-0.10 % (dry weight) of the alga (*G. lichenoides*) and PGE_2 (**3**) 0.05-0.7 (dry weight of the alga).

3. Mammalian-Type Prostaglandins in Marine Organisms

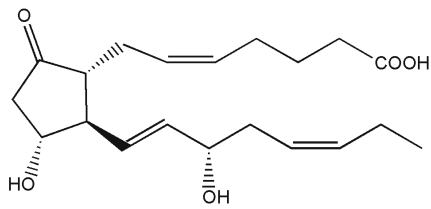
Prostaglandin E_2 and $PGF_{2\alpha}$ have also been isolated from the other marine microorganisms. The mammalian prostaglandin $F_{2\alpha}$ (**36**), and prostaglandin E_2 (**84**) were found in marine algae and invertebrates (Table 1). This is particularly interesting because the biosynthetic studies have shown that some marine organisms utilize completely different pathways than those of mammals for the formation of these prostaglandins. The marine invertebrate species were found to have a modest ability to convert exogenous eicosatrienoic acid to PGE_1. The PGE_2 **(84)** was isolated from the Australian red alga *Gracilaria lichenoides* as 0.05-0.07% of the dry weight of the alga.[73] Structure of PGE_2 **(84)** was determined by chemical studies. The structure of methyl ester of PGE_2 (**84**) was confirmed from the combination of spectroscopic data, including ^{1}H NMR and optical rotation. Furthermore, it was hypothesized that the algae may use a lipoxygenase-initiated pathway to synthesize these prostanoids,[73] in similarity to the pathway now believed to occur in *Plexaura homomalla*[74-76] and in contrast to the route in mammalian system.

The Japanese red alga *Gracilaria uerrucosa* is commonly eaten, but its ingestion causes a severe gastrointestinal problems known as ŏgonori poisoning.”The constituents responsible for the these type of problems were isolated from *Gracilaria uerrucosa* using a mouse bioassay. The main agent shown to be identical to PGE_2 (**84**) by spectroscopic methods and partial degradations however, the absolute stereochemistry was not investigated.[77] Prostaglandins are known as an emetic agents in many animal systems, and it is for this reason that corals apparently produces various prostaglandins as an antipredatory defense.[78] A patented process for the isolation of PGE_2 (**84**)

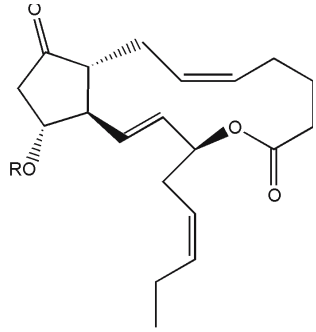
Table 1. Mammalian-type prostaglandins in marine organisms

S. No	Organism	Organism type	Prostaglandin derivatives	References
1	*Plexaura homomalla*	Soft coral	PGA/PGB/PGE/ PGF_{α}	86-89
2	*Euplexaura erecta*	Soft coral	PGF_{α}	74
3	*Gracilaria uerrucosa*	Red alga	PGE	74
4	*Anthoplexaura* sp.	Soft coral	PGE	90
5	*Terpios zeteki*	Sponge	PGF, PGE	81
6	*Cymphoma gibbosum*	Mollusc	PGA/PGB	91
7	*Modiolus demissus*	Mollusc	PGA/PGE	91
8	*Aplysia californica*	Mollusc	PGF, PGE	81
9	*Tethys fimbria*	Mollusc	PGA/PGE/ PGF_{α}	83-85, 92
10	*Mytilis* sp.	Mussel	PGE	90
11	*Homarus* sp.	Lobster	PGE	90
12	*Strongylocentrotus intermedius*	Sea urchin	PGF, PGE	93
13	*Strongylocentrotus nudus*	Sea urchin	PGF, PGE	93
14	*Crassostrea gigas*	Oyster	PGF_{α}	94
15	*Pactinopectin yessoensis*	Scallop	PGE	86
16	*Halocynthia roretzi*	Tunicate	PGF1, PGE	86
17	*Lobophytum depressum*	Soft coral	PGF_{α}	95
18	*Lobophytum carnatum*	Soft coral	PGA/PGB	96

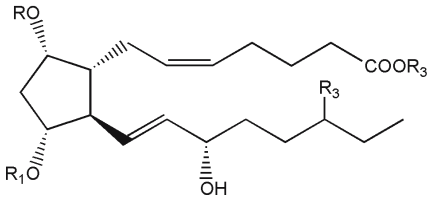
from *G. uerrucosa* has appeared.[79] *Euglena gracilis* an marine organism with both animal and plant like biochemical characteristics, was found to contain PGE_2.[80] It has been observed that the production of PGE_2 (**14**) as well as other eicosanoids in *E. gracilis* (PGF_2, **36**) is greater in cells grown in the dark than those grown in the light. It has been suggested that their production may be the result of this organism's animal type metabolic capacities. PGE_2 (**84**) have also been isolated from aqueous extracts of the sponge *Terpios zeteki*.[81] The mantles of the nudibranch *Tethys fimbria* were found to contain a rich diversity of prostanoids, including PGE_2 (**84**) and PGES as well as the corresponding lactones, PGE_3 1,15 lactone (**85**) and PGE_3 1, l5 Blactone 11-acetate (**86**). Structures of all these compounds were determined on the basis of spectroscopic analysis and comparison with their synthetic derivatives. Japanese coral *Euplexaura erecta,* was also found be a source of $PGF_{2\alpha}$ derivative (**36**).[74] The compounds were identified by comparison of spectroscopic data with the analytical data of the authentic sample of PGF_2 and several derivatives using TLC and GC-MS. The 9-O-acetate of methyl $PGF_{2\alpha}$ (**35**) and methyl-11-acetoxy- PGF_2, (**37**) were isolated from the Red Sea soft coral *Lobophyton depressum* and their structure were determined principally by ^{1}H NMR. The *S* stereochemistry at C15 was established by the combined use of biological and chemical properties of the hydrolysis product of PGF_2 (**36**). Three additional prostanoids such as methyl 11,18-diacetoxy-PGF_2 (**38**), 11-acetoxy-PGF_2 (**39**), and 11,18-diacetoxy-PGF_2 (**87**) were also isolated and characterized by the use of spectroscopic methods.



84



85, R = H
86, R = Ac



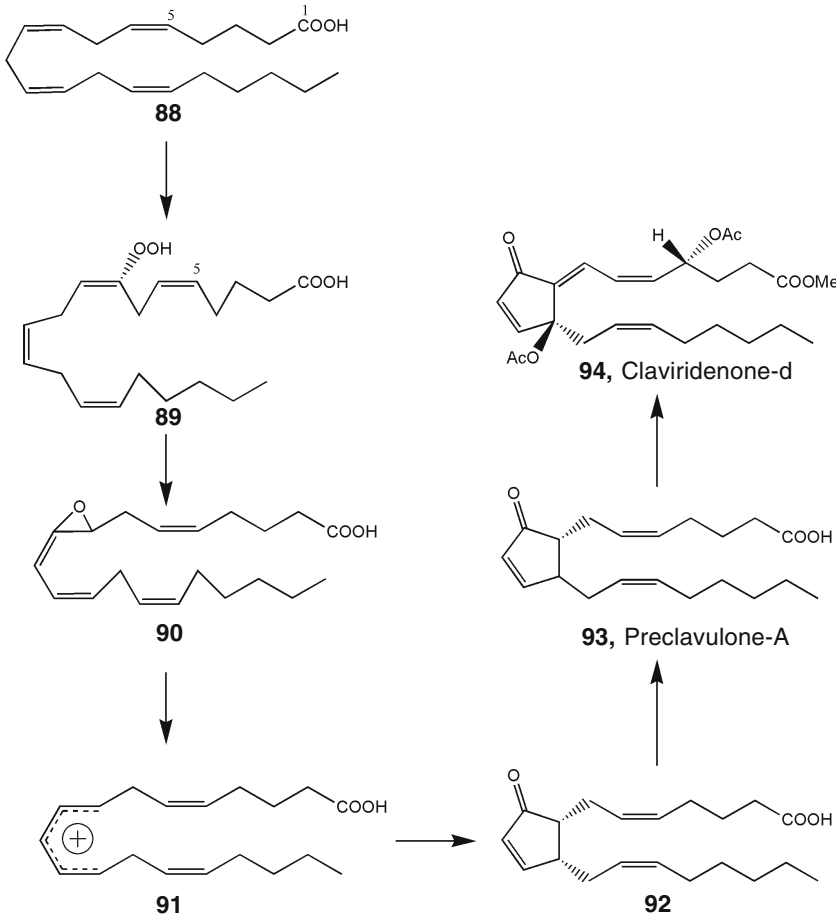
87, R = H, R_1 = Ac, R_2 = H, R_3 = H

PGF_2 (**36**) was also isolated along with PGE_2 from the Australian red alga, *Gracilaria lichenoides* and the structure was identified by detailed spectroscopic analysis of its methyl ester derivatives. The aqueous extracts of the sponge *Terpios zeteki* were found to contain $PGF_{2\alpha}$ (**36**) and 6-keto-PGF_1 along with PGE_2 (**84**).[81] Same two prostanoids were isolated using similar methodology from the aqueous extracts of the mollusc *Aplysia californica.* It has been observed that branchial tissues of the tunicate *Halocynthia roretzi* metabolize exogenous 14C-labeled eicosa-8, 11, 14-trienoic acid to PGF1-like compounds.[82] The nudibranch *Tethys fimbria* contains prostaglandin-1, 15-lactones of the A and E series. In addition of these prostaglandins they contains F series as well, including PGF_2, 1,15-lactone-11-acetate and PGF_3, 1,l-blactone-11-acetate.[83] In addition of this, the reproductive tissues and eggs of this nudibranch were shown to contain high concentrations of long-chain fatty acid esters of PGF_2 and PGF_3.[84,85]

4. Biosynthesis

The biosynthesis of mammalian prostaglandins is well established. Although marine organisms use the same precursors for the biosynthesis of marine prostaglandins, the mechanisms of the formation of these compounds are different. Corey et al[97] have shown that under suitable conditions 8,11,14-eicosatrienoic acid (**24**) or arachidonic acid (**25**) is converted to PGA (**11**) or PGA_2 (**12**) respectively by homogenates of the gorgonian *Plexaura homomalla* (Espers). These workers[98] have further studied the role of symbiotic algae of *P. homomalla* in prostaglandins biosynthesis. It was found that no appreciable amounts of prostaglandins were detected in the whole cells of the algae. There could be two possible roles of the algae to the coral. The synthesis of

fatty acid precursors, such as arachidonic acid and then release them to the coral for the biosynthesis of the prostaglandins. The second possibility is to biosynthesize the prostaglandins themselves. The former role seems more reasonable in view of the widespread occurrence of C20 polyunsaturated acids found in marine algae. The experiments conducted revealed that the prostaglandin synthetase is contained in the coral cells, not in the algal cells. The delineation of the biosynthesis of PGA_2 (**12**) in *P. homomalla* had been complicated by several practical difficulties stemming mainly from the enormous speed of proteolytic self digestion. Biosynthesis of claviridenone-d (**92**) in the Pacific coral *Clavularia viridis* was studied.[99] It has been shown that the coral converts arachidonic acid (**88**) to 8-(R~~h~~ydroxyperoxy-5) 11,14-(Z), 9-(E)-eicosatetraenoic acid (**89**), [(*R*)-HPETE] and, hence, to preclavulone-A (**93**) from which claviridenone-d (**92**) could arise by a series of three hydroxylation (at C4, C7 and C12) followed by esterification and elimination of C7 oxygen by analogy with the biosynthesis of the plant regulation *cis*-jasmonic acid[100-102] it appears probable that the conversion of



(**89**) to (**93**) occurs by way of allene oxide (**90**) and oxido-pentadienyl cation (**91**) intermediate. 8-Epoxygination-oxide pentadienyl cation cyclization route is believed to be general for the biosynthesis of marine prostanoids. Preclavulone-A (**93**) is possibly a precursor not only of claviridenones but of other prostanoids as well, and thus may serve as a Primary' marine prostanoid. The identification and role of these prostanoids is of unusual interest since they may well serve a crucial biochemical function in marine biology.

Plausible biosynthetic sequence, arachidonic acid 8,l5-*bis*-HPETE corresponding 8,9-allene oxide corresponding 9-*oxo*-8,9,10,1l,12-pentadienyl cation PGA_2.

5. Concluding Remarks

Prostaglandins play important regulatory role in many normal cellular functions. They are synthesized locally on demand, perform a tissue-specific function, and then rapidly inactivated by metabolic enzymes. They display a wide range of biological effects in concentrations as low as 10^9 .

Weinheimer and Spraggins reported the first high yield isolation of nonmammalian-type (15*R*) PGA_2 and (15*S*) PGA_2 methyl ester acetate from the Caribbean gorgonean *Plexaura homomalla.* Smaller quantities of PGB_2 and PGE_2 are also isolated from this source. Prostaglandin derivatives are also reported from other marine organisms, including a red alga. There is great interest in *P. homomalla* as a potential commercial scale source of PGs either by pruning in natural or by marine farming. *Plexaura homomalla* (Esper) occurs in two forms, *P. homomalla* var. (*R*) and *P. hommalla* var (*S*). The former yields biologically inactive (15*R*)-PGA_2 whereas the latter gives the biologically active (15*S*) PGA_2. Synthetic procedures have been developed for the conversion of (15*R*) PGA_2 and (15*S*) PGA_2 into biologically important prostaglandins E_2 and $F_{2\alpha}$. It is now possible to synthesize PGE_2 from (15*R*) PGA_2 in three steps and $PGF_{2\alpha}$ in four steps. Marine prostanoids are, thus, rich source for synthesizing biologically useful prostaglandins and their analogues.

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