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Preface

In this volume, seven topics of general interest to medicinal chemists, microbiologists, pharmacologists and clinicians are reviewed. During the past few years, much interest has been shown in phosphodiesterase Type IV inhibitors, mainly for their therapeutic potential in the treatment of asthma; these are reviewed in Chapter 1.

Progress in organ transplantation is largely dependent on controlling the body's natural tendency to reject the new organ. The mode of action and biosynthesis of the immunosuppressant cyclosporins are reviewed in Chapter 2. Carbapenems are chemically closely related to penicillins and in Chapter 3 their present status in the treatment of bacterial infection is surveyed.

Recent progress in the treatment of breast cancer by inhibiting aromatase is reviewed in Chapter 4. The search for better drugs to treat schizophrenia continues. Central dopamine D_2 antagonists with associated and predominant serotonin $5HT_2$ antagonism, as illustrated by risperidone, may represent a new class of potentially useful antipsychotics and are reviewed in Chapter 5.

Our understanding of the role of protein tyrosine kinases and their inhibition of cell multiplication by a wide variety of compounds is incomplete; the review in Chapter 6 suggests that further work in this field may well be rewarding. Work on a recently synthesized series of compounds (based on a natural product) which showed promise as inhibitors of squalene synthase – an enzyme involved in the formation of cholesterol – is described in Chapter 7.

We wish to thank our authors for reviewing the often extensive literature of their topics. We hope that their efforts will be appreciated by both newcomers and those who wish to update their knowledge. Finally, we are grateful for permission to reproduce material which is protected by copyright, and for the co-operation of our publisher.

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G.P. Ellis
D.K. Luscombe

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1 Phosphodiesterase Type IV Inhibitors

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INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs*; E.C. 3.1.4.17) which were discovered more than 30 years ago [1], are the enzymes which degrade the second messengers cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) and cyclic 3′, 5′-guanosine monophosphate (cyclic GMP) to 5′-AMP and 5′-GMP, nucleotides which are unable to activate the protein kinase cascade. As second messengers, cyclic AMP and cyclic GMP play key roles in the functional responses of cells to many hormones and neurotransmitters. The cyclic AMP cascade is depicted in *Figure 1.1*. Seven cyclic

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*The following abbreviations are used in this review: AIDS, acquired immune deficiency syndrome; AMP, 3′, 5′-adenosine monophosphate; ARDS, adult respiratory distress syndrome; BAL, bronchial alveolar lavage; BHR, bronchial hyper-reactivity; con-A, concanavalin A; COPD, chronic obstructive pulmonary disease; CNS, central nervous system; DRL, differential reinforcement of low rate; ECP, eosinophil cationic protein; FEV_1, forced expiratory volume (1 minute); fMLP, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage-colony stimulating factor; GMP, 3′, 5′-guanosine monophosphate; HCP-1, high affinity, cyclic AMP-specific phosphodiesterase I; IBMX, 3-isobutyl-1-methylxanthine; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; MBP, major basic protein; MEPI (ENHA) erythro-9-[2-hydroxy-3-nonyl]adenine; MNL, mononuclear leukocyte; PAF, platelet activating factor; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; PMN, polymorphonuclear neutrophils; RT-PCR, reverse-transcription polymerase chain reaction; SAR, structure-activity relationship; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; TNF, tumour necrosis factor; TSH, thyroid stimulating hormone; VLA_4, very late antigen-4.

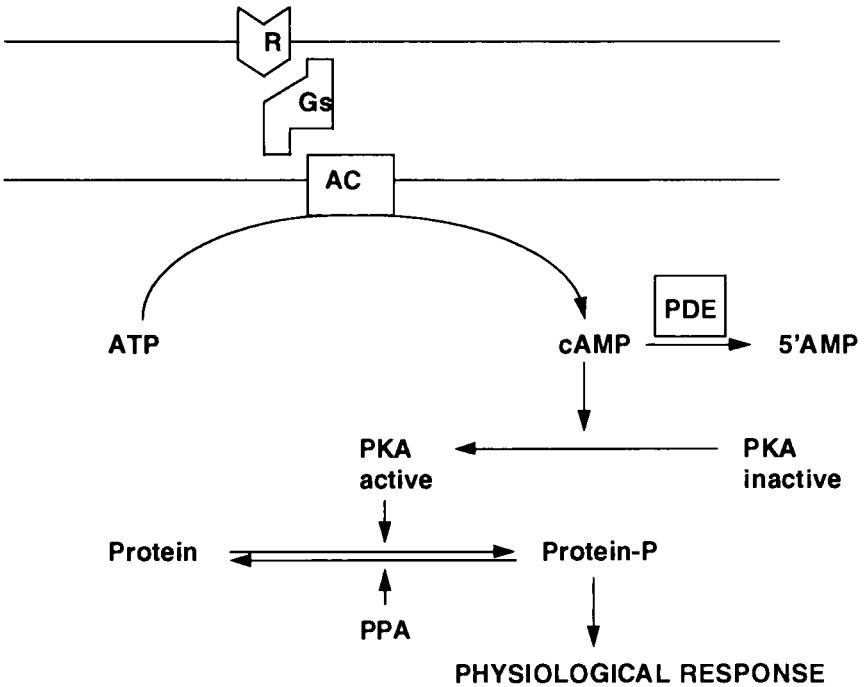


Figure 1.1. The cyclic AMP cascade showing the role of cyclic AMP PDE. Hormones, neurotransmitters, mediators, etc. bind to specific receptors (R) coupled to adenylate cyclase (AC) through a stimulatory G-protein (Gs). AC catalyzes the conversion of ATP to cyclic AMP which binds to the regulatory subunit of cyclic AMP-dependent protein kinase (PKA), so uncoupling it from the catalytic moiety. The activated PKA phosphorylates specific intracellular protein substrates which alters their activities. This results in an altered cellular response, such as smooth muscle relaxation or suppression of inflammatory cell functions (see text). The action of cyclic AMP is abrogated by cyclic AMP phosphodiesterase (PDE) which hydrolyses the 3'-phosphodiester bond on cyclic AMP, so converting it to the inactive 5'-AMP. Protein phosphatases (PPA) act to dephosphorylate proteins and counteract the actions of cyclic AMP.

nucleotide PDE families have been identified each of which comprises several distinct but related gene products [2]. Regulation of cyclic nucleotide metabolism is thus far more complex than originally envisaged permitting more selective interactions with pharmacological agents.

Major current interest centres around selective inhibitors of cyclic AMP-specific PDE IV, mainly as a result of their potential utility in inflammatory diseases such as asthma. A market opportunity in this disease may be offered by the perceived liabilities of β-adrenoceptor agonists [3, 4]

and glucocorticoids [5]. Furthermore, the inhibitory effects of PDE IV inhibitors on the release of Tumour Necrosis Factor (TNFα) [6, 7], a cytokine that has been implicated in pathological conditions including septic shock [8], cachexia [9], and arthritis [10], indicates wide-ranging therapeutic potential for this class of compounds.

This review summarizes the biochemistry, molecular biology, regulation and pharmacology of PDE IV. The structure-activity relationships (SARs) of known groups of compounds against PDE IV are discussed in detail, as are the *in vitro* and *in vivo* actions of PDE IV inhibitors which have led to the current guarded optimism of their therapeutic potential. Finally, the side-effects of these compounds which may have an important impact on the eventual clinical utility of PDE IV inhibitors are critically assessed.

BIOCHEMISTRY

CLASSIFICATION OF PDE ISOENZYMES

As alluded to in the introduction, multiple PDEs have been identified [11, 12]. These isoenzymes differ in their substrate specificity, kinetic properties, responsiveness to endogenous regulators (Ca^{2+}/calmodulin, cyclic GMP) and susceptibility to inhibition by various compounds. Molecular biological studies have demonstrated that many cyclic nucleotide PDEs are separate gene products with multivariant regulatory (Ca^{2+}/calmodulin, cyclic GMP binding sites) and other ill-defined domains linked to highly conserved (>60% amino acid identity) and homologous catalytic sequences (approximately 270 amino acids) which are located near the carboxyl terminus of the enzyme. Several attempts to categorize PDE isoenzymes have been made, the most widely adopted system of nomenclature being that proposed by Beavo and Reifsnyder [11] which was recently updated by Michaeli *et al.* [12] and Beavo [2]. Seven major families have been proposed, although it is likely that these will be added to. The families are designated by the Roman numerals I, II, III, IV, V, VI and VII and correspond to Ca^{2+}/calmodulin-stimulated-, cyclic GMP-stimulated-, cyclic GMP-inhibited-, cyclic AMP specific-, cyclic GMP-specific-, photoreceptor- and rolipram-insensitive (cyclic AMP-specific)-PDEs, respectively. Members of one family share 20-25% sequence homology with members of another. These families contain two or more related subfamilies (designated with a capital letter) which are derived from similar (70-90% homology) but distinct genes. Furthermore, several of the subfamilies have multiple members (designated

with Arabic numerals) produced by alternative mRNA splicing or different start sites for translation of the protein. A brief description of the seven PDE families identified thus far is presented below.

PDE I Isoenzyme Family

PDE I, or Ca^{2+}/calmodulin-stimulated PDE, is a family of isoenzymes which, as the name implies, is activated by calmodulin; micromolar concentrations of Ca^{2+}/calmodulin can induce a marked increase in catalytic activity (5 to 20-fold) [13, 14]. Kinetic analysis has demonstrated that this is due to an increase in V_{max} as well as slight decrease in K_m [13, 14]. At least four different members of the PDE I family (59 kDa, 61 kDa, 63 kDa, 75 kDa forms) have been described [11] and cDNAs encoding these proteins have been isolated and characterized [2].

PDE II Isoenzyme Family

Cyclic GMP-stimulated PDEs display a low affinity for both cyclic AMP (K_m: 30-100μM) and cyclic GMP (K_m: 10-30 μM) and exhibit positive homotropic co-operativity with respect to substrate [15, 16]. This effect is related to the high-affinity binding of cyclic GMP to an allosteric site (K_d: ~0.1 μM). Relatively low concentrations of cyclic GMP (1-5 μM) stimulate cyclic AMP hydrolysis. A 4.2 -kilobase pair cDNA encoding a cyclic GMP-stimulated PDE has been cloned from a bovine adrenal cortex library [17].

PDE III Isoenzyme Family

The cyclic GMP-inhibited PDE family is characterized by its high-affinity for cyclic AMP (K_{ms}: 0.1-0.5 μM) and cyclic GMP (K_{ms}: 0.1-0.5 μM) (V_{max} greater for cyclic AMP than for cyclic GMP) and competitive inhibition of its cyclic AMP hydrolytic activity by cyclic GMP and certain positive inotropic agents [18]. PDE IIIs with similar characteristics have been isolated from a variety of tissues. Molecular biological studies have provided evidence for multiple molecular forms of PDE III [19, 20].

PDE IV Isoenzyme Family

Cyclic AMP-Specific PDE exhibits a high-affinity for cyclic AMP (K_m 0.5-2 μM) but has weak affinity for cyclic GMP (K_m > 50 μM) [21]. Since PDE IV as a possible target for therapeutic intervention is the basis of this review, a

discussion of the properties, molecular biology and regulation of PDE IV will be allocated a separate section (see below).

PDE V Isoenzyme Family

Cyclic GMP PDEs have been purified from lung and platelets [22, 23]. They display substrate selectivity for cyclic GMP (K_m: 4-6 μM), exhibiting little activity on cyclic AMP. As with PDE II, a high-affinity, non-catalytic cyclic GMP binding site exists on PDE V, the function of which is uncertain. Recently, the structure of a bovine lung cyclic GMP-binding, cyclic GMP-specific PDE was deduced from a cDNA clone [24].

Table 1.1. PROPERTIES AND SELECTIVE INHIBITORS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOENZYMES

	PDE Family [a]	*Km (μM)*		*Selective Inhibitors*	*Reference*
		cAMP	*cGMP*		
I.	Ca^{2+}/calmodulin-stimulated	2-70	2-20	vinpocetine trifluoperazine[b] KS-505a	[27] [28] [29]
II.	cGMP-stimulated	30-100	10-30	MEPI (ENHA)[c]	[30]
III	cGMP-inhibited	0.1-0.5	0.1-0.5	siguazadon, SK&F 94120 SK&F 95654 milrinone	[31] [32] [33]
IV	cAMP-specific	0.5-2	>50	rolipram Ro 20-1724 nitraquazone denbufylline RP 73401	[34] [34] [35] [36] [37]
V	cGMP-specific	>40	1.5	zaprinast SK&F 96231	[27] [38]
VI	Photoreceptor	>500	17-20	zaprinast	[39]
VII	Rolipram-insensitive cAMP-specific	0.2	-	None	

[a] Nomenclature based on Beavo and Reifsnyder [11]
[b] Trifluoperazine inhibits PDE I by acting as a calmodulin antagonist.
[c] MEPI/ENHA: erythro-9-[2-hydroxy-3-nonyl]adenine

PDE VI Isoenzyme Family

Photoreceptor PDEs are located exclusively in retinal rods and cones where they play an important role in visual transduction, hydrolysing cyclic GMP upon stimulation by transducin [25]. They display selectivity for cyclic GMP (K_{ms}: 17-20 μM) although they are less discriminating than PDE V (K_m: cyclic AMP: 600-2000 μM). As with PDEs II and V, non-catalytic cyclic GMP binding sites are associated with rod PDE VI [11, 25]. Three retinal PDEs have been isolated: membrane-bound rod, soluble rod and cone PDEs. They are composed of several subunits in which catalytic (α, β and α') or inhibitory activity (γ) reside. A δ-subunit of unknown function also co-purifies with PDE VI [26]. cDNA clones for rod α-, β-, γ and δ subunits as well as the cone α- and γ subunit of PDE VI have been sequenced, cloned and expressed [2, 26].

PDE VII Isoenzyme Family

A gene recently isolated from a human glioblastoma cDNA library was expressed in a cyclic AMP PDE-deficient strain of *Saccharomyces cerevisiae* [12]. This novel gene, named *HCP-1* (*h*igh affinity, *c*yclic AMP-specific *p*hosphodiesterase **1**), encodes a cyclic AMP PDE (K_m = 0.2 μM) which is insensitive to cyclic GMP and inhibitors of the PDE III (amrinone, milrinone) and PDE IV (rolipram, Ro 20-1724) isoenzyme families. Although *HCP-1* shares sequence homology with the catalytic domain of all cyclic nucleotide PDEs, it does not share extensive homology to the Drosophila dunce cyclic AMP PDE. Thus, based upon these unique characteristics, *HCP-1* appears to represent a member of a previously unrecognised PDE family designated PDE VII [12] (see Table 1.1).

PROPERTIES OF PDE IV ISOENZYMES

PDE IV has been considered to be a relatively poorly defined enzyme due to it being present in only trace amounts within cells, its apparent localization in different subcellular compartments and its disposition to proteolysis during purification procedures. As a consequence, purification has proved to be difficult. The availability of inhibitors such as rolipram which selectively inhibit PDE IV have greatly aided the study of this family of enzymes; however, the greatest advances in our understanding of the nature of mammalian PDE IV have emanated from recent molecular biological studies.

Molecular biology

The characterization of the 'dunce' mutation in *Drosophila melanogaster* and the ras complementation studies in yeast [40] have provided invaluable tools for the characterization of mammalian PDE IV. Using 'dunce' PDE IV cDNA probes, four rat clones were isolated which have now been confirmed as transcripts of four different genes [33]. The four rat PDE IVs have highly conserved central sequences corresponding to the catalytic domain but have less homologous N- and C-termini [40]. The original rat cDNA clones, designated rpdes 1-4, have been reclassified according to the nomenclature of Beavo and Reifsnyder [11] and are now referred to as PDE IV_A (rpde2), PDE IV_B (rpde4), PDE IV_C (rpde1) and PDE IV_D (rpde3) (Table 1.2). More recently, four human PDE IV subtypes (hPDE IV_{A-D}) have been identified and characterized [41, 42] which bear great structural homology to their rat counterparts (Table 1.2). Using human cDNA probes, the chromosome locations of the different PDE IV genes have been determined in humans and mice (Table 1.2). Northern blotting and reverse-transcription polymerase chain reaction (RT-PCR) studies have

Table 1.2. HUMAN (h) AND RAT (r) PDE IV SUBTYPES

PDE IV subtype	*Alternative nomenclature*	*Human chromosome location*	*Reference*
hPDE IV_A	monocyte PDE IV*	19 (mouse 9)	[61]
	DPDE2 (PDE46*/TM3)		[42]
rPDE IV_{A1}	RD1, rpde2*		[49]
rPDE IV_{A2}	RD2*		[62]
rPDE IV_{A3}	RD3*		
hPDE IV_B	brain PDE IV*	1p31 (mouse 4)	[63]
	DPDE4 (PDE32*/TM72)		[42]
rPDE IV_{B1}	DPD*		[64]
rPDE IV_{B2}	rpde4* (same as IV_{B1}?)		[49]
hPDE IV_C	DPDE1 (PDE21)	19 (mouse 8)	[42]
rPDE IV_C	rpde1		[49]
hPDE IV_D	DPDE3 (PDE39/PDE43)	5q12 (mouse 13)	[42]
rPDE IV_{D1}	rpde3.1*		
rPDE IV_{D2}	rpde3.2(internal deletion of pde3.1?)		[49]

Information on the chromosomal locations of hPDE IV subtypes in humans and mice (in parenthesis) are from [149] and [150].
* Product of 1 or more alternatively spliced mRNAs of subfamily gene.
RD, 'rat dunce-like'. DPD, 'dunce-like' phosphodiesterase.

demonstrated that transcripts for the four PDE IV variants of different sizes are differentially expressed between tissues [42, 43].

The molecular size of purified PDE IV, as revealed by separation on polyacrylamide gel electrophoresis, varies between 26 and 82 kDa [34, 40, 44–47]. Although limited proteolysis of the native protein undoubtedly accounts for some of these discrepancies, the recent appreciation of the molecular basis for the marked PDE IV heterogeneity within and between tissues provides an alternative explanation. Northern blot analysis of mRNA from different cells and tissues demonstrated the existence of transcripts of different sizes for each of the four PDE IV subtypes [48–51]. Five different transcripts each for PDE IV_C and PDE IV_A have been demonstrated in germ cells and three classes of PDE IV_A with different 5′ ends have been obtained from brain cDNA libraries [48, 52]. Furthermore, at least three PDE IV_D cDNAs with different 5′ ends have been detected [53]. Multiple transcriptional start sites and alternative splicing of the newly transcribed nuclear RNA are responsible for producing the heterogeneity of the PDE IV subtype mRNAs [53].

The physiological significance of the resultant different N-termini of the translated PDE IV subtypes is currently being elucidated. The unique N-terminal domain of PDE IV_A appears to target the protein to the membrane [54, 55]. No evidence has emerged indicating that the membrane association is due to the existence, on the N-terminal extension, of a target site for post-translational modification such as farnesylation which is important in the membrane binding of PDE VI [56]. Certainly, association with the particulate fraction is not dependent upon acylation of cysteine residues within this domain [57]. Studies on the organization of the PDE IV_D gene [58] have demonstrated that different transcriptional units code for proteins regulated by cyclic AMP through different mechanisms. One transcriptional unit directs the synthesis of a protein rapidly activated by phosphorylation, while the other directs the synthesis of a truncated protein which is constitutively active [58]. The *de novo* synthesis of the latter can be induced by cyclic AMP (see below).

The quaternary structure of the native PDE IV is uncertain. Although early studies failed to detect any association of the kidney PDE monomer (60 kDa) [44], analysis of purified rat brain or rat heart cytosolic PDE IV by gel filtration or SDS-PAGE indicates that the the enzyme can exist as a monomer (~44 kDa) or dimer (89 kDa) [34]. In crude preparations, a more complex quaternary structure may exist [34]. Similar studies on human recombinant PDE IV_A reveal protein molecular sizes of either 320 or 88 kDa, suggesting that this enzyme can exist as a homotetramer [59]; however, the lack of co-operativity demonstrated in kinetic studies on this enzyme do

not support this conclusion. Indeed, two recent studies have provided evidence that the quaternary structure of human PDE IV_A is indeed dimeric [47, 60].

High-affinity rolipram binding site

Until recently, rolipram was considered to be a relatively weak (K_i ~0.5-1 μM), competitive inhibitor of PDE IV; however, the existence of a stereoselective, high-affinity (K_d ~1 nM) binding site for rolipram has been appreciated since 1987 [65]. Although its nature was (and still is) uncertain, the failure of protein purification techniques to separate it from cyclic AMP hydrolytic activity strongly suggests that it is associated with PDE IV [65]. Furthermore, its importance is indicated by the close correlation between the potency order of diverse cyclic AMP PDE inhibitors in displacing [^{3}H] rolipram from brain membranes and their central [66] and peripheral [67, 68] actions.

That high-affinity rolipram binding is associated with cyclic AMP PDE was demonstrated unambiguously in studies on human recombinant PDE IV_A and PDE IV_B [63, 69]. Interestingly, the nature of binding differs between the two PDE IV subtypes. Only a single high-affinity (K_d ~2 nM), non-interacting (n_H = 1) binding site for rolipram was demonstrated on PDE IV_A, although a low-affinity binding site (K_d = 40 nM) is also present [69]; however, similar studies performed on hPDE IV_B, revealed two non-interacting high-affinity rolipram-binding sites (K_{ds} = 0.4 and 6 nM) [63].

Studies on recombinant PDE IV suggest that the rolipram binding site and catalytic site are distinct entities [69]. Firstly, the affinity of rolipram for its binding site on human PDE IV_A is approximately 100-fold greater than for inhibition of catalytic activity [69]. Furthermore, the rank order of potency of structurally dissimilar compounds in displacing [^{3}H] rolipram does not correlate with their inhibition of cyclic AMP hydrolysis [69]. Interestingly, replacement of catalytically important histidine residues on PDE IV_A by site-directed mutagenesis decreases enzyme activity, increases the proportion of low-affinity binding and decreases the proportion of high-affinity rolipram binding [70].

The relevance of the high-affinity rolipram binding site remains a mystery. One possibility is that it represents an allosteric site. An alternative paradigm proposes that PDE IV can exist in at least two non-interconvertible tertiary or quaternary forms, with similar catalytic properties, against which rolipram and similar compounds bind with widely different affinities [71]. The possibility that the high-affinity site does not

influence catalytic activity and serves some other function cannot be totally eliminated. This latter possibility would appear unlikely, however, in view of studies on the eosinophil PDE IV. In these studies, it was demonstrated that the rank order of potencies of compounds in inhibiting the tightly membrane-bound PDE IV in this cell-type and displacing [^{3}H] rolipram binding to brain membranes are poorly correlated [68]; however, the relationship is greatly improved when the PDE IV inhibitory potencies of compounds are tested against enzyme that has been solubilized or exposed to vanadate/glutathione [68]. The altered kinetic properties of the eosinophil PDE IV induced by these treatments suggest that it can exist in different conformational states [68]. Thus, although [^{3}H] rolipram binding to the eosinophil PDE IV has yet to be demonstrated, these results support the conclusion that the influence which the high-affinity rolipram binding site exerts on catalytic activity is dependent upon the enzyme conformation.

REGULATION OF PDE IV

Regulation by cyclic AMP

Agents that elevate cyclic AMP accumulation exert two types of control on cyclic AMP PDE - long-term and short-term upregulation. Short-term activation of cyclic AMP PDE is one of several mechanisms to rapidly turn-off the cyclic AMP signal, whereas enzyme induction occurs as cells attempt to adapt to prolonged elevation of the second messenger. The well-documented induction of cyclic AMP PDE occurs after prolonged (2 or more hours) exposure of cells to cyclic AMP analogues or agents that elevate intracellular levels of the second messenger and is blocked by inhibitors of mRNA or protein synthesis [72]. Upon removal of the stimulus, cyclic AMP PDE activity decays slowly to basal levels over several hours; for example, PDE IV is induced by incubating U937 cells with the β-adrenoceptor agonist, salbutamol, prostaglandin E_2, or 8-bromo-cyclic AMP [72]. The magnitude of the response of activators of adenylate cyclase is enhanced by inhibiting cyclic AMP hydrolysis with rolipram [72]. Northern analysis of mRNA has demonstrated that multiple PDE IV subtypes (PDE IV_B and PDE IV_D) are induced following exposure of rat Sertoli cells by a variety of agents that elevate cyclic AMP [51].

Several studies [73, 74] have demonstrated activation of cyclic AMP PDE by agents that increase cyclic AMP which, because of the rapidity of the response (minutes), cannot possibly be attributed to *de novo* synthesis of protein. Although the most obvious mechanism for this short-term

activation is protein phosphorylation (as has been demonstrated for PDE I, PDE III and PDE V), only recently has the phosphorylation of PDE IV been unequivocally demonstrated [58, 74]. In FRTL-5 thyroid cells, thyroid stimulating hormone (TSH) activates a 93 kDa cyclic AMP PDE through cyclic AMP-dependent phosphorylation [74]. The phosphorylated protein appears to correspond to a splice variant of rat PDE IV_D (rpde3.3) which possesses an extended N-terminus with a consensus sequence for phosphorylation by PKA [58, 74]. Indeed, phosphorylation and activation of recombinant rpde3.3 by the catalytic subunit of PKA has been demonstrated in a cell-free system [58]. Two other splice variants of PDE IV_D (rpde3.1 and rpde 3.2) which do not possess the extended N-terminus (translated products ~70 kDa) are not phosphorylated by PKA [51]. To underscore the complexity of PDE IV regulation by agents that elevate cyclic AMP, mRNA for both rpde 3.1 and rpde 3.2 are induced by exposing FRTL-5 cells to TSH, whereas mRNA levels of rpde3.3, the constitutive PDE IV_D splice variant in these cells, remains unaltered [58].

Regulation by cytokines and other pro-inflammatory stimuli

Increased cyclic AMP PDE activity is observed in mononuclear cells (monocytes and macrophages) in response to histamine [73], lipopolysaccharide (LPS) [75] and cytokines (IFNγ/Il-4) [76, 77]. The effects are observed within 1 hour, suggesting that the mechanism leading to cyclic AMP PDE activation is distinct from the induction of PDE IV by cyclic AMP observed in U937 cells and Sertoli cells. Indeed, histamine induces cyclic AMP PDE activation in mono nuclear leukocytes (MNL) within minutes [73]. Interestingly, although this inflammatory mediator stimulates cyclic AMP accumulation, PKC rather than PKA has been implicated in its activation of cyclic AMP PDE [78]. Activation of cyclic AMP PDE in human peripheral blood lymphocytes and rat thymocytes has been observed in response to proliferative agents, concanavalin A (con A) and phytohaemaglutanin (PHA) [79, 80].

The increased cyclic AMP PDE activity may facilitate the pro-inflammatory actions of cytokines and proliferative agents by lowering the levels of a second messenger known to antagonize their actions. In monocytes from atopic individuals, cyclic AMP activity is elevated [81–83]. The reason for this is uncertain, although a genetic defect (cyclic AMP PDE may be novel) or response to the allergen-associated inflammation have been offered as possible explanations [84]. Reduced β-adrenoceptor responsiveness [81], as well as enhanced release of histamine, IL-4, PGE_2

and IgE have been linked to the increased cyclic AMP PDE in MNL from atopic individuals [85–88].

Regulation by phosphatidic acid

Incubation of one but not the other of the two forms of PDE IV from U937 cells for 5 min results in increased cyclic AMP hydrolysis [89]. In contrast, lysophosphatidic acid, which (like phosphatidic acid) has been proposed to have signalling functions, inhibits PDE IV [89]. Although the K_i of rolipram against U937 cell PDE IV was in the order of 1 μM, it inhibited the phosphatidic acid-induced activation at concentrations as low as 10 nM [89]. The lipid A component of LPS, bears structural similarities to phosphatidic acid and some of its actions have been attributed to mimickry of this bioactive lipid [90]. Incubation of murine macrophages with LPS inhibits PGE_2-stimulated cyclic AMP accumulation by activating PDE IV [75]; however, it is unlikely that this is related to the activation seen with phosphatidic acid in a cell-free system since a prolonged incubation period (30-60 min) is required for LPS to elicit its effect in macrophages.

ASSAY OF PDE IV

The PDE IV isoenzyme is found in many tissues and cell types and especially in inflammatory and immunocompetent cells (see p.20). In studies on cells or tissues containing several PDE isoenzymes, purification or partial purification is required before further investigation can proceed. Typically, PDE IV is separated from other isoenzymes by one or more column chromatography techniques and the PDE activity measured by one of several assay methods. A commonly used assay is the two-step radioisotope method first described by Thompson *et al.* [45]. In this procedure, PDE IV activity is assayed at 30°C in a reaction mixture containing: 10 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, [^{3}H]-cyclic AMP, together with any other addition (endogenous regulator, inhibitors, etc.). In the first stage of the assay, [^{3}H] cyclic AMP is converted to 5′-AMP by added cyclic AMP PDE. In the second stage, the [^{3}H] 5′-AMP is dephosphorylated to [^{3}H] adenosine by the 5′-nucleotidase activity in snake venom which is added to the assay mixture. The [^{3}H] adenosine formed at the end of this second stage of the assay is thus a measure of the cyclic AMP PDE activity. [^{3}H] Adenosine is separated from [^{3}H] cyclic AMP substrate on an anion exchange column (Dowex) and quantified by liquid scintillation spectrometry.

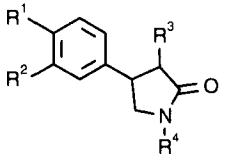
STRUCTURE -ACTIVITY RELATIONSHIPS OF PDE IV INHIBITORS

ROLIPRAM ANALOGUES

Rolipram and close structural analogues

Although the archetypal PDE IV inhibitor rolipram (1) was first described as a PDE inhibitor in 1976 [91], detailed SAR data were not reported until 1989 [92]. Indeed the first SAR report of a PDE IV inhibitor in 1971 [93]

Table 1.3. ROLIPRAM STRUCTURE-ACTIVITY RELATIONSHIPS
Values represent measurements made on PDE IV from bovine aorta [92]. Bz = benzyl; IA = inactive.



Compound	R1	R2	R3	R4	IC-50 μM
(1)	MeO	cPentylO	H	H	5
(3)	H	H	H	H	IA
(4)	H	nBuO	H	H	IA
(5)	nBuO	H	H	H	IA
(6)	nBuO	HO	H	H	IA
(7)	nBuO	MeO	H	H	IA
(8)	MeO	MeO	H	H	53
(9)	MeO	nBuO	H	H	13
(10)	MeO	isoBuO	H	H	18
(11)	MeO	BzO	H	H	96
(12)	MeO	MeO	Me	H	22
(13)	MeO	MeO	COOEt	H	21
(14)	MeO	MeO	COOH	H	IA
(15)	MeO	MeO	H	Ph	19
(16)	MeO	MeO	H	Bz	17

related to Ro-20-1724 (2). Rolipram and Ro-20-1724 share similar SARs at their catechol ring substituents and the data for rolipram and analogues [92] are summarized in Table 1.3. The unsubstituted (3) and monobutoxy (4, 5) derivatives were inactive, as was the free phenol (6). These results (when compared with the 3, 4-dimethoxy analogue (8)) clearly indicate that alkoxy groups must be present in both the meta and para positions relative to the pyrrolidinone ring. The inactivity of the 4-butoxy derivative (7), as well as that of other analogues with rather bulky para substituents, emphasise the steric constraints at this position. It was found that steric bulk could be tolerated at the 3-position, the highest potency being conferred by a cyclopentyloxy group as in rolipram (1). A benzyloxy group, as in (11) was less favourable. Given its easy accessibility, the 3, 4-dimethoxy substituent pattern in (8) was retained whilst changes were made to the pyrrolidinone ring substitution. Substitution as 3-methyl (12) and 3-COOEt (13) increased activity but the acid (14) was devoid of activity. Surprisingly the *N*-phenyl (15) and *N*-benzyl (16) analogues were also more active than the parent (8). Removal of the carbonyl group in the pyrrolidinone ring abolished all activity.

In the investigation of SARs in Ro-20-1724 (2) and analogues [93], activity was found to be optimal with the bulky 3-butoxy group (IC_{50} 0.1 μM; rat erythrocytes). It was also found that the 2, 3-dimethoxy compound and the addition of a methoxy group at position 5 led to the abolition of activity. On the imidazolidinone ring, the addition of an acid group at the ring junction with the catechol group, also abolished activity. Both enantiomers were active, most activity residing in the (-) form. When rolipram and Ro-20-1724 were tested alongside each other in the bovine aorta assay [92], rolipram was found to be five times more active.

Further compounds, including rigid analogues of (8), were then investigated [92] and their activities are collected into Table 1.4. The most active compound was found to be the *trans* isomer (20), but still only half as active as (8). Modelling studies of overlays generated with rolipram, Ro-20-1724, (20) and the potent, non-selective PDE inhibitor trequensin (21), led to a pharmacophoric model for potent PDE IV inhibition [92]. An adapation of this to a more general model [94] is shown in *Figure 1.2*. This model has the carbonyl dipole (a′) orientated nearly coplanar with the catechol ring at a distance of 8.6Å from the 4-methoxy oxygen atom. A small alkyl group is allowed at (f′) and a larger one at (e′), (preferably cyclopentyl) and (b′), (preferably benzyl). Medium sized, preferably non-polar groups, are allowed at the α-lactam position (c′) and unsaturation at the benzylic position (d′) is not imperative.

This pharmacophoric model also fits for a series of rolipram/Ro-20-1724

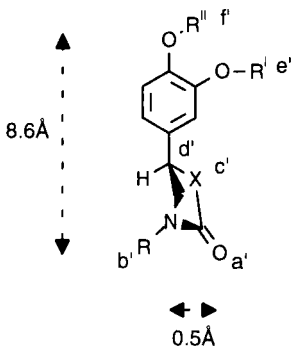
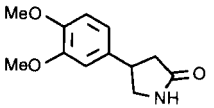
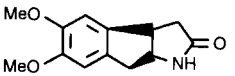
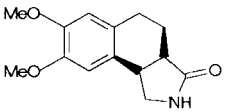
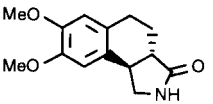
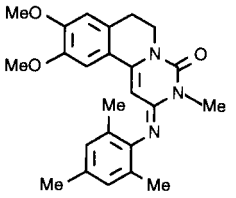
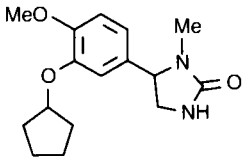


Figure 1.2. Pharmacophoric model proposed by Torphy et al. [94] for PDE IV inhibition

Table 1.4. RIGID ANALOGUES OF ROLIPRAM
Values represent measurements made on PDE IV from bovine aorta [92]

Compound		IC-50 μM
(8)		53
(18)		800
(19)		180
(20)		138
(21)		0.4

hybrids [95] (X = NH), typified by the 1-methyl-2-imidazolidinone (22). When tested on an enzyme preparation from rat cerebral cortex, (22) was found to have a PDE IV activity of 0.33 μM (rolipram IC_{50} 0.49 μM). SAR studies were also undertaken to further examine the steric requirements at



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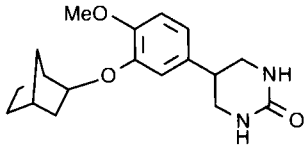

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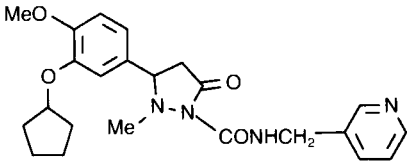

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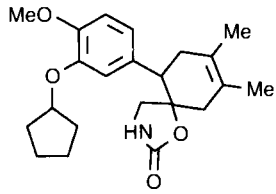

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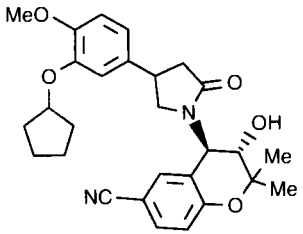

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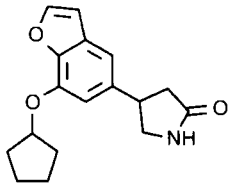


(27)

the (e') position of the model (*Figure 1.2*). Activity was found to be optimal for an *exo*-norbornyl group (IC_{50} 0.22 μM). In addition to their activity at the PDE IV catalytic site, the imidazolidinones also bind to the rolipram binding site (see Biochemistry section) with a very high affinity (K_d = 2-10 nM). As an extension of this work, it was found that PDE IV catalytic activity could be retained [96] when the imidazolidinone group was expanded to the 3, 4, 5, 6-tetrahydropyrimidin-2-(1H)-one (23). The PDE IV activity (canine tracheal muscle) of a series of 5-pyrazolidin-3-ones has been reported [97], a typical analogue being (24) (IC_{50} 85 nM). It has subsequently been reported [98] that the S-form of (24) is the eutomer (IC_{50} 9 nM) isolated as as the maleate salt (WAY-127093A). The pulmonary anti-allergic and anti-inflammatory properties of S-(24) are also described.

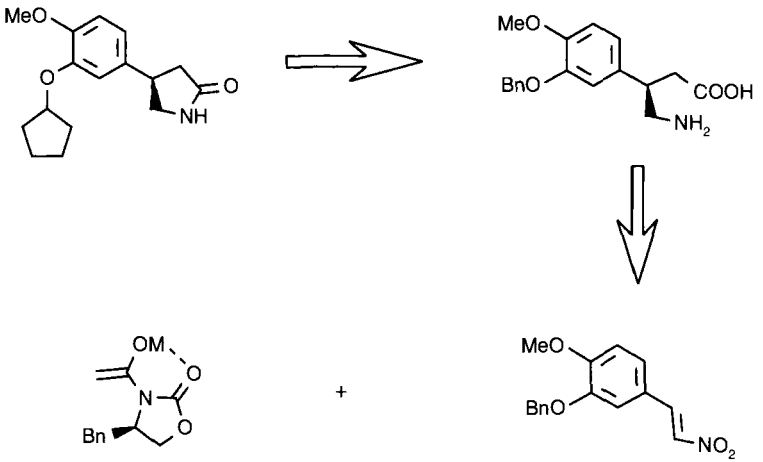


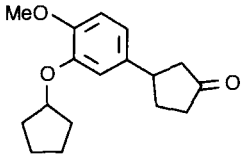
Figure 1.3. An enantiospecific retrosynthesis of rolipram [109]

The novel spirolactam (25) has been reported [99] to have potent PDE IV inhibitory activity (IC_{50} 100 nM). Rolipram analogues with a variety of pyrrolidinone *N*-substituents have been reported, for example, *N*-hydroxy [100], *N*-methoxymethyl [101] and *N*-(4-aminobenzyl) [102], with PDE IV activity in the μM region. The wide range of possible rolipram *N*-substituents is further exemplified by the report [103] of the cromakalim (a potassium channel activator)/rolipram hybrid (26) with PDE IV activity (IC_{50} 2μM). In a study designed [104] to explore further the interaction of the catechol ethers of rolipram with the PDE IV enzyme, the groups were combined as cyclic ethers and this led to a loss in activity. It was argued that this loss stemmed from the unfavourable orientation of the electron lone pairs associated with the oxygen atoms of the catechol; optimal binding occurring when the dipoles are orientated in the same direction. In order to test this hypothesis, cyclic analogues, in which the favourable orientation of the lone pairs is present, were synthesized and the benzofuran (27) restored PDE IV activity (K_i 0.26 μM; rolipram 0.19 μM; recombinant human PDE IV enzyme).

It was found that the (-) enantiomer of rolipram (presumed to be the *R*-configuration) is the antipode primarily responsible for the pharmacological effects [105–107]. The first direct evidence of the *R*-absolute stereochemistry has been obtained from an X-ray determination of the *N*-(4-bromobenzyl) derivative of the rolipram enantiomers [108]. Interestingly the *R*-(+) enantiomer, derived from the *R*-(-) enantiomer of rolipram,

was found to be a poor inhibitor of PDE IV (IC_{50} 74 μM; human monocytes), whilst the (*S*)-(-) enantiomer was much more potent (IC_{50} 0.17 μM); obviously the bulky group on the rolipram nitrogen has a profound effect upon the active conformation of the respective enantiomers. Further studies using other substituents on nitrogen are required to confirm that this is a general effect.

Two chiral syntheses of rolipram have appeared [109, 110]; the more versatile synthesis of Mulzer *et al.* [109] employs as the main step an enantioselective Michael addition of a chiral enolate to a Ω-nitrostyrene. The retrosynthetic sequence is depicted in *Figure 1.3*.



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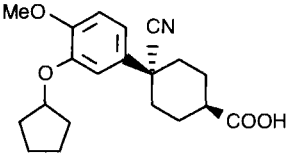

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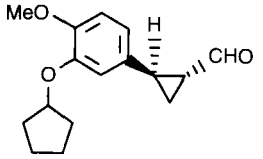

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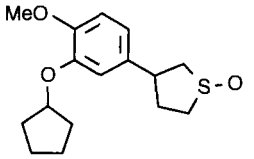
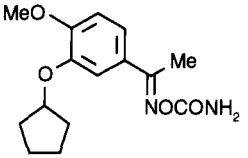


(31)

Cycloalkanes

A series of SmithKline Beecham patents [111–117] disclose novel cycloalkanone analogues of the rolipram pyrrolidinone heterocycle. The earlier patents [111, 112] describe cyclopentanones and cyclohexanones, for example (28), with PDE IV activity in the low μM range (human monocytes). In a series of five later patents [113–117], cyclohexanes with a cyano group α to the phenyl ring together with a variety of 4-substituents were described. The compounds demonstrated the inhibition of TNFα production *in vitro* (human monocytes) and PDE IV inhibition values were claimed to be in the low μM-nM range but without any precise values. The compound of most interest is the cyano acid (29) [115], which has been designated SB 207499. As well as being a potent inhibitor of PDE IV (K_i 92 nM), (29) also inhibited the LPS-induced release of TNFα from human peripheral monocytes (IC_{50} 0.11 μM) [118].

Rhone Poulenc Rorer workers [119] have reported a series of cyclopropane analogues, typified by (30) to have PDE IV inhibitory activity in the nanomolar region (pig aorta). The same group [120] has also reported cycloalkane analogues, in which the carbonyl group has been replaced by sulfoxide. Again the activity is in the nanomolar region and the analogue (31) is a preferred example. It is interesting to speculate that the sulphoxide of (31) binds at the enzyme in a similar fashion to the carbonyl group of cyclopentanone (28) and rolipram.



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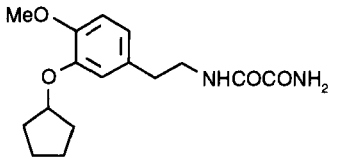


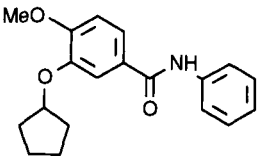
(33)

Acyclic analogues

A molecular modelling analysis of rolipram led to the identification of a novel series of oxime-carbamates and carbonates [121]. The analogue PDA-641 (32) possesses high PDE IV inhibitory activity (IC_{50} 48 nM, canine trachealis) and has entered Phase1 clinical trials for the prophylactic treatment of asthma. SmithKline Beecham have reported [122] a series of oxamide analogues e.g. (33) to be selective PDE IV inhibitors with IC_{50} values ranging between 50 nM and 40 μM (human monocytes).

Benzamides

Workers at Rhone Poulenc Rorer [37] have described a series of very potent benzamide analogues designed from the lead compound (34). The SAR's for substituent changes in the *N*-phenyl ring are summarized in Table 1.5. Introduction of single substituents into the *N*-phenyl ring gave up to a 20-fold increase in Type IV inhibitory potency whilst retaining good

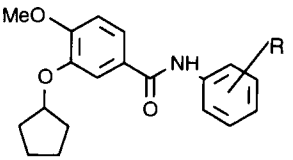


(34)

selectivity for the isoenzyme (when compared to types I, III, and V). The rank order of potency was found to be ortho > meta > para substitution for the chloro compounds (35-37). 2, 6-Disubstitution in the *N*-phenyl ring, as in (38), led to a further increase in potency. This substitution pattern was superior to both 2, 3- (39), and 2, 5- (40) dichloro substitution. Other disubstituted analogues, for example, 2, 6-dimethyl (41), 2, 6-dibromo (42) and 2, 6-difluoro (43) also retained high potency. Changes to the catechol groups led to similar SAR's as that seen for rolipram [92] and Ro-20-1724 [93], with maximal potency residing in a 4-methoxy substituent together with a bulky 3-cycloalkoxy group, for example, cyclopentyloxy and *exo*-2-norbornyloxy [37]. Up to a further ten-fold increase in potency was observed when the *N*-phenyl ring was replaced by a pyridyl group; the most potent compound being the 3, 5-dichloro-4-pyridyl benzamide (44) (RP 73401) with a PDE IV IC_{50} inhibitory activity of 1 nM. This aortic PDE IV inhibition correlated well with the eosinophilic PDE IV (IC_{50} 0.8 nM). RP 73401 also possesses bronchodilating activity [37] and these PDE IV selective benzamides offer the exciting possibility of a dual anti-inflammatory/bronchodilator mode of action.

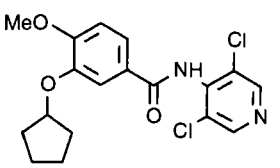
Whereas replacement of the benzamide linkage with thioamide retained activity [37], a homologous amide ($CONHCH_2$) and ester (COO) led to a complete loss of activity, indicating the tight SAR's in this part of the

Table 1.5. BENZAMIDE STRUCTURE-ACTIVITY RELATIONSHIPS
Values represent measurements made on PDE IV from pig aorta [37].



Compound	R	IC-50 μM
(34)	H	2.6
(35)	4-Cl	>1000
(36)	3-Cl	1.3
(37)	2-Cl	0.19
(38)	2,6-diCl	0.023
(39)	2,3-diCl	>200
(40)	2,5-diCl	0.5
(41)	2,6-diMe	0.1
(42)	2,6-diBr	0.4
(43)	2,6-diF	0.07

molecule. In a subsequent publication [123], a much wider range of linkages were investigated and the most potent found to be the styryl (CH = CH) and ketomethylene ($COCH_2$) groups (unpublished data). Given that these two groups most closely resemble the amide linkage, they can be considered to be isosteric replacements. Pfizer [124] and Celltech [125] have also disclosed similar styryl analogues. Celltech [126] also disclose compounds in which the styryl group is further substituted with a phenyl group as in (45) and with a cyano group [125] as in (46). Unfortunately, no PDE IV data are



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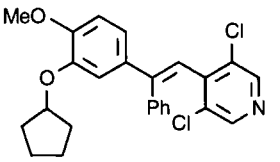

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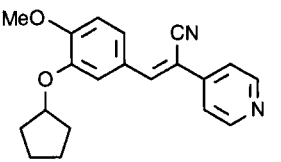
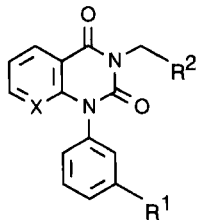


(46)

divulged in these patent publications. Pfizer [124] and Celltech [127] have also disclosed compounds in which the aryl rings are directly connected as biphenyl analogues. Although no specific PDE IV inhibition data are divulged for these analogues, their SARs must be different from those of the styryl analogues.

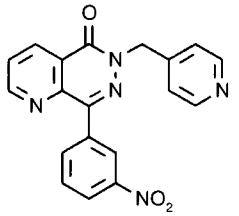
QUINAZOLINE DIONES AND ANALOGUES

Nitraquazone (47) (PDE IV IC_{50} 1.9 μM (rat brain)) is the archetypical quinazoline dione, representative of a structurally distinct family of PDE IV inhibitors (47-51) to rolipram. It was hypothesized [128] that the nitro group of (47) could overlay the lactam moiety of rolipram to explain its PDE IV activity. The nitro group was successfully replaced with an ester (48), but an acid or *N*-methylamide were inactive. Although the analogous pyridopyrimidine dione (49) was less potent than (48), replacement of R^2 with groups of



	X	R^1	R^2
(47)	CH	NO_2	Me
(48)	CH	COOMe	Me
(49)	N	COOMe	Me
(50)	N	COOMe	Ph
(51)	N	NO_2	4-pyridyl

higher lipophilicity substantially increased potency, the highest potency residing in the *N*-benzyl analogue (50)(PDE IV (rat brain) IC_{50} 40 nM). Subsequently [129], reintroduction of a nitro group together with a 4-pyridyl group at R^2 as in (51) increased potency even further (PDE IV (human lymphocyte) IC_{50} 0.28 nM). Activity against PDE IV is not limited to quinazoline diones; as shown by the pyridopyridazinone (52) [130] (IC_{50} 5 nM; human lymphocyte), the naphthyridin-2(1H)-one (53) [131] (IC_{50} 0.38 μM; porcine cardiac ventricle) and the very potent quinoline (54) [132] (IC_{50} 0.023 nM; human lymphocyte). Furthermore, a series of 2-aryloxypyridine-3-carboxamides (55), effectively ring open variants of (50), have shown PDE IV activity between 0.2 and 10 μM [133]. A wide variety of substituents R can be accommodated in the meta and para positions of the aromatic ring.



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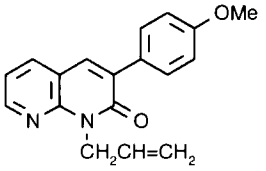

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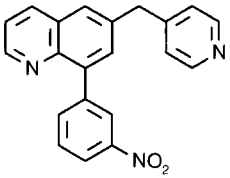

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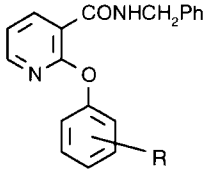

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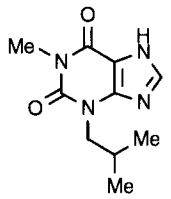
XANTHINES

Xanthines have been used in the treatment of bronchial asthma for over a century [134] and were the first group of compounds reported to possess PDE inhibitory activity. The most potent xanthine IBMX (56) is a relatively weak and non-selective PDE inhibitor (Table 1.6) [135]. The xanthines are also potent adenosine receptor antagonists [136] and this property contributes to their narrow therapeutic margin of efficacy. Much effort has been put into the search for more potent and selective analogues. Denbufylline (57) shows surprisingly selective PDE IV activity (Table 1.6) [137] without the adenosine receptor activity common to many alkyl xanthines. Derivatization at the *N*-7 or C-8 amino sites of the selective PDE



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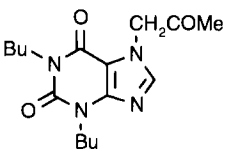

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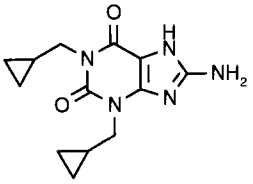

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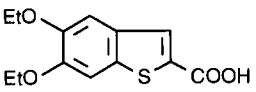

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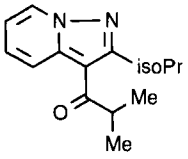

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IV inhibitor BRL 61063 (58) provided PDE IV inhibitors with varying degrees of PDE V, adenosine receptor and high-affinity rolipram binding site activity [138]. More recently [138a] workers at Almiral have reported a series of xanthines, typified by LAS-31025 (structure not disclosed), to be selective PDE IV inhibitors.

MISCELLANEOUS PDE IV INHIBITORS

Tibenalast (59), a weak inhibitor of PDE IV (IC_{50} = 14.9 μM; guinea-pig PMN) is active in models of pulmonary function [139] in the guinea-pig.

Table 1.6. PDE INHIBITION BY XANTHINES

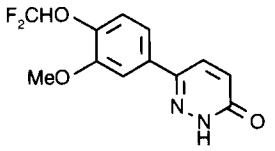
	IC_{50} μM			
	PDE I	*PDE III*	*PDE IV*	*PDE V*
IBMX (56)	7	3.4	12	11
Denbufylline (57)	> 100	> 100	0.8	10.8

Support for its potential anti-inflammatory activity is indicated by an inhibition of superoxide generation [140] from human neutrophils pre-treated with certain priming agents. Tibenalast has also shown a modest bronchodilator effect in severe asthmatics [141].

Ibudilast (60) is marketed in Japan as an anti-asthma drug [142], the mechanism of action of which is not fully understood. Although only a moderately potent inhibitor [143] of PDE IV (IC_{50} = 12 μM; bovine tracheal smooth muscle), ibudilast shares with rolipram several biological actions on eosinophils and airways smooth muscle which can be attributed to cyclic AMP PDE inhibition. It is likely that this action accounts, at least in part, for the anti-asthma properties.

MIXED PDE III/IV INHIBITORS

No detailed SARs have been described for these compounds. However, in general, compounds which possess dual PDE III/IV activity contain the catechol ether moiety of PDE IV inhibitors together with the flat topography required for potent PDE III inhibition [144]. The pyridazine zardaverine (61) has been found to be equiactive on PDE III (IC_{50} = 0.58 μM; human platelet) and PDE IV (IC_{50} = 0.79 μM; canine trachealis) [145]. Benzafentrine (62) is probably the most studied hybrid analogue [146]. It is



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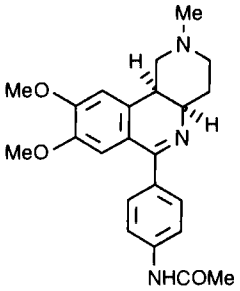

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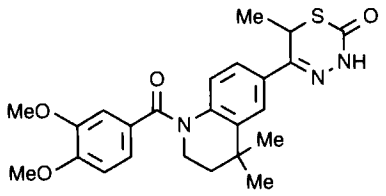

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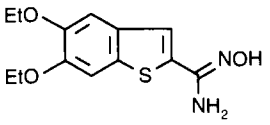

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unique amongst PDE III inhibitors in possessing a protonated amine moiety (at physiological pH) rather than the usual amide for interaction with the enzyme. Dual PDE III/IV activities for EMD 54662 (63) and the tibenalast analogue Org 30029 (64) have been reported [147, 148].

ANTI-INFLAMMATORY EFFECTS OF PDE IV INHIBITORS

DISTRIBUTION OF PDE ISOENZYMES IN INFLAMMATORY AND IMMUNOCOMPETENT CELLS

The distribution of PDE isoenzymes in inflammatory cells is shown in Table

Table 1.7. PDE ISOENZYME PROFILES IN INFLAMMATORY/IMMUNO-COMPETENT CELLS

Cell-type	*Species and Source*	*PDE isoenzymes present*	*References*
Mast cells	Murine - bone derived	I, IV	[151]
	Rat-peritoneal	V	[152]
Basophils	Human peripheral blood	III, IV	[153]
Eosinophils	Guinea-pig peritoneal	IV	[154,155]
	Human peripheral blood		[156]
Neutrophils	Human peripheral blood	IV	[157]
Monocytes	Human peripheral blood	IV	[158,159]
Macrophage	Guinea-pig peritoneal macrophage	IV	[160]
	Murine peritoneal macrophage	II, III, IV	[75]
B-lymphocytes		Not known	
T-lymphocytes	Rat T-cells	II,III,IV	[79]
	Rat T-cells	II, III, IV,V	[161]
	Human peripheral blood	IV	[80]
	Human peripheral blood	III, IV	[162,163]
	Human T-cell clones	IV, VII	[164,165]
Platelets	Human peripheral blood	II, III, V	[31,32]
			[166]
			[167]
Endothelial Cells	Bovine aorta, Porcine aorta	II, IV	[168,169]
	Foetal bovine aorta	II, IV, V	[170]
Epithelial cells	Bovine trachea	I, II, III, IV, V	[171]

1.7. PDE IV is widely distributed in cell-types implicated in chronic inflammatory diseases. Amongst the bone-marrow derived cells, only platelets do not express this isozyme. PDE III, which has been identified in basophils [153], murine (but not guinea-pig) macrophages [75], T-lymphocytes [79, 161–163], platelets [31, 32, 166, 167] and epithelial cells [171] is less widely distributed. PDE I (murine mast cells [151], epithelial cells [171]), PDE II (murine macrophages [75], rat T-lymphocytes [79, 161], platelets [31, 32, 166, 167], endothelial cells [168–170], epithelial cells [171]), PDE V (rat T-cells [161], platelets [31, 32, 166–167], epithelial cells [171]) and, perhaps, PDE VII (T-cells [165]) are also represented. Not surprisingly, PDE IV inhibitors have wide-spread effects on inflammatory cells.

ACTIONS OF PDE IV INHIBITORS ON INFLAMMATORY/IMMUNOCOMPETENT CELLS *IN VITRO*

PDE IV inhibitors suppress the functions of several cell-types implicated in allergic and inflammatory disorders (see Table 1.8).

Granulocytes

Activation of mast cells and eosinophils is thought to play an important role in the pathogenesis of allergic disorders such as asthma. The release of mediators (histamine, LTD_4) from sensitized mast cells is believed to be a primary cause of the acute bronchoconstriction in response to antigen exposure [187]. Evidence has also emerged that mast cells can release cytokines (IL-4, TNFα) which may contribute to the inflammatory responses in asthmatic late-phase reactions [188, 189]. The effects of PDE IV inhibitors on mast cells varies considerably depending on the tissue and species from which the cells are isolated. In murine bone-marrow-derived mast cells, rolipram inhibits antigen-induced LTC_4 release [151]; however, in functional studies on rat peritoneal mast cells, the PDE V inhibitor, zaprinast, but not rolipram suppresses mediator release [152]. Recently, studies in human lung mast cells have shown that rolipram only modestly inhibited histamine release although a significant elevation in cyclic AMP levels was observed [190]. In contrast, SK&F 95654, a PDE III inhibitor, elicited only a slight increase in cyclic AMP, but was more effective than rolipram in blocking histamine release [190]. There is no information on the effects of PDE IV inhibitors on mast cell-derived pro-inflammatory cytokines. In human peripheral blood basophils, rolipram inhibits antigen- and anti-IgE-induced mediator (histamine, LTC_4) release; an effect which is

Table 1.8. EFFECTS OF PDE IV INHIBITORS ON INFLAMMATORY CELLS

Cell Type	*PDE IV inhibitor effect*	*Comments*	*Reference*
Mast Cells	Inhibition of mediator release (M)	PDE V but not PDE IV inhibitors inhibit mediator release from rat peritoneal mast cells	[151,152]
Basophils	Inhibition of mediator release	PDE III inhibitors enhance the actions of PDE IV inhibitors	[153]
T-lymphocytes	Inhibition of proliferation (H)	PDE III inhibitors enhance the inhibitory effect of PDE IV inhibitors	[163]
	Inhibition of Il-2 expression (H)		[172,173]
B-lymphocytes	Not known		
Monocytes	Inhibition of:		
	Arachidonic acid breakdown (H)		[174]
	Phagocytosis (H)		[175]
	Superoxide generation (H)	PDE inhibitors weak suppressors of IL-1β release	[175]
	TNFα release (H)		[9,176,177]
Macrophages	Inhibition of:		
	Superoxide generation (GP)		[95,178]
	Mediator release (M)		[179]
	TNFα release (M)		[179]
Eosinophils	Inhibition of:		
	Superoxide generation (H/GP)		[155,154,156]
	Thromboxane release (GP)		[143]
	Chemotaxis (GP)		[180]
	Degranulation (MBP, ECP, EDN) (GP/H)		[181,182]
Neutrophils	Inhibition of:		
	Superoxide generation (H)		[183]
	Chemotaxis (H)		[184]
	Phagocytosis (H)		[175]
	Mediator release (H)		[185]
	Degranulation (H)		[182]
Endothelial cells	Reduction of permeability		[186]

GP - Guinea-pig, H - Human, M - Mouse.

potentiated by SK&F 95654 and forskolin [153]. Interaction studies of this nature have yet to be reported on human lung mast cells.

Eosinophilia is a prominent pathological feature of allergic diseases and is thought to contribute to the airways damage observed in asthma [187, 191]. The cytotoxic potential of the eosinophil derives from its ability to generate reactive oxygen species, peroxidase and cationic proteins [187, 191]. Eosinophil products induce epithelial damage and increase airway reactivity in experimental animals *in vivo* [187], fuelling speculation that they may contribute to the pulmonary hyper-reactivity observed in asthmatics. Several studies demonstrate that the cytotoxic potential of eosinophils is potently reduced by PDE IV inhibitors. Rolipram suppresses the generation of reactive oxygen species in both human [156] and guinea-pig [154, 155] eosinophils in response to particulate (serum opsonized zymosan) and several soluble stimuli. In a comparative experiment, RP 73401 was found to be six times more active [37] than rolipram in inhibiting guinea-pig eosinophil superoxide generation. In addition, LTB_4-induced release of thromboxane [143], major basic protein (MBP) and eosinophil cationic protein (ECP) [182] as well as hrC5a, platelet activating factor (PAF) and formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis [180] are potently inhibited by PDE IV inhibitors.

Neutrophils are important in host defence, accumulating rapidly at sites of injury or infection. The aberrant release of proteases and reactive oxygen species can lead to extensive tissue damage [192]. Neutrophils play important roles in acute and chronic inflammatory diseases, although their functions in allergic conditions is unclear. PDE IV inhibitors suppress several neutrophil responses including degranulation [182], superoxide anion generation [183], phagocytosis [175], chemotaxis [184] and mediator production [185].

Monocytes and macrophages

Tissue macrophages and their circulating precursors, blood monocytes, play three distinct but interrelated functions [193]. They recognize and remove inflammatory stimuli, act as antigen-presenting cells and release several pro-inflammatory factors, including hydrolytic enzymes, growth factors, lipid mediators, reactive oxygen species and cytokines. The capacity of mononuclear phagocytes to remove inflammatory stimuli determines whether an inflammatory response progresses to overt expression of the disease [193]. In comparison with their effects on eosinophils and neutrophils, PDE IV inhibitors are generally weak inhibitors of monocyte and macrophage functions. Suppression by PDE IV inhibitors of ara-

chidonic acid breakdown [174, 179], phagocytosis [175] and production of reactive oxygen species [78, 160, 175] has been reported. In contrast to the relatively weak inhibition of these functions, the generation of TNFα by both monocytes [6, 7, 176] and macrophages [179] is exquisitely sensitive to the inhibitory effects of PDE IV inhibitors. The mechanism by which these compounds and other agents that elevate cyclic AMP levels exert this effect is uncertain, although it is clear that suppression of TNFα mRNA expression occurs [7]. Inhibitory effects of PDE IV inhibitors at the transcriptional and post-transcriptional levels have been suggested [7]. Selective inhibitors of PDE III weakly inhibit LPS-induced TNFα generation, whereas PDE I and PDE V inhibitors are inactive [176]. In comparison with their effects on TNFα, PDE IV inhibitors exert little or no effect on the release of IL-1β or IL-6 [7, 176]. Inhibitory effects of PDE IV inhibitors on LPS-induced TNFα-release have also been reported in whole blood [177]. The large number of pathological conditions associated with excessive TNFα production, including arthritis, AIDS and endotoxic shock [194], suggest that PDE IV inhibitors have considerable therapeutic potential.

T- and B-lymphocytes

T-cells release several cytokines which are, at least in part, responsible for co-ordinating the cellular responses in inflammatory disorders such as asthma [195]. Studies in mice [196] and humans [197] have demonstrated that CD-4 positive T-helper (Th) lymphocytes can be functionally categorized into two groups based on the cytokines they elaborate: the Th-1 subtype is defined by its restricted cytokine production of IL-2, interferon (IFN)γ and TNFβ. These cells mediate delayed-type hypersensitivity reactions and are the major isolates from non-atopic donors [198]. The Th-2 subtype releases IL-4, IL-5, and IL-10 and constitutes the major isolates from atopic donors. In cognate and non-cognate interactions with B-lymphocytes Th-2 cells support IgE synthesis [199, 200].

PDE IV inhibitors (Ro 20-1724, rolipram) suppress PHA-stimulated proliferation of human peripheral blood T-lymphocytes [163]. In purified CD4+/CD8+ T-cells, incomplete inhibition is observed with rolipram alone; however, a much greater suppression of [^{3}H] thymidine incorporation is observed in combination with the PDE III inhibitor, CI-930 [163].

A number of studies have demonstrated that cyclic AMP PDE inhibitors suppress the release of IL-2 from T-cells [172, 173]. Whether this is the predominant mechanism by which PDE IV inhibitors exert their effects on

[^{3}H] thymidine incorporation into T-lymphocytes is unlikely since both rolipram and CI-930 inhibit con A-induced murine splenocyte proliferation at concentrations below those required to suppress IL-2 generation [173]. Indeed, cyclic AMP elevating agents may have multiple inhibitory effects on T-cell proliferation [173].

Few studies have investigated the specificity of PDE IV inhibitor actions on T-helper 1 and T-helper 2 derived cytokines. The majority of data suggest that agents that elevate cyclic AMP, including PDE inhibitors, are more effective suppressors of T-helper 1 cytokines (IL-2, IFNγ) than T-helper 2 elaborated cytokines (IL-4, IL-5) [201–205]. For example, the weak and non-selective PDE inhibitor, pentoxifylline, reduces IL-2, but not IL-4, release from rat T-cells [206]. However, recent studies paint a less clear picture of the relative effectiveness of PDE IV inhibitors in blocking the release of cytokines from these two classes of T-helper cells. For example, rolipram blocks the proliferation of peripheral blood mononuclear cells elicited by tetanus toxoid (Th-1) and ragweed (Th-2) antigens [207, 208]. Surprisingly, rolipram was more effective at suppressing the ragweed-driven proliferative response [207, 208]. Reverse transcription polymerase chain-reactions showed that the PDE IV inhibitor attenuated IL-5 and IFNγ, but not IL-4, gene expression following allergen provocation [208]. Somewhat at odds with these results is the finding that the selective PDE IV inhibitor, WAY PDA-641, suppresses the generation of IL-4 induced by anti-CD3 [209].

The effects of PDE IV inhibitors on B-lymphocyte function is poorly studied. Although Ro 20-1724 inhibits the spontaneous IgE generation by MNL from individuals with atopic dermatitis, the effect is probably indirect via monocytes [88].

Neural cells

Stimulation of bronchial C-fibres induces bronchoconstriction and inflammation, by means of a central reflex pathway and local release of the sensory neuropeptides, including substance P and neurokinin A [210]. Studies on the contractile response of guinea-pig bronchi to electrical field stimulation *in vitro* demonstrated that rolipram, but not siguazodan or zaprinast, reduces the release of pro-inflammatory peptides from C-fibre endings [210]. Since these neuropeptides increase microvascular permeability, mediator release, inflammatory cell recruitment and mucus secretion as well as contracting bronchial smooth muscle, this property of PDE IV inhibitors may have important implications for their *in vivo* effects.

Other cell-types

Although agents that elevate cyclic AMP generally dampen the functions of other cells associated with inflammatory disorders, such as platelets [211, 212], endothelial cells [213–215], and epithelial cells [216, 217], the effects of PDE IV inhibitors have not been extensively studied. Of interest is the report demonstrating that rolipram reduces albumin flux across endothelial monolayers [186], an effect which may explain the inhibition by PDE IV inhibitors of pulmonary capillary-ischaemia reperfusion oedema [218], and, *in vivo*, of microvascular leakage [219, 220].

ANTI-INFLAMMATORY EFFECTS OF PDE INHIBITORS *IN VIVO*

PDE IV and mixed PDE III/IV inhibitors exhibit anti-inflammatory actions in several animal models of inflammation. Early studies demonstrated that rolipram and similar compounds reduce the swelling in skin elicited by a number of stimuli [221, 222]. Responses to antigen, particularly in the airways, are effectively inhibited by PDE IV and mixed PDE III/IV inhibitors, fuelling speculation that these compounds will be effective anti-asthma agents [223–227]. In these studies, compounds were tested against the acute effects of antigen on mediator release [223], inflammatory cell accumulation [223, 224], microvascular leakage [225] and the consequential airways hyper-reactivity [224]. The relevance of these acute effects to the chronic human disease remains to be seen. Of great interest are studies in rodents [7, 226] and in humans [227] which demonstrate that PDE IV inhibitors potently suppress endotoxin-induced TNFα generation *in vivo,* thus extending their therapeutic potential to the many diseases in which this inflammatory cytokine is believed to be an important pathological factor [194].

Inhibition of mediator release

Rolipram, administered i.v. to sensitized guinea-pigs, inhibits antigen- but not LTD_4- or histamine-induced bronchoconstriction, suggesting that it exerts its inhibitory influence at the level of the mast cells and, perhaps, basophils rather than by relaxing bronchial smooth muscle [228]. In contrast, the PDE III inhibitor, CI-930, inhibits both allergen- and LTD_4-induced bronchoconstriction under identical conditions indicating a smooth muscle effect of this compound [228]. *In vitro* studies on tracheae isolated from sensitized guinea-pigs have shown that rolipram inhibits ovalbumin-elicited release of PGD_2 but not of histamine [223]. It should be

noted that PDE IV inhibitors do suppress mediator-induced bronchoconstiction when administered into the airways of guinea-pigs and rats as dry powders [229]. Reduction of passive cutaneous anaphylaxis (PCA) in the skin of rats, mice and guinea-pigs by PDE IV inhibitors, as well as PDE V inhibitors [230, 231], is also suggestive of inhibition of mediator release *in vivo*. Direct evidence for inhibition of mediator release by PDE IV inhibitors derives from studies in mice in which application of arachidonic acid to the ears of mice results in oedema and the production of lipid mediators (LTB_4 and PGE_2) [232]. The modest inhibitory effect of rolipram on mediator release was probably insufficient to account for its anti-inflammatory effect in this model.

Inhibition of microvascular leakage

The endothelium of post-capillary venules normally constitutes a tight barrier to blood cells and plasma proteins; however, during inflammatory episodes, endothelial cells contract in response to the release of mediators such as tachykinins, PAF and histamine following antigen challenge [233], leaving gaps through which cells and large molecular weight molecules can escape into the surrounding tissues. The increased microvascular permeability allows plasma proteins, including complement and blood-clotting factors with pro-inflammatory potential, to escape and the accompanying fluid exudation causes oedema [233].

Rolipram effectively suppresses allergen-induced microvascular leakage into the lungs of sensitized guinea-pigs [225]. Furthermore, PDE IV (rolipram) and PDE V (zaprinast) inhibitors, administered via the oral, intratracheal or intravenous routes, markedly attenuate microvascular leakage into the small and large airways tissue as well as into the bronchiol alveolar lavage (BAL) fluid [219, 220]. Inhibitors of PDE III (siguazodan, milrinone) and PDE I (vinpocetine) do not inhibit the microvascular leakage in guinea-pig airways elicited by PAF [219, 220]. A recent, preliminary report documents evidence showing that inhibitors of either PDE IV or PDE III inhibit the pulmonary oedema induced by LPS aerosols in guinea-pigs and it was suggested that, based on these results, such compounds might be useful in the treatment of adult respiratory distress syndrome (ARDS) [234]. In contrast to its effects in lung, rolipram does not inhibit oedema formation in guinea-pig skin following exposure to histamine, zymosan-activated plasma or PAF [235]. However, both rolipram and denbufylline suppress the rat ear swelling induced by arachidonic acid or croton oil [221, 222]. A preliminary report also demonstrates that in a hamster cheek pouch model, PDE IV and PDE

III/IV inhibitors attenuate the microvascular permeability evoked by bradykinin [236].

Inhibition of inflammatory cell accumulation

Antigen exposure leads to the accumulation of eosinophils in the lungs of asthmatics [187], a response that can be replicated in a variety of sensitized animals [223, 224, 229, 237]. Oral administration of selective PDE IV inhibitors or mixed PDE III/IV inhibitors reduces antigen-induced eosinophilia in the BAL fluid and lung tissue of guinea-pigs and Brown Norway rats [229]. Other routes of administration are also effective in reducing inflammatory cell influx. For example, subcutaneous injection of rolipram significantly inhibits the antigen-induced accumulation of eosinophils and neutrophils in cynomolgus monkeys [224], and administration of a micronized, dry-powder formulation of rolipram or RP 73401 directly into the airways of guinea-pigs suppresses the appearance of leukocytes into BAL fluid [229]. A wide-variety of mediators, cytokines and chemokines are chemoattractants for eosinophils [195]. Recent studies demonstrate that PDE IV and PDE III/IV inhibitors attenuate the eosinophilia induced in guinea-pig lungs by PAF, IL-5, IL-3, GM-CSF and TNFα [238–241]. Furthermore, zardaverine, a mixed PDE III/IV inhibitor, inhibits endotoxin-induced neutrophil accumulation in the lungs of rats, an effect which may be mediated by suppression of TNFα release into the BAL fluid [239] (see below).

Eosinophils and other inflammatory cells accumulate at sites other than the lungs following exposure to allergen and other stimuli. These responses are also attenuated by inhibitors of cyclic AMP PDE. For example, in a guinea-pig eye model of eosinophilia, rolipram (p.o.) suppresses the accumulation of eosinophils elicited by histamine and a combination of LTB_4 and LTD_4 in the conjunctival epithelium [242]. Rolipram also inhibits the accumulation of indium-labelled eosinophils, but not neutrophils, into the skin of guinea-pigs in response to zymosan-activated plasma, PAF and histamine [235]. Inhibitors of PDE III (SK&F 94120) and PDE V (zaprinast) are ineffective in this model of inflammation [235]. Finally, urate crystal- or LTB_4-evoked neutrophil accumulation into the peritoneum of mice is also attenuated by rolipram [232].

The mechanism(s) by which cyclic AMP PDE inhibitors suppress the accumulation of inflammatory cells elicited by allergen or other stimuli at sites of inflammation is uncertain. Inhibition of chemotaxis through a direct effect on leukocytes is one possibility for which there is some supportive *in vitro* evidence [180]. Alternatively, cyclic AMP PDE inhibitors may inhibit

the release of chemoattractant agents. Agents that elevate cyclic AMP reduce the number of endothelial cell surface adhesion molecules, either directly [243] or, perhaps, indirectly by inhibiting the release or the elaboration of cytokines such as TNFα [6, 176, 179] which upregulate their expression. Data from an eosinophilic cell line (EoL-1) demonstrate that cyclic AMP down-regulates expression of several integrins including VLA_4 [244]. This will effectively reduce the movement of leukocytes into inflammed tissues since adhesion is an important step in the process of diapedesis. Finally, there is evidence that rolipram may exert an effect on the development of eosinophil progenitors [245].

Inhibition of bronchial hyper-reactivity (BHR)

Selective PDE IV inhibitors as well as mixed PDE III/IV inhibitors suppress hyper-reactivity induced by antigen and other stimuli in animal models. In conscious, unrestrained guinea-pigs, i.p. administration of rolipram, as well as Org 20241 (PDE III/IV inhibitor) and theophylline, attenuate hyper-reactivity after both the early and late reactions [246]; however, in another study [237], the mixed PDE III/IV inhibitor, benafentrine, and theophylline were ineffective in reducing PAF-induced BHR although eosinophilia was substantially attenuated. Rolipram, administered by subcutaneous injection, although ineffective in blocking antigen-induced bronchospasm, significantly reduces BHR and eosinophilia in cynomolgus monkeys following repeat antigen challenge [224]. RP 73401, administered via the i.v. route, to anaesthetized guinea-pigs, inhibits PAF-induced bronchial hyper-reactivity to bombesin [229]. Studies in the clinic with ibudilast, which exhibits PDE IV inhibitory activity [143], suggest that compounds with cyclic AMP PDE inhibitory activity can suppress airway hypersensitivity in asthmatic subjects.

Inhibition of cytokine release

TNFα is a cytokine released from several inflammatory/immunocompetent cells, particularly monocytes and macrophages, whose over- production has been implicated as an important pathological factor in many diseases, including arthritis, AIDS and septic shock [194]. For this reason, intensive effort is being directed to the identification of pharmacological agents that can suppress the elaboration of TNFα. Infusion of pentoxifylline, a very weak and non-selective inhibitor of cyclic AMP PDE, into normal human volunteers decreases serum TNFα levels evoked by by injecting endotoxin [247]. No effects on IL-6 levels were observed. These findings have

prompted the clinical evaluation of this xanthine in AIDS patients [248]. Oral administration of rolipram suppresses LPS-induced hypothermia and production of TNFα in the serum of CD-1 mice [194, 226]. Antigen-induced increases in TNFα and IL-8 (but not IL-1β or IL-6) in the airways of cynomolgus monkeys are significantly reduced by subcutaneous injection of rolipram [224]. Recently, the results of a clinical study in which the xanthine cyclic AMP PDE inhibitor, BRL 61063, was tested for tolerability and TNFα production in blood stimulated *ex vivo* with LPS was reported [227]. An inhibitory effect of this compound was observed but only at doses where side-effects (nausea, vomiting and headaches) occurred [227, see below]. Whether the inhibition of TNFα production through cyclic AMP PDE inhibition is sufficient to explain the activities of theophylline [249] and ibudilast [250] in animal models of arthritis is uncertain.

ACTIONS OF PDE IV INHIBITORS ON AIRWAY SMOOTH MUSCLE

PROFILE OF PDE ISOENZYMES IN AIRWAY SMOOTH MUSCLE

The complement of PDE isoenzyme subtypes in airways smooth muscle from different species is shown in Table 1.9. Although early studies failed to detect the full complement of PDEs, it is now clear that in canine, bovine and human tissue, five isoenzyme families are represented [135, 251–255]. Only in one study on canine trachealis has the subcellular localization of PDEs in airway smooth muscle been investigated [251]. All five isoenzyme families are represented in the cytosolic fraction whereas only PDE I (β), PDE III and, perhaps PDE V are associated with membranes [251].

SMOOTH MUSCLE RELAXANT EFFECTS OF PDE IV INHIBITORS *IN VITRO*

Relaxant effects of PDE IV inhibitors have been reported on airway smooth muscle preparations from a variety of species. Their effectiveness depends upon the nature and concentration of the contractile agonist employed. Furthermore, the relaxant potencies of both PDE IV and PDE III inhibitors differ considerably in airway smooth muscle preparations from different species and airways levels [256]. Isoprenaline-induced relaxation and cyclic AMP accumulation are potentiated by treatment of canine trachealis with Ro 20-1724 and the PDE III inhibitors, SK&F 94120 and siguazodan [257, 258]. None of these cyclic AMP PDE inhibitors influence the relaxant effects of the guanylate cyclase activator, nitroprus-

side [257]. Additive or even synergistic relaxant effects of PDE IV inhibitors in combination with PDE III inhibitors have been reported in tracheal smooth muscle from a variety of species in the absence or presence of β-adrenoceptor agonists [257]. In human airway smooth muscle, no clear pattern for the spasmolytic activity of rolipram has emerged [256]. For example, employing smooth muscle preparations from all airways levels, evidence for [253, 259, 260] and against [256, 261, 262] a spasmolytic effect of rolipram has been documented.

In guinea-pig trachea, a poor correlation exists between the relaxant and PDE IV inhibitory potencies of compounds with diverse structures [67]. In contrast, an excellent correlation exists between the relaxant effects of these compounds and their abilities to displace [^{3}H]rolipram from its high-affinity binding site in brain membranes [67]. Possible reasons for this are discussed elsewhere in this review.

BRONCHODILATING ACTIONS OF PDE IV INHIBITORS

PDE IV inhibitors protect against the bronchoconstricting effects of contractile agonists in a number of different species including guinea-pig

Table 1.9. KINETIC PROPERTIES OF PDE ISOENZYMES IN TRACHEALIS FROM DIFFERENT SPECIES

		K_m app (μM)		
Isoenzyme	*Substrate*	*Canine*	*Bovine*	*Human*
PDE Iα	cAMP	absent[a]	absent[a]	10-20
	cGMP	absent[a]	absent[a]	0.5-1.5
PDE Iβ	cAMP	1	2.9	0.5-2
	cGMP	2	ND	1-5
PDE II	cAMP	93	56-105	70-100
	cGMP	60	51-85	10-50
PDE III	cAMP	0.3	absent[a]	0.6
	cGMP	8	absent[a]	5
PDE IV	cAMP	4	2.6-2.9	1.2
	cGMP	40	30	>100
PDE V	cAMP	135	126	>100
	cGMP	4	2.6-6	2-7

ND: Not determined

[a] Isoenzyme either absent or expressed in an amount too low to evaluate kinetically. Data taken from [251] (canine), [252] (bovine), [135] (bovine), [253] (human), [254] (human) and [255] (human).

[67, 229, 263], cat [264], rat [265] and dog [266–268]. However, as alluded to previously, although anti-inflammatory activity and suppression of antigen-evoked responses are routinely observed with rolipram administered via the oral route, suppression of the bronchoconstriction elicited by several contractile agonists in guinea-pigs is not always observed [223, 224]. Indeed, in cynomolgus monkeys, even antigen-induced bronchoconstriction is not inhibited by subcutaneous delivery of rolipram, although hyper-reactivity and eosinophilia are suppressed [224]. In contrast, rolipram and other PDE IV inhibitors suppress the bronchospasm evoked by a number of contractile agonists when administered to guinea-pigs as aerosols or dry powders directly into the airways [229]. Bronchodilating activity is observed when PDE IV inhibitors such as WAY PDA-641 are administered to rats (p.o., i.v. or i.d.) [265] and dogs (i.v.) [267, 268]. At the time of writing, the clinical results on only one relatively weak PDE IV inhibitor, tibenalast (LY 186655), have been reported [269]. In that study, a single oral 150 mg dose of tibenalast produced a slight but non-significant increase in FEV_1 in 40 asthmatic subjects.

Several studies have evaluated the bronchodilating activities of mixed PDE III/IV inhibitors. Inhalation of benzafentrine (2-3 mg/kg) reversed methacholine-induced bronchoconstriction in non-asthmatic volunteers [270]. Bronchodilating effects of benzafentrine are also observed following i.v. administration but not via the oral route [270]. Inhalation of zardaverine, also elicited a slight increase in FEV_1 in asthmatics [271], but had no effect in patients with chronic obstructive pulmonary disease (COPD). Zardaverine is a weaker bronchodilator when applied directly into guinea-pig lungs by inhalation of dry powder or intra-tracheal (i.t.) installation than when administered via the intrajejunal (i.j.) route [272]. In the same studies, B9004-070 was shown to be a more effective bronchodilator after local administration into the guinea-pig lung, a finding attributed to poor bioavailability.

As in *in vitro* studies, no correlation exists between inhibition of partially purified PDE IV activity and suppression of histamine-induced bronchoconstriction in anaesthetized guinea-pigs [67]. In contrast, and in accord with *in vitro* studies an excellent relationship is obtained when suppression of histamine-induced bronchoconstriction by a range of PDE inhibitors is correlated with their ability to displace [^{3}H]rolipram from brain membranes [67]. As discussed earlier, an explanation for these results may be provided by the possible existence of a site on the airways smooth muscle PDE IV, distinct from the catalytic site, at which some (rolipram, nitraquazone), but not all (3-isobutyl-1-methylxanthine-IBMX), inhibitors potently interact to inhibit cAMP hydrolytic activity.

CNS ACTIONS OF PDE IV INHIBITORS

Numerous reports indicate that PDE IV participates in the biochemical processes controlling the behaviour of mammals, including humans, and lower organisms. Indeed, the great advances in the molecular biology of mammalian PDE IV owes much to studies on the *Drosophila melanogaster* 'dunce' gene which encodes a cyclic AMP-specific PDE [273]. In these insects, mutations at this locus cause learning defects, memory defects, or both, depending on the specific learning situation.

In mammals, the profile of behavioural and physiological effects of PDE IV inhibitors resembles those observed previously with known anti-depressant drugs: rolipram, and other cyclic AMP PDE inhibitors, reduce locomotor activity in mice and rats [105, 274], antagonize reserpine-induced hypothermia and locomotor activity [275, 276], reduce mobility in the forced-swim test in mice [95], enhance yohimbine-induced lethality in mice [276] and, upon repeat administration, down-regulate the density of β-adrenoceptors in rat brain [277]. Furthermore, rolipram reduces response rates and increases reinforcement rates in a differential reinforcement of low rate (DRL) behavioural schedule in rats [278]. Anti-depressant activity of rolipram in the clinic has been documented [279, 280]; however, because its efficacy, relative to tricyclic antidepressants, has been questioned [281] and in view of the pronounced side-effects profile (see below), interest in its therapeutic potential for the treatment of CNS disorders has waned.

The mechanism by which rolipram elicits its anti-depressant effects is uncertain. Some [282], but not all [283] studies indicate that the behavioural effects of PDE inhibitors correlate closely with their efficacies in displacing [^{3}H]rolipram from its high-affinity binding site in brain membranes. Cyclic AMP levels in brain are known to be elevated following i.v. administration of PDE IV inhibitors to rats [284]. Rolipram and other PDE inhibitors increase noradrenaline turnover [285]; they stimulate release of noradrenaline from slices of rat cerebral cortex *in vitro* [286, 287] and the firing of neurones in the locus ceruleus [288]. However, it is uncertain whether these presynaptic effects contribute greatly to the behavioural and neuropharmacological actions of rolipram [276, 288, 289].

Rolipram and closely related analogues, decrease locomotor activity and rearing in mice confronted with a free exploratory procedure which indicates behavioural sedation [290]; however, in a light/dark choice test, no evidence for anxiolytic activity was revealed [290]. Early studies in rodents suggested that rolipram might be useful for the treatment of Parkinson's disease [275], but this has not been confirmed in monkeys [291] or in the

clinic [292]. The amnesia for inhibitory avoidance response induced by the protein synthesis inhibitor, anisomycin, is reversed by rolipram, suggesting that PDE IV inhibitors can affect memory processes in mammals [293]. Analgesic effects of PDE IV inhibitors, as assessed in tail-flick, paw-pinch and hot-plate tests, have been observed following microinjection of Ro 20-1724 at sites in the caudal reticular formation of the rat brainstem [294]. Finally, studies in rats and cats with denbufylline, suggest that PDE IV inhibitors may be useful in the treatment of cerebral ischaemia [295]. This, to some extent, has been confirmed in the clinic, where denbufylline improved symptom scores in patients with chronic organic brain syndrome [296].

SIDE-EFFECTS OF PDE IV INHIBITORS

From the previous discussion, it is clear that PDE IV inhibitors have considerable therapeutic potential. Furthermore, they are generally devoid of the cardiovascular side-effects such as increased cardiac contractility, vasodilatation and potential arrhythmogenic activity associated with PDE III inhibitors [297]. However, they do exhibit a number of side-effects which may limit their potential therapeutic utility, especially if they are to be administered orally. This, perhaps, is not surprising in view of the wide-ranging tissue distribution of PDE IV.

Several gastrointestinal side-effects have been observed with PDE IV inhibitors in the clinic. Nausea and vomiting have been reported following administration of rolipram [298], tibenalast [269], BRL-61063 [227] and zardaverine (even by the inhalation route) [271] to human subjects. The mechanism(s) by which PDE IV inhibitors induce these side-effects is/are uncertain but studies demonstrating the potentiation of apomorphine-induced emesis in dogs [299] by Ro 20-1724 suggest that the nausea and vomiting is likely to be produced, at least in part, via the emesis centres in the brain. PDE IV inhibitors are very potent stimulators of acid secretion [300] from gastric parietal cells and this, by producing local irritation, may exacerbate gastro-intestinal disturbances. Rolipram has been shown to cause a significant but transient fall in plasma osmolality in ten healthy male volunteers [301]. This may be due to an antidiuretic effect of rolipram by increasing cyclic AMP levels in renal tubules. Whether the anti-inflammatory potential of PDE IV inhibitors will be compromised by unacceptable CNS effects (see above) remains to be determined.

CONCLUSIONS AND FUTURE PROSPECTS

The next few months will reveal whether the current, guarded optimism in the therapeutic potential of PDE IV inhibitors will be fulfilled. At the time of writing, only a limited amount of information on the clinical efficacy of relatively weak PDE IV inhibitors in asthma and skin disorders (psoriasis) [302] has been published. Several companies currently have, or will have, compounds undergoing clinical evaluation in asthma patients. These compounds include RP 73401 (Rhone-Poulenc Rorer) [37], LAS-31025 (Almirall; structure unknown) [138a], WAY-PDA-641 (Wyeth-Ayerst) [121], SB 207499 (SmithKline Beecham) [118], and CDP 840 (Celltech/ Merck; structure unknown). Although a large body of information from acute animal studies suggests that PDE IV inhibitors will be effective in the treatment of asthma, and bronchodilating activity has been demonstrated in the clinic with weak PDE IV inhibitors and mixed PDE III/IV inhibitors, long term studies with the newer, highly potent compounds will be required to determine whether they suppress the mucosal inflammation associated with this chronic disease. This will ultimately determine whether they will assume a position of importance in prophylactic asthma therapy.

The evidence implicating TNFα in autoimmune and viral diseases [131] together with the impressive inhibitory effects of PDE IV inhibitors in suppressing release *in vitro* [110, 111, 114, 130] and *in vivo* [131, 162] suggest therapeutic potential for these compounds in the wide number of pathological conditions associated with over elaboration of this cytokine. Although compounds such as pentoxifylline have demonstrated some activity in TNFα-associated conditions [185], the concept still has to be tested with the new PDE IV inhibitors in the development pipe-line. Finally, whether information on the distribution of PDE IV subtypes in the CNS and the development of PDE IV subtype selective inhibitors will result in renewed interest in their potential for treatment of psychotic disorders remains to be seen.

The pharmaceutical goal is to identify orally active compounds which exhibit potent *in vivo* efficacy but with a reduced side-effect profile. Whether this is achievable is currently not known. Targeting specific PDE IV subtypes or designing compounds which interact weakly at the high-affinity rolipram binding site whilst retaining potent inhibitory effects on cyclic AMP hydrolysis offer rational although uncertain ways forward. Cellular selectivity may be achievable as indicated by the preliminary results on SB 207499, which is generally equipotent with rolipram on functional responses in several inflammatory cells but is far less effective in stimulating acid secretion in isolated parietal cells [303]. Alternatively, the answers to these

problems may be solved by pharmacokinetic strategies. If unsuccessful, then it might be impossible to deliver PDE IV inhibitors orally and direct application to the skin or lungs may be the preferred routes. Limited information suggests that, while therapeutic activity is retained by these routes of administration, problems with side-effects may be diminished [304–306].

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2 Biosynthesis and Mechanism of Action of Cyclosporins*

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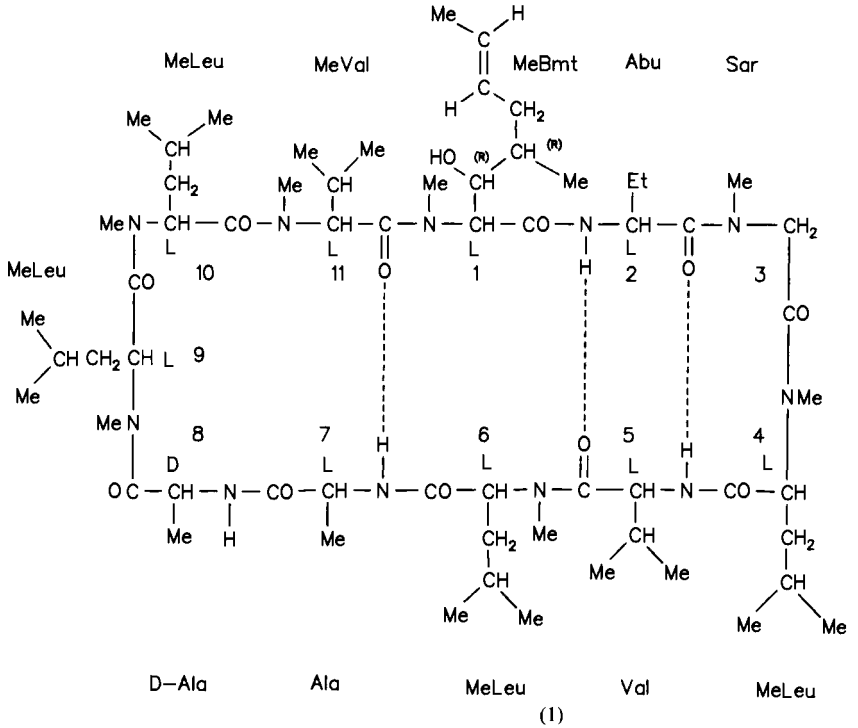
* Dedicated to Professor Dr. Horst Kleinkauf on the occasion of his 65th birthday.

INTRODUCTION

The topics of this article have already been reviewed in this series several years ago [1]. Since then, major progress has been made in understanding the biosynthesis of cyclosporins as well as the mechanism of action of cyclosporin A (CyA). The emphasis of this review, therefore, will be on recent studies on the biosynthesis and mechanisms of action of immunosuppressants.

The first specific immunosuppressant discovered was cyclosporin A (*Figure 2.1*, 1). This cycloundecapeptide is produced by fungi imperfecti of a great variety of taxa (reviewed in [2]) and exerts several biological properties, including antifungal, anti-parasitic, anti-inflammatory and immunosuppressive activities [3]. It is currently the drug of choice for the prevention of organ and bone marrow transplant rejection and therefore widely used in transplantation surgery [4]. A second indication for the use of CyA is the treatment of autoimmune diseases [5, 6]. Until now, the nephrotoxicity of the drug (and, in part, also its immunosuppressivity) prevents its wider use in many other indications described for cyclosporin A. Such indications include type I diabetes [7], psoriasis [8–12], malaria [13], multi-drug resistance in cancer chemotherapy [14–18] and anti-human immune deficiency virus activity [19–21]. Therefore, the search for new cyclosporin A analogues that are either immunosuppressive and non-nephrotoxic, or non-immunosuppressive but still exerting one or more of the other properties of cyclosporin A, remains an important objective. Recently some approaches have been made to produce new cyclosporins either by chemical synthesis [22–30], by precursor-directed biosynthesis [31, 32] or by *in vitro* biosynthesis [33, 34]. Only the latter two approaches are discussed in this review.

Besides the clinical importance of CyA, this drug has played a pivotal role in the last few years in the uncovering of cellular signalling events in T-cell activation. Using CyA together with immunosuppressants of the macrolide type (FK506 and rapamycin) and with antagonists of both classes of immunosuppressive drugs, a putative mechanism for the action of these compounds has been elucidated (for recent reviews see [35–38]).



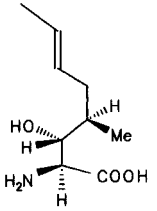
(1)

Figure 2.1. Structure of cyclosporin A (1). Abu, L-2-aminobutyric acid; Bmt, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (=(2S, 3R, 4R, 6E)-2-amino-3-hydroxy-4-methyloct-6-enoic acid); Sar, sarcosine

BIOSYNTHESIS OF CYCLOSPORIN A PRECURSORS

BIOSYNTHESIS OF BMT

The most uncommon amino acid of cyclosporin A is (2*S*, 3*R*, 4*R*, 6*E*)-2-amino-3-hydroxy-4-methyloct-6-enoic acid [(4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine, Bmt, 2]. So far, the only metabolites for which Bmt has



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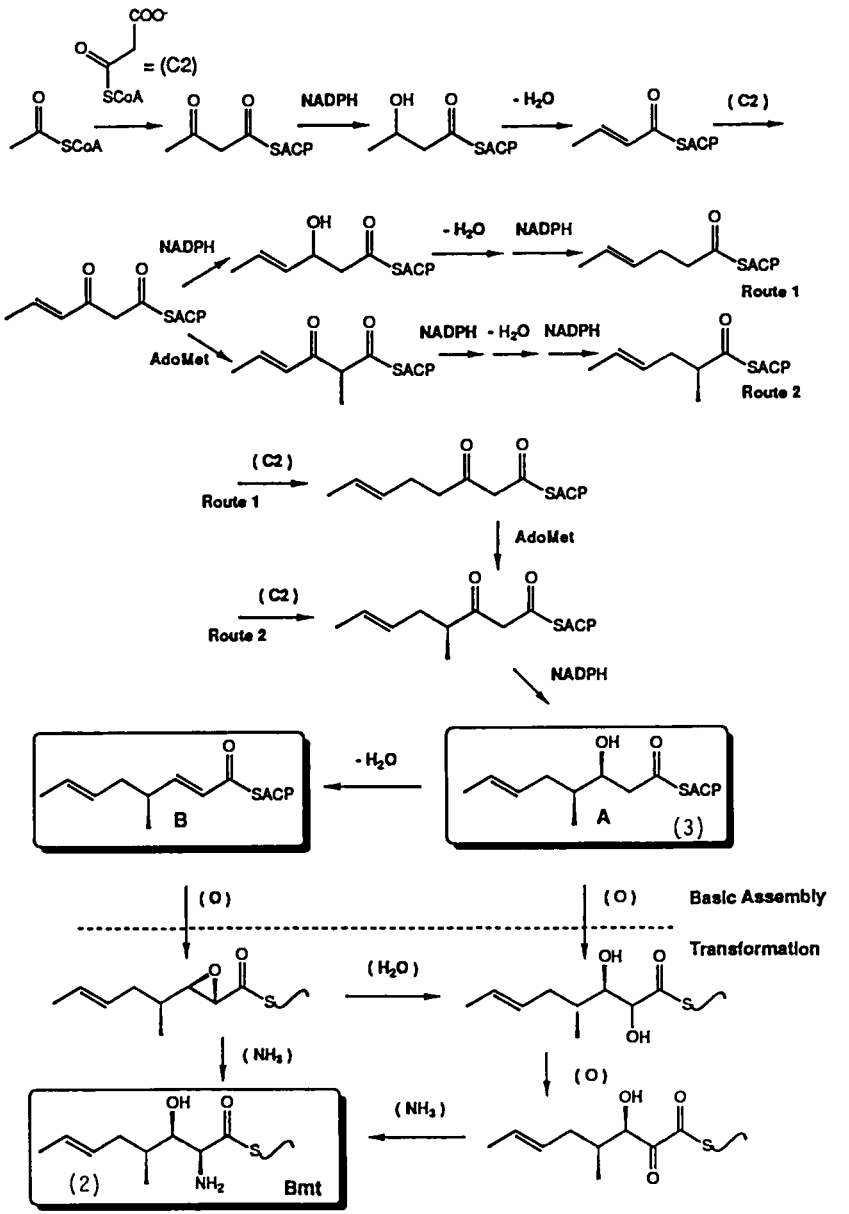
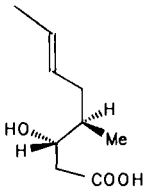


Figure 2.2. Possible routes for the biosynthetic pathway to Bmt (2). Polyketide biosynthesis of type I is assumed to take place in an enzyme-bound form (ACP = acyl carrier protein). The product (3) is proposed to be released from the Bmt synthase as a coenzyme A thioester which allows the enzyme to enter the next biosynthetic cycle (with permission from [43]).

been described to be a building block are the cyclosporins [34, 39] and the related peptolide SDZ 214-103 [40] and its analogues [34].

Preliminary incorporation studies [41] using ^{13}C-labelled precursors had suggested that Bmt was synthesized by head-to-tail condensation of four acetate units and the addition of one methyl group from a methionine. Recent ^{13}C-labelling studies with selectively labelled ^{13}C-glucose gave the final proof for this biosynthetic origin [42].

These data gave Offenzeller *et al.* [43] reason to design two putative pathways for the biosynthesis of the polyketide backbone of Bmt (*Figure 2.2*) which were investigated by *in vivo* incorporation studies of appropriate precursors and by *in vitro* biosynthetic studies. Using this approach, the authors have been able to purify and characterize the Bmt-polyketide synthase. They showed that 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoic acid (3) is a key intermediate of Bmt biosynthesis. On the basis of their data, the



(3)

authors propose a mechanism by which the product (3) is released as a coenzyme A thioester. They speculate that the final transformation reactions to form (2) could take place on this thioester so that the *in vivo* substrate for cyclosporin biosynthesis would be the already-activated amino acid [43]. Nevertheless, as will be outlined in the following sections, the *in vitro* systems were clearly capable of activating Bmt (2) and of incorporating non-activated amino acids in position 1 of the cyclosporin ring [33, 34, 44].

BIOSYNTHESIS OF THE OTHER CONSTITUENT AMINO ACIDS OF CyA

By labelling CyA with ^{13}C-glucose, Senn *et al.* [42] furthermore showed that the other constituent amino acids of cyclosporin are synthesized by classical biosynthetic pathways. This was deduced by comparison of the expected labelling of the constituent amino acids of CyA with the experimentally obtained ones as depicted in *Figure 2.3*.

The non-proteinogenic L-2-aminobutyric acid (Abu) results from oxalo-

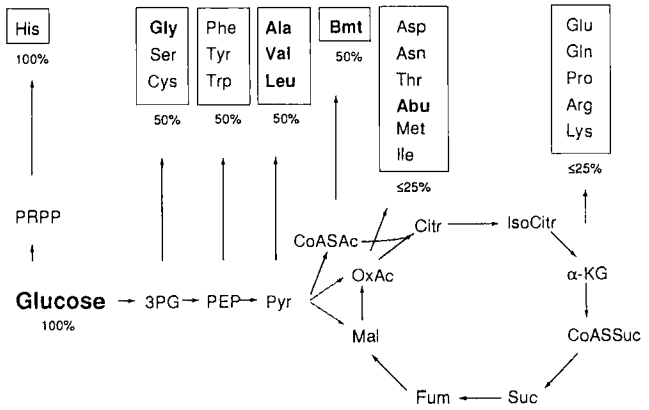


Figure 2.3. Biosynthesis of constituent amino acids of cyclosporin A from glucose with expected maximum label incorpotation at a given position when grown on 100% selectively enriched ^{13}C-glucose. Key intermediates of glycolysis and the Krebs cycle are depicted in abbreviated form. Flux of carbon atoms through the pentose phosphate pathway is neglected. The amino acids occurring in CyA are shown in bold type; for abbreviations refer to Figure 2.1. *CoAsSuc, succinyl coenzyme A; Citr, citrate; Isocitr, isocitrate; α-KG, α-ketoglutarate; Fum, fumarate; Mal, malate; OxAc, oxaloacetate; Pyr, pyruvate; Suc, succinate; PEP, phospho*enol*pyruvate; PG, 3-phosphoglycerate; PRPP, phosphoribosylpyrophosphate (with permission from [42]).*

acetate (*Figure 2.3* [42]), and D-alanine (D-Ala) is generated from L-Ala by a fungal racemase [45].

BIOSYNTHESIS OF CYCLOSPORINS

There are four general mechanisms known for the biosynthesis of peptides, two of which are nucleic acid-dependent, namely, the classical ribosomal translation (for recent reviews see [46–48]) and the ribosomal-independent bacterial pentaglycine-peptide biosynthesis [49]. The other two are nucleic-acid independent, namely, the enzymatic biosynthesis of short peptides (such as glutathione [50], carnosine [51] and pantotheine [52]), which is performed by single enzymes; and the thiotemplate mechanism involving multienzymes (for a recent review see [53]).

Also in [1] the first preliminary data from specifically labelled cyclosporins yielded by precursor feeding have already been argued to give strong evidence for a biosynthetic pathway involving multienzyme thiotemplates.

PRECURSOR-DIRECTED BIOSYNTHESIS OF ANALOGUES OF CyA

By feeding the appropriate amino acids, several new cyclosporins could be obtained by precursor-directed biosynthesis *in vivo* [31, 54], thus supporting the hypothesis that the unmethylated amino acids are substrates of the cyclosporin synthetase complex. These new cyclosporins, listed in *Figure 2.4*, are [Me-cyclohexylalanine[1]]CyA (4), [allylglycine[2]]CyA (5), [D-Ser[8]]CyA (6), [D-Ser[8]]CyC (7), [Ser[8]]CyD (8), [D-Ser[8]]CyG (9) [31], [3-fluoro-D-Ala[8]]CyA (10) [54] and [2-deutero-3-fluoro-D-Ala[8]]CyA (11) [32].

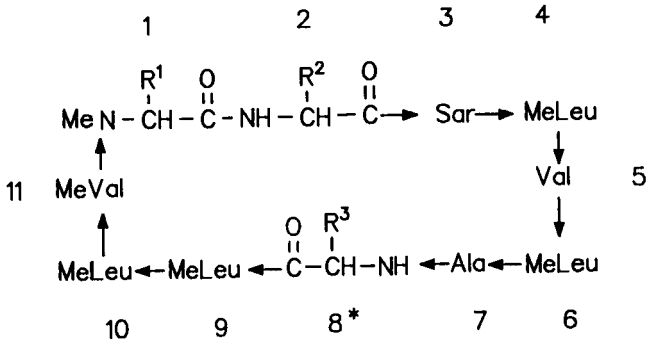
CYCLOSPORIN SYNTHETASE

In 1990, we described an enzyme, called 'cyclosporin synthetase', capable of synthesizing cyclosporin A using unmethylated constituent amino acids of (1), ATP/Mg^{2+}, and *S*-adenosyl-L-methionine as substrates [44]. This enzyme was isolated from the cyclosporin-producing fungus imperfectus *Beauveria nivea* (previously designated *Trichoderma polysporum*, *Tolypocladium inflatum*, and *Tolypocladium niveum*). To our surprise, it behaves as a single polypeptide chain under all circumstances examined, including boiling in a buffer which contains sodium dodecylsulphate (SDS), urea and β-mercaptoethanol. Its molecular mass is clearly higher than the textbook limit for an enzyme subunit, represented by tyrocidine synthetase 3 with a molecular mass of about 450 kDa [55].

Molecular mass of cyclosporin synthetase

In order to determine the apparent molecular mass of cyclosporin synthetase, we used, beside tyrocidine synthetase 3, enniatin synthetase with a molecular mass of 250 kDa [56] and linear gramicidin synthetase 2 (350 kDa [57]) as references in a 3% SDS-polyacrylamide gel. Extrapolation from the molecular masses of these calibration proteins resulted in a molecular mass between 650 and 800 kDa for cyclosporin synthetase [44].

In the year that we published the first characteristics of cyclosporin synthetase, the sequence of the open reading frame of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, the first enzyme in penicillin biosynthesis, was also published [58, 59]. In contrast to the molecular mass of 230 kDa obtained from extrapolation in SDS-polyacrylamide gels [60] (similar to the estimation of the molecular mass of cyclosporin synthetase), the molecular mass of ACV synthetase derived from the open reading frame of the nucleotide sequence was 420 - 425 kDa. Thus, at least the molecular mass of this polypeptide synthetase had been drastically underestimated.



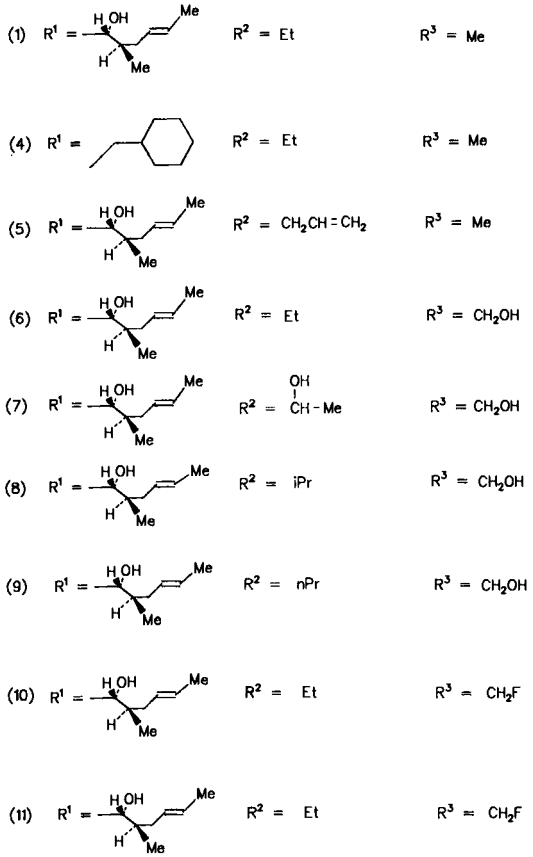


*Figure 2.4. Structures of cyclosporin A analogues obtained by precursor-directed biosynthesis. *In all cyclosporins obtained by precursor-directed biosynthesis, position 8 has the* D*-configuration. In (11), the hydrogen atom at C-2 is replaced by a deuterium atom (^2H).*

Using ACV synthetase with a molecular mass of 420 kDa as a reference, we re-estimated the molecular mass of cyclosporin synthetase to be about 1.5 MDa [61, 62]. The value of 570 kDa obtained from the same experiments for gramicidin S synthetase 2 has been in good agreement with data obtained from laser desorption mass spectroscopy (556 + 5 kDa; Vestal, M. and von Döhren, H., unpublished results) and the value of 510 kDa deduced from the sequence of the open reading frame published in 1992 [63], indicating the general validity of this method.

However, the value obtained for cyclosporin synthetase by linear regression analysis of the mobilities of the reference proteins was also a result of extrapolation. The basic difficulty in determining very high molecular masses of proteins like cyclosporin synthetase in SDS-polyacrylamide gels is the lack of appropriate standards (the highest of which was gramicidin S synthetase reported in 1992). Our efforts were, therefore, then directed to estimating the molecular mass of cyclosporin synthetase by a method which is independent of reference proteins. In collaboration with Bettina Schmidt and Detlev Riesner [64, 65] we determined the molecular mass of cyclosporin synthetase by CsCl density gradient centrifugation experiments in an analytical ultracentrifuge. To this end, we established fluorescence labelling of cyclosporin synthetase with 4′-{[(iodoacetyl)amino]methyl}fluorescein to enable measurements at low concentrations, since otherwise, the enzyme tends to aggregate [65]. The data obtained from these experiments again confirmed the observation that cyclosporin synthetase is a single polypeptide of high molecular mass. From nine measurements, obtained from samples with concentrations ranging from 10 to 44 nM at the beginning of the ultracentrifuge run, an average molecular mass of 1.4 MDa with a maximum error of ± 160 kDa was derived from the band widths of the macromolecular distributions, as is exemplified for one concentration in *Figure 2.5*. This value was in good agreement with the value of 1.5 MDa obtained from SDS-polyacrylamide gel analyses [62, 65].

Recently, the open reading frame of the cyclosporin synthetase gene has been established [66, 67]. Most importantly, this work confirms our data showing that cyclosporin synthetase is a single polypeptide. The gene does not contain any introns and codes for a protein of 1, 689, 243 Da. Hence, also our second approach to determining the molecular mass of this enormous enzyme resulted in a slight underestimation.

From data available so far on peptide synthetases, it appears to be a common rule that all bacterial enzymes like gramicidin S synthetase, tyrocidine synthetase and actinomycin synthetase (all reviewed in [47]) have subunit structure, whereas fungal enzymes, such as SDZ 214-103 synthetase [68], SDZ 90-215 synthetase [69] and cyclosporin synthetase consist of single

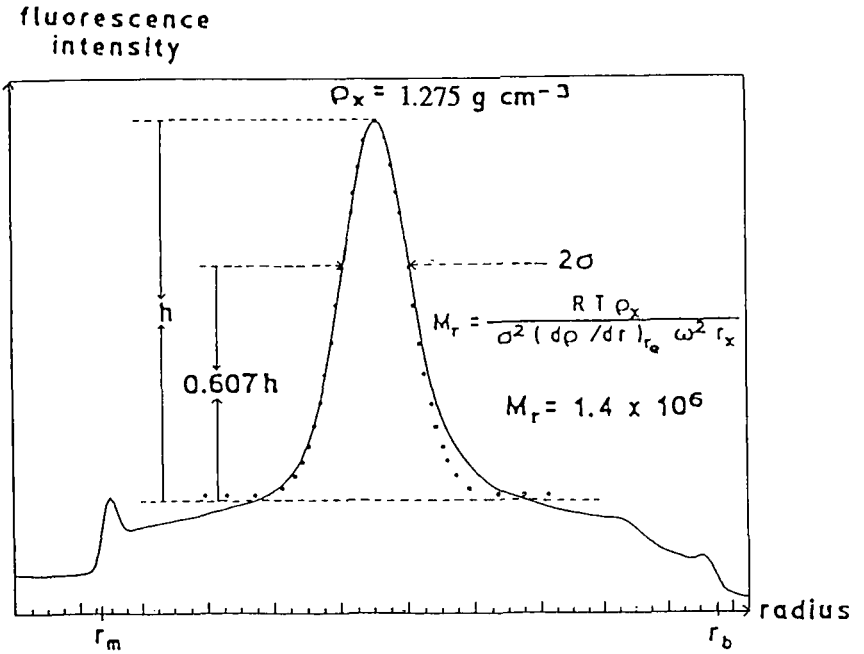


Figure 2.5. Molecular mass estimation of cyclosporin synthetase by density gradient centrifugation [65a]. Cyclosporin synthetase purified by glycerol gradient ultracentrifugation [44] has been labelled with 4'-{[(iodoacetyl)amino]methyl}fluorescein. 8.8 µg of this enzyme preparation (end concentration 25 µg/ml) were analysed by CsCl density gradient centrifugation at 40,000 rpm in an analytical ultracentrifuge equipped with a fluorescence detection system. The profile shows the distribution of fluorescence intensity monitored after 9 h. Open circles designate the best fit to the Gaussian distribution equation:

$$f(r) = \frac{1}{\sigma \sqrt{2\,\pi}} \cdot e^{-\frac{(r-r_x)^2}{2\,\sigma^2}}$$

r_m represents the meniscus, r_b the bottom of the measuring cell, r_x the radial position of the protein band, σ the bandwidth (from [64], with permission).

polypeptide chains. ACV synthetase is the simplest member of this family, whereas cyclosporin synthetase represents the most complex member, so far. According to the IUPAC nomenclature [70], these enzymes are designated 'multifunctional polypeptides'; the bacterial enzymes on the other hand are called 'multienzyme complexes'.

Mechanism of biosynthesis of CyA

Already in our first publication on the characterization of cyclosporin

synthetase [44], we gave strong evidence that this enzyme follows a thiotemplate mechanism. Hence, we could demonstrate, that the enzyme is capable of activating all constituent amino acids of CyA in their unmethylated form (including Bmt (2); cf. [43] and the discussion above) as aminoadenylates. This was done by measuring the amino acid-dependent ATP-pyrophosphate exchange reactions [44]. In a more recent communication [71], we showed furthermore that cyclosporin synthetase is capable of forming CyA when chemically synthesized aminoadenylates of its constituent unmethylated amino acids are used as substrates without supplying ATP/Mg^{2+}.

The second step in CyA biosynthesis is the transesterification of the enzyme-bound activated amino acids onto reactive thiol groups of the enzyme. This interpretation is supported by the observations that all these amino acids can be bound covalently to the enzyme [44] and split off again by performic acid oxidation [71]. Cyclosporin synthetase can be radiolabelled by incubation with ^{14}C-labelled substrate amino acids, ATP and Mg^{2+}, as exemplified in *Figure 2.6* for leucine and valine. Furthermore, the binding of all amino acids onto cyclosporin synthetase can be inhibited by specific SH-reagents like 2, 2′-dithiodipyridine and this inhibition can be reversed by 1, 4-dithioerythritol [71].

N-Methylation of substrate amino acids occurs while they are bound to the enzyme as thioesters. *S*-Adenosyl-L-methionine acts as the methyl donor [44]. The methyl transferase activity or activities of cyclosporin synthetase is (are) an integral part of the enzyme as has been demonstrated by photolabelling of the methyl transferase entity with radiolabelled *S*-adenosyl-L-methionine [44]. Biosynthesis of an unmethylated cyclosporin is not possible, most probably due to the need for sarcosine at position 3 for the formation of a β-turn [72]. Nevertheless, it was possible to synthesize the unmethylated diketopiperazine *cyclo*-(D-Ala-Leu) using a cyclosporin synthetase prepared from a mutant strain of *B. nivea* [73, 74, and see below], though its formation is about 20 times slower than that of the methylated product. This result shows, however, that cyclosporin synthetase can be considered as a peptide synthetase joined to one or more methyltransferase unit(s). From the recent data of the open reading frame of cyclosporin synthetase [67], it has become evident that each of the seven active centres of the enzyme which catalyzes an *N*-methylation reaction contains a separate methyltransferase entity.

The possibility of a single multifunctional polypeptide synthesizing a cycloundecapeptide raised the question of whether its synthesis involves condensation of at least two precursor peptides. The thiotemplate mechanism, as it was proposed by Lipmann [75], suggests a central

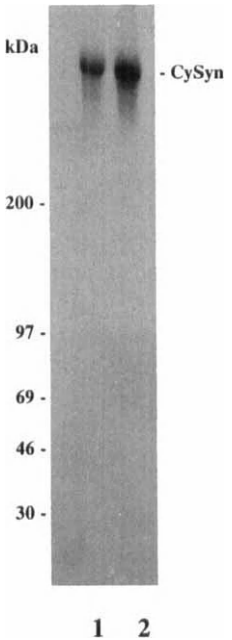
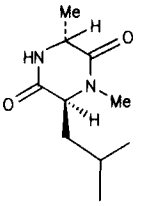


Figure 2.6. Gel electrophoresis of cyclosporin synthetase with covalently bound amino acids. Cyclosporin synthetase has been incubated for 10 min together with $MgCl_2$, ATP, and [^{14}C]-valine (lane 1) or [^{14}C]-leucine (lane 2). The incubation mixture together with sodium dodecylsulphate was separated in a gradient gel [44]. The gel was fluorographed, dryed and exposed to a X-ray film. The position of cyclosporin synthetase (CySyn) is indicated on the right hand; the positions and molecular masses of radioactive reference proteins are indicated on the left hand.

4′-phosphopantetheine arm which functions as an internal transport system transferring the growing peptide chain from one active centre on the enzyme to the next. The 4′-phosphopantetheine molecule having a length of 20 Å is able to reach up to 6 of the active centres of an enzyme [76]. As CyA consists of 11 amino acids, it would be expected that one molecule of cyclosporin synthetase is equipped with at least two 4′-phosphopantetheine molecules and will catalyze the synthesis of at least two linear precursor peptides. Although we could show that 4′-phosphopantetheine is a prosthetic group of cyclosporin synthetase and we found, in several estimations, values higher than one molecule of 4′-phosphopantetheine per cyclosporin synthetase molecule, we were not able to determine the exact stoichiometry of 4′-phosphopantetheine bound to cyclosporin synthetase [44].

Therefore, in order to study the mechanism of cyclosporin formation in more detail we examined a blocked mutant of the producer strain which is no longer capable of synthesizing cyclosporin A [74]. The cyclosporin synthetase we isolated from this mutant also proved to be unable to synthesize CyA *in vitro*. However, formation of the diketopiperazine



(12)

cyclo-(D-Ala-MeLeu) (12) was catalyzed. This compound, which has also been shown to be produced *in vivo* by the mutant [74], represents a partial sequence of CyA (1), namely positions 8 and 9, indicating that position 8 could be at least one starting point of precursor peptide synthesis. This assumption was confirmed by verifying that D-Ala is an N-terminal amino acid of enzyme-bound precursor peptides [71]. However, surprisingly, we could not identify any further N-terminal amino acids. We therefore started to isolate and identify some of the enzyme-bound precursor peptides. We finally identified four such linear peptides [77], namely H-D-Ala-MeLeu-OH, H-D-Ala-MeLeu-MeLeu-OH, H-D-Ala-MeLeu-MeLeu-MeVal-OH, and H-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-OH. All these peptides start with D-Ala. The latter, which has nine of the 11 amino acids of the CyA molecule, indicates that CyA is synthesized stepwise via a single linear precursor peptide with D-Ala at position 8 as the only starting point. Interestingly, the first total chemical synthesis of CyA by Wenger also started with D-Ala [72]. The linear undecapeptide, H-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OH, could not be isolated, suggesting that the cyclization reaction must be very fast and almost simultaneous with the formation of the undecapeptide [71]. A summary of our recent knowledge on enzymic CyA formation is given in *Figure 2.7*. The enzyme catalyzes a least 40 partial reaction steps: eleven aminoadenylation reactions, eleven transthiolation reactions, seven *N*-methylation reactions, ten elongation reactions and the final cyclization reaction, thus representing the most complex multifunctional polypeptide known. The difficulty of imagining the stepwise formation of a single undecapeptide by the thiotemplate mechanism can be overcome by a slight modification of this

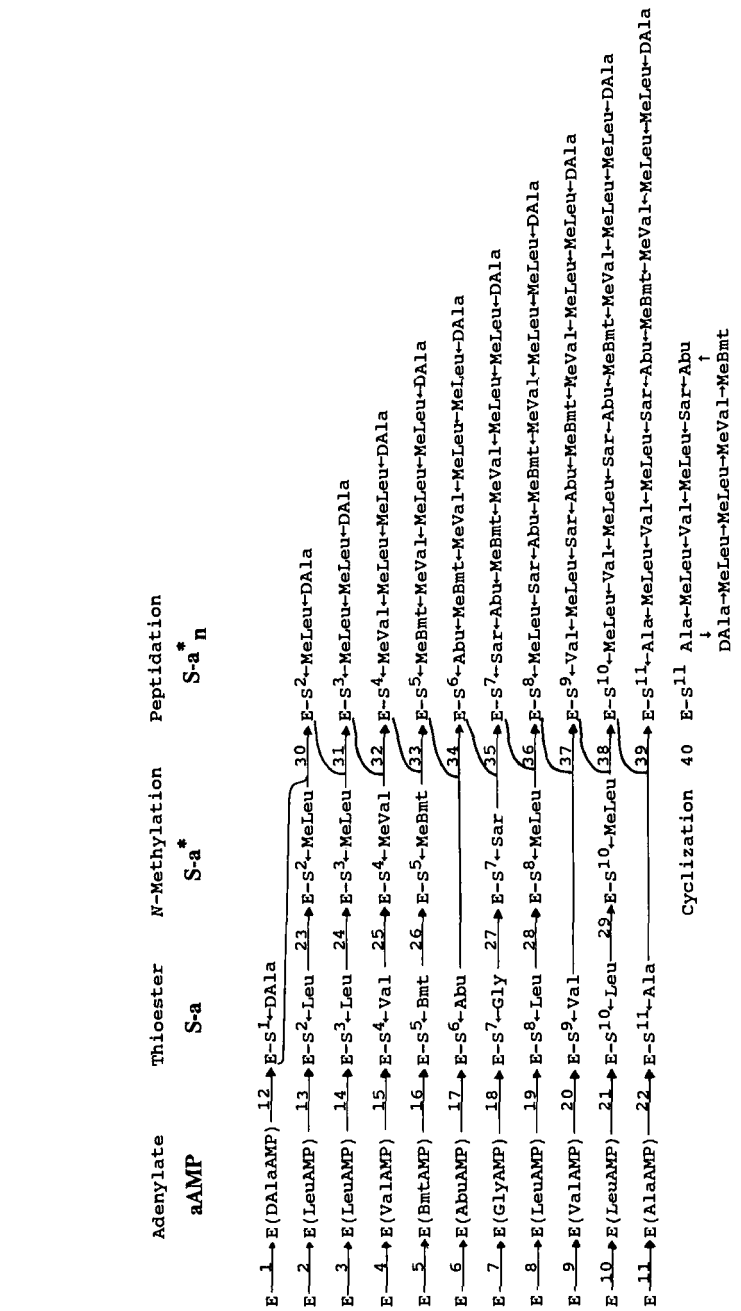


Figure 2.7. Hypothetical reaction scheme for the biosynthesis of cyclosporin A (1). The numbers above the arrows indicate the partial reaction step. E stands for cyclosporin synthetase, the other abbreviations are the same as in Figure 2.1.

model. Recent data on the open reading frames coding for different peptide synthetases provide evidence that these enzymes have a serine at each of their active sites and not a cystidine residue as previously thought [78]. The easiest way to interpret all these data is to suppose a 4′-phosphopantetheine to be bound at each active centre which is consistent with the sequence data of the open reading frame [67]. Then, no central 4′-phosphopantetheine is needed and the geometry of 4′-phosphopantetheine is no longer a limitation.

Molecular shape of cyclosporin synthetase

The knowledge of the molecular mass of cyclosporin synthetase enabled us to roughly estimate the molecular shape of the enzyme by sedimentation centrifugation experiments in an analytical ultracentrifuge. We found an average sedimentation coefficient of 26.3, the expected S-value for a globular protein of 1.4 MDa would be 36. From these values, we could calculate the enzyme's shape to be a discus of 330 Å in diameter with a thickness of 46 Å [65]. Revision of the calculations for the molecular mass of 1.69 MDa [67] resulted in a discus of 322 Å in diameter and a thickness of 53.6 Å [Schmidt, B., personal communication].

As outlined elsewhere [79, 80], the prokaryotic chaperonine/chaperone machineries form cavities which are large enough to accommodate up to a 90 kDa globular protein. Although no comparable data exist for eukaryotic cells, one has to assume that a protein-like cyclosporin synthetase would not have enough space in these cavities. It appears therefore most likely that each domain (about 140 kDa and not globular) is folded on itself rather than the whole protein. Some preliminary evidence for such a mechanism is given by data from experiments trying to denature cyclosporin synthetase with urea; we were able to discriminate at least between two stages of denaturation. When we used low urea concentrations (about 0.8 M), the enzyme was still able to synthesize the diketopiperazine *cyclo*(D-Ala-MeLeu), but not CyA; higher urea concentrations led to a totally inactive enzyme preparation [81, and Dittmann and Lawen, unpublished results].

Together with the data from Vater's group obtained for gramicidin S synthetase 2 by electron microscopy (showing a four-domain ring) [82], and the data of the sequence from the open reading frame (showing eleven homologous modules which are supposed to be responsible for the activation of the eleven constitutive amino acids of CyA) [67], all the evidence strongly suggests a ring structure consisting of eleven domains with the overall dimensions given above.

PEPTOLIDE SDZ 214-103 SYNTHETASE

In 1988, the structure of a new cyclic peptolide (SDZ 214-103) produced by the fungus *Cylindrotrichum oligospermum* (Corda) Bonorden was published [40]; it exerts similar biological activities to CyA. Peptolide SDZ 214-103 (*Figure 2.8*, 13) is closely related to CyA and has as the main structural difference a D-2-hydroxyisovaleric acid in the ester linkage at position 8 instead of D-alanine in the cyclosporins. According to the conventional nomenclature, it can be designated as [L-threonine^{2}, L-leucine^{5,10}, D-2-hydroxyisovaleric acid^{8}]CyA. We were able to prepare an enzyme fraction from crude extracts of the mycelium of *Cylindrotrichum* Bonorden, which is capable of synthesizing peptolide SDZ 214-103 *in vitro*. Cyclosporin A is not a reaction product of the peptolide synthetase and cyclosporin synthetase does not synthesize peptolide SDZ 214-103 *in vitro* [68]. Peptolide 214-103 synthetase is clearly distinct from cyclosporin synthetase; however, both enzymes have in common the large size (about 1.65 MDa for peptolide SDZ 214-103 synthetase) and, as far as studied, the principal biosynthetic mechanism. Peptolide SDZ 214-103 synthetase catalyzes at least 39 partial reaction steps, namely, eleven aminoadenylation reactions, eleven transthiolation reactions, six *N*-methylation reactions, ten elongation reactions and the final cyclization reaction. The hydroxy acid is activated like the amino acids first as an aminoadenylate and transthioesterified thereafter.

IN VITRO SYNTHESIS OF CyA AND PEPTOLIDE SDZ 214-103 ANALOGUES

The first report on *in vitro* biosynthesis of CyA showed that with a partially purified cyclosporin synthetase preparation CyA and several homologues (which either occur naturally or had been obtained by precursor feeding experiments) are accessible [83]. But in the light of our later results, which never yielded the high specificity shown in this report, it is very likely that the radioactivity co-migrating with the cyclosporin references was just a contamination of these rather than trace amounts of *in vitro* formed cyclosporins. The authors do not give data on the elution profile of their enzyme, so that a very likely explanation would be that they were dealing with a preparation lacking intact cyclosporin synthetase. With an enzyme preparation obtained from an improved producer strain (7939/45), we were able to establish a method for reproducible *in vitro* biosynthesis of μg-amounts of cyclosporins [33, 34]. With this method it became possible to synthesize *in vitro* a number of new cyclosporins which were not obtainable *in vivo* [33, 34, 84]. This method could also be adapted for the biosynthesis of new peptolide SDZ 214-103 analogues [34].

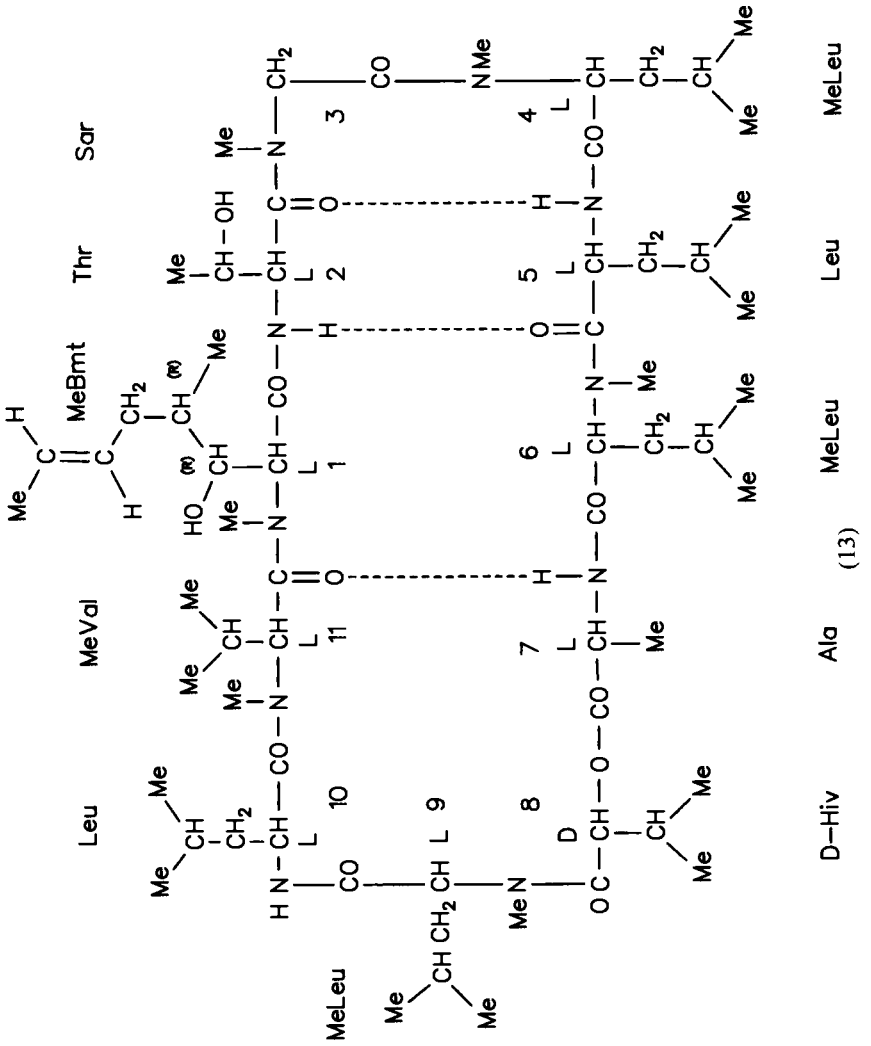


Figure 2.8. Structure of peptolide SDZ 214–103 (13). D-Hiv, D-2-hydroxyisovaleric acid; all other abbreviations as in Figure 2.1.

These experiments were carried out by incubating at suboptimal temperature for seven days partially purified enzyme preparations together with all the necessary amino acids in their unmethylated form (including one

D-amino acid or D-hydroxy acid), ATP, $MgCl_2$ and *S*-adenosyl-L-methionine. Suboptimal temperature was chosen in order to stabilize the enzyme and to minimize *S*-adenosyl-L-methionine degradation. The cyclopeptides were extracted with ethyl acetate and purified chromatographically.

As is exemplified for [D-Abu^8]CyA in *Figure 2.9*, this method helps to synthesize new cyclosporins not obtainable *in vivo*. The new cyclosporin could be obtained enzymatically in sufficient amounts for structural analysis, whereas experiments to obtain [D-Abu^8]CyA by feeding D-2-aminobutyric acid to the fungus only resulted in stimulation of CyA production, probably due to the action of the D-alanine racemase [85]. Since no reference compounds exist for the new cyclosporins, it was necessary to produce sufficient quantities for structural analysis. Preliminary structural proof was done by fast atom bombardment mass spectrometry and the result is shown for [D-Abu^8]CyA in *Figure 2.9b*.

A second criterion used to confirm a cyclosporin structure is the immunosuppressive activity of this class of cyclopeptides. So far, all new cyclosporins synthesized *in vitro* exert immunosuppressive activity *in vitro*.

Although we have analyzed the substrate specificity for all of the 11 amino (hydroxy) acid sites as far as they are experimentally accessible [34], I want to restrict myself here for the most part to positions 1 and 8 of the cyclosporin A and peptolide SDZ 214-103 molecules as two examples of a special interest: position 1 carries the unique amino acid Bmt (2), and position 8 carries a D-amino acid in the cyclosporins but a D-hydroxy acid in the peptolides.

Incorporation of amino acids into position 1

Most natural cyclosporins and all natural peptolide SDZ 214-103 analogues contain the new amino acid Bmt (2) at position 1 of the cyclopeptide ring. Among the natural cyclosporins there are two containing the deoxy-analogue (CyF and CyK), one containing Leu (CyO) and a further one containing L-2-amino-octanoic acid (CyZ) instead of Bmt. Incorporation of the latter amino acid indicates that neither the double bond nor the 3-hydroxy group is essential for substrate recognition of the enzyme. Both presumptions were verified *in vitro*: cyclosporin synthetase is capable of incorporating into position 1 L-2-amino-octanoic acid, L-2-amino-3-hydroxy-4-methyloctanoic acid (dihydro-Bmt) and both L-3-cyclohexylalanine and L-3-hydroxy-3-cyclohexylalanine, mimetics of deoxy-Bmt and Bmt, respectively. On the other hand, for position 1 the aromatic amino acids phenylalanine and tyrosine are not substrates of the enzyme; by

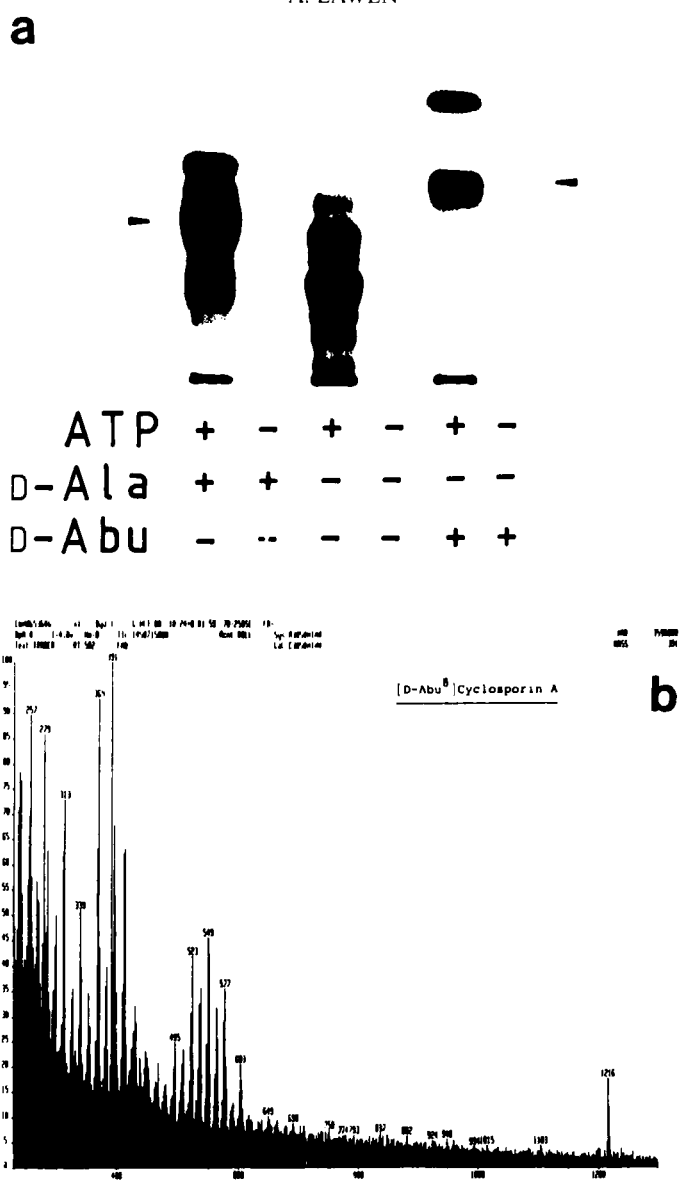


Figure 2.9. In vitro *synthesis of [D-Abu⁸]CyA. (a) When all constitutive amino acids of CyA are incubated together with ATP, Mg^{2+}, S-adenosyl-L-[^{14}C-methyl]methionine and the enzyme fraction, the main product synthesized is CyA (left arrow). When D-alanine (D-Ala) is replaced by D-Abu, the new compound, [D-Abu⁸]CyA, is synthesized (right arrow). The TLC separation of EtOAc-extractable reaction products is shown as autoradiogram. (b): Fast atom bombardment mass spectrum of [D-Abu⁸]CyA, showing the correct molecular ion peak (1216 (M + H)⁺) (from [64], with permission).*

contrast, tyrosine totally inhibits the biosynthetic activity of cyclosporin synthetase [34].

As the *in vitro* incorporation of L-2-amino-octanoic acid and L-2-amino-3-hydroxyoct-6-enoic acid demonstrates, the 4-methyl group is also not essential for substrate recognition. Furthermore, both steric positions are allowed for the 4-methyl group, as has been shown by the incorporation of L-2-amino-3-hydroxy-4, 4-dimethyloctanoic acid [33]. The steric requirements for the substituents at C-4 appear to be minimal: we found strong evidence that L-2-amino-3-hydroxy-4-butyloctanoic acid is incorporated into cyclosporin *in vitro* [34].

The length of the alkyl chain, as far as we tested, may range from three C-atoms (Leu) to seven (L-2-amino-3-hydroxy-4, 8-dimethylnonanoic acid, the amino acid with the longest alkyl chain we tested). For intermediate lengths, we found good evidence for the incorporation of norleucine and L-2-amino-4-methylhex-4-enoic acid, both containing a four-carbon chain at C_α. Threonine (two-carbon chain) is not an effective substrate of the enzyme at this position; serine, on the other hand, probably does serve as a substrate, in spite of its carrying only a hydrogen atom instead of the alkyl chain of Bmt. Interestingly, the hydrolysis artefact of Bmt, *cyclo*-(dihydro-Bmt) [39], is also accepted by the enzyme [34].

Introduction of charges other than the 3-hydroxy group leads to the loss of substrate recognition, as has been shown for phosphinothricin (2-amino-4-(hydroxymethylphosphinyl)butanoic acid) and N-ε-Boc-L-Lys. The incorporation experiments with various precursors in position 1 of cyclosporin are summarized in *Table 2.1*.

Since the related cyclosporins and SDZ 214-103 and its homologues are, to our knowledge, the only naturally occurring peptides containing the new amino acid Bmt as a building unit, we also expected to find a very similar substrate specificity for both synthetases, especially for position 1 containing the unique MeBmt. Surprisingly, the two enzymes showed great differences in their substrate recognition; in general, cyclosporin synthetase recognizes a broader pattern of amino acids at the majority of the active sites than does peptolide SDZ 214-103 synthetase.

As for cyclosporin synthetase, at position 1 peptolide SDZ 214-103 synthetase recognizes the saturated Bmt, L-2-amino-3-hydroxy-4-methyloctanoic acid (dihydro-Bmt), as a substrate to form dihydro-SDZ 214-103. But, unlike cyclosporin synthetase, peptolide SDZ 214-103 synthetase cannot incorporate L-2-amino-3-hydroxy-4, 4-dimethyloctanoic acid, demonstrating that the stereochemistry of the 4-methyl group is probably critical for substrate recognition by peptolide SDZ 214-103 synthetase. It also appears from our experiments that substrates devoid of a 4-methyl

Table 2.1. INCORPORATION STUDIES WITH VARIOUS AMINO ACIDS IN POSITION 1 OF CYCLOSPORIN A.

N-Methylated amino acid	*New substance detected*	*Co-chrom. with ref.*[1]	*TLC R_f-value*[2]	*HPLC α-value*[3]	*New cyclosporin isolated*[4]	*FAB-value ($M+H^+$)*	*Immunosuppressive activity*
L-2-Amino-3-hydroxy-4-methyloctanoic acid	+	+	0.40	12.18	–	n.d.	n.d.
L-2-Amino-3-hydroxy-4,4-dimethyloctanoic acid	+	+	0.43	13.74	+	1217	n.d.
L-3-Cyclohexylalanine	+	+	0.48	19.79	–	n.d.	n.d.
Leu	+	+	0.43	11.04	–	n.d.	n.d.
L-2-Amino-octanoic acid	+	+	0.55	24.58	–	n.d.	n.d.
3-Hydroxy-3-cyclohexyl-L-alanine	+	n.a.	0.37	12.01	+	1203	+
L-2-Amino-3-hydroxyoct-6-enoic acid	+	+	0.40	9.00	–	n.d.	n.d.
L-2-Amino-3-hydroxy-4,8-dimethylnonanoic acid	+	+	0.37	27.73	–	nd.	n.d.
L-2-Amino-3-hydroxy-4-butyloctanoic acid	+	n.a.	0.40	21.86	–	n.d.	n.d.
cyclo-(dihydro-Bmt)	+	+	0.47	10.82	–	n.d.	n.d.
Nle	+	n.a.	0.42	n.d.	–	n.d.	n.d.
L-2-Amino-4-methylhex-4-enoic acid	+	n.a.	0.36	13.82	–	n.d.	n.d.
Ser	+	+	0.10	4.10	–	n.d.	n.d.
allo-Thr	+	n.a.	0.22	4.50	–	n.d.	n.d.
Cys	+	n.a.	0.21	5.36	–	n.d.	n.d.
D-2-Amino-3-hydroxy-4,4-dimethyloctanoic acid	no						
Thr	no						
Phe	no						
Tyr	no						
Phosphinothricin	no						
N-ε-Boc-L-Lys	no						

For each amino acid, three assays were performed: one with all constitutive amino acids of CyA, a second in which Bmt was omitted and a third with the new amino acid replacing Bmt. The extracts of the incubation mixtures were chromatographed on TLC (with permission from [34]).

[1] Co-chromatography by TLC and HPLC.

[2] TLC system: silica gel 60 HPTLC plates, water-saturated EtOAc, 2 x 10 cm.

[3] The α-factor is defined as relative retention time $[(t_{R,1}-t_0)/(t_{R,2}-t_0)]x10$, whereat $t_{R,1}$ and $t_{R,2}$ mean the corresponding retention times, and t_0 is the void retention time. CyA is taken as reference compound ($\alpha = 10.00$).

[4] Isolation of unlabelled cyclosporins by preparative HPLC was performed as described in [33].

n.d., not done; n.a., reference not available.

moiety, or carrying an enlarged alkyl group at this position, are not incorporated by this enzyme (*Table 2.2*). The 3-hydroxy group appears not to be essential as demonstrated by the incorporation of both L-3-cyclohexylalanine and L-3-hydroxy-3-cyclohexylalanine. As in the case of cyclosporin biosynthesis, addition of tyrosine to the incubation mixture is inhibiting [34].

Incorporation of amino and hydroxy acids into position 8.

Position 8 of the cyclosporin molecule is the starting position of the biosynthetic process [71] and the only position in cyclosporin with a D-amino acid. All substrates for this position with the exception of glycine have to be in the D-configuration for recognition by the enzyme. Only slightly longer side-chains than in D-Ala are allowed: D-Abu, D-vinylglycine and probably the branched amino acid D-valine are substrates for position 8. Nevertheless, exchange of D-Ala in the incubation mixture by the last one does not lead to the synthesis of [D-Val^8]CyA as indicated by the FAB ($M + H^+$)-value of 1216 for the reaction product instead of the expected value of 1230. We proposed that, as a result of the branched side-chain of valine, the enzyme selects a smaller neighbouring side-chain leading to the synthesis of [Gly^7, D-Val^8]CyA [34]. Since the D-valine-dependent synthesis of a new substance can be observed when D-Ala is omitted from the incubation mixture, D-Val is obviously incorporated at position 8 of cyclosporin. On the other hand, the incorporation of the D-Ala-analogue D-cyclopropylglycine, which should not have the same space requirements as D-Val, yields, although in small amounts, [D-cyclopropylglycine^8]CyA. The β-position of D-Ala may carry a hydroxyl- (D-Ser), a sulphydryl-(D-Cys) or a halogeno-group (3-chloro-D-Ala, 3-fluoro-D-Ala), whereas the double- and three-fold-substituted amino acids 3, 3-difluoro-D-Ala and 3, 3, 3-trifluoro-D-Ala are not substrates of cyclosporin synthetase. Amino acids with longer side-chains substituted by polar residues at C-3, as in 3-chloro-D-vinylglycine, both, D-Thr and D-allo-Thr and at C-4 (D-homoserine) do not serve as building blocks for enzymatic incorporation into cyclosporins. Interestingly, the aromatic amino acid D-Phe is a minor substrate of the enzyme (*Table 2.3*).

The SDZ 214-103 synthetase substrates D-2-hydroxyisovaleric acid and D-lactic acid (the latter one is the isoster hydroxy acid to D-Ala) are not substrates of cyclosporin synthetase [68]. Thus, cyclosporin synthetase is not capable of introducing an ester linkage between positions 7 and 8, as SDZ 214-103 synthetase does [34].

As far as has been analyzed, peptolide SDZ 214-103 synthetase recognizes

Table 2.2. INCORPORATION STUDIES WITH VARIOUS AMINO ACIDS IN POSITION 1 OF PEPTOLIDE SDZ 214–103.

N-*Methylated amino acid*	*New substance detected*	*Co-chrom. with ref.*	*TLC R_f-value*	*HPLC α-value*	*New peptolide isolated*	*FAB-value ($M+H^+$)*	*Immuno-suppressive activity*
L-2-Amino-3-hydroxy-4-methyl-octanoic acid	+	+	0.43	23.4	–	n.d.	n.d.
L-3-Cyclohexyl-alanine	+	n.a.	0.57	35.0	+	n.d.	+
Leu	+	n.a.	0.48	23.0	+	n.d.	+
3-Hydroxy-3-cyclo-hexyl-L-alanine	+	n.a.	0.38	n.d.	–	n.d.	n.d.
cyclo-(dihydro-Bmt)	+	n.a.	0.76	31.3	+	n.d.	+
L-2-Amino-octanoic acid	no						
L-2-Amino-3-hydroxy-4,4-dimethyl-octanoic acid	no						
L-2-Amino-3-hydroxy-4,8-dimethyl-nonanoic acid	no						
L-2-Amino-3-hydroxy-4-butyl-octanoic acid	no						
L-2-Amino-3-hydroxyoct-6-enoic acid	no						
L-2-Amino-4-methylhex-4-enoic acid	no						
Nle	no						
Ser	no						
Thr	no						
Phe	no						
Tyr	no						
N-ε-Boc-L-Lys	no						
Phosphinothricin	no						
D-2-Amino-3-hydroxy-4,4-dimethyloctanoic acid	no						

For each amino acid, three assays were performed: one with all constitutive amino acids of peptolide SDZ 214–103, a second in which Bmt was omitted and a third with the new amino acid replacing Bmt. The extracts of the incubation mixtures were analyzed as described in the legend of *Table 2.1* (with permission from [34]).

Table 2.3. INCORPORATION STUDIES WITH VARIOUS AMINO ACIDS IN POSITION 8 OF CYCLOSPORIN A.

Amino or hydroxy acid	*New substance detected*	*Co-chrom. with ref.*	*TLC R_f-value*	*HPLC α-value*	*New cyclosporin isolated*	*FAB-value ($M+H^+$)*	*Immunosuppressive activity*
D-Abu	+	n.a.	0.43	13.09	+	1216	+
D-Ser	+	+	0.26	5.45	–	n.d.	n.d.
Gly	+	+	0.21	7.58	+	1188	+
3-Fluoro-D-alanine	+	+	0.46	9.84	+	1220	+
3-Chloro-D-alanine	+	+	0.49	11.73	+	n.d.	+
D-Vinylglycine	+	n.a.	0.45	12.11	+	1214	+
D-Cys	+	+	0.40	12.06	–	n.d.	n.d.
D-Phe	+	+	0.63	35.49	+	n.d.	+
D-Cyclopropylglycine	+	n.a.	0.48	15.20	+	n.d.	+
D-Val[1]	+	–	0.33	11.68	+	1216	+
D-tert-Butylalanine	+	n.a.	0.34	n.d.	–	n.d.	n.d.
D-Lys	+	+	0.32[2]	5.43	–	n.d.	n.d.
1-Chloro-D-vinylglycine	+	n.a.	0.45[3]	n.d.	–	n.d.	n.d.
D-Nva	no						
D-Ile	no						
D-allo-Ile	no						
DL-tert-Butylglycine	no						
2-Hydroxyethyl-D-serine	no						
DL-Homoserine	no						
D-Thr	no						
D-allo-Thr	no						
3,3-Difluoro-DL-alanine	no						
3,3,3-Trifluoro-DL-alanine	no						
D-Cyclohexylalanine	no						
D-Pro	no						
N-ε-Boc-D-Lys	no						
D-Orn	no						
D-2-Hydroxyisovaleric acid	no						
D-Lactic acid	no						

For each amino acid, three assays were performed: one with all constitutive amino acids of CyA, a second in which D-Ala was omitted and a third with the new amino or hydroxy acid replacing D-Ala. The extracts of the incubation mixtures were analyzed as described in the legend of *Table 2.1* (with permission from [34]).

[1] The data obtained argue strongly against the synthesis of [D-Val[8]]CyA; nevertheless they indicate incorporation of D-Val into position 8. We prefer the interpretation that [Gly[7],D-Val[8]]CyA is made by the enzyme because of steric hindrance due to the valine in position 8.

[2] For separation of this basic cyclosporin another solvent mixture had to be used: $CHCl_3$:MeOH:AcOH = 80:20:2 (R_f for CyA = 0.86).

[3] Since the product in the preparation of 1-chloro-D-vinylglycine used in the assay contained some 20% of D-vinylglycine, [D-vinylglycine[8]]CyA was made by the enzyme.

D-2-hydroxy acids carrying alkyl chains at the α-carbon from methyl (D-lactic acid) to butyl (D-2-hydroxycaproic acid). Methyl branches at C-3 (D-Hiv, D-2-hydroxy-3-methylvaleric acid) and C-4 (D-2-hydroxy-4-methylvaleric acid (= D-2-hydroxyisocaproic acid)) do not hinder incorporation of the hydroxy acid into the peptolide. On the other hand, the α-branched D-2-hydroxyisobutyric acid cannot serve as a substrate.

In the case of cyclosporin synthetase, D-Ala is the building block of all natural cyclosporins [39]. The isoster D-lactic acid is a good substrate of peptolide SDZ 214-103 synthetase, leading to a peptolide with high immunosuppressive activity (*Table 2.4*). Glycolic acid, the isoster of the

Table 2.4. INCORPORATION STUDIES WITH VARIOUS HYDROXY ACIDS IN POSITION 8 OF PEPTOLIDE SDZ 214-103.

Hydroxy acid or amino acid	*New substance detected*	*TLC R_f-value*	*HPLC α-value*	*New peptolide isolated*	*FAB-value ($M + H^+$)*	*Immunosuppressive activity*
D-Lactic acid	+	0.37	10.5	+	1219	+
D-2-Hydroxybutyric acid	+	0.41	15.1	+	1233	+
D-2-Hydroxy-n-valeric acid	+	0.42	18.5	+	1247	+
D-Hiv	+	0.42	19.0	+	n.d.	+
D-2-Hydroxy-3-methylvaleric acid	+	0.42	23.6	+	1261	+
D-2-Hydroxyisocaproic acid	+	0.43	23.9	+	1261	+
D-2-Hydroxycaproic acid	no					
D-2-Hydroxyisobutyric acid	no					
Glycolic acid	no					
DL-Isoserine	no					
D-Glyceric acid	no					
DL-Tartaric acid	no					
DL-3-Phenyl-lactic acid	no					
D-Ala	no					
D-Val	no					

For each hydroxy acid, three assays were performed: one with all constitutive amino acids of SDZ 214-103 and D-Hiv, a second in which D-Hiv was omitted and a third with the new hydroxy or amino acid replacing D-Hiv. The extracts of the incubation mixtures were analyzed as described in the legend of *Table 2.1* (with permission from [34]).

good substrate for position 8 of the cyclosporins, Gly, is not a substrate of peptolide SDZ 214-103 synthetase, which prefers longer alkyl side-chains. Both synthetases efficiently accept the ethyl side-chain (D-Abu or D-2-hydroxybutyric acid), whereas the propyl analogue is only a substrate for peptolide SDZ 214-103 synthetase (D-2-hydroxy-n-valeric acid; not D-Nva in the cyclosporins). Likewise, D-Val, the isoster of the naturally occurring SDZ 214-103 hydroxy acid D-Hiv, is not incorporated into cyclosporin to form [D-Val^8]CyA; this may be due to steric hindrance at neighbouring sites rather than the specificity of the D-Ala site, as discussed above.

On the other hand, the aromatic D-phenyllactic acid, the isoster of D-Phe, which is a substrate for cyclosporin synthetase, is not incorporated into peptolide SDZ 214-103 analogues. Incorporation of hydroxy acids with charged side-chains appears not to be successful at all; thus, the D-Ser isoster D-glyceric acid does not serve as a substrate. Likewise D-isoserine and the multiply charged D-tartaric acid are not incorporated. D-Amino acids are, in general, not substrates of SDZ 214-103 synthetase (*Table 2.4*, [68]).

Peptides are not substrates of cyclosporin synthetase [34], as has been described for gramicidin S synthetase 2 [86]. Nevertheless, one very exciting result of our recent work was the proof that this enzyme is capable of introducing a β-alanine into position 7 or 8 of the ring instead of the α-alanines present at these positions in cyclosporin A. This leads to 34-membered rings in contrast to the 33-membered ring of the cycloundecapeptide cyclosporin A. To our knowledge, this is the first time that such a phenomenon has been described. We assume that the position of the final cyclization reaction is the less restricted in this enzyme, but again, this does not hold true for the peptolide SDZ 214-103 synthetase, which was not capable of synthesizing ring-extended peptolides [84]. At present, it remains an open question, whether it is an unusual characteristic of cyclosporin synthetase to synthesize ring-extended peptides or whether the failure of peptolide SDZ 214-103 synthetase to do so is the exception within this class of multienzyme polypeptides. Both [βAla^7]CyA and [βAla^8]CyA show immunosuppressive activity [84].

β-Ala is a rather rare structural element in naturally occurring peptides. The list of β-Ala-containing peptides so far comprises besides pantothenic acid [87], the bursaphelocides [88], leucinostatines [89, 90], destruxins [91], roseotoxins [92], efrapeptins [93], leualacin [94], the Theonella-peptolide [95], carnosine and β-alanylornithine [96] and sarcophagine [97].

Besides providing knowledge on the enzymology of the unusual large enzymes cyclosporin synthetase and peptolide SDZ 214-103 synthetase, these studies also provide information as to whether trials to produce cyclosporins or peptolides of special interest by precursor-directed biosyn-

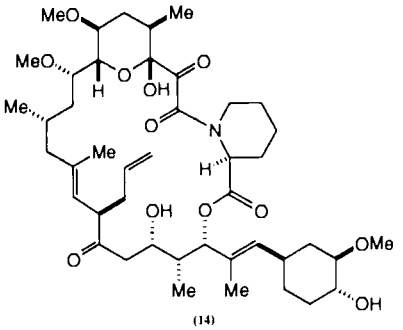
thesis are worth doing or not. These data will therefore be important for all further studies on fermentation of cyclosporin analogues.

BIOLOGICAL ACTIVITIES OF *IN VITRO* SYNTHESIZED CYCLOSPORINS

With one exception, all *in vitro* synthesized cyclosporin and peptolide SDZ 214-103 analogues exhibit immunosuppressive activity. *Table 2.5* shows immunosuppressive activities of a representative selection of cyclosporins and peptolides measured *in vitro*. The structure-activity relationship of the cyclosporins will be discussed below. So far, the only known cyclosporin without immunosuppressive activity accessible *in vitro* is [MeIle^4]CyA [98]. Interestingly, this compound exerts a very potent anti-HIV activity [99]. In a series of experiments to revert multidrug resistance *in vitro* involving several *in vitro* synthesized cyclosporins, we found peptolide SDZ 214-103 to be the most active compound [100, 100a].

BIOSYNTHESIS OF FK506

Although the biosynthesis of the macrolide FK506 (14) is not directly related to the peptide biosyntheses discussed above, recent knowledge should be briefly summarized. Evidence has been published for the existence



(14)

of a multienzyme complex responsible for FK506 biosynthesis [101]. One enzyme of this complex, a 170 kDa pipecolic acid-activating enzyme, has been purified. This enzyme activates pipecolic acid as an aminoadenylate and subsequently forms a pipecolic acid thioester [101]. It appears to be involved in the first steps of FK506 biosynthesis. A second enzyme involved in FK506 biosynthesis, namely a 32 kDa [102] 31-*O*-demethylimmunomycin *O*-methyltransferase has also been reported [103].

Table 2.5. IMMUNOSUPPRESSIVE ACTIVITY OF SOME IN VITRO SYNTHESIZED CYCLOSPORIN A AND PEPTOLIDE SDZ 214–103 ANALOGUES

Cyclopeptide	*Biosynthesis*	*Activity*
Cyclosporin A (Sandimmun^{R})	natural	+ + +
Cyclosporin A	enzymic	+ + +
[Me-3-hydroxycyclohexylalanine^{1}]CyA	enzymic	+ +
[Nva^{2,5},MeNva^{11}]CyA	enzymic	+ +
[MeIle^{4}]CyA	enzymic	–
[Nva^{5},MeNva^{11}]CyA	enzymic	+ +(+)
[aIle^{5},aMeIle^{11}]CyA	enzymic	+ +
[aIle^{5,11}]CyA	enzymic	+
[Ile^{5},MeIle^{11}]CyA	enzymic	+
[Cyclopropylglycine^{5},MeCyclopropylglycine^{11}]	enzymic	+ +(+)
[Gly^{7}]CyA	enzymic	+ +
[βAla^{7}]CyA	enzymic	+ +
[Gly^{7,8}]CyA	enzymic	+ +
[Abu^{7},D-Abu^{8}]CyA	enzymic	+ +
[ß-chloro-D-Ala^{8}]CyA	enzymic	+ +
[ß-fluoro-D-Ala^{8}]CyA	enzymic	+ +
[D-Abu^{8}]CyA	enzymic	+ +
[D-Phe^{8}]CyA	enzymic	+
[D-Cyclopropylglycine^{8}]	enzymic	+ +(+)
[Gly^{8}]CyA	enzymic	+ +(+)
[βAla^{8}]CyA	enzymic	+ +
SDZ-214–103	natural	+ + +
SDZ-214–103	enzymic	+ + +
[MeCyclohexylalanine^{1}]-SDZ 214–103	enzymic	+ +
[3-Hydroxynva^{2}]-SDZ-214–103	enzymic	+ + +
[Abu^{7}]-SDZ-214–103	enzymic	+ +(+)
[D-Lactic acid^{8}]-SDZ-214–103	enzymic	+ + +
[D-2-Hydroxybutyric acid^{8}]-SDZ-214–103	enzymic	+ + +
[D-2-Hydroxy-n-valeric acid^{8}]-SDZ-214–103	enzymic	+ + +
[D-2-Hydroxy-3-methylvaleric acid^{8}]-SDZ-214–103	enzymic	+ +(+)
[D-2-Hydroxyisocaproic acid^{8}]-SDZ-214–103	enzymic	+ +
[MeAbu^{11}]-SDZ 214–103	enzymic	+ +(+)
[MeaIle^{11}]-SDZ 214–103	enzymic	+ +(+)
[MeLeu^{11}]-SDZ 214–103	enzymic	+ +

+ + + = strong immunosuppressive activity
+ + = moderate activity
+ = weak activity
– = no activity

IMMUNOPHILINS

CYCLOPHILIN A

In 1984, Handschumacher *et al.* discovered a cytosolic cyclosporin A-binding protein, which he called 'cyclophilin' [104]. Cyclophilin has been detected in all organisms and organs examined (for a review see [105]) and has a molecular mass of 17.7 kDa [106]. It has been demonstrated that the immunosuppressive activity of various cyclosporins is proportional to their affinity for cyclophilin; therefore the inhibition of an unknown enzymatic activity of the latter was likely to be the reason for the immunosuppressive activity of CyA [107, 108].

Also in 1984, Fischer and co-workers detected an enzyme which is likely to be involved in the folding of prolyl-containing proteins. They called it 'peptidyl-prolyl-*cis/trans*-isomerase' (PPIase) [109]. Fischer's group, and independently Takahashi and colleagues, were able to show in 1989 that PPIase is identical with the CyA-binding protein cyclophilin [110, 111]. This discovery prompted a significant increase in research activities on the biochemistry of cyclophilin and since then various distinct cyclophilins have been detected; the 17.7 kDa protein therefore is now called 'cyclophilin A'.

PEPTIDYL-PROLYL-*CIS/TRANS*-ISOMERASE ACTIVITIES

The PPIase activity is usually measured by an assay developed by Fischer *et al.* [109]. The assay makes use of the fact that the chromophore in the substrate tetrapeptide succinyl-alanyl-alanyl-prolyl-phenyl-*para*-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA) can only be split off by chymotrypsin when the Ala-Pro bond has the *trans* conformation. In aqueous solution, that is the case for about 90% of this peptide. When the peptide is incubated together with an excess of chymotrypsin, 90% (the portion in the *trans* conformation) are digested during the time of mixing, which can be followed by an instantaneous increase of the extinction. The remaining 10% of the peptide in the *cis* conformation then begins to isomerize in order to restore the equilibrium. This reaction is catalyzed by the PPIase. The enzymatic activity is inhibited by CyA [110, 111]. PPIase itself does not change the equilibrium of the isomers.

Soon after the discovery that cyclophilin A is identical with PPIase, it was shown that the 'FK506 binding protein' (FKBP) also exerts PPIase activity [112, 113]. The activity of FKBP is inhibited by the binding of FK506 and the related rapamycin, but not by CyA, whereas cyclophilin's PPIase activity is not inhibited by FK506 or by rapamycin [112, 113]. Both the

cyclophilins and FKBP's of different origins are highly conserved, whereas there exists almost no homology between the two families [114]. Very recently, the first member of a third group of PPIases, called 'parvulins' has been described [115]. The present knowledge on the PPIase activities has been summarized [116].

IMMUNOPHILINS

When Harding *et al.* [112] described the first FKBP (now called 'FKBP-12') they introduced the name 'immunophilin' as a common name for both classes of PPIases, the cyclophilins and the FKBP's. To date, at least five different cyclophilins and four different FKBP's, which can be distinguished by their molecular masses, cellular and tissue distribution (*Table 2.6*) and the sequences of their genes (for a review see [133]) have been characterized from higher eukaryotes. Both, cyclophilins and FKBP's have been found to occur across species from bacteria to man (for review see [133]). Interestingly, the strains of both cyclosporin A- and peptolide SDZ 214-103-producing fungi contain both cyclophilins and FKBP's [Bang, H., Lawen, A. and Fischer, G., unpublished results]; the same holds true for the producers of macrolide immunosuppressants [134, 135].

Table 2.6. VERTEBRATE IMMUNOPHILINS

Immunophilin	*Molecular mass (kDa)*	*Localization*	*References*
Cyclophilin A	18	cytoplasm, nuclear membranes	103, 117 118
Cyclophilin B	23	membranes, organelles	119–121
Cyclophilin C	22	tissue specific	122
Cyclophilin D	22	membranes, organelles	120
Cyclophilin-40	40	associated with the steroid receptor	123–125
FKBP-12	12	cytosolic	112, 126
FKBP-13	13	membranes	126, 127
FKBP-25	25	nucleus	128, 129
FKBP-59	59	associated with steroid receptor and heat shock proteins	130 131
		cytoskeleton, nucleus	132

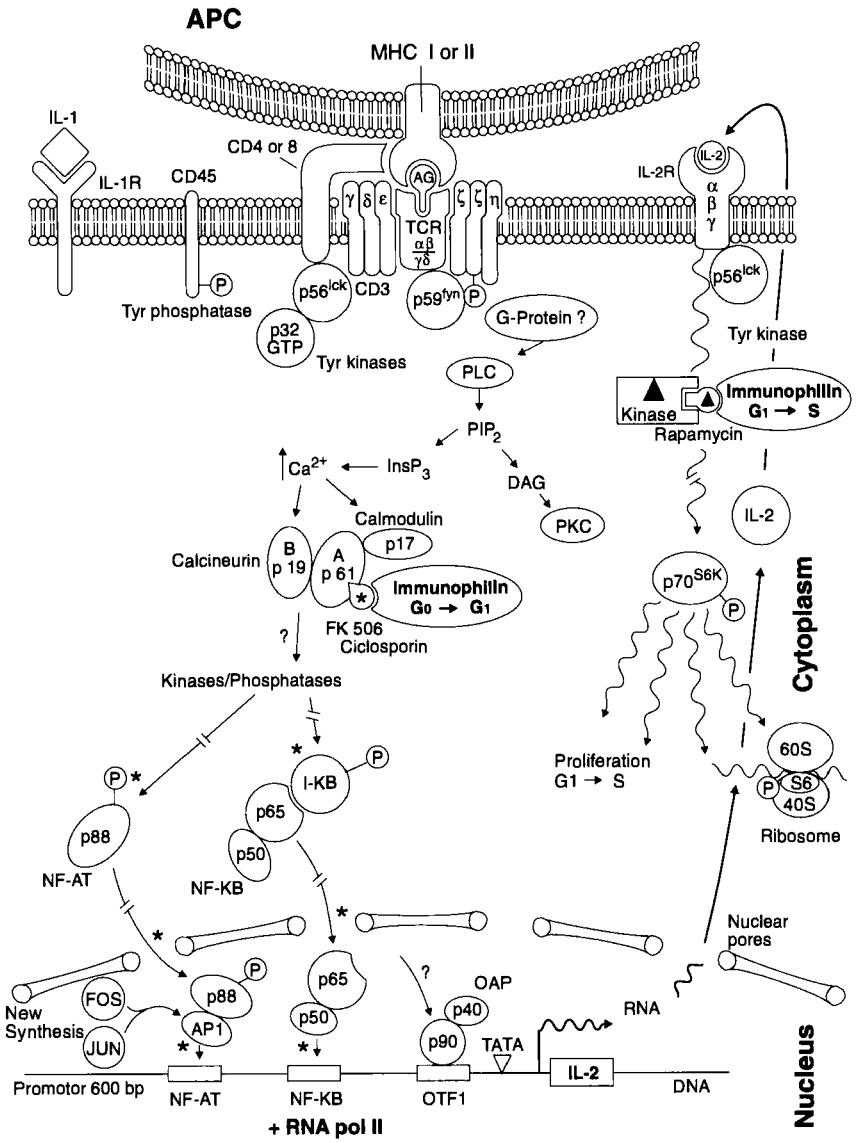
CALCINEURIN-INHIBITION AND IMMUNOSUPPRESSION

The fact that the immunophilins are almost ubiquitous proteins strongly argues against the hypothesis that inhibition of the PPIase activity could be the reason for the specific inhibition of T cell proliferation by the immunosuppressants. Furthermore, the doses of these compounds needed *in vivo* are only sufficient to inhibit about 10% of the total respective PPIase activity in the blood. In addition, some analogues of the immunosuppressants have been found which show no correlation between their affinity for the immunophilin and their immunosuppressive activity [122, 136]. For the cyclosporins, these were the non-immunosuppressive analogue [MeAla^6]CyA, which binds well to cyclophilin, and [(2*S*, 3*R*, 6*E*)-4, 4-dimethyl-3-hydroxy-6-octenoic acid^1]CyA ([MeBm$_2$t]CyA), which exerts *in vitro* immunosuppressive activity but does not inhibit cyclophilin [122]. Finally, it was shown that the pathway which is inhibited by rapamycin is a Ca^{2+}-independent one, whereas FK506 (like CyA) inibits a Ca^{2+}-dependent pathway [136]. Since both FK506 and rapamycin bind to the same immunophilins (FKBP's) and inhibit their activity, it became evident that the reason for the immunosuppression could not be the inhibition of the PPIase activity.

In 1991, two groups reported the detection of a protein which specifically binds the complexes CyA-cyclophilin and FK506-FKBP, but not the complex rapamycin-FKBP or the uncomplexed immunophilins or immunosuppressants [137, 138]. This 61 kDa protein was identified as subunit A of the serine/threonine protein phosphatase 2B (calcineurin). Calcineurin is a Ca^{2+}/calmodulin-dependent phosphatase which is inhibited by the binding of the immunosuppressant-immunophilin complexes.

A second important insight into the mechanism of action of the immunosuppressants was reported in the same year by Crabtree's group [139]: They demonstrated that the translocation of the cytosolic subunit (p88) of the transcription factor NF-AT to the nucleus is inhibited by CyA and FK506, leading consequently to the inhibition of interleukin-2 (IL-2) transcription.

Further experiments gave proof of the involvement of calcineurin inhibition in immunosuppression: (1) Overexpression of calcineurin in T cells renders these more resistant to the effects of CyA and FK506 [140]; (2) The complex [MeAla^6]CyA-cyclophilin does not bind to calcineurin, whereas the complex [MeBm$_2$t]CyA-cyclophilin binds and inhibits calcineurin[141, 142]; (3) phosphorylated p88 has been shown to be a substrate of calcineurin [143]. The recent understanding of the mechanism of action of the immunosuppressants cyclosporin A, FK506 and rapamycin is summa-



rized in *Figure 2.10*. Very recently, a rapamycin-FKBP-binding protein, 'FKBP-rapamycin-associated protein (FRAP)', has been reported [144]. Whether or not it exerts phosphatidylinositol-3-kinase activity, as predicted from some sequence homology is not yet clear. Furthermore, rapamycin has

←

Figure 2.10. Mechanisms of action of immunosuppressants. CyA and FK506 both interfere, by binding to their cognate immunophilins, with molecules of the calcium-associated signalling pathway from the T-cell receptor (TCR) to the activation of interleukin 2 (IL-2) transcription in the nucleus. TCR is activated by binding of the major histocompatibility complex (MHC) (which has bound an antigen peptide (Ag)) of an antigen-presenting cell (APC). Transcriptional regulation of the IL-2 gene is modulated by a combination of transcription factors, such as nuclear factor of activated T-cells (NF-AT), nuclear factor of immunoglobulin K light chain in B-cells (NF-KB) and octamer transcription factor (OTF) 1, interacting with their corresponding recognition sites at the IL-2 promoter. These DNA/protein complexes, together with RNA polymerase II (RNA pol II), are necessary for IL-2 transcription. Potential interaction sites of the pentameric complex [calcineurin A (p61), B (p19), calmodulin (p17), immunophilin and immunosuppressant], including for example, sites for modification and translocation of antigen-inducible transcription factors [NF-AT (p88), NF-KB (p50, p65)], are indicated by asterisks. CyA and FK506 interfere with the G_0 to G_1 transition of the cell cycle (Ca^{2+}-dependent), whereas rapamycin (bound to its cognate immunophilin FKBP) interferes with the (Ca^{2+}-independent) G_1 to S transition of the cell cycle, inhibiting the stimulation of p70 S6 kinase ($p70^{S6K}$), perhaps by inhibiting a kinase upstream. The G_1 to S transition is stimulated by the binding of IL-2 to the IL-2 receptor (IL-2R) (adapted from [146] with permisson.).

been shown to prevent the IL-2-stimulated dissociation of the inhibitor protein $p27^{Kip1}$ from cyclin-E associated cyclin-dependent kinase 2 [145].

Structure-activity relationships

The discovery of calcineurin as target for the CyA-cyclophilin and FK506-FKBP complexes, but not for the complex of rapamycin and FKBP, has led to a dual domain concept for the immunosuppressants [146] (*Figure 2.11*). This concept has meanwhile gained considerable support from studies using CyA analogues and from the solutions of the structures of immunophilin-immunosuppressant complexes.

As was discussed earlier in this series [1], the amino acids 10, 11, 1, 2 and 3 of the CyA molecule are involved in cyclophilin binding, a hypothesis derived from binding studies using a series of CyA analogues [106]. This has recently been confirmed by solutions of the NMR [147] and X-ray [148] structure of the CyA-cyclophilin A complex.

Interestingly, not only crystals of a monomeric CyA-cyclophilin A complex have been grown and their structure solved, but it has also been possible to solve the X-ray structure of a decameric CyA-cyclophilin A complex, consisting of two pentamers in a 'sandwich-like' association [149]. A monomeric unit of such a decameric complex is shown in *Figure 2.12*.

[$MeBm_2t^1$]CyA has been demonstrated to exert about 30% of the immunosuppressive activity of CyA, whereas it binds only with about 1% of the efficiency of CyA to cyclophilin A [122]. The X-ray structure of the

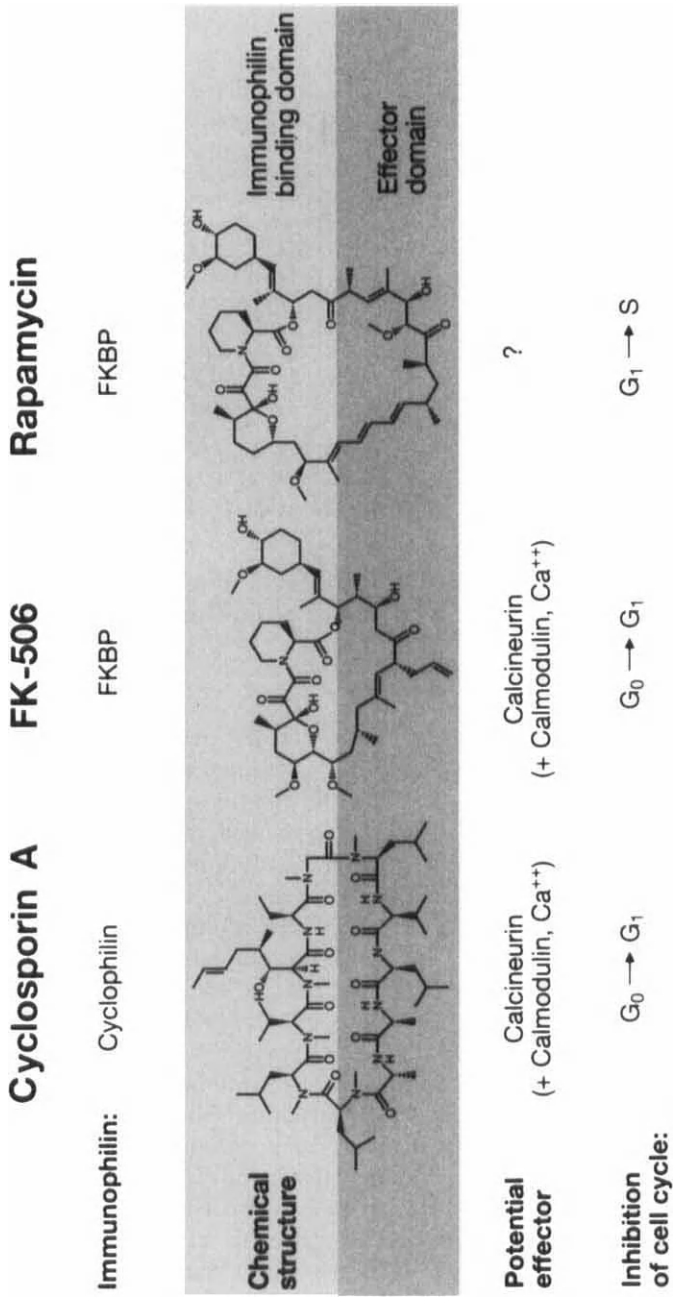


Figure 2.11. Dual domain concept for immunosuppressive compounds (from [146] with permission).

complex [MeBm$_2$t^1]CyA-cyclophilin A has also been solved [150], showing only small structural changes when compared with the structure of the CyA-cyclophilin A complex. These structural changes (concerning the side-chains of amino acids 1 and 10) obviously are beneficial for the binding to calcineurin.

[MeIle^4]CyA [98] inhibits cyclophilin with the same activity as CyA, but does not exert any immunosuppressive activity. Obviously this change from an unbranched to a branched C-β at position 4 abolishes the capability of binding to calcineurin, which as has been discussed, has a 'tight-binding' pocket for this region [151].

CALCINEURIN-INHIBITION IN OTHER BIOLOGICAL SYSTEMS

CyA and FK506 (and their analogues) have also been tools to investigate the involvement of calcineurin in pathways other than immunosuppression. Using analogues which inhibit their cognate immunophilins but do not inhibit calcineurin (like [MeIle^4]CyA and rapamycin), the involvement of calcineurin in various signalling pathways could be demonstrated: calcineurin has been shown to be involved in the recovery from the α-factor arrest in yeast [152], in the regulation of K^+ channels in guard cells [153], in exocytosis (serotonin release) of mast cells [154] and in the apoptotic signalling in B lymphocytes induced by inhibitors of the plasma membrane oxidoreductase system (for example, capsaicin) [155], the ionophore ionomycin [156] or by crosslinking of surface immunoglobulin M [157].

OTHER *IN VIVO* ACTIVITIES OF THE IMMUNOPHILINS

Peptidyl-prolyl cis/trans-*isomerase acitivities*

Although all immunophilins described so far have the PPIase activity in common, this activity is obviously not involved in the mechanism of immunosuppression. The role of PPIase activity has not yet been determined *in vivo*, but evidence is increasing that it does play a role. Thus, the transcription of the FKBP-13 mRNA in yeast is up-regulated in response to accumulation of unfolded precursor proteins in the endoplasmic reticulum [158] and CyA has been shown to inhibit an initial step in transferrin folding within the endoplasmic reticulum of Hep G2 cells [159].

In Drosophila, the cyclophilin homologue ninaA is required for the transport of rhodopsin 1 from the endoplasmic reticulum [160]; analysis of ninaA mutants has shown that the active site of the PPIase is required for its function [161].

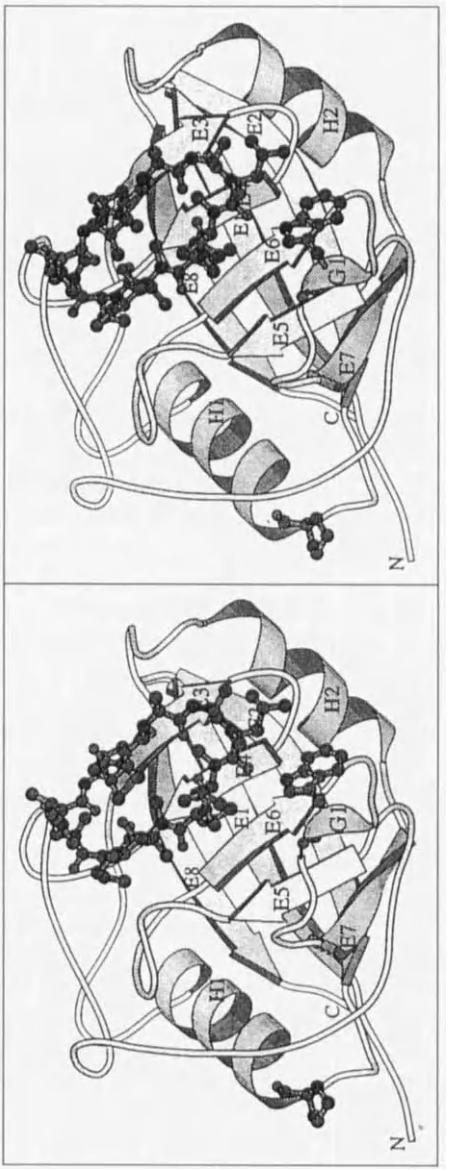


Figure 2.12. Stereodrawing of one of the ten CyA-cyclophilin A complexes. The ribbon representation has helices (H1 and H2), strands (E1-E8) and turns (white). CyA is shown as a dark shaded circular molecule (right-handed) (adapted from [148] with permisson).

The velocity of folding of type IV procollagen triple-helix has been shown to be significantly increased by cyclophilin A *in vitro* [162]. *In vivo*, collagen triple-helix formation is slowed by CyA, indicating the involvement of cyclophilin's PPIase activity in this process [163].

Transmission of HIV activity

The anti-HIV activity of CyA may possibly be due to its inhibition of HIV-induced T-cell apoptosis [21]. In the light of the finding that [MeIle^4]CyA is an even better anti-HIV drug than CyA itself, this mechanism is very unlikely. [MeIle^4]CyA does not bind as a complex with cyclophilin to calcineurin and is therefore unable to inhibit apoptosis [98, 99, 155, 164]. Luban *et al.* [165] have shown that both cyclophilin A and B specifically bind to the viral Gag protein. This binding is inhibited by CyA. Whether the cyclophilins have to act as PPIases or as chaperones for maintaining Gag functional is still unclear. Two recent publications report that cyclophilin A is specifically incorporated into HIV-1 virions [166, 167]. Binding of cyclophilin A to Gag requires a proline-rich region in the Gag protein. Replacing of Pro 222 by alanine blocks *in vitro* cyclophilin A binding and inhibits HIV-1 replication [166]. Incorporation of cyclophilin A into HIV-1 virions can be inhibited by CyA and [MeIle^4]CyA [166, 167].

Chaperone/heat shock protein-like activity

Besides its PPIase activity, cyclophilin A has also been suggested to exert a chaperone activity. This interpretation results from testing cyclophilin A in the *in vitro* folding of carbonic anhydrase [168, 169], although a recent report gives evidence for a misinterpretation of these results and suggests that the observed effects can be attributed to PPIase activity [170]. Indirect evidence for its function as a chaperone is the presence of the decameric unit of cyclophilin A-CyA complexes in crystals of the complex. Each decamer is composed of two pentamers in a sandwich-like manner, a theme well-known from other chaperones and heat shock proteins [148]. Furthermore, cyclophilin expression can be up-regulated by mercuric chloride treatment of maize leaves [171] and by heat shock in yeast [172].

FKBP59 binds to the heat shock protein hsp90 [130, 131, 173] and binds as this complex together with cyclophilin-40 [124, 125] to the steroid receptor. FKBP59 can furthermore regulate the activity of the heme-regulated eIF-2α kinase directly by affecting its conformational state [174].

Other activities affecting the immune system

Both cyclophilin A and FKBP12 have been shown to possess chemotactic activities for leukocytes; these activities are inhibited by the cognate immunosuppressants [175, 176]. Cyclophilin A is secreted by macrophages in response to endotoxin [177], thus possibly acting itself as a cytokine.

CyA, as well as to the cyclophilins, can bind specifically to interleukin-8 (IL-8) [178], which, although sharing some sequence homology with cyclophilin, has no PPIase activity. Whether or not binding to IL-8 plays a role in the anti-inflammatory action of CyA is not yet known.

A cyclophilin-related protein is also involved in the activity of natural killer cells [179]. A 150 kDa surface tumour recognition protein of natural killer cells is homologous with cyclophilin in its N-terminus.

CONCLUSIONS

The mechanism of biosynthesis of the cyclosporins is now better understood and the open reading frame of the giant gene has been cloned. An *in vitro* system for biosynthesis of new analogues which allows *in vitro* screening of new drugs for various activities has been established. Finding CyA analogues with better biological activities, either immunosuppressive or non-immunosuppresive, still remains a major objective. Genetic work should allow us to construct new synthetases by making genetic chimeras of several modules involved in amino acid activation. The consequent construction of an enzyme mainly producing [MeIle^4]CyA would be of scientific and commercial interest.

Our understanding of the mechanism of action of the immunosuppressants has advanced considerably during the last few years. Nevertheless, several uncertainties still remain, for example, the identity of natural substrates of the PPIase and chaperone activities of immunophilins, and the exact mechanism of the anti-HIV activity of the cyclosporins.

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3 Recent Advances in the Chemistry and Biology of Carbapenem Antibiotics

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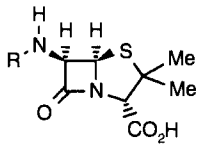
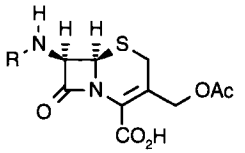
INTRODUCTION

The discovery of the antibacterial properties of a culture of the fungus *Penicillium notatum* by Alexander Fleming in 1929 [1] was a milestone in modern medicine. However, it was not until 1940 that a research team at Oxford University, led by Abraham, Chain and Florey, isolated penicillin G (1) in pure form and identified its immense potential for the treatment of a wide range of life threatening bacterial infections [2].



(1) R = $PhCH_2CO$; Penicillin G
(2) R = H; 6-APA



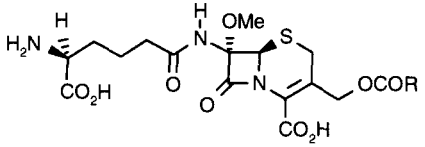
(3) R = $H_2NCH(CO_2H)CH_2CH_2CH_2CO$; Cephalosporin C
(4) R = H; 7-ACA

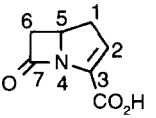
Antibacterial chemotherapy received a further impetus in 1959, when Beecham scientists recognized 6-aminopenicillanic acid (2) (6-APA) as a precursor of the bioactive penicillins during the fermentation of *Penicillium chrysogenum* [3]. These results prompted the production of 6-APA on a large scale, principally by the enzymatic deacylation of penicillin G. Chemical reacylation of 6-APA then led to the preparation of many new penicillins and the clinical use of compounds such as methicillin, ampicillin, amoxycillin, carbenicillin, ticarcillin, and flucloxacillin [4].

The successful development of semi-synthetic 6-APA derivatives also prompted considerable effort in cephalosporin research. Cephalosporin C (3) had been isolated by Abraham and colleagues from a strain of *Cephalosporium acremonium* supplied by Professor Brotzu [5]. Prior to the discovery of 6-APA, Brotzu had suggested that from micro-organisms associated with sewage outflows, one could expect to obtain compounds antagonistic to sewage bacteria; *Cephalosporium acremonium* was indeed such an organism. Chemical deacylation of cephalosporin C, followed by reacylation of the cephalosporin nucleus, 7-ACA (4), led to the clinical development of antibiotics such as cephalothin, cephaloridine and cephalexin, the so-called first generation cephalosporins [6, 7].

By the mid-1960's, with the widespread use of penicillins and cephalosporins, bacterial resistance became a major concern in β-lactam chemotherapy. During their early studies on the purification and evaluation

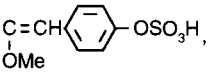
of penicillin, Abraham and Chain recognized that bacteria could produce an enzyme that was capable of inactivating the antibiotic [8]. This enzyme, which they named penicillinase, is one of a class of hydrolytic enzymes now referred to as β-lactamases.

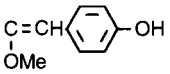




(6)

(5) R = NH_2 ,

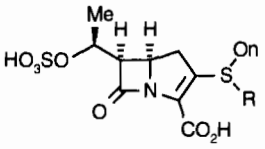


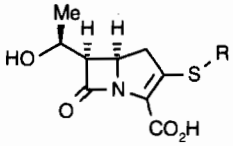


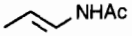
The year 1971 proved to be another landmark in β-lactam research when the β-lactamase-stable cephamycins (5) were reported simultaneously by Lilly [9] and Merck [10] workers. This discovery was of particular significance because these 7α-methoxy-cephalosporin derivatives were isolated from *Streptomyces* species*; all naturally occurring β-lactam antibiotics had previously been produced by fungi. Since then, extensive screening of soil micro-organisms by many researchers led to the discovery of many new 'non-classical' β-lactams, differing widely in structure from penicillins and cephalosporins. These include a novel family of β-lactam antibiotics based upon the carbapen-2-em-3-carboxylic acid (6) nucleus** and are represented by the olivanic acids (7-13) [11–14] and the thienamycins (14-16) [15]. Their structures are distinct from the classical β-lactam antibiotics in that (a) they have a highly strained 4, 5-bicyclic ring system consisting of an unsaturated five-membered ring in which a

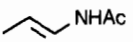
* *Streptomyces* are Gram-positive, aerobic bacteria of the Actinomycetales. They differ from fungi in that they are procaryotic, have bacterial-type cell walls and are inhibited by antibacterial agents. The streptomycetes form long chain spores, many species are pigmented and are found in soil.

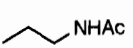
** The numbering system in this review is based upon the 'trivial' carbapenem nomenclature. The systematic nomenclature defines this ring system as 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.

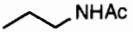


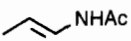


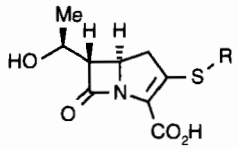
(7) R =  , n = 0; MM 13902

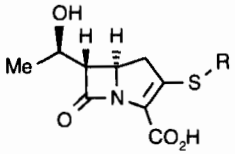
(8) R =  , n = 1; MM 4550

(9) R =  , n = 0; MM17880

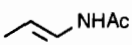
(10) R =  ; MM 22380

(11) R =  ; MM 22382

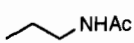




(12) R =  ; MM 22381

(13) R =  ; MM 22383

(14) R =  ; Thienamycin

(15) R =  ; N-Acetylthienamycin

(16) R =  ; N-Acetyldehydro-thienamycin

methylene replaces the sulphur atom at position 1, and (b) the C-6(7) acylamino-substituent of the bicyclic β-lactam ring is replaced by a carbon substituent, which invariably is an α-hydroxyethyl group. The so-called 'carbapenem' family of antibiotics display potent activity against a broad range of Gram-positive and Gram-negative bacteria, including β-lactamase-producing organisms. Furthermore, several members of this class of β-lactam antibiotics are potent inhibitors of β-lactamase enzymes.

These developments prompted considerable effort directed towards the chemical modification of these natural products, as well as their total syntheses. Much of this work has been the subject of numerous reviews [16–19] and will be discussed only briefly on this occasion. The present review focuses upon the development of the newer generation of totally synthetic carbapenem derivatives with improved metabolic stability.

β-LACTAM ANTIBIOTICS

MODE OF ACTION

The bacterial cell requires a rigid structure external to its cell membrane in order to maintain the integrity of the organism in hypotonic environments. This framework is provided by peptidoglycan, which is synthesized by all bacteria. β-Lactam antibiotics exert their lethal effect by inhibiting the synthesis of the bacterial peptidoglycan, thereby disrupting the morphology of the cell and eventually resulting in cell lysis and cell death [20–23].

Peptidoglycan is synthesized by all bacterial cells, but not by eukaryotic cells, and it is this property which provides the β-lactam antibiotics with their selective toxicity, *i.e.* their ability to kill bacterial cells without damaging mammalian cells.

The cell wall peptidoglycan is a network structure and all the peptidoglycans are built on the same general pattern [22, 24–27]. Linear glycan strands of alternative β, 1-4 linked *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M) pyranoside residues are substituted through the D-lactyl group of *N*-acetylmuramic acid, by L-Ala-γ-D-Glu-L-Xaa-D-Ala tetrapeptide units, where L-Xaa is most often a diamino acid, such as *meso*-diaminopimelic acid in *Escherichia coli* (see *Figure 3.1*)*. Peptide units substituting adjacent glycan strands are linked together by means of bridges that extend from the C-terminal D-alanine of one peptide to the ω-amino group of the diamino residue of another peptide.

Of the various discrete stages that have been identified for the biosynthesis of the bacterial cell wall, the final stage concerns the incorporation of newly formed disaccharide peptide units into pre-existing wall peptidoglycan; new peptide bonds must be made between nascent (uncross-linked) glycan strands and existing peptidoglycan. This reaction is called transpeptidation and is carried out by serine proteases, known as transpeptidases. Only when cross-links are formed does peptidoglycan become an insoluble matrix capable of maintaining the structural integrity of the wall. The sequence of events leading to the formation of cross-links in *E. coli* has several distinct stages. The transpeptidases operate by an acyl-enzyme mechanism, involving a serine residue at the active site. Initial formation of a non-covalent Michaelis complex between the enzyme and the terminal D-alanyl-D-alanine unit of a pentapeptide chain linked to muramic

* In Gram-positive bacteria, such as *Staphylococcus aureus*, the dibasic amino acid is L-Lysine and the amide cross-link has a Gly_5 peptide linker.

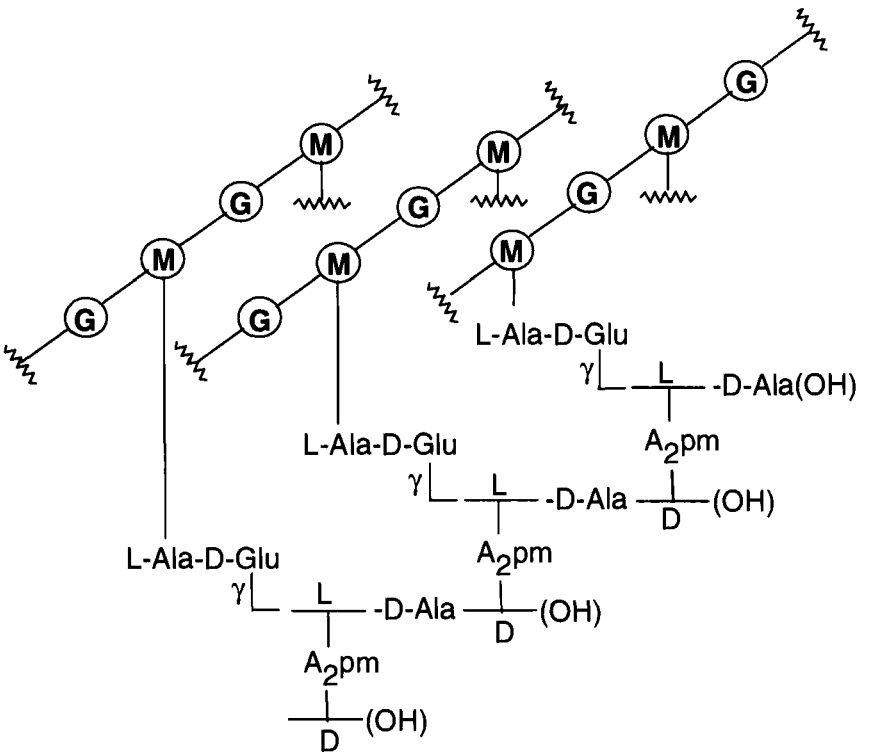


Figure 3.1. Wall peptidoglycan in E. coli showing cross-linking [24, 26]; G = N-acetylglucosamine, M = N-acetylmuramic acid, A_2pm = meso-diaminopimelic acid.

acid on a nascent glycan strand is followed by the formation of a covalent acyl-enzyme and elimination of the terminal D-alanine. Attack at the acyl-enzyme ester bond by a suitably positioned ω-amino group of a *meso* diamino-pimelic acid residue on an adjacent glycan strand provides the cross-link between the nascent glycan and the existing peptidoglycan of the wall; similar transpeptidations serve to cross-link the glycan strands at other points and firmly link the network together (*Figure 3.2*).

In *E. coli*, only 20-30% of the peptidoglycan is cross-linked; the remaining peptides which are not cross-linked are all tetrapeptides, lacking the fifth amino acid. An enzyme called D, D-carboxypeptidase is responsible for removing the terminal D-alanine from the pentapeptides on nascent glycan chains. The activity of this enzyme is similar to that of the transpeptidase, in that it binds to the D-alanine-D-alanine portion, cleaves the peptide linkage

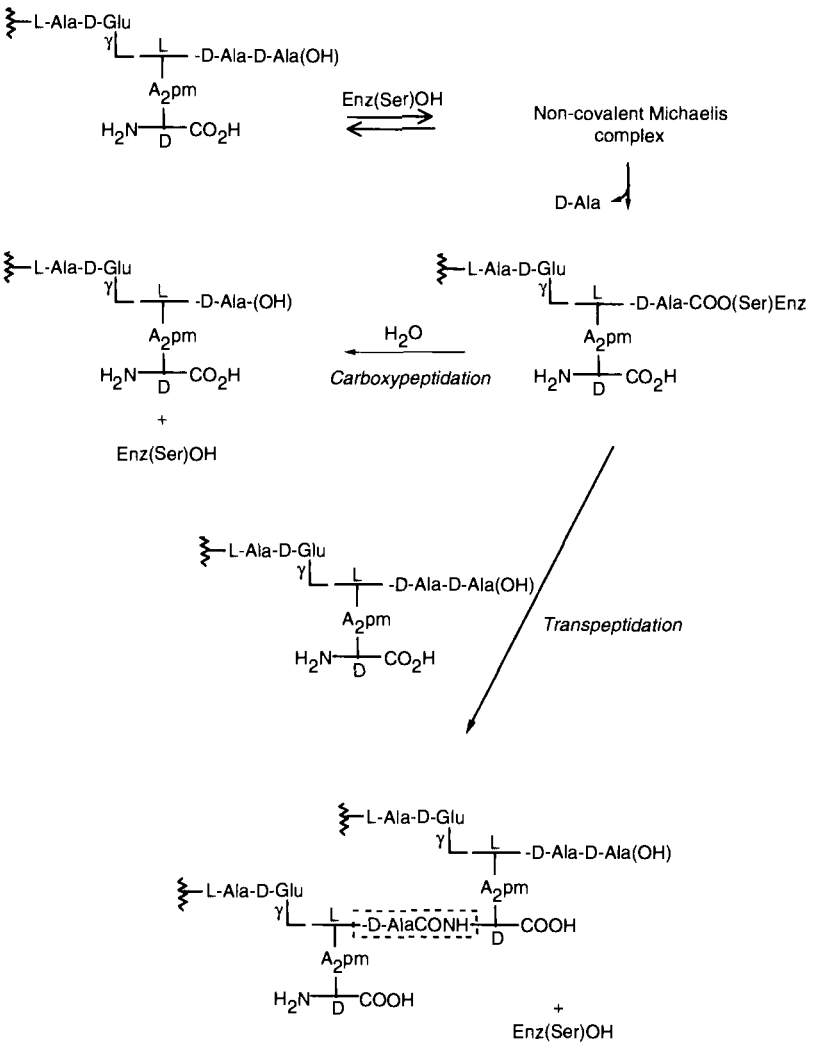
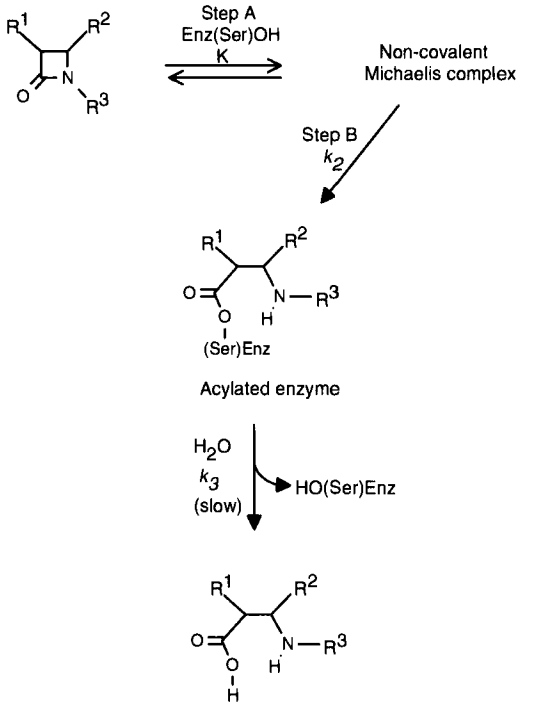


Figure 3.2. Transpeptidation and carboxypeptidation catalysed by D,D-peptidases in E. coli *[24-27]*

and releases the terminal D-alanine (*Figure 3.2*). However, instead of carrying out the transpeptidation, the carboxypeptidase leaves the peptide as an uncross-linked tetrapeptide. Organisms such as *Staphylococcus aureus* which have highly cross-linked peptidoglycan have very low carboxypepti-



Scheme 3.1. Interaction between a β-lactam antibiotic and a serine transpeptidase [24, 38]

dase activity. It therefore seems that the enzymes combine to control cross-linking.

Another D, D-peptidase involved in peptidoglycan biosynthesis is the D, D-endopeptidase enzyme. This enzyme breaks the cross-links in the peptidoglycan strands; that is, it reverses the action of transpeptidases. This activity is believed to provide local sites for peptidoglycan expansion and wall extension, which may be important at the point of cell division.

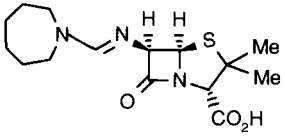
β-Lactam antibiotics exert their antibacterial activity by irreversibly inhibiting the serine transpeptidases; they simulate the D-Ala-D-Ala portion of the pentapeptide substrate and acylate the serine hydroxyl group themselves. As in the interaction of the transpeptidase with the nascent pentapeptide, the first stage is the formation of a non-covalent Michaelis complex (*Scheme 3.1*; Step A). This is followed by the formation of an acylated enzyme (*Scheme 3.1*; Step B). Because the amide bond that is cleaved is endocyclic, there is no loss of a fragment corresponding to D-Ala. The resulting complex is very stable and resistant to attack by an external nucleophile (H_2O or RNH_2). Consequently, k_3 has a very small value and

the reaction effectively stops at this stage. The net effect is that the transpeptidase is prevented from exercising its normal function of completing the peptide cross-linkages in the cell wall. The cell wall weakens as the cell grows and eventually bursts (lysis), resulting in cell death [24, 25].

β-Lactams bind not only with transpeptidases, but also with D, D-carboxypeptidases and D, D-endopeptidases. These enzymes are therefore collectively referred to as penicillin-binding proteins (PBPs). Each bacterial species has its own specific assortment of PBPs, which are numbered in order of decreasing molecular mass. The several PBPs present in a single bacterial cell may fulfil distinct cellular functions; they do not exhibit the same degree of essentiality and they show widely varying sensitivity to β-lactam antibiotics.

In *E. coli*, the most studied bacterium, seven PBPs are consistently found in the cytoplasmic membrane, ranging in molecular weights of 91,000 to 40,000 (PBP 1A, PBP 1B, PBPs 2-6). Two further PBPs with molecular weights 32,000 (PBP-7) and 29,000 (PBP-8) are occasionally detected [25]. The higher molecular weight PBPs, PBP 1A, 1B, 2 and 3 are believed to function as transpeptidases and are vital to cell viability; hence they are often referred to as essential PBPs. Each of these enzymes has a distinct role in peptidoglycan biosynthesis. PBPs 1A and 1B are believed to play vital parts in maintaining the integrity of the cell and possibly controlling the extension of the cell walls during growth. PBP 2 is involved in controlling the shape of the cell and PBP 3 is involved in cell division. The most abundant PBPs are those of lowest molecular weight, that is PBPs 4, 5, and 6. They have D, D-carboxypeptidase activity and, whilst they possibly control the degree of cross-linking of the peptidoglycan, they are not considered to be essential for the viability of the cells.

β-Lactam antibiotics differ in their affinities for penicillin binding proteins. Acylaminopenicillins and cephalosporins exert their antibacterial effect on Gram-negative bacteria by binding to PBPs 1 and 3. Amidinopenicillins such as mecillinam (17) bind only to PBP 2; this results in the formation of large spheroplasts which lyse at antibiotic concentrations that are considerably higher than the minimum inhibitory concentrations



(17) Mecillinam

(MICs). Thienamycin and imipenem (*N*-formimidoylthienamycin) meanwhile have been found to bind with highest affinity to PBP 2, and then to PBP 1A and 1B [28–30]. This leads to the formation of spheroplasts which rapidly lyse and bactericidal concentrations are similar to inhibitory concentrations. In contrast to β-lactams which bind to PBP 3, no filaments are produced.

These differences in mode of action between the carbapenem, imipenem, and other β-lactam antibiotics may explain the observation that imipenem, in contrast to penicillins and cephalosporins, has a post-antibiotic effect (PAE) when used against Gram-negative bacteria [31, 32]. This means that when a bacterial culture is exposed to imipenem, which is then removed, growth will not resume until after a lag-period. If clinically relevant, this would permit the use of longer dose intervals even if the drug concentration at the site of infection falls below inhibitory levels.

BACTERIAL RESISTANCE TO β-LACTAM ANTIBIOTICS

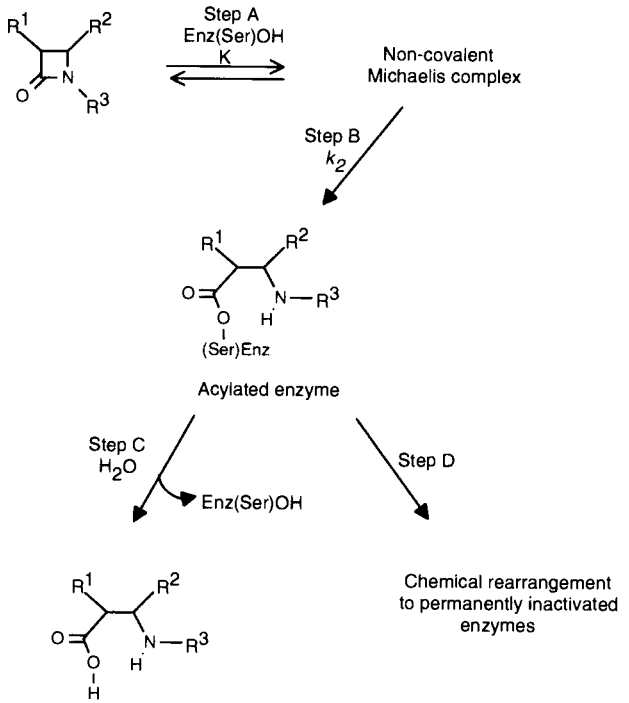
The discovery and developement of penicillin revolutionised the therapy and control of bacterial infections. Yet, even before penicillin was used in the clinic, there was evidence that the potential value of the drug was under threat from bacterial resistance. The existence of a major resistance mechanism, inactivation by a bacterial enzyme, was described soon after the first report of the successful isolation of penicillin [2]. Within a decade, the majority of strains of *Staph. aureus* isolated in hospitals were resistant to penicillin G.

The discovery of the penicillin nucleus, which made possible the preparation of the semi-synthetic penicillins and which also provided the impetus for cephalosporin research, led to the introduction into the clinic of a variety of β-lactam antibiotics with differing antibacterial and pharmacological properties. Many of these agents were designed with the specific aim of being effective against bacteria which were resistant to existing penicillins and cephalosporins. The development of each new agent was however soon followed by the appearance of strains of bacteria possessing one or more resistance mechanisms [33]. As a consequence, the development of resistance seriously threatened the clinical usefulness of β-lactam antibiotics in both hospital and community practice.

Bacteria may exhibit resistance to β-lactam antibiotics by one or more mechanisms [33]. One mechanism is the production, by Gram-negative and Gram-positive bacteria, of modified target sites with reduced affinities for β-lactam antibiotics (modified PBPs). Another way by which bacteria may display resistance is modification of the cell wall, resulting in the reduction

in the rate of passage of the antibiotic into the cell and to the target site. This is of importance in the case of Gram-negative bacteria only, as the cell walls of Gram-positive bacteria lack the permeability barriers to β-lactam antibiotics. The major mechanism of bacterial resistance however is due to the production of bacterial β-lactamases [34–36].

With the exception of one rather uncommon class of zinc-requiring β-lactamases [37], initial non-covalent binding at the active site frequently leads to acylation of a serine hydroxyl group by the β-lactam (*Scheme 3.2,* step B) which, irrespective of the fate of the protein, results in destruction of the β-lactam. A β-lactam is a substrate for the β-lactamase if acylation is followed by hydrolysis of the serine ester linkage (*Scheme 3.2,* step C), which regenerates the active enzyme and at the same time a ring-opened form of the β-lactam, which is antibacterially inactive; hence the term β-lactamase. Alternatively, formation of the acylated enzyme may be followed by



Scheme 3.2. Interaction of β-lactams with serine β-lactamases [38]

chemical rearrangement to a permanently inactivated β-lactamase (*Scheme 3.2*, step D); in this situation the β-lactam agent acts as an inhibitor of the β-lactamase. In combination with β-lactamase-susceptible antibiotics, β-lactamase inhibitors protect the antibiotic from inactivation by the β-lactamase enzyme, thereby producing a synergistic effect against β-lactamase producing bacteria [38].

NATURALLY OCCURRING CARBAPENEM ANTIBIOTICS

ISOLATION

Application of a screen, designed by Beecham scientists to discover naturally occurring inhibitors of bacterial β-lactamases, resulted in the isolation of a group of β-lactamase inhibitors named the olivanic acids from soil micro-organisms [11]. This assay, which came to be known as the KAG assay, involved seeding an agar plate containing penicillin G with a β-lactamase-producing strain of *Klebsiella aerogenes*. The test solution was then introduced into wells in the agar and the plate was incubated overnight at 37°C. Solutions containing a diffusible β-lactamase inhibitor gave zones of inhibition around the wells, resulting from protection of the penicillin present in the agar. In the absence of β-lactamase inhibitor, bacterial growth occurred as a result of the inactivation of the penicillin by the β-lactamase.

The first β-lactamase inhibitors to be discovered using this assay were the olivanic acids, MM 13902 (7), MM 4550 (8) and MM 17880 (9), so named as they were produced by a strain of *Streptomyces olivaceus* [11–13]. Not only are they potent β-lactamase inhibitors, but are also powerful antibacterial agents. The discovery of these sulphate esters was soon followed by the identification of the closely related hydroxy compounds, MM 22380 (10), MM 22382 (11), MM 22381 (12) and MM 22383 (13) [14].

At the same time, Merck researchers isolated the thienamycin* family of antibiotics (14-16) from *Streptomyces cattleya*, using an assay designed to detect inhibitors of peptidoglycan synthesis [15, 39]. Subsequently, a whole series of carbapenem derivatives, represented by PS-5 (18) [40], carpetimycin A (19) [41], and asparenomycin A (20) [42] have been isolated from *Streptomyces* spp. Many are potent inhibitors of β-lactamases, as well as antibacterial agents. Their occurrence and biological properties have been the subject of several recent reviews [16–19].

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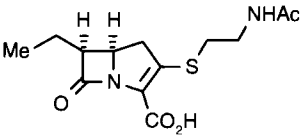
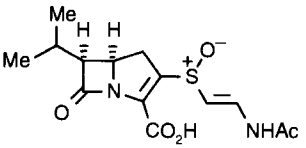
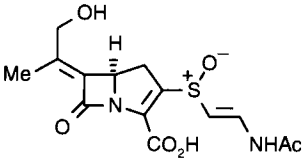
*So named to denote the then novel β-thioenamine chromophore.



(18) PS-5



(19) Carpetimycin A



(20) Asparenomycin A

BIOLOGICAL ACTIVITY OF THE CARBAPENEM ANTIBIOTICS

The carbapenems are a family of extremely potent naturally occurring antibiotics, with thienamycin being the most active. They display potent activity against a broad range of Gram-positive and Gram-negative bacteria, including penicillin-resistant strains of *Staph. aureus* and *Haemophilus influenzae* and organisms that are often resistant to other β-lactam antibiotics, such as indole-positive *Proteus, Enterobacter* spp., *Serratia marcescens* and the anaerobe *Bacteroides fragilis*. The antibacterial properties of the olivanic acids (7-13) are shown in *Table 3.1*, whilst those of thienamycin (14) and *N*-acetylthienamycin (15) are outlined in *Table 3.2*. The antibacterial activities of some other representative carbapenems are given in *Table 3.3*.

The major structural difference between the olivanic acids and the thienamycins is the absolute stereochemistry of the chiral centre at C-8, which is (*S*) for the olivanic acids and (*R*) for the thienamycins. In the olivanic acid series, it is those compounds with the *cis* orientated β-lactam (*i.e.* 5*R*, 6*R*), as in MM 13902 (7), MM 17880 (9), MM 22380 (10) and MM 22382 (11), that are most potent.* Whilst the corresponding (8*S*) hydroxyethyl compounds with the *trans* β-lactam (*i.e.* 5*R*, 6*S*), MM 22381 (12) and MM 22383 (13), also have a broad spectrum of activity, they are less potent than the *cis* compounds.

*The decreased activity of the *cis* carbapenem sulphoxide, MM 4550 (8) has been attributed to its chemical instability [43].

Table 3.1. ANTIBACTERIAL SPECTRUM OF SEVEN OLIVANIC ACID DERIVATIVES; MEAN MIC (mg/l) [43]

Organism	*MM 4550*	*MM 13902*	*MM 17880*	*MM 22380*	*MM 22381*	*MM 22382*	*MM 22383*
Escherichia coli	12.5	0.2	0.2	0.2	6.2	0.2	12.5
Escherichia coli *	25	1.6	1.6	25	6.2	25	12.5
Klebsiella aerogenes	12.5	0.4	0.4	0.8	6.2	0.4	6.2
Klebsiella aerogenes *	50	3.1	3.1	50	6.2	100	12.5
Proteus mirabilis	12.5	0.2	0.4	0.8	12.5	0.2	12.5
Proteus mirabilis *	12.5	0.2	0.4	0.8	12.5	0.2	12.5
Proteus morganii	25	0.4	0.8	1.6	12.5	0.8	25
Proteus rettgeri	25	0.4	0.8	3.1	12.5	1.6	12.5
Proteus vulgaris	12.5	0.4	0.8	1.6	6.2	0.8	6.2
Enterobacter aerogenes	25	3.1	3.1	1.6	6.2	1.6	12.5
Enterobacter cloacae	100	12.5	6.2	3.1	12.5	3.1	25
Serratia marcescens	25	3.1	3.1	3.1	12.5	3.1	12.5
Pseudomonas aeruginosa	>100	25–50	100	>100	>100	>100	>100
Haemophilus influenzae	6.2	0.1	0.2	0.2	6.2	0.1	6.2
Haemophilus influenzae *	6.2	0.1	0.2	1.0	6.2	0.5	6.2
Bacteroides fragilis	3.1	0.4	0.4	0.4	3.1	0.4	3.1
Staphylococcus aureus	25	1.6	1.6	0.4	3.1	0.4	6.2
Staphylococcus aureus *	50	1.6	1.6	0.8	3.1	0.4	6.2
Staphylococcus aureus **	100	12.5	12.5	6.2	100	6.2	100
Streptococcus pyogenes	6.2	0.2	0.1	0.1	0.8	0.05	1.6
Streptococcus faecalis	50	6.2	6.2	1.6	25	1.6	50

*β-lactamase producing strains
** methicillin-resistant strains

Table 3.2. ANTIBACTERIAL ACTIVITIES OF N-ACETYLTHIENAMYCIN AND THIENAMYCIN; MIC VALUES (mg/l) [43]

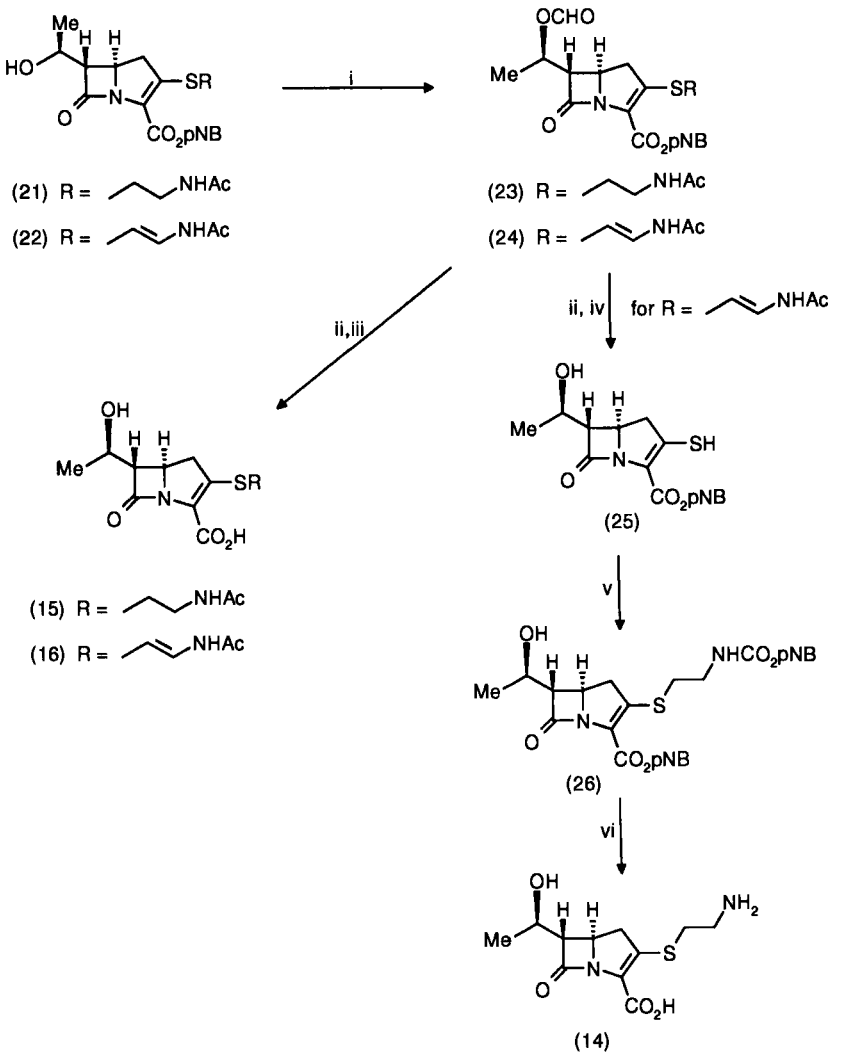
Organism	*N-Acetylthienamycin*	*Thienamycin*
Escherichia coli (0111)	0.8	0.2
Escherichia coli JT39 (RTEM)	0.8	0.4
Klebsiella aerogenes A	0.4	0.4
Klebsiella aerogenes VA2 (RTEM)	0.8	0.4
Proteus mirabilis 977	3.1	3.1
Proteus rettgeri WM16	3.1	3.1
Enterobacter cloacae T753	3.1	3.1
Serratia marcescens US20	3.1	1.6
Pseudomonas aeruginosa NCTC 10662	25	3.1
Bacteroides fragilis BC-16	NT	0.4
Staphylococcus aureus Russell	0.2	0.04
Streptococcus faecalis I	6.2	1.6
Streptococcus pyogenes CN10	0.05	0.01

Thienamycin (14) and *N*-acetylthienamycin (15) have the same *trans* β-lactam orientation as MM 22381 (12) and MM 22383 (13) but have the (*R*) configuration at C-8. They have much improved antibacterial potency compared to MM 22381 and MM 22383, being similar to the *cis*-isomer, whilst retaining the stability of the (5*R*, 6*S*, 8*S*) compounds towards R-TEM β-lactamases. Furthermore, thienamycin (14) displays exceptional activity against *Pseudomonas aeruginosa* and this is attributed to the basic amino group of the C-2 cysteaminyl side-chain. This combines to make thienamycin one of the most potent, broad spectrum antibacterial agents to be isolated from natural sources. Thienamycin was shown to have pronounced activity against *E. coli, Proteus mirabilis, Klebsiella pneumoniae, Serratia marcescens, Streptococcus pyogenes,* penicillin G sensitive and penicillin G resistant *Staph. aureus* and *Enterobacter* spp., as well as *Ps. aeruginosa* [47]. A number of studies have shown it to be superior to other semi-synthetic, as well as naturally occurring penicillins and cephalosporins, including amoxycillin, carbenicillin, ticarcillin, apalcillin, mezlocillin, azlocillin, piperacillin, cephalothin, cefoxitin and cefotaxime [48–52].

Table 3.3. *IN VITRO* ACTIVITIES (MIC, mg/l) OF PS-5 (18) [44], CARPETIMYCIN A (19) [45] AND ASPARENOMYCIN A (20) [46]

Organism	*Strain*	*PS-5 (18)*	*Strain*	*Carpetimycin A (19)*	*Strain*	*Asparenomycin A (20)*
Escherichia coli	K-12	1.56	JC-2	0.05	JC-2	1.56
Escherichia coli *			EC-1	0.2	377	0.39
Klebsiella pneumoniae	K2	3.13	PCI-602	0.2	SRL-1	0.78
Klebsiella pneumoniae *			25	0.78	363	0.78
Proteus mirabilis	P-6	6.25	21100	1.56	PR-4	3.13
Proteus mirabilis *			TN 265	2.5		
Proteus morganii			IFO 3168	0.39		
Proteus rettgeri	P-7	6.25				
Proteus vulgaris	109	12.5	IID 874	0.39	CN-329	12.5
Proteus vulgaris *	P-5	6.25	69	0.78		
Enterobacter cloacae	45	6.25	IID 977	0.78	233	1.56
Enterobacter cloacae *	E-16	3.13	3	3.13		
Serratia marcescens	S-18	3.13	NHL	0.2	13880	12.5
Serratia marcescens *			4	3.13		
Pseudomonas aeruginosa	NC5	>100	10490	6.25	25619	25
Staphylococcus aureus	209P	0.02	209P	0.39	JC-1	1.56
Staphylococcus aureus *	Russell	0.20			C-14	1.56
Streptococcus pyogenes	NY5	0.08			C-203	1.56

*β-lactamase producing strain



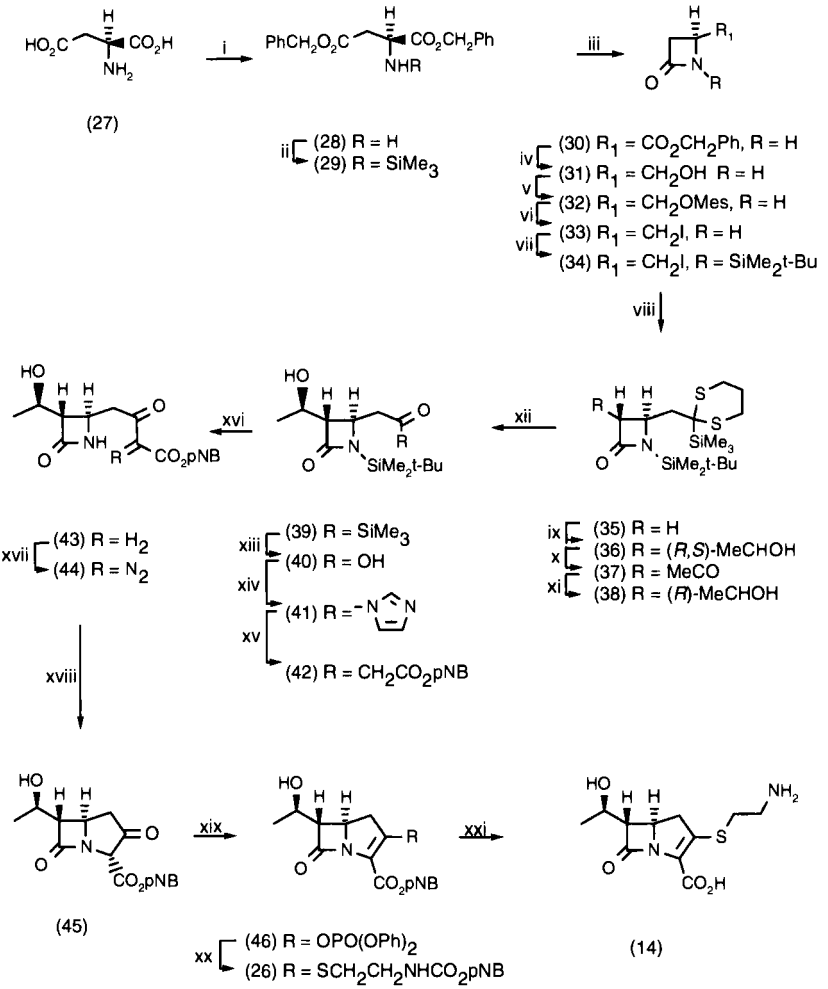
Scheme 3.3. Interconversion of the olivanic acids and the thienamycins [53].
Reagents: (i) Diethyl azodicarboxylate, PPh_3, HCO_2H; (ii) NaOH, aq. 1,4-dioxan; (iii) (a) H_2, 5% Pd/C, aq. 1,4-dioxan, (b) $NaHCO_3$; (iv) N*-bromoacetamide, aq. 1,4-dioxan; (v) 2-*p*-nitrobenzyloxycarbonylaminoethyl bromide, K_2CO_3, DMF; (vi) H_2, 5% Pd/C, aq. 1,4-dioxan, pH 7.0 phosphate buffer.*

INTERCONVERSION OF THE OLIVANIC ACIDS AND THE THIENAMYCINS

Structure-activity studies revealed that the stereochemical configuration required for optimum antibacterial activity was that found in the thienamycin series, that is (5*R*, 6*S*, 8*R*). The olivanic acids, MM 22381 (12) and MM 22383 (13) differ from *N*-acetylthienamycin (15) and *N*-acetyldehydrothienamycin (16) respectively by virtue of the stereochemical configuration at C-8. In order to inter-relate the two series, the configuration of the α-hydroxyethyl side-chain of MM 22381 and MM 22383 was inverted by way of a Mitsunobu reaction sequence [53]. Thus, treatment of the *p*-nitrobenzyl esters (21) and (22) of MM 22381 and MM 22383 with diethyl azodicarboxylate, triphenylphosphine and formic acid provided the formate esters (23) and (24), respectively. Alkaline hydrolysis of the formate esters, followed by hydrogenolysis provided *N*-acetylthienamycin (15) and *N*-acetyldehydrothienamycin (16) (*Scheme 3.3*). The combination of this inversion process with a procedure developed for the variation of the C-2 alkylthio side-chain in the olivanic acids [54] provided a preparation of thienamycin (14) from MM 22383 (13). Addition of hypobromous acid to the double bond of the C-2 acetamidoethenylthio substituent in MM 22383, as well as MM 22382, resulted in the formation of a bromohydrin, which fragmented to provide the C-2 thiol (25); subsequent alkylation or addition to propiolate esters provided a wide range of C-2 substituents with either the (5*R*, 6*S*, 8*S*), (5*R*, 6*R*, 8*S*) or (5*R*, 6*S*, 8*R*) stereochemistry, depending on whether MM 22383, MM 22382 or *N*-acetyl-dehydrothienamycin was chosen as starting material [55, 56]. Thus, thienamycin was prepared from MM 22383 by elaboration to the thiol (25) and alkylation with 2-*N*-*p*-nitrobenzyloxycarbonylaminoethyl bromide, followed by hydrogenolysis of the resulting ester (26).

TOTAL SYNTHESIS OF (+)-THIENAMYCIN

Owing to the chemical instability of the carbapenem antibiotics at high concentrations, fermentation procedures failed to provide sufficient quantities of natural products for clinical evaluation and production. For Merck scientists, the solution to this problem was to devise an efficient, high yielding and cost-effective synthetic route to thienamycin and related clinically important compounds. Initial efforts were directed towards the synthesis of racemic thienamycin [16, 18]. In their first chiral synthesis of (+)-thienamycin, which proceeded from L-aspartic acid (27), the pivotal reaction was the carbene insertion reaction to provide the bicyclic ring system by formation of the C-3–N-4 bond [57] (*Scheme 3.4*).



Scheme 3.4. Synthesis of (+)-Thienamycin (14) from L-Aspartic Acid [57].
Reagents: (i) $PhCH_2OH$, ptsa.H_2O, benzene, reflux; (ii) Me_3SiCl, Et_3N, Et_2O; (iii) t-BuMgCl, Et_2O; (iv) $NaBH_4$, MeOH; (v) $MeSO_2Cl$, Et_3N, CH_2Cl_2; (vi) NaI, acetone, reflux; (vii) t-$BuMe_2SiCl$, Et_3N, DMF, (viii) 2-lithio-2-(trimethylsilyl)-1,3-dithiane, THF, -78°C; (ix) MeCHO, LDA, THF, -78°C; (x) TFAA-Me_2SO, Et_3N, CH_2Cl_2, -78°C; (xi) K-selectride, KI, ether; (xii) $HgCl_2$, HgO, aq. MeOH, reflux; (xiii) H_2O_2, aq. MeOH; (xiv) carbonyldiimidazole, THF; (xv) Mg salt of mono-p-nitrobenzyl malonate, THF; (xvi) methanolic HCl; (xvii) p-carboxybenzenesulphonyl azide, Et_3N, MeCN; (xviii) rhodium (II) acetate, toluene, 80°C; (xix) $ClP(O)(OPh)_2$, cat. DMAP, i-Pr_2NEt, MeCN; (xx) N-p-nitrobenzyloxycarbonyl-cysteamine, i-Pr_2NEt, MeCN, -5°C; (xxi) H_2, 10% PdlC, 40 psi.

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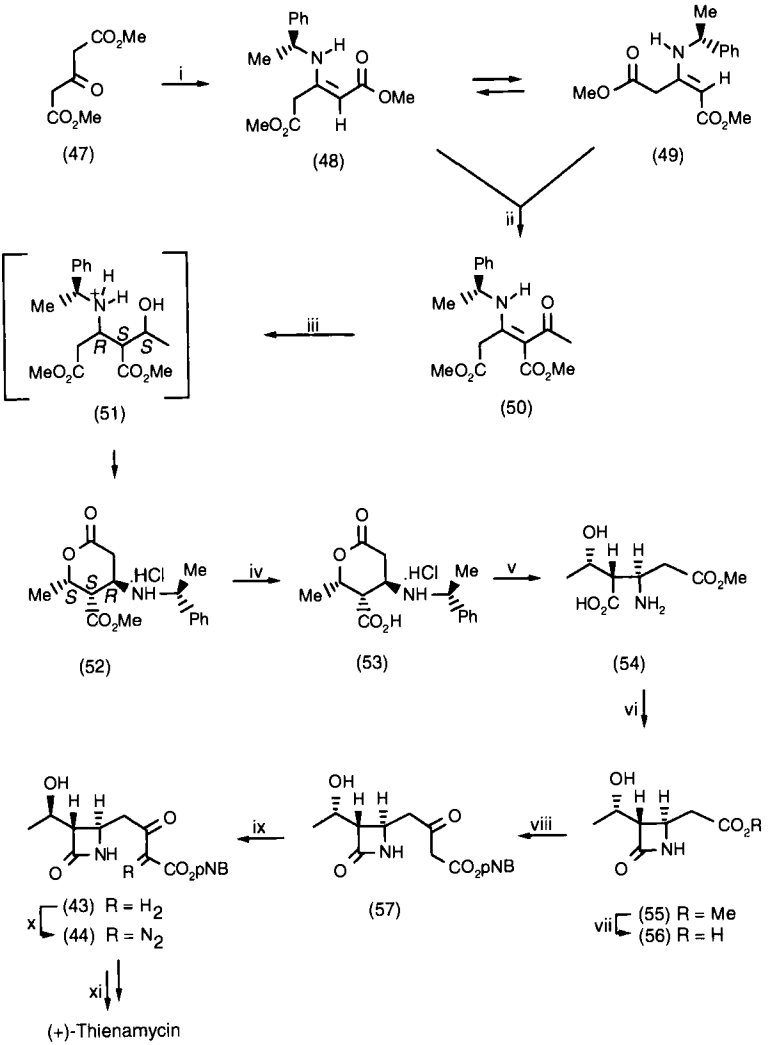
Sodium borohydride reduction of the azetidinone ester (30), obtained by *t*-butyl magnesium chloride-mediated cyclization of the *N*-trimethylsilyl derivative of dibenzyl aspartate (29), provided alcohol (31), which was elaborated to the iodomethyl derivative (33), *via* the mesylate (32). *N*-Silylation of (33), followed by reaction with 2-lithio-2-trimethylsilyl-1,3-dithiane gave the substituted dithiane (35). Condensation of the lithium enolate derived from (35) with acetaldehyde gave a 1:1 mixture of *trans-(R)* and *trans-(S)* alcohols (36). Oxidation of the diastereoisomeric mixture of alcohols to the *trans*-acetyl derivative (37), followed by potassium selectride reduction gave predominantly the *trans-(R)* alcohol (38). Dithiane hydrolysis provided the trimethylsilyl ketone (39), which underwent Baeyer-Villiger oxidation to the acid (40). The requisite ketoester intermediate (42) was then obtained by condensing the imidazole derivative (41) with the magnesium salt of mono-*p*-nitrobenzyl malonate. Removal of the *N*-silyl protecting group followed by diazo exchange from *p*-carboxybenzenesulphonyl azide provided the cyclization precursor (44). Thermolysis of (44) in the presence of rhodium acetate then provided the bicyclic ketoester (45). Introduction of the cysteamine side-chain was accomplished by converting ketoester (45) to the enol phosphate (46) and reaction with *N*-(*p*-nitrobenzyloxycarbonyl)-cysteamine. Finally, hydrogenolytic removal of the *p*-nitrobenzyl protecting groups of (26) provided (+)-thienamycin (14).

A commercially viable, enantioselective route was subsequently developed by the Merck Process Research group [58, 59]. This approach, which is also based upon the carbene insertion reaction, is characterized by the early introduction of the hydroxyethyl group through the formation of a highly functionalized acyclic derivative with three contiguous asymmetric centres (*Scheme 3.5*).

Condensation of dimethyl 1,3-acetonedicarboxylate (47) with (*R*)-(+)-α-methyl- benzylamine provided an equilibrium mixture of enamines (48) and (49) in nearly quantitative yield, without the need for purification. Acylation of this equilibrium mixture using ketene then furnished the enamino ketone (50) in excess of 95% yield. This product (50) displayed a rigid structure by virtue of an internal hydrogen bond, thus setting the stage for a stereoselective reduction. Hydrogenation of (50) in acetic acid containing 2 molar equivalents of phosphoric acid and 5% platinum-on-carbon catalyst, followed by lactonization of alcohol (51) gave the triply asymmetric, chiral lactone (52) in 80-90% yield, as the hydrochloride salt. The relative stereochemistry of (52) had already been established by conversion of racemic intermediates to model azetidinones [60] and was confirmed by X-ray crystallography. Unfortunately, the relative stereochemistry at the methyl substituted carbon was incorrect and necessitated



Scheme 3.5. Practical synthesis of (+)-thienamycin (14) from dimethyl acetonedicarboxylate and (+)-α-methylbenzylamine [59].

Reagents: (i) (R)-(+)-α-methylbenzylamine, toluene, AcOH (6 mol %); (ii) ketene, toluene; (iii) H_3PO_4 (2 mol equiv.), AcOH, 5% Pt/C, H_2, 90-1000 psi., 20°C; (iv) conc. aq. HCl, 85° C; (v) (a) MeOH, H_2, Pd/C, 40 psi., 60°C; (b) n-Bu_3N, CH_2Cl_2; (vi) DCCI, aq. MeCN; (vii) aq. NaOH; (viii) (a) carbonyldi-imidazole; (b) Mg salt of mono p-nitrobenzyl malonate; (ix) (a) diethyl azodicarboxylate, PPh_3, HCO_2H; (b) dil aq. HCl; (x) p-dodecylbenzenesulphonyl azide; (xi) see Scheme .4.

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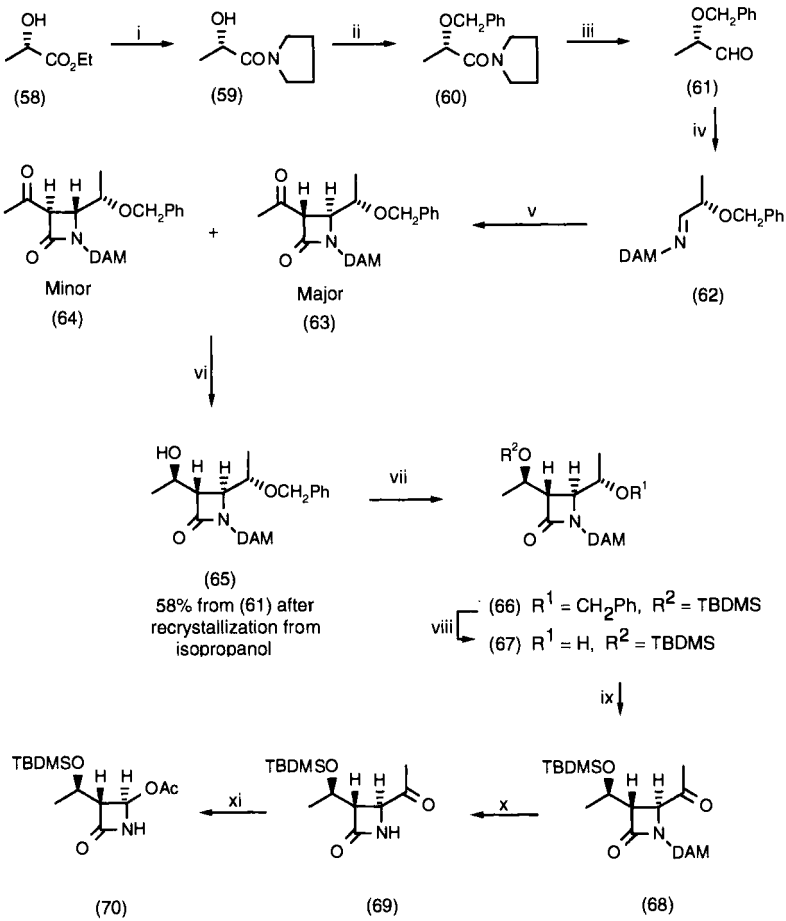
an inversion step at a later stage in the synthetic route. Hydrolysis of (52) to the carboxylic acid (53), followed by hydrogenolysis of the α-methylbenzyl group and methanolysis of the lactone ring then provided the highly functionalized, chiral amino acid (54) in high yield [>90% from (52); 50% overall yield] and excellent purity with the need for only one purification stage. Amino acid (54) was then cyclized in high yield to azetidinone (55) using dicyclohexylcarbodi-imide. Saponification provided the sodium salt of acid (56), which was converted to ketoester (57) by the previously described method. Inversion of the 1-hydroxyethyl group was then achieved by treating azetidinone (57) with formic acid in the presence of triphenylphosphine and diethyl azodicarboxylate; hydrolysis of the resulting inverted formate ester provided (43). Diazo transfer from *p*-dodecylbenzenesulphonyl azide yielded the diazo ketoester (44), which was converted to (+)-thienamycin by the previously described methodology.

Numerous asymmetric syntheses of thienamycin and structurally related carbapenems have since been reported. Most strategies have focussed upon the elaboration of the correct stereochemistry at the three chiral centres of a monocyclic precursor [61]. Subsequent progression has usually been by way of the diazo-ketone approach and the hydroxyethyl side-chain has often been introduced by way of a stereoselective aldol condensation [62]. In other cases, the substituent has been incorporated before construction of the β-lactam ring using chiral building blocks derived from D-glucose [63], L-threonine [64, 65], D-allothreonine [66], and (*S*)-(-)-hydroxyethylbutyrate [67, 68]. Functionalized β-lactams derived from the penicillin nucleus have also been elaborated to (+)-thienamycin and related compounds [69–71].

A particularly versatile and much used intermediate for asymmetric carbapenem syntheses is (3*R*, 4*R*)-4-acetoxy-3-[(*R*)-1-(*t*-butyldimethylsilyloxy)ethyl]-2-azetidinone (70). A practical synthesis of this key intermediate has been reported by Ito and co-workers (*Scheme 3.6*) [72]. The [2 + 2] cycloaddition reaction of diketene with the chiral imine (62), derived from the inexpensive (*S*)-ethyl lactate (58), proceeded in a highly stereoselective manner to provide the desired 3, 4-*trans*-3-acetylazetidinone (63) as the major product (diastereoselectivity 7 ~ 10:1). Potassium triethylborohydride reduction of the crude diastereoisomeric mixture of azetidinones (63) and (64) gave the pure 3-[(1*R*)-hydroxyethyl]azetidin-2-one (65) in good yield after recrystallization from isopropanol. The alcohol (65) was converted to the ketone (68) by way of the silyl ether (66) and the alcohol (67). Removal of the di-*p*-anisylmethyl group in ketone (68) was cleanly effected by treatment with sodium peroxydisulphate in the presence of disodium hydrogen phosphate buffer; oxidation of (69) with *m*-chloroperbenzoic acid furnished the chiral 4-acetoxy azetidinone derivative (70). The elaboration

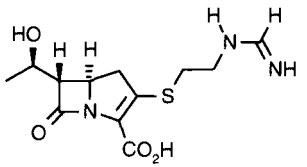


Scheme 3.6. Chiral synthesis of the key intermediate, (3R, 4R)-4-Acetoxy-3-[(R)-1-(t-butyldimethylsilyloxy)ethyl]azetidin-2-one (70) from (S)-Ethyl lactate [72].
Reagents: (i) pyrrolidine; (ii) benzyl chloride, NaH; (iii) $NaAl(OCH_2CH_2OMe)_2H_2$, toluene; (iv) di-p-anisylmethylamine (DAM-NH_2), $MgSO_4$, toluene; (v) Diketene (4 equiv.), CH_2Cl_2, -35°C, 3 days; (vi) $KBEt_3H$, THF, -78°C; (vii) TBDMSCl (t-$BuMe_2SiCl$), DMAP, DMF; (viii) H_2, Pd/C, EtOAc; (ix) N-*chlorosuccinimide, Me_2S, Et_3N, toluene; (x) $Na_2S_2O_8$, Na_2HPO_4, aq. acetone; (xi)* m-*chloroperbenzoic acid, EtOAc.*
Reprinted from Tetrahedron, 45, 5767-5790, Ito, Y., Kobayashi, Y., Kawabata, T., Takase, M. and Terashima, S., 1989, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

of this versatile intermediate to a variety of optically active carbapenem derivatives is discussed in later sections of this review.

IMIPENEM (*N*-FORMIMIDOYLTHIENAMYCIN, MK 0787)

The unique structure and exceptional antibacterial potency, particularly against *Pseudomonas spp.*, created considerable interest in thienamycin. Unfortunately, its novel, highly strained carbapenem nucleus, together with the basic aminoethylthio side-chain contributed to its chemical instability. It is believed that this concentration-dependent instability, which made thienamycin less attractive as a parenterally administered drug, is due to the intermolecular aminolysis of the β-lactam ring by the cysteamine side-chain. Merck scientists therefore investigated the derivatization of the amino group of thienamycin and found that the *N*-formimidoyl derivative (71) (imipenem, MK 0787), prepared by the reaction of thienamycin with methyl formimidate in aqueous solution at pH 8.2, was 5-fold more stable than thienamycin in concentrated aqueous solution [73]. Furthermore, the crystalline *N*-formimidoylthienamycin (71) retained the antibacterial spectrum of thienamycin and exhibited enhanced potency against *Ps. aeruginosa.*

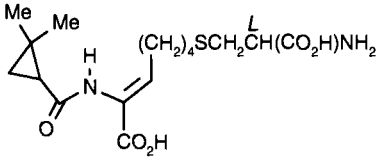


(71) Imipenem

PRIMAXIN (IMIPENEM IN COMBINATION WITH CILASTATIN)

In view of the improved chemical stability, excellent antimicrobial activity, stability to bacterial β-lactamases and accessibility in large quantities by total synthesis, *N*-formimidoylthienamycin (71) was selected for clinical evaluation. However, as in the case of other naturally occurring carbapenem antibiotics, only a low recovery of imipenem was evident in the urine after parenteral administration in animal models, as well as man [74]. Subsequently, detailed studies revealed that this class of antibiotics is extensively degraded by the renal dipeptidase enzyme (DHP-1) [75, 76] located in the brush borders of the kidney [77] (*Table 3.4*). Enzymatic turnover of imipenem *in vivo* therefore posed a serious threat to its clinical efficacy and inhibitors of this zinc-containing hydrolytic enzyme were therefore sought. Indeed, coadministration of imipenem with a potent, competitive inhibitor of the DHP-1 enzyme, cilastatin (MK 0791, 72) [78, 79], in a 1:1 ratio

resulted in a 10-fold improvement in the urinary recovery of imipenem in chimpanzees and humans [77, 80]. Furthermore, the nephrotoxic potential of the antibiotic was significantly reduced. This led to an intensive evaluation of the imipenem-cilastatin combination [81, 82], which was ultimately marketed as Primaxin, an injectable product for the treatment of lower respiratory tract, intra-abdominal, genito-urinary, gynaecological, bone and joint and skin and soft tissue infections, septicaemia and prophylaxis of post-operative infections.



(72) Cilastatin

Most of the research since the discovery of Primaxin has concentrated upon the development of a single compound that possesses all, or most of the properties of the imipenem/cilastatin combination. This work is the subject of the following sections of the present review.

Table 3.4. RELATIVE SUSCEPTIBILITY TO DHP-1 OF NATURALLY OCCURRING CARBAPENEM ANTIBIOTICS AND ITS CORRELATION WITH LOW URINARY RECOVERY [77]

Antibiotic	*Susceptibility to DHP-1 (hog renal) relative to thienamycin*	*% Dose recovered in urine (mice)*
Thienamycin	1.0	25
N-Formimidoylthienamycin	0.9	28
N-Acetylthienamycin	4.2	11.4
MM 22380	12	1.0
MM 22381	20	ND
MM 22383	51	ND
MM 13902	30	<1.0
MM 17880	8.3	ND
PS 5	43	ND

ND = Not determined

TOTALLY SYNTHETIC NON-NATURAL CARBAPENEMS

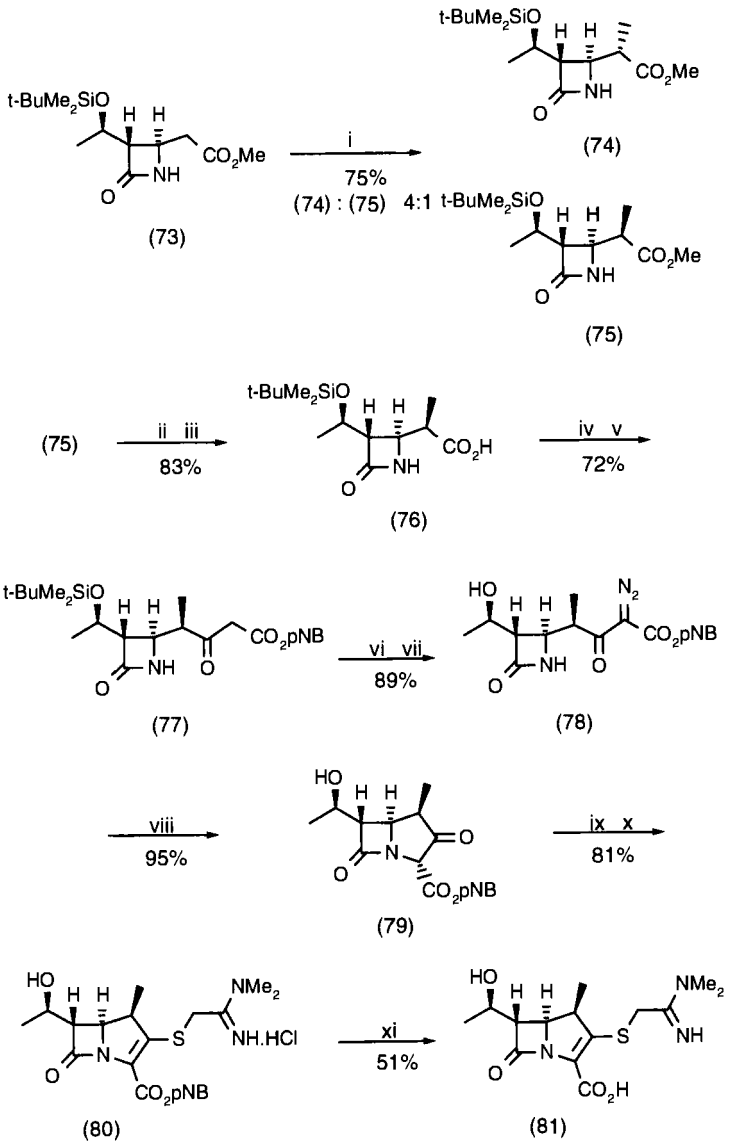
By the time that imipenem was under development, the scope for semi-synthetic modification of natural carbapenems appeared to have been practically exhausted. The next phase of carbapenem research was therefore directed at the total synthesis of non-natural carbapenem structures, largely based on systematic variations of the thienamycin nucleus. Over the past decade a considerable effort has been devoted to this task. A major objective of this research was the discovery of a carbapenem with the antimicrobial spectrum and potency of imipenem, but with significantly enhanced chemical and metabolic stability.

MODIFICATIONS AT C-3, C-5, AND C-6

Analogues of thienamycin in which the 3-carboxy group has been replaced by a 3-(5-tetrazolyl) [83] or a 3-(methylphosphonyl) group [84] have much improved stability to DHP-1, but greatly diminished antibacterial activity; the 5-tetrazolyl compound has about one-tenth of the potency of thienamycin, and the methylphosphonyl derivative is even less active.

Racemic 5-methylthienamycin and a number of C-2 modified analogues were prepared and, in comparison with the 5-unsubstituted carbapenems, were found to have improved chemical stability and much greater stability to mouse kidney homogenate [85]. Unfortunately, the improved stability had been gained at the cost of significantly reduced antibacterial activity. For racemic *N*-acetyl-5-methylthienamycin, the various epimers at C-6 and C-8 were also prepared, and the same order of activity relative to stereochemisty was observed as in the 5-unsubstituted series: 8*R*, 6*S* > 8*S*, 6*R* > 8*S*, 6*S* > 8*R*, 6*R*.

A variety of small alkyl groups, sometimes substituted by a hydroxy group or sulphated hydroxy group, are found at C-6 in natural carbapenems, with both 6*R* and 6*S* stereochemistries being known; introduction of larger or more functionalised 6-alkyl substituents generally resulted in reduced potency [86–92]. (6*R*)-[(*R*)-1-Fluoroethyl] carbapenems have improved water solubility over their 6-(1-hydroxyethyl) couterparts, but similar antibacterial activity and stability to DHP-1 [93, 94]. 6-Aminomethyl- and 6-[(*R*)-1-aminoethyl)]- 1β-methylcarbapenems show good activity against Gram-negative organisms *in vitro*, but have relatively poor chemical stability [87]. 6-Acylamino- [95, 96] and 6-carbamoyl-1β-methylcarbapenems [97] also have poor chemical stability, and consequently showed rather weak activity in MIC testing. Disubstitution at C-6, as for example in 6-hydroxy and 6-methoxy *epi*-PS-5 [98], resulted in



Scheme 3.7. 1β-Methylcarbapenem synthesis [99]

Reagents: (i) lithium diisopropylamide (2 equiv.); MeI; (ii) 2.5 M *NaOH, MeOH; (iii) 6* M *HCl; (iv) carbonyl di-imidazole; (v) magnesium* p*-nitrobenzyl malonate; (vi) 6* M *HCl; (vii) dodecylbenzenesulphonyl azide, Et_3N; (viii) rhodium(II) octanoate; (ix) $(PhO)_2POCl$, $(iPr)_2NEt$; (x) $HSCH_2C(NMe_2)NH.HCl$, $(iPr)_2NEt$; (xi) H_2, Pd/C, $NaHCO_3$ (1 equiv.).*

increased chemical and metabolic stability, but at the expense of drastically reduced antibacterial activity.

In conclusion, modifications at C-3, C-5, and C-6 have so far failed to give any overall improvement on the properties of the natural carbapenems in terms of stability and antibacterial activity. At these positions, the thienamycin structure is therefore regarded as being optimal. Modifications at the remaining positions, C-1 and C-2, are discussed in the following sections.

1β-METHYL AND OTHER 1-SUBSTITUTED CARBAPENEMS

The Merck group described the first synthesis of a 1β-methylcarbapenem, starting with the optically active ester (73), an intermediate in the chiral synthesis of imipenem (*Scheme 3.7*) [99]. The resulting 1β-methylcarbapenem (81), which also contains the novel 2-dimethylamino-2-iminoethylthio side-chain at C-2, is chemically stable, resistant to DHP-1, and retains the potent antibacterial activity of imipenem (*Table 3.5*). The introduction of 1α-methyl, 1β-ethyl, 1α- and 1β-hydroxy, and 1α- and 1β-methoxy groups also increased stability to DHP-1 over that of the 1-unsubstituted carbapenems, but antibacterial activity was diminished [86].

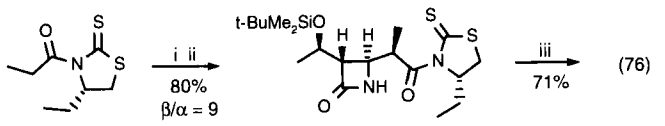
The improved stability imparted to the carbapenem nucleus by introduction of a 1β-methyl group aroused considerable interest. Consequently, the stereoselective synthesis of the later-stage intermediates (76) - (79) in the

Table 3.5. RELATIVE IN VITRO ANTIBACTERIAL POTENCY AND DHP-1 SUSCEPTIBILITY OF IMIPENEM (71) AND 1β-METHYLCARBAPENEM (81) IN COMPARISON WITH THIENAMYCIN (14) [99]

Organism (n)	*Thienamycin Mean MIC (mg/l)*	*Imipenem Potency*[a]	*Compound (81) Potency*[a]
Staphylococcus aureus (5)	0.04	1.2	0.5
Enterococcus (2)	3.80	1.3	0.8
Escherichia coli (7)	0.36	1.6	3.5
Enterobacter spp. (6)	2.04	2.1	8.0
Klebsiella spp. (5)	1.77	1.2	3.5
Serratia spp. (2)	2.50	2.6	7.0
Proteus spp. (6)	7.08	1.5	4.6
Pseudomonas aeruginosa (5)	9.13	3.5	5.3
DHP-1 susceptibility[b]	1.0	0.90	0.026

[a] Antibacterial potency relative to thienamycin = 1

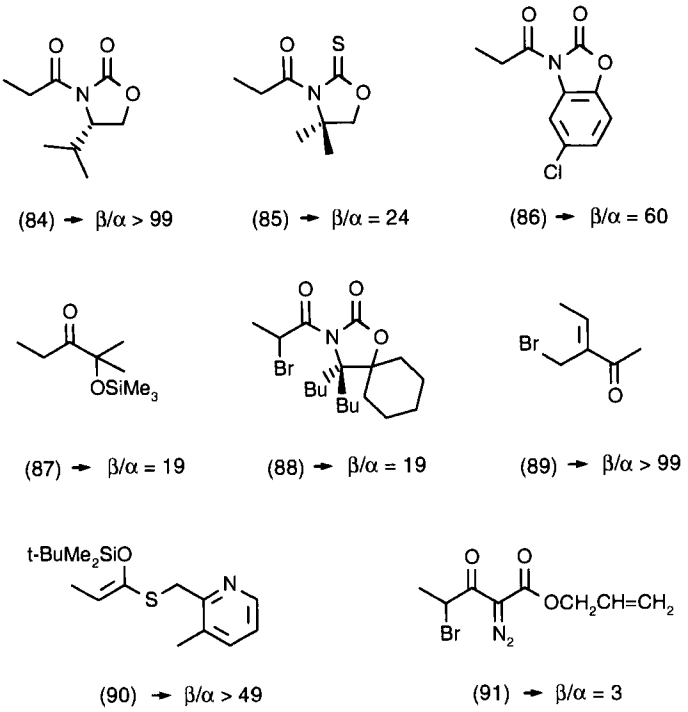
[b] Relative susceptibility to hog renal DHP-1 [77]



Scheme 3.8. Stereoselective synthesis of the Merck carboxylic acid (76) [100]. Reagents: (i) $Sn(OSO_2CF_3)_2$, N-ethylpiperidine, THF, -50°C; (ii) azetidinone (71); (iii) 1M NaOH, THF.

Merck synthesis (*Scheme 3.7*) became an important goal in carbapenem research.

Aldol-type condensations between the chiral acetoxy-azetidinone (70) and enolates of propionic acid derivatives have been used by several research groups seeking stereoselective syntheses of the acid (76). Nagao *et al.* [100, 101] used the tin enolate of the chiral *N*-propionylthiazolidinthione (82) to give the 4-substituted azetidinone (83) which was hydrolysed to the

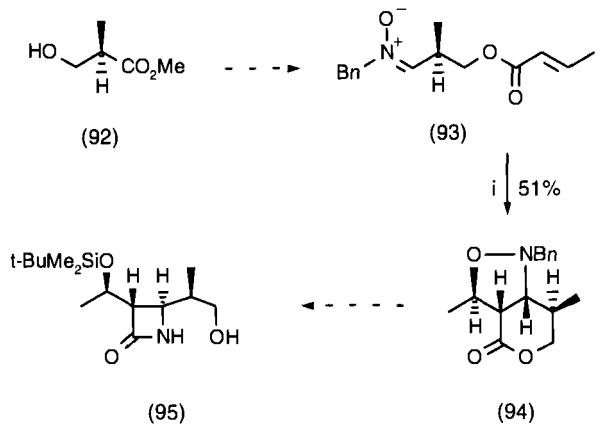


carboxylic acid (76) (*Scheme 3.8*). The Merck group [102] similarly used the boron enolate of the chiral oxazolidinone (84) (giving $\beta/\alpha > 99$). A number of achiral derivatives have been used to equally good effect, providing stereoselective syntheses of intermediates which are convertable into the acid (76): Bristol-Myers researchers used the tin enolate of the oxazolidinthione (85) [103, 104] and zirconium enolates of various *S*-alkyl and *S*-aryl thiopropionates [105, 106]; Sankyo scientists used the boron enolate of the oxazolidinone (86) [107]; and Shirai and Nakai used the tin enolate of the α-silyloxy-ketone (87) [108]. In a similar vein, stereoselective Reformatsky-type reactions have been used: Ito *et al.* used the α-bromopropionate (88) [109]; and Mori and Oida used the allylic bromide (89) [110]. Thiolesters of the acid (76) have been prepared by Lewis acid-mediated condensation of (70) with *O*-silyl enol ethers of thiopropionates [111–113], and in this respect the ether (90), which gave a high yielding (90%) and stereoselective ($\beta/\alpha >$ 49) condensation, proved particularly useful [112].

Stereoselective aldol-type reactions have also been used to convert the azetidinone (70) directly to β-ketoester intermediates analogous to (77) and (78). Metal salt-mediated condensations beween (70) and the dianion or bis-silyl enol ether of methyl 3-oxopentanoate gave the methyl ester corresponding to (77) [114]. Although the yield and stereochemical outcome in these reactions could be independently optimised by varying the metal salt, no method emerged from this study in which both were high. Better results were obtained using the tin enolate derived from (91), which gave, after *O*-desilylation, the allyl ester corresponding to (78) (> 98% pure; 45% overall yield) [115].

Using the aldol condensation, a number of practical, stereoselective methods have been developed for the routine preparation of 1β-methylcarbapenems. The literature contains many more excellent routes to these compounds [116–139], but not all of these will be described in detail; a few examples will be given by way of illustrating the variety of methods that are now available.

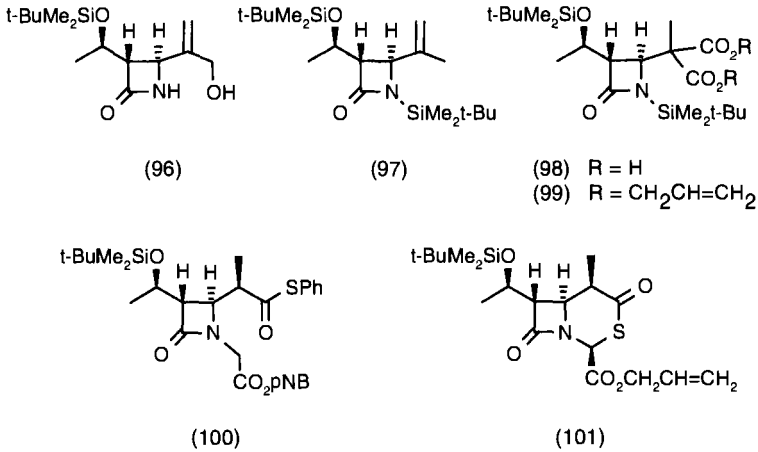
Ihara *et al.* [116] used the (*R*)-2-methylhydroxypropionate (92) to construct the nitrone (93) which underwent a completely sterospecific, intramolecular cycloaddition to give the isoxazolidine (94) (*Scheme 3.9*). Compound (94) was converted into the alcohol (95), which could be oxidized to the acid (76). The alcohol (95) has also been prepared from the azetidinone (70), by way of stereoselective hydrogenation of the olefin (96) over a ruthenium catalyst [117], and by stereoselective hydroboration-oxidation of the olefin (97) [118]. Two high-yielding and stereoselective syntheses of (76) from (70) have been developed using malonic acid derivatives: the diacid (98) was decarboxylated to (76) in refluxing formic



Scheme 3.9. Nitrone cycloaddition route to 1β-methylcarbapenems [114]. Reagents: (i) reflux in t-amyl alcohol. Bn = benzyl.

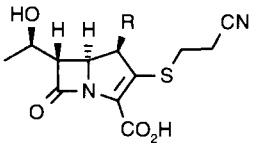
acid/ethyl acetate [119, 120]; and the diallyl ester (99) was converted into (76) in a palladium mediated deallylation-decarboxylation reaction [121].

Although for the most part, the methods outlined in *Scheme 3.7* have been used to complete the synthesis of 1β-methylcarbapenems from intermediates such as (76), some studies have been described on alternative procedures for carrying out these later synthetic steps. For example, the Sumitomo group prepared the thiolester (100) and used this in a Dieckmann cyclization to prepare the β-ketoester (79) [140]; and the Tanabe group converted the acid (76) into the thiazinone (101) and used the Eschenmoser

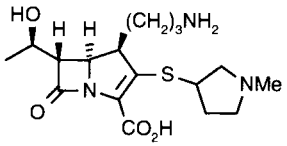


sulphide contraction to convert this into various 1β-methylcarbapenem allyl esters [141].

In addition to the considerable efforts devoted to 1β-methylcarbapenems, a few studies have been described on 1-substituted-alkyl carbapenems, with the object of obtaining compounds that possess the favourable stability properties of the 1-methyl derivatives but improved antibacterial properties. The Shionogi group prepared 1α- and 1β-substituted (fluoro, cyano, hydroxy, and acetoxy)-methyl carbapenems [142]. The 1β-fluoromethyl derivatives showed the best antibacterial activity, but overall offered little improvement over their 1β-methyl couterparts. The Bristol-Meyers Squibb group undertook a more detailed study of 1β-substituted-alkyl carbapenems [143–147]. Incorporation of larger hydrocarbon groups (ethyl, n-propyl, vinyl, allyl) in place of the 1β-methyl group in (102) had a detrimental effect on antibacterial activity. In an attempt to overcome the loss of activity associated with increased steric bulk, alkyl groups carrying various polar substituents were introduced. Hydroxy, ether, thioether, azido, cyano, formyl (and derivatives), and carboxy (and derivatives) substituents had little effect on antimicrobial activity; however, amino substituents, as in (103), did restore activity against Gram-negative organisms to the level seen in the 1β-methyl analogue and showed particularly good anti-pseudomonal activity [143]. This activity was even more pronounced in analogues



(102) R = Me
(103) R = $(CH_2)_2NH_2$



(104)

carrying an aminoalkylthio substituent at C-2; compound (104), for example, showed good activity against Gram-positive and Gram-negative organisms and better activity than imipenem against *Ps. aeruginosa*. Although these compounds have useful antibacterial activity and stability to DHP-1, they have only limited chemical stability in solution at physiological pH. In order to overcome this poor stability, various mono- and di-peptide derivatives of the 1β-aminoethyl compound (103) were prepared [145]. These showed much increased chemical stability, but reduced activity *in vitro*. However, *in vivo* the peptide derivatives showed similar or slightly improved activity compared with the parent amine (103), suggesting that they were acting as prodrugs of (103) [145]. The 1β-aminoalkyl carbapen-

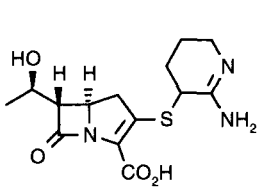
ems are an interesting development, although it still remains to be seen whether their weakness in terms of chemical instability can be overcome.

Introduction of the 1β-methyl group into the carbapenem nucleus gave the desired improvements in chemical and metabolic stability, and a number of practical syntheses of this class of compound have been developed. The next stage in finding an improvement on the imipenem-cilastatin combination was the optimisation of the C-2 substituent for antibacterial potency.

MODIFICATION AT C-2

The availability of the ketoesters (45) and (79) has enabled the synthesis of a wide variety of carbapenems with thio-linked substituents at C-2. Early structure-activity studies had indicated that for potent, broad spectrum activity, and especially for activity against *Ps. aeruginosa*, a basic or positively charged group was required in the C-2 substituent, and consequently many of the compounds described in the literature are of this type.

Compounds containing an amidinoalkylthio group in place of the cysteamine side-chain of thienamycin have excellent antibacterial activity, comparable to that of imipenem [86, 148]; compound (81) (*Scheme 3.7*), which also contains a 1β-methyl group, is an example of this type. Compounds in which the amidino group is incorporated in a five- or six-membered ring, as in (105), also have excellent activity [149].



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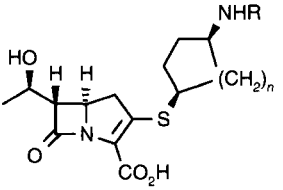
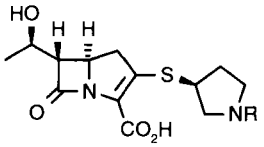


(106) n = 1 or 2
R = H or CH=NH

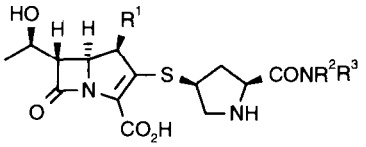
The Bristol-Myers group [150] investigated the effect of replacing the flexible ethyl chain in the cysteamine moiety by a more rigid cyclopentyl or cylohexyl ring, as in (106). Overall, the introduction of a rigid ring structure between the nucleus and the basic functionality did not significantly change the antibacterial potency. The Sankyo group [151] have also investigated the effect of using a ring system between the amino group and the nucleus, but in this case the amino function was incorporated in a five-membered ring; the amino compound (107) and its amidino derivative (108) are more active

than thienamycin against many Gram-negative organisms, including *Ps. aeruginosa*. Compound (108), known as panipenem, has recently been launched in Japan as a broad spectrum, injectable antibiotic. Although panipenem has better stability than imipenem towards renal DHP-1, it is still extensively metabolized by this enzyme *in vivo*, and is therefore used in a 1:1 combination with *N*-benzoyl β-alanine (betamipron), an inhibitor of renal tubular uptake which prevents nephrotoxicity [152].



(107) R = H

(108) R = CMe=NH; Panipenem

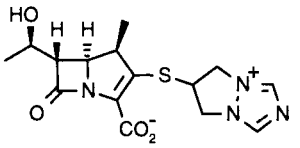


(109) R^1 = H; R^2 and R^3 = various [153]

(110) $R^1 = R^2 = R^3$ = Me: Meropenem

The 2-pyrrolidinothiocarbapenem series was developed further by the Sumitomo group, who investigated the effects of adding an aminocarbonyl group at the pyrrolidine 5′-position [153]. Compounds (109, $R^2 = R^3$ = H and $R^2 = R^3$ = Me) and their (3′*R*, 5′*S*), (3′*R*, 5′*R*), and (3′*S*, 5′*R*) isomers were studied to determine the effect of stereochemistry on activity. All were highly active antibacterials, but the *cis* compounds had the better anti-pseudomonal activity, with the (3′*S*, 5′*S*) isomer (109) being the most active. Activity in the (3′*S*, 5′*S*) series was also dependent on the nature of the aminocarbonyl substituent, with the dimethyl compound being one of the most active. Finally, the effect of a 1β-methyl group was investigated; in comparison with (109, $R^2 = R^3$ = Me), compound (110) has improved activity against Gram-negative bacteria and greatly improved stability to DHP-1 (half-life in the presence of hog renal DHP-1 was increased from 16 to 310 minutes) [153]. Compound (110), known as meropenem, is an extremely potent, broad spectrum antibacterial [154]; activity against Gram-negative organisms is better than that of imipenem, whereas that against Gram-positive organisms is slightly worse (*Table 3.6*). Meropenem was developed jointly by Sumitomo and ICI, and has recently been launched as a parenteral antibiotic for treatment of severe bacterial infections; unlike imipenem, meropenem has sufficient stability to DHP-1 to be used without co-administration of a DHP-1 inhibitor.

C-2 Heterocyclyl-alkylthio carbapenems carrying a highly basic [155] or quaternised nitrogen or sulphur [156] are also potent antibacterials. Studies on these series revealed a correlation between the basicity of the C-2



(111) Biapenem

side-chain and activity against certain organisms, especially *Ps. aeruginosa*. Likewise, compounds containing a quaternised nitrogen or sulphur have much better anti-pseudomonal activity than the corresponding unquaternised derivatives. One notable compound of this type is the triazolium derivative (111) [101, 157, 158], known as biapenem, which is currently being developed by Lederle as a broad spectrum, DHP-1 stable carbapenem. Biapenem has better stability to DHP-1 than either imipenem or meropenem [159], and appears to fall beween these two antibiotics in terms of antibacterial activity (*Table 3.6*).

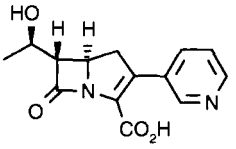
To date, panipenem, meropenem, and biapenem represent the ultimate developments in terms of C-2 thio-substituted carbapenems; the latter two

Table 3.6. COMPARATIVE IN VITRO ANTIBACTERIAL ACTIVITIES FOR IMIPENEM (71), MEROPENEM (110), AND BIAPENEM (111) [157]

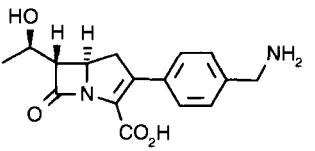
Organism (n)	*MIC90 (mg/l)*		
	Imipenem	*Meropenem*	*Biapenem*
Staphylococcus aureus (19) (methicillin sensitive)	0.03	0.12	0.06
Staphylococcus aureus (27) (methicillin resistant)	16	8	8
Coagulese-negative staphylococci (26)	0.5	2	2
Streptococcus pyogenes (20)	≤0.008	≤0.008	≤0.008
Streptococcus pneumoniae (20)	0.03	0.06	0.03
Enterococcus faecalis (20)	1	4	4
Escherichia coli (24)	0.25	0.03	0.25
Klebsiella spp. (24)	0.25	0.06	0.25
Enterobacter spp. (24)	1	0.12	0.25
Serratia marcescens (22)	1	0.12	2
Proteus mirabilis (24)	2	0.12	2
Morganella morganii (26)	2	0.12	2
Pseudomonas aeruginosa (25)	8	8	4
Xanthomonas maltophilia (18)	>128	128	>128
Haemophilus influenzae (34)	2	0.12	1
Moraxella catarrhalis (25)	0.06	≤0.008	0.03
Bacteriodes fagilis (31)	0.5	1	0.5

derivatives also incorporating the 1β-methyl modification. Interestingly, all these compounds contain the S-C-C-N arrangement in the C-2 group, in common with the natural carbapenem thienamycin and its derivative imipenem.

Although thio-linked substituents have been the major area of study in terms of C-2 modified carbapenems, carbon-linked substituents have also attracted some attention. The Merck group [160–162] prepared a number of 2-aryl carbapenems, such as (112). These compounds have good antibacterial activity (except against *Ps. aeruginosa*), and, more importantly,



(112)



(113)

they showed better stability to DHP-1 than thienamycin (*Table 3.7*). The antibacterial potency and stability to DHP-1 were increased by addition of a basic or quaternised nitrogen substituent, as in (113) (*Table 3.7*), but anti-pseudomonal activity was still less than that of thienamycin [163]. The effects of 1α- and 1β-methyl substituents in these 2-aryl derivatives were also studied [164], but, counter to expectations, these modifications resulted in a reduction in both antimicrobial activity and stability to DHP-1.

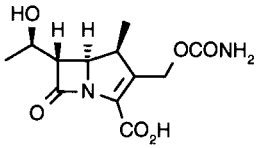
Table 3.7. RELATIVE IN VITRO ANTIBACTERIAL POTENCY AND DHP-1 SUSCEPTIBILITY OF 2-ARYL-CARBAPENEMS (112) AND (113) IN COMPARISON WITH THIENAMYCIN (14) [164]

Organism (n)	*Thienamycin Mean MIC (mg/l)*	*Compound (112) Potency*[a]	*Compound (113) Potency*[a]
Staphylococcus aureus (5)	0.04	1.5	1.5
Enterococcus (3)	5	6	9.2
Escherichia coli (5)	0.3	9	3.5
Enterobacter spp. (6)	1.4	20	11
Klebsiella spp. (5)	1.3	7	3.2
Serratia spp. (2)	2.5	28	5.3
Proteus spp. (5)	7.5	17	6.5
Pseudomonas aeruginosa (5)	5.8	0.05	0.3
DHP-1 susceptibility[b]	1.0	0.4	0.02

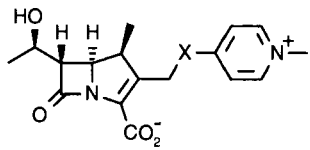
[a] Antibacterial potency relative to thienamycin = 1

[b] Relative susceptibility to hog renal DHP-1 [77]

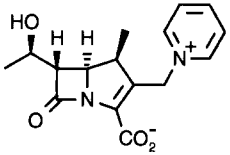
Variously substituted 2-alkyl carbapenems have also been investigated. Fujimoto *et al.* [165] prepared dethiathienamycin and found it to be about half as potent as thienamycin. Merck workers [166] prepared a number of 2-(*O*-functionalized methyl)-1β-methyl- carbapenems; the carbamate (114) is the most active of these and exceeds the activity of thienamycin against most organisms, except *Ps. aeruginosa*. The Shionogi group [167, 168] prepared a series of 2-(*S*-functionalized methyl) derivatives, and ICI Pharma [169] prepared a series of 2-(*N*-functionalised methyl) carbapenems; the 4-pyridinium compounds (115) and (116) were the best antibacterials in these series, although, like the *O*-functionalized-methyl compounds, they had weaker anti-pseudomonal activity than imipenem. Scientists at Shionogi [170] also prepared 2-(*N*-functionalised methyl)- and 2-(*N*-functionalised vinyl)-carbapenems containing a quaternised nitrogen substituent. Compounds (117) and (118) have the best activity, being similar to imipenem, including activity against *Ps. aeruginosa*.



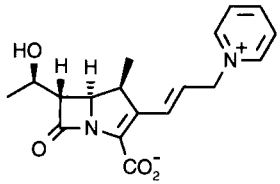
(114)



(115) X = S
(116) X = NH



(117)

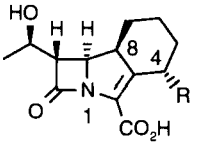


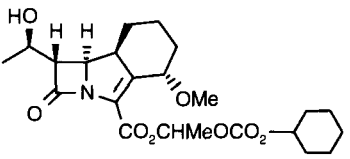
(118)

Carbapenems with carbon-linked substituents at C-2 have not received the same attention as those with a C-2 sulphur-linked substituent. Nevertheless, work in this series has resulted in compounds with broad-spectrum activity comparable to sulphur-linked derivatives such as imipenem. An advance over the sulphur-linked compounds is provided by the 2-aryl carbapenems which have good stability to DHP-1 without the addition of a 1β-methyl group.

TRI- AND TETRA-CYCLIC CARBAPENEMS

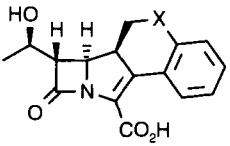
A natural progression from carbapenems carrying carbon-linked substituents at C-1 and C-2 was to join these groups together so as to form a further ring structure. Glaxo scientists [171] have described a series of such tricyclic carbapenem structure, known as tribactams. Compound (119), GV 104326,





(119) R = OMe; GV 104326
(120) R = H, SMe, SOMe, NHMe

(121) GV 118819



(122) X = CH_2
(123) X = S

was found to be substantially more active than its C-4 and C-8 stereoisomers, and, in terms of its overall antibacterial activity and stability to DHP-1, was also preferred to its analogues (120) [172]. This tribactam has broad-spectrum activity (excluding *Ps. aeruginosa*) (*Table 3.8*) and is completely stable to DHP-1; its hexetil prodrug ester (121), GV 118819, is orally absorbed in mice (bioavailability of GV 104326 = 77%) and shows good efficacy in murine infection models [174]. Recently, the pharmacokinetics of GV 104326 have been determined following single intravenous and oral (as its prodrug GV 118819) administration in man [175]. The kinetics of GV 104326 were linear up to doses of 1 g (i.v.) and 0.5 g (p.o.), oral bioavailability was about 40%, and the compound was well tolerated in both studies.

Scientists at Hoechst [176] and Bayer [177] prepared a number of tetracyclic carbapenems, and found that, as in the tribactams, the β-stereochemistry is required at the carbapenem C-1 position for optimal antibacterial activity. Compounds (122) and (123) are two of the more

active members of this series, and show good activity against Gram-positive bacteria, but only moderate activity against Gram-negative organisms and no activity against *Ps. aeruginosa*. The pivaloyloxymethyl ester of (123) was orally absobed in mice [177].

Tri- and tetra-cyclic structures are an interesting development in the area of non-natural carbapenems. These compounds have good stability to DHP-1, but to date have not shown the potent, truly broad-spectrum activity that distinguishes carbapenems such as imipenem and meropenem. However, the demonstration of good oral absorption by way of pro-drug esters is a notable advance.

CLINICAL AND COMMERCIAL ASPECTS

Since its introduction into clinical use in 1985, the imipenem-cilastatin combination (Primaxin) has largely lived up to its initial promise. Imipenem has the broadest spectrum of antibacterial activity of all antibiotics

Table 3.8. COMPARATIVE IN VITRO ANTIBACTERIAL ACTIVITIES FOR IMIPENEM (71) AND TRIBACTAM GV 104326 (119) [173]

Organism (n)	MIC_{90} *(mg/l)*	
	Imipenem (71)	*GV 104326 (119)*
Staphylococcus aureus (27) (methicillin sensitive)	≤0.015	0.12
Staphylococcus aureus (15) (methicillin resistant)	4	16
Staphylococcus epidermidis (12)	≤0.015	0.006
Streptococcus pyogenes (9)	0.003	0.015
Streptococcus pneumoniae (11)	0.003	0.007
Enterococcus faecalis (10)	2	4
Escherichia coli (10)	0.25	0.5
Klebsiella pneurmoniae (11)	0.5	2
Enterobacter cloacae (10)	1	2
Serratia marcescens (10)	>32	16
Proteus mirabilis (11)	4	2
Proteus vulgaris (9)	8	1
Morganella morganii (11)	4	4
Haemophilus influenzae (11)	1	0.25
Haemophilus parainfluenzae (7)	1	1
Moraxella catarrhalis (8)	0.25	0.5

available for systemic use in humans. It is used as initial monotherapy of a variety of moderate to severe infections, with a clear role in empirical treatment of mixed infection. Administration is generally in 500mg to 750mg intravenous doses every 6 hours. The daily cost of imipenem is one of the highest of the currently available parenteral antibiotics, although this is approximately the same as that of most double-antibiotic regimens and less than that of most conventional triple-antibiotic regimens. In 1993, Primaxin was the 10th best selling antibiotic worldwide and the total audited sales exceeded £277 million [178].

Imipenem is not, however, without its drawbacks. The most common adverse effects are nausea and vomiting, which are often associated with over-rapid intravenous infusion, and seizures, the incidence of which is unacceptably high for patients with underlying neurological disease or impaired renal function. Imipenem is not recommended for therapy of meningitis. Another shortcoming of imipenem is in the treatment of infections involving *Ps. aeruginosa*, where clinical failures have occurred as a result of development of resistance (*i.e.* selection of insensitive strains) during therapy.

Meropenem addresses at least some of these deficiencies. It is reported to be less epileptogenic than imipenem and has better activity against *Ps. aeruginosa* [154]. Imipenem is a powerful inducer of chromosomal β-lactamase, and moderate level resistance in *Ps. aeruginosa* has been attributed to slow hydrolysis by β-lactamase combined with reduced permeability; the overall result of this being that, at therapeutic concentrations, insufficient number of molecules reach the target PBPs [179]. Meropenem, being a poorer inducer of β-lactamase, is subject to less hydrolysis in strains with reduced permeability, and is thus better able to combat moderately imipenem-resistant pseudomonas. Highly imipenem-resistant *Ps. aeruginosa,* however, are also highly resistant to meropenem.

Although imipenem and meropenem are resistant to most serine β-lactamases, they are subject to rapid hydrolysis by a number of bacterial zinc metallo-β-lactamase enzymes. Such enzymes have been found in *Xanthomonas maltophila*, *Aeromonas hydrophila*, and *Bacteroides fragilis* [180]. All strains of *Xanthomonas maltophila* produce a metallo-β-lactamase, and are consequently resistant to carbapenems. The possible emergence of further strains which produce carbapenem-hydrolysing enzymes represents a potential threat to the future use of these otherwise extremely potent antibiotics.

SUMMARY

The discovery of the olivanic acids and thienamycin aroused considerable interest amongst medicinal chemists and microbiologists around the world. The susceptibility of these agents to metabolic degradation has, however, been a major obstacle in their development. For many years the only notable success from such intensive research was the combination of imipenem with cilastatin, an inhibitor of the renal dipeptidase enzyme DHP-1. The enormous success of Primaxin for the treatment of a range of life-threatening bacterial infections provided the impetus for the discovery of totally synthetic, non-natural carbapenem derivatives that combine the broad spectrum of antimicrobial activity with stability to enzymatic degradation. This has indeed been realised in the development of meropenem; it possesses the broad spectrum of activity and resistance to β-lactamases that are embodied in imipenem as well as displaying increased stability to human dehydropeptidases.

Most recent research has focused upon the development of carbapenem antibiotics which combine broad spectrum antimicrobial activity and metabolic stability with oral absorption, for the treatment of community-acquired infections. Indeed, the pro-drug esters of the tricyclic carbapenems represent the first significant advance in this respect.

However, the increased use of carbapenem antibiotics would undoubtedly accelerate the emergence of carbapenem-hydrolysing enzymes. The ultimate challenge could therefore be the design and synthesis of carbapenem derivatives that are resistant to these metallo-β-lactamases.

Due to the enormous problems encountered in the development of the carbapenem antibiotics, this area of research has, in the past, been described as a battlefield that did not bode well for the future [181]. Primaxin and meropenem proved however that these problems were not insurmountable, and are therefore a testimony to the persistence and dedication of those scientists in their war against bacterial infection.

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4 Inhibition of aromatase

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INTRODUCTION

This chapter is a review of recent progress in the chemistry of inhibitors of the P450 enzyme aromatase, specifically the human placental oestrogen synthetase. Researchers requiring background to both the general strategy of inhibition of aromatase in the potential treatment of oestrogen-dependent disease states [1–6] and the biochemical aspects of the enzyme mechanism [7–16] are referred to previous reviews [1, 17–31]. More recently, excellent overviews of the more important aspects of the molecular biology of aromatase have appeared [17, 22] together with discussions on the potential multiple functionality of this enzyme [31] and in the role of an oestrogen-2-hydroxylase.

For this review, the inhibitors have been divided into different classes based on their structural characteristics, or specific groupings such as aminoglutethimide analogues and wider divisions such as natural products. It is hoped that development of the inhibitors that appear within each category follows a broadly chronological order and that the most important aspects of their activity, synthesis and structural features have been highlighted where deemed appropriate.

MECHANISTIC ASPECTS OF THE AROMATASE ENZYME

This section places in perspective current views on the mechanistic aspects of the aromatase enzyme and directs readers toward some excellent work on recent biochemistry and molecular biology.

Tan has outlined the areas in which general agreement has been reached [7], and concluded that (i) aromatase has single enzyme status, (ii) the enzyme is a NADPH, oxygen-dependent mono-oxygenase, (iii) the enzyme reaction is stoichiometric with respect to NADPH (iv) reaction is by initial geminal dihydroxylation of the 19-methyl group on the androgen substrate followed by (v) elimination of the 19-carbon as formic acid which follows the third processing of NADPH/O_2 and (vi) the 2β-H and not the 2α-H from the androgen substrate is incorporated into water after completion of the enzymic reaction. Disagreement continues concerning (i) the stepwise or concerted reaction mechanism (ii) the exact nature of the third step (iii) the number of isozymes in the human placenta and (iv) the conformation and keto-enol equilibrium position of the A-ring of the androgen substrate within the enzyme on catalysis (see *Figure 4.1*). The first, second and disputed third steps of the enzyme's mechanism also were reviewed in some depth. The debated 1- and 2-position hydrogen elimination that occurs

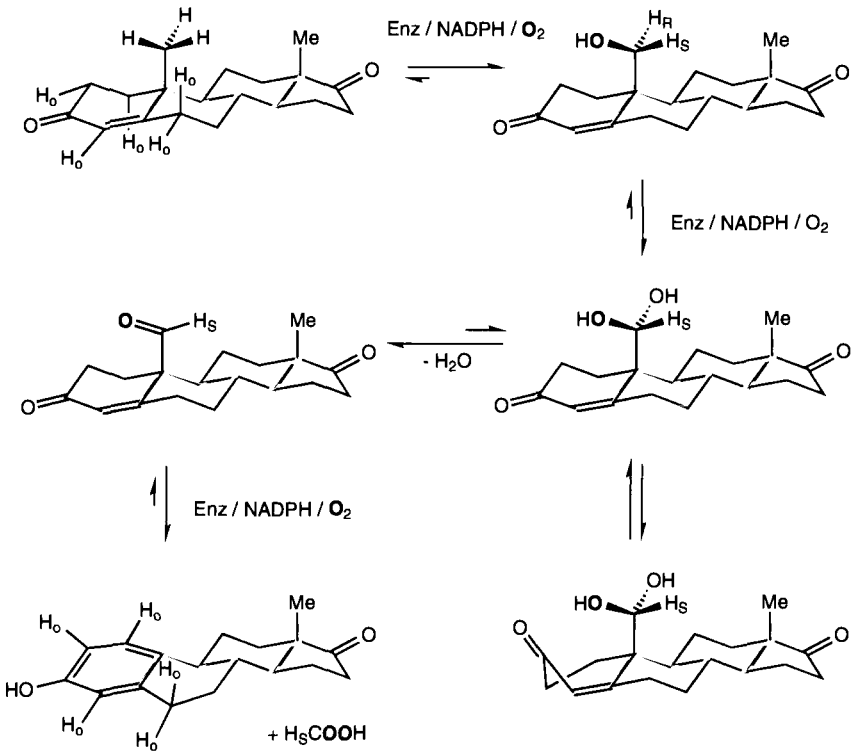


Figure 4.1 The currently accepted scheme for the mechanism of aromatisation of androstenedione by aromatase. Conformational equilibrium for the 19-dihydroxy substrate is included and pro-S and pro-R positions on the substrate are labelled as S and R, the labels remain to indicate their fate. Enz = enzyme, H_o indicates key A- and B-ring hydrogens.

during enzymic conversion of the androgen to oestrogen is viewed as almost settled with a proposed mechanism by Covey *et al.* [32] (see *Figure 4.2*) in conjunction with the existing evidence [12-22].

The rôle of amino acid residues within the active site during the catalysis is gradually unfolding from mutation studies in conjunction with computer-aided molecular modelling (CAMM) of the tertiary structure of aromatase. Studies based on a recently solved X-ray structure of cytochrome $P450_{BM3}$ has afforded, along with $P450_{CAM}$, working models of the tertiary structure of $P450_{XIXA1}$ by complementarity of sequence alignments and definition of putative α-helices and β-sheeting [28, 29]. *Figures 4.3 and 4.4* show details from the work of Zhou and co-workers which is still in the process of further

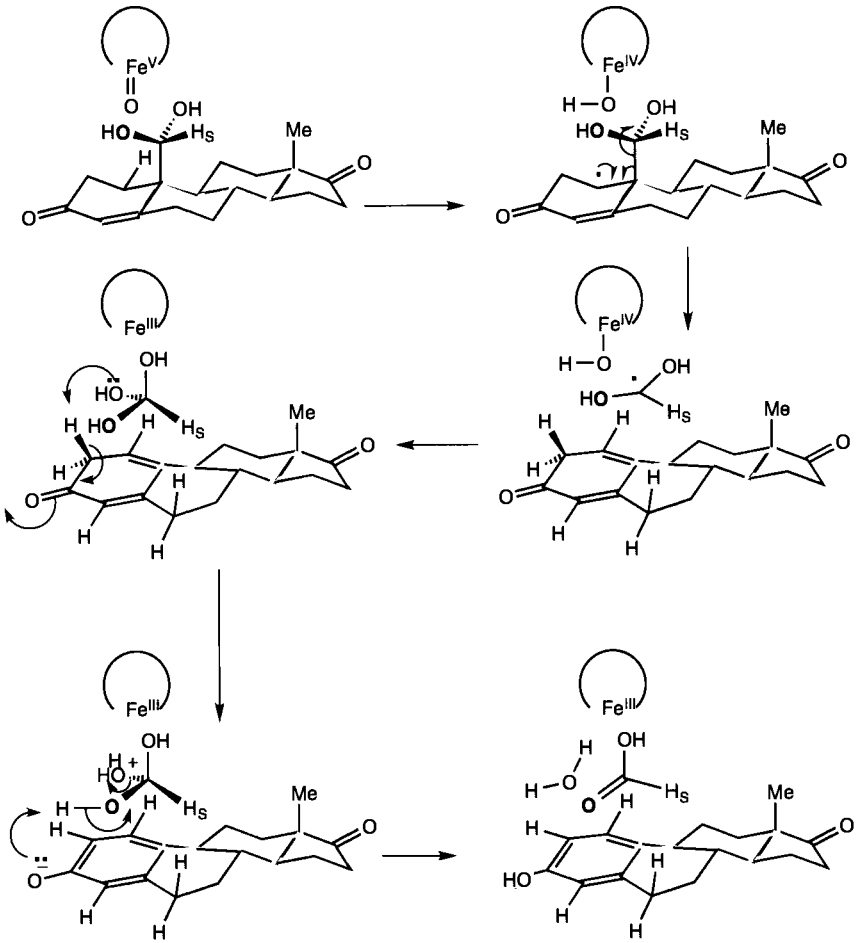


Figure 4.2 Radical mechanism as proposed by Covey [32], generation of a reactive orthoformate and the consequent abstraction of the 2-hydrogen of the steroid and dehydration of the orthoformate to yield formic acid and water. (Note: this does not include any role for specific amino acid residues within the active site of aromatase) (Diagram adapted from Covey)

refinement. *Figure 4.3* shows a stereographic view of the native enzyme detailing the α-helical and the β-sheet regions; *Figure 4.4* reveals specific residue contacts with androstenedione and 7-APTA within an enzyme bound complex model. Residues Gly-121, Iln-125 and Phe-235 are conserved in human, rat, mouse, chicken and trout aromatase, whilst

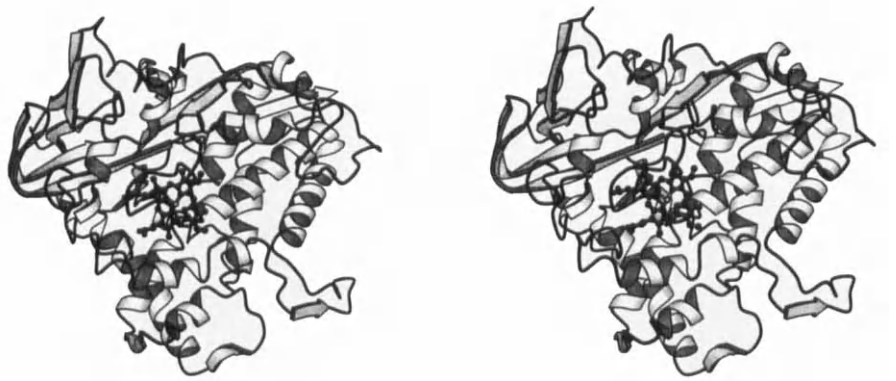


Figure 4.3 Diagram depicting the computer modelled tertiary structure, including apo-protein porphyrin, of human placental aromatase.

Iln-474 is unique to the human enzyme. This region defines the area of interest to molecular biologists for their mutation studies. It appears to be an 'extra' hydrophobic pocket roughly orthogonal to the plane of the substrate extending from the 4- and 7-positions of the steroid in the bound complex models (see *Figure 4.4a and 4.4b*). Armarneh *et al.* [30] have suggested that Thr-310 is the residue involved in proton donation to bound oxygen on the haem moiety and that it is intimately involved in the hydroxylation process. Glu-302 has been implicated in the 2-hydrogen abstraction [23] with strong supporting evidence of loss of activity on mutation to non-functional amino acids [30]. These mutation studies have provided excellent data for the model refinement process previously mentioned *in lieu* of the X-ray structural data for aromatase.

STEROIDAL INHIBITORS OF AROMATASE

1- AND 2-SUBSTITUTED SUBSTRATE ANALOGUES

Work has progressed on taking a prototypical suicide inhibitor (1) and extending previous structure-activity studies [32] to include D-ring modifications. These modifications were not only designed to modulate aromatase inhibition but also to address the problem of the biological transformation, by reduction generally, of the 17-keto group of the androstenedione analogues *in vivo*. Compounds (1) to (4), (5) and (6) were found to be mechanistically based inhibitors that required enzyme turnover to be

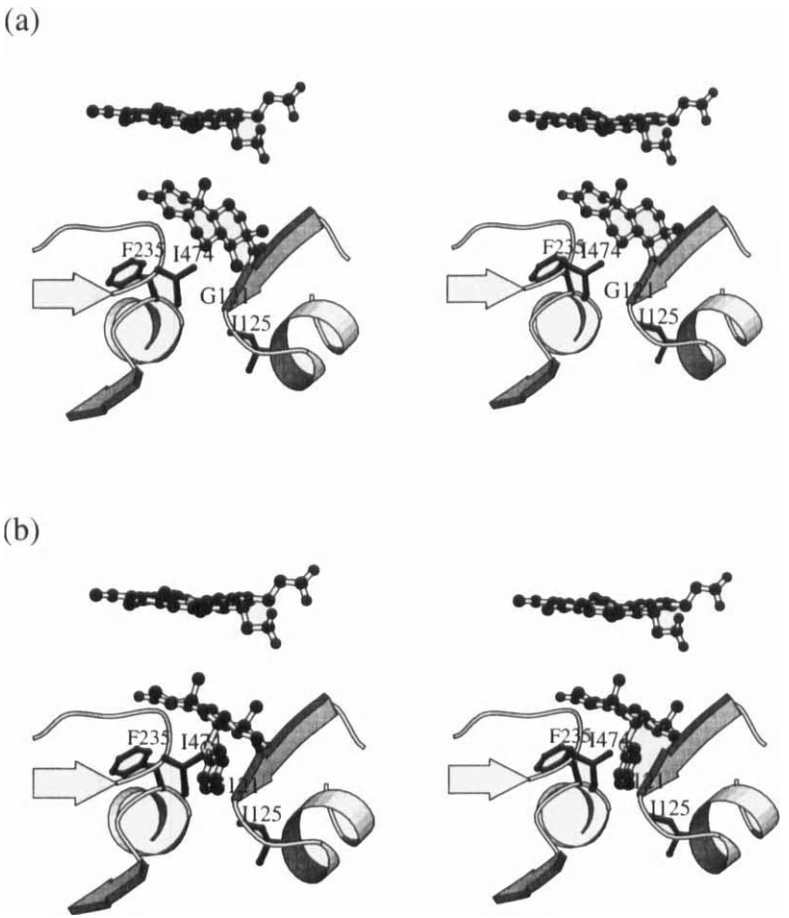
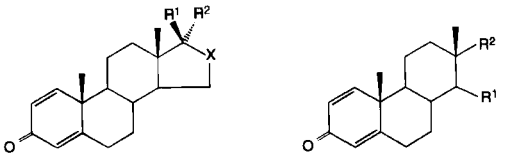
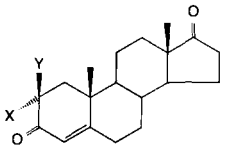


Figure 4.4 Stereoscopic views of the putative structural detail of human placental aromatase with (a) 'located' substrate androstenedione and (b) the steroidal inhibitor 7-APTA [21, 67].



(1) $R^1 = R^2 = O$, $X = CH_2$
(2) $R^1 = OH$, $R^2 = H$, $X = CH_2$
(3) $R^1 = R^2 = O$, $X = CH_2$
(4) $R^1 = R^2 = H$, $X = CH_2$
(5) $R^1 = H$, $R^2 = CHO$
(6) $R^1 = H$, $R^2 = COCH_3$
(7) $R^1 = R^2 = O$, $X = CH_2$

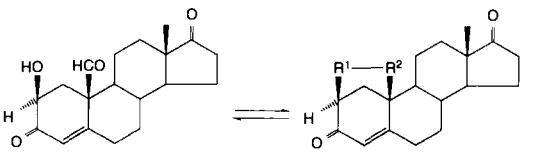
activated; these analogues displayed a range of $t_{1/2}$ inhibition of aromatase ($t_{1/2}$ from 372 to 1290 s) and binding affinity (K_i from 260 to 8560 nM). Conclusions reached by this research were that (i) as long as the D-ring remains intact, modifications to the D-ring functionality caused only minor changes to the affinity and rates of inactivation and (ii) the 17-keto group is *not* essential for relatively tight binding of these substrate analogues. D-Ring-opened analogues may loose binding affinity by as much as 300-fold (7) but this may be retrieved by shortening the chain length of (7) to that of (6) [33]. The loss of binding was ascribed to the increased conformational flexibility of the ethyl analogue and its accessibility to certain conformations of the ethyl substituent.



(8) X = H , Y = OH
(9) X = H , Y = SH

Utilizing the potential intermediacy of (8) in the biosynthesis of oestrone from androstenedione, 2α-SHA (9) has been prepared and was found to be a poor inhibitor (K_i = 3100 nM) [34]. This loss of binding was explained by an expected structural perturbation of the A-ring of the analogue (9) with respect to androstenedione but the compound did inactivate aromatase ($k_{inact} = 2 \times 10^{-3}$ s ^{1}). Modifications of (8) were discussed in the same study at the 10-/19-positions and are covered later.

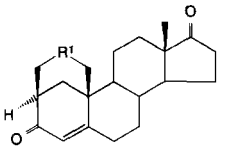
A logical extension of analogue work based on (8) followed by extrapolating the concept from (8) to (10) and the proposed oxidized intermediate lactol (11) [35]. Since (11) is chemically labile, being in equilibrium with its open chain tautomer, the carbocyclic analogue of the isomers of (11), (12) and (13), were synthesized and both the (*R*)-(12,



(10)

(11) R^1 = O , R^2 = CHOH
(12) R^1 = CH_2 , R^2 = CHOH (R)
(13) R^1 = CH_2 , R^2 = CHOH (S)
(14) R^1 = R^2 = CH_2

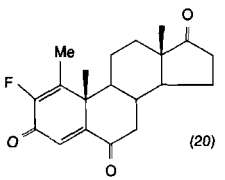
K_i = 870 nM) and (*S*)-(13, K_i = 926 nM) alcohols were found to be competitive inhibitors of aromatase; interestingly, (14, K_i = 70 nM) had a higher activity than either (12) or (13). A continuation of this work by Merrell Dow led to some simple but elegant chemistry in the synthesis of a 2,19-methyleneoxy bridged analogue (15) [36, 37]. The hypothesis was that (15) would be activated by aromatase-mediated hydroxylation and that formaldehyde would be released by a retro-aldol reaction thereby potentially hydroxymethylating the enzyme at nucleophilic residues within the active site leading to inactivation of aromatase.



(15) R^1 = O
(16) R^1 = NH
(17) R^1 = CH_2
(18) R^1 = S
(19) R^1 = $NCOCF_3$

The ether (15) exhibited reasonable competitive inhibition (K_i = 17.6 nM) but slow mechanism-based inactivation of aromatase. Compounds (16) to (18) have also been reported as time-dependent inhibitors of aromatase [38], but (19) showed no such activity. The biological activity of (16, K_i = 259 nM, k_{inact}/K_i = 16,760) has been reported in the patent literature [39]. The methylene analogue (17) exhibited the highest inhibitory activity, but its time-dependent inhibition was not NADPH-dependent, i.e., it was not enzyme-activated and (16) was thought to be a non-competitive inhibitor of aromatase [40].

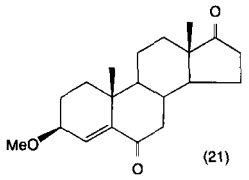
2-Fluoro-1-methylandrosta-1,4-dien-3,17-dione (20, K_i = 58 nM) was found by Schering AG to have fair competitive inhibition of aromatase [41].



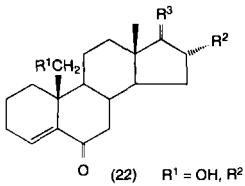
(20)

3-SUBSTITUTED SUBSTRATE ANALOGUES

3α-Methoxy steroid (21) shows moderate binding to aromatase (K_i = 700 nM) but with time-dependent inactivation of the enzyme, ($k_{inact} = 6 \times 10^{-4}$ s^{-1}), in which NADPH and oxygen were required with androstenedione protecting against the action of (21). The 3β-methoxy isomer had an IC_{50} > 100μM and hence its K_i was not determined [42].



(21)



(22) R^1 = OH, R^2 = H, R^3 = O
(23) R^1 = R^2 = H, R^3 = O
(24) R^1 = OH, R^2 = H, R^3 = H,17β-OH
(25) R^1 = H, R^2 = Br, R^3 = O
(26) R^1 = R^2 = H, R^3 = H,17β-OH

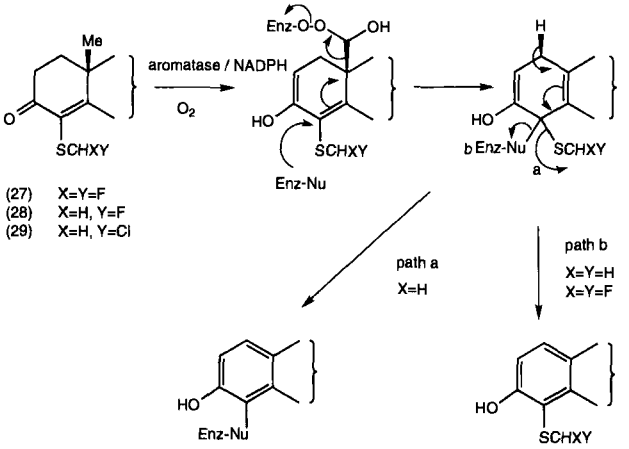
In contrast to much earlier findings [43], a series of 3-deoxy steroids (22) to (26) has shown that potent inhibition of aromatase can be achieved by this class of steroid (22, K_i = 13 nM). This study also demonstrated a change in relative potency of (22) to (26) depending on the substrate used for the assay, androstenedione compared with [1β-^{3}H] 16α-hydroxyandrostenedione [44]. In an earlier paper [45], compound (22) was found to be a strong competitive inhibitor of aromatase, as potent as 4-hydroxyandrostenedione (K_i = 12.5 nM) but it did not display any inactivation of the enzyme.

4- AND 5-SUBSTITUTED SUBSTRATE ANALOGUES

With the success of 4-hydroxyandrostenedione (4-OHA), work on analogues has continued in an attempt to improve 4-OHA's shortcomings. 2,2-Dimethyl-4-OHA has been synthesized and has been shown to be a powerful competitive inhibitor of aromatase (K_i = 11.4 nM) but does not cause time-dependent inactivation in contrast to 4-OHA; in the same study, 2,2-dimethylandrostenedione (K_i = 13 nM) was also found to be an excellent inhibitor [46].

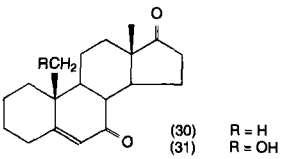
Scientists at Roussel-UCLAF described an interesting group of 4-substituted steroids [47] some of which were employed as potential 'double jeopardy suicide inhibitors' [48] of aromatase. A range of 4-alkoxy- and 4-alkylthio-androstenediones (27–29) were synthesized and the nature of their inhibition evaluated. Roussel regarded compounds (27) to (29) as the most interesting, (27, IC_{50} = 2700 nM) being a moderate competitive inhibitor but reversible, whereas (28, IC_{50} = 80 nM, K_i = 30 nM, k_{inact} = 3.1 × 10^{-3} s^{-1},) and (29, IC_{50} = 94 nM, K_i = 30 nM, k_{inact} = 3.8 × 10^{-3} s^{-1},) were potent and time-dependent requiring enzyme activation. An hypothesis was proposed to explain the nature of the behaviour of (28) and (29) with respect to (27); this is summarized in *Scheme 4.1* [47].

A most unusual substitution pattern for a steroidal inhibitor of aromatase



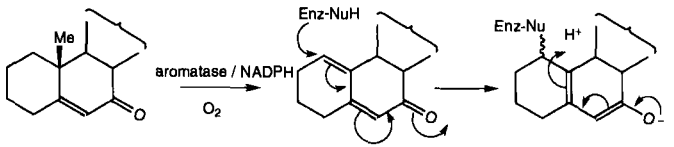
Scheme 4.1 Proposed inactivation of aromatase by (27) to (29).

was investigated by Numazawa and co-workers [49]. A 5-en-7-one (30, K_i = 143 nM, $k_{inact} = 1.15 \times 10^{-3}$ s^{-1}) was found to be a mechanism-based inhibitor of aromatase along with analogue (31, K_i = 11,100 nM, $k_{inact} = 0.9 \times 10^{-3}$ s^{-1}), and a hypothesis for its action formulated (see *Scheme 4.2*). An interesting comparison can be made with the 3-deoxy compounds (22) and (23).

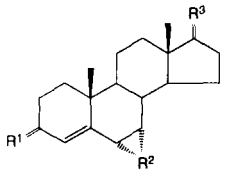


6-SUBSTITUTED SUBSTRATE ANALOGUES

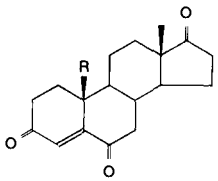
Numazawa and co-workers have investigated various substitutions at the 6- and 7-positions of the B-ring, in compounds such as the 6α,7α-cyclopropanes (32) to (34). These were found to be competitive inhibitors of



Scheme 4.2 Proposed mechanism of inactivation of aromatase by (30) and (31)



(32) R^1 = O, R^2 = CF_2, R^3 = O
(33) R^1 = H,H, R^2 = CH_2, R^3 = O
(34) R^1 = H,H, R^2 = CH_2, R^3 = H,17β-OH



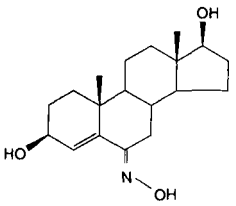
(35) R = Me
(36) R = CH_2OH
(37) R = CHO

aromatase showing no time-dependent inactivation of the enzyme; (33, K_i = 5 nM) exhibited a high potency [50].

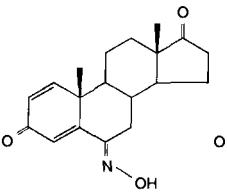
The androst-4-ene-3,6,17-triones are still providing valuable information both on structure-activity relationships and mechanistic aspects of aromatase inhibition [51, 52]. Androstenetrione (AT) (35) was found to be a mechanism-based inactivator of the enzyme whereas the 3-deoxyAT was not, but 19-OHAT (36) and 19-oxoAT (37) were. Both (36, K_i = 610 nM, $k_{inact} = 3.7 \times 10^{-3}\ s^{-1}$) and (37, K_i = 7500 nM, $k_{inact} = 1.2 \times 10^{-3}\ s^{-1}$) reacted readily with *N*-acetyl-L-cysteine with $t_{1/2}$ = 1500 s and 1200 s respectively and their inactivation of aromatase was probably due to their inherent reactivity. The 4β,5β-epoxyandrostane-3,6,17-trione synthesized as a possible intermediate in the activation of AT was not a time-dependent inactivator of aromatase. It was suggested that 19-oxygenation and not 4β,5β-epoxidation as previously put foward [53, 54] was the route for activation of AT.

A novel biotransformation using *Arthrobacter simplex* ATCC6946 converted (38) to (39). Direct chemical oxidation of (38) yielded products (40) and (41); alcohol (38) was converted by $MnO_2/CHCl_3$ into (40) and by CrO_3/pyridine to (41) . The aromatase inhibitory properties of (39) are yet to be evaluated, but (40, K_i = 471 nM) and (41, K_i = 610 nM) were found to be competitive and reversible.

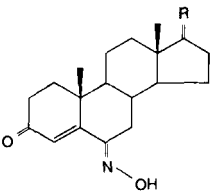
Androstenedione has been biotransformed by *Acremonium strictum*



(38)



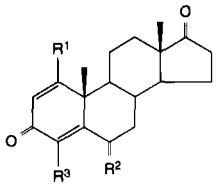
(39)



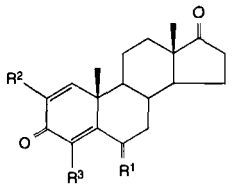
(40) R = H,17β-OH
(41) R = O

NN106 (FERM P-9143) into 14α-hydroxy-4-androstene-3,17-dione. This compound was 3-ethoxylated and oxidized chemically by various reagents but was converted into 14α-hydroxy-4-androstene-3,6,17-trione and released in the workup when Jones oxidation was employed (no data)[21].

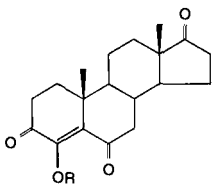
Atamestane (SH489, 42) and exemestane (FCE24304, 43) have both been involved in phase 1 trials [57, 58] and are showing promising profiles.



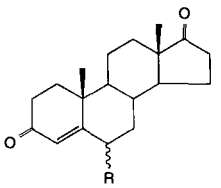
(42) R^1 = Me, R^2 = H,H , R^3 = H
(43) R^1 = H, R^2 = CH_2 , R^3 = H



(44) R^1 = O, R^2 = OH , R^3 = H



(45) R = Me
(46) R = H

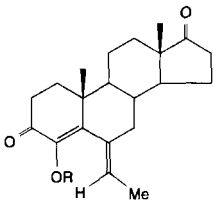


(47) R = α or β alkyl or aryl

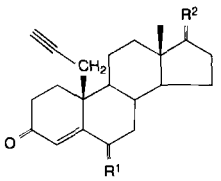
The solvolytic cleavage of 4β,5β-epoxyandrostane-3,6,17-trione has been exploited as a route to (44), (45) and (46) as potential aromatase inhibitors (no data) [59].

Structure-activity studies on the 6-substituted androstenediones have been extended to a series of 6-alkyl- and 6-aryl-androst-4-ene-3,17-diones (47). All were found to be competitive inhibitors of aromatase; the 6-n-alkyl steroids had K_i values from 1.4 to 12 nM. The 6β-vinyl, 6α-benzyl (47) steroids and the 6-methylene steroid (48) were very powerful inhibitors (K_i = 5.1,10 and 4.9 nM respectively) whereas the 6α- and 6β-isopropyl, 6-phenyl, 6β-benzyl and 6β-ethynyl were relatively weak [K_i = 22(α), 31(β), 63(β) and 62 nM, respectively]. The 6β-ethyl derivative was the most potent compound synthesized (K_i = 1.4 nM). The results seem to indicate that aromatase has a hydrophobic pocket with a limited volume being accessible in the active site in a region corresponding to the β-side rather than the α-side at the 6-position [60].

Potential metabolites of (49) MDL 18,962, 10-(2-propynyl)oestr-4-ene-



(48) R = OH or Me

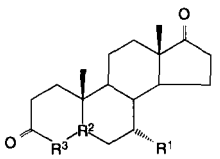


(49) $R^1 = H,H$, $R^2 = O$
(50) $R^1 = H,H$, $R^2 = H$, 17β-OH
(51) $R^1 = H$, 6β-OH, $R^2 = O$
(52) $R^1 = R^2 = O$

3,17-dione, a mechanism-based inhibitor of aromatase, were synthesized and screened for activity. All the potential metabolites were time-dependent mechanism-based inactivators of the enzyme. Metabolic action of 17β-hydroxy steroid oxidoreductase on (49) would lead to (50); compound (50) was synthesized and found to possess moderate activity (50, K_i = 28 nM, $t_{1/2}$ = 468 s at IC_{50}). Metabolism of (49) by 6β-hydroxylase would give (51) and the action of 6β-alcohol dehydrogenase on (51) would yield (52); (51, K_i = 13 nM, $t_{1/2}$ = 2220 s at IC_{50}) and (52, K_i = 167 nM, $t_{1/2}$ = 366 s at IC_{50}). The triketone (52) which from primate studies is a confirmed metabolite, is 25% as efficient as the parent compound (49) as an aromatase inhibitor, (50) 11% and (51) 5%, hence metabolism of (49) was deemed not to cause obligatory loss of aromatase inhibiting properties of (49) [61]. Isomerisation of androsta-4,6-diene-3,17-dione to androsta-4,7-diene-3,17-dione afforded the starting material for the synthesis of the androsta-4,6,8(14)-triene-3,17-dione (FCE 24917) and the isomeric androsta-4,6,8(9)-triene-3,17-dione (FCE 24918). This was achieved by treating the 7,8α-epoxyandrost-4-ene-3,17-dione derived from the above diene, with 4-toluenesulphonic acid at room temperature. FCE 24918 was found to be a time-dependent enzyme-activated inhibitor of aromatase (IC_{50} = 375 nM, K_i = 72 nM, $k_{inact} = 0.17 \times 10^{-3}$ s^{-1}), whereas FCE 24917 was a competitive reversible inhibitor (IC_{50} = 102 nM). This was expected because of the enhancement of the nucleophilicity at position 4 and the subsequent aromatisation of the B-ring on enzyme activation and nucleophilic attack on FCE 24918 by the enzyme [62]. Recently, potential oxygenated metabolites of exemestane (43) that involve modifications of the 6-methylene, have been investigated and of the 6-oxo series, activity followed this sequence: 6-methylene > 6-spiro-oxiranes > 6β-hydroxymethyl > 6-hydroxy-6-hydroxymethyl > 6β-carboxy with IC_{50} values of ca. 27, 206, 295, 2,300 and 7,200 nM, respectively; members of the 17β-hydroxy series were approximately 3-8-fold less potent [63].

7-SUBSTITUTED SUBSTRATE ANALOGUES

A patent describes unusual examples of a *cis*-fused A/B ring steroid, 7α-allyl-5β-androstan-3,17-dione (53, K_i = 900 nM) [64] and the *trans*-fused isomer (54, K_i = 600 nM) as potential inhibitors of aromatase. The most potent compound (55, K_i = 200 nM) is the more usually configured androst-4-ene-3,17-dione. All these compounds were competitive reversible inhibitors of aromatase with moderate activity.

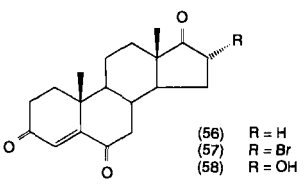


(53) $R^1 = CH_2CH{=}CH_2$, R^2 = α-CH, $R^3 = CH_2$
(54) $R^1 = CH_2CH{=}CH_2$, R^2 = β-CH, $R^3 = CH_2$
(55) $R^1 = CH_2CH{=}CH_2$, R^2-R^3 = CH=C

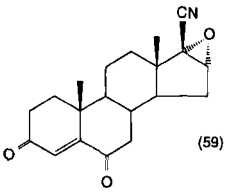
Bruggemeier and co-workers have extended the work on 7α-substituted 1,4- and 4,6-androstadiene-3,17-dione analogues previously studied [65,66] to include the synthesis of further analogues [67] and a study of their ability to covalently bind to aromatase. They have demonstrated, by analysis of a protein complex digest, that covalent linking takes place onto a lipophilic fragment of aromatase. Computer-aided molecular modelling of the 4,6-androstadiene-3,17-diones has provided further information about the active site requirements of aromatase proximal to the α-face of these substrate analogues [67].

16-SUBSTITUTED SUBSTRATE ANALOGUES

Numazawa and co-workers have examined the potential of various 16-substituted steroids as suicide substrates of aromatase. 16α-Substitution of AT has produced reasonably potent enzyme inhibitors, (56, K_i = 45 nM), (57, K_i = 150 nM), (58, K_i = 1180 nM) and (59, K_i = N.D., IC_{50} = 10,000 nM). The bromoketone (57, $k_{inact} = 6.95 \times 10^{-3}\ s^{-1}$) showed a biphasic loss of time-dependent aromatase activity in the presence of NADPH. This is not surprising since the highly reactive α-bromo ketone may react (chemically or biochemically) with the enzyme incubate. The alcohol (58)



(56) R = H
(57) R = Br
(58) R = OH



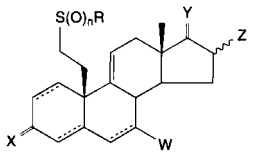
(59)

showed no evidence of suicide inhibition of aromatase [42]. The deoxy analogues of (56) and (58) have also been studied (see earlier) [45].

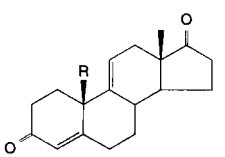
16α-Hydroxyoestrone (K_i = 9500 nM) has been cited as a natural inhibitor of aromatase and hence plays a role with oestrone (K_i = 1500 nM), oestradiol (K_i = 2200 nM) and oestriol (K_i = 162,000 nM), in modulation of the enzyme's activity. A suggestion has been made that aromatase also acts as the oestrogen 2-hydroxylase of these oestrogens to yield the catechol-oestrogens, based on the observation of potent competitive inhibition of 2-hydroxylase activity by CGS16949a [68].

10- AND 19-SUBSTITUTED SUBSTRATE ANALOGUES

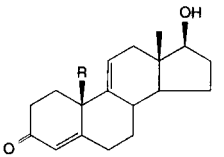
Since the discovery of potent inhibitors of aromatase centred on 10- and 19-substituted natural substrates, research activity has been intense in this area. Roussel-UCLAF has patented several compounds based on the general structure (60) with attention centred on the 10β-[2-(alkylthio)ethyl)] substituent. Compounds (61) to (66) have all shown good activity with IC_{50} values of 20, 870, 50, 500, 165 and 5.5 nM, respectively [69, 70]. Additional studies have indicated that 9,11-epoxy-6-hydroxy-10-[2-(methylthio)ethyl]oestr-4-ene-3,17-dione is a useful inhibitor of aromatase [71]. The same team patented the carbaldehyde (67) recording an IC_{50} = 85 nM



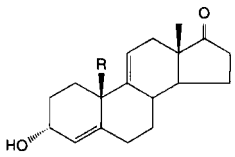
(60) X = O, CH_2 etc., W = H, alkyl, thioalkyl etc., Y = O, OH, NOR etc., Z = H, alkyl etc., R = alkyl



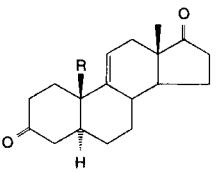
(61) R = $MeSCH_2CH_2$
(62) R = $EtSCH_2CH_2$



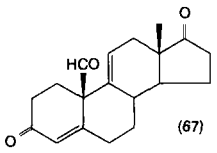
(63) R = $MeSCH_2CH_2$
(64) R = $FSCH_2CH_2$



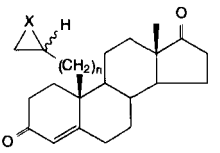
(65) R = $MeSCH_2CH_2$



(66) R = $MeSCH_2CH_2$



(67)

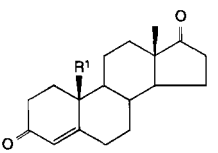


(68) X = O, n = 0
(69) X = S, n = 0
(70) X = O, n = 1
(71) X = S, n = 1

[70]. RU54115 (61, K_i = 0.5 nM) has an extremely tight binding with the enzyme *in vitro*; *in vivo* it has had success in controlling DMBA-induced mammary tumours in female rats [72].

In an attempt to build on the successes of the 10β-oxiranyl and thiiranyl inhibitors (68) and (69), homologues (70) and (71) have been synthesized [73]. The thiiranes were found to be more potent than the oxiranes and the 2′(*S*) were better inhibitors than the 2′(*R*) diastereoisomers, (70, (*R*)-, K_i = 753 ± 113 nM), (70, (*S*)-, K_i = 86 ± 4 nM), (71, (*R*)-, K_i = 206 ± 41 nM) and (71, (*S*)-, K_i = 22 ± 1 nM) when screened against aromatase. Spectroscopic studies using purified enzyme, suggest that the 2′(*S*) compounds co-ordinate to the Fe of the haem site as with the lower homologues (68) and (69); however the potencies of the 2′(*S*) isomers were reversed in (70-(*S*)) and (71-(*S*)) [73]. The 10β-aziridinyloestr-4-ene-3,17-diones have also been made and yielded potent competitive inhibitors with the same sterospecificity of potency as the corresponding oxiranes and thiiranes, the 19(*R*)-isomer was demonstrated as a slow but tight binding (K_i = 3.4 nM) inhibitor of aromatase [74].

In the process of synthesizing the lactol analogue (11), the Merrell Dow group chose a route that generated other 19-substituted androst-4-ene-3,17-diones and determined their activities towards aromatase. The halohydrins (72-(*R*), K_i = 27 nM, k_{inact}/K_i = 88220, τ_{50} = 291s) and (72-(*S*), K_i = 1130 nM, k_{inact}/K_i = 1900, τ_{50} = 319.8 s) and the bromoketone (73, K_i = 190 nM, k_{inact}/K_i = 1860, τ_{50} = 1962 s) demonstrated time-dependent inhibition with aromatase with low nanomolar affinity [35, 36].



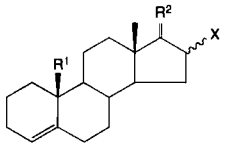
(72) R^1 = $XCH_2CH^*(OH)CH_2$, X = bromine
(73) R^1 = $XCH_2C(=O)CH_2$, X = bromine
(74) R^1 = CH_2SSR^2, R^2 = Et
(75) R^1 = CH_2SH

Organon workers have shown the *in vivo* efficacy of the 19-(ethyldithio)androst-4-ene-3,17-dione (74, R^1 = Et). ORG 30365 was found to be a potent competitive inhibitor of aromatase, but its pharmaceutical stability was poor; dithio analogues of (75) were synthesized and selected for

in vivo trials. The disulphide (74, R^1 = Et) was also found to be a potent inhibitor *in vivo* and it is suspected that biochemical cleavage of the disulphide releases (75). In addition, (74, R^1 = Et) displays an excellent hormonal profile with no undesirable androgenic activity. Other dithio compounds including (74, R^1 = Me, Pr, Bu, pentyl, $-CH(CH_2)_5$, $-CH(CH_2)_7$, t-Bu) were screened; (74, R^1 = Et) was the most potent in *in vivo* tests [75].

Numazawa and co-workers have investigated a series of 19-substituted substrate analogues (76–85) for their activity against aromatase. The 17-oxo series (76) to (80) were found to be effective competitive inhibitors of aromatase, for example, (79, $k_{inact} = 3.55 \times 10^{-4}\ s^{-1}$); the weak inhibitor (80, $k_{inact} = 1.76 \times 10^{-3}\ s^{-1}$) displayed time-dependent inhibition and androstenedione prevented the inactivation which required NADPH and oxygen. L-Cysteine did not prevent inhibition which suggests strongly that both are activated by 19-position bioconversions [76].

Precedent for the probable mechanism of action of (79) and (80) exists for the 3-oxo analogues [77]. The presence of the 17-oxo functionality in these compounds seems to be extremely important for their potency [78]. Some indication of the stereospecificity in the inhibition of aromatase of the isomers of (80) has been forthcoming although it is not conclusive [79].



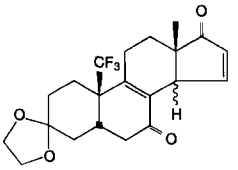
(76) $R^1 = CH_2OCOMe$, $R^2 = O$, X = H
(77) R^1 = Me, $R^2 = O$, X = α-Br
(78) R^1 = Me, $R^2 = O$, X = β-Br
(79) $R^1 = CHF_2$, $R^2 = O$, X = H
(80) R^1 = CH(OH)C CH, $R^2 = O$, X = H

(81) $R^1 = CH_2OH$, R^2 = β-OH, H , X = H
(82) $R^1 = CH_2OCOMe$, R^2 = β-OH, H , X = H
(83) $R^1 = CH_2OH$, R^2 = β-OCOMe, H , X = H
(84) $R^1 = CHF_2$, $R^2 = O$, X = H
(85) $R^1 = CH_2OCOMe$, R^2 = β-OCOMe, H , X = H

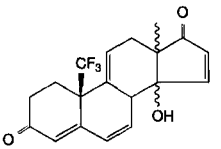
10β-Trifluoroacetyl and 10β-allyl analogues of androstenedione have been studied for their potential inhibition of aromatase [80]. The 10β-trifluoroacetylandrostenedione was not a substrate for the enzyme although it did act as a weak competitive inhibitor (K_i = 1700 nM) which is surprising considering that the corresponding methyl ketone was shown to be a rather potent inhibitor (K_i = 120 nM) [81]. 10β-Allylandrostenedione was not converted to the oxirenes as expected; this suggests that inhibition of the propargyl analogue (49) may not proceed through the proposed oxirenyl intermediate as suggested [82].

Further studies with the 19-thiomethyl- and 19-azido-androstenediones revealed both to be 'slow binding' competitive reversible inhibitors of aromatase. The formation of an initial enzyme-inhibitor complex was

detectable, and this complex 'rearranged' to a tighter bound enzyme-inhibitor complex with both compounds. For 19-thiomethylandrostenedione, the $t_{1/2}$ was 756 s with an initial K_i = 2.4 nM and final K_i = 1.4 nM. Constants for the 19-azidoandrostenedione could not be determined. It is suggested that 19-azidoandrostenedione binds *via* the primary nitrogen atom in a bent $-CH_2-\underline{N}^{-}-N^{+}{}_2$ system [83].

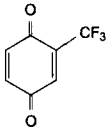


(86) (13α,14α)
(87) (13β,14β)

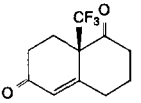


(88) (13α,14β)
(89) (13α,14β)

Primarily to exercise a synthetic methodology, some interesting structures have been forthcoming that place a 19-trifluoromethyl substituent at the angular position (86) and (87) [84]. A second group with a somewhat more practical approach achieved the synthesis of (88) and (89) *via* the Wieland-Miescher ketone (91) using (90) in the key step [85].



(90)

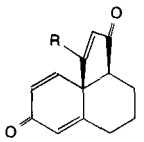


(91)

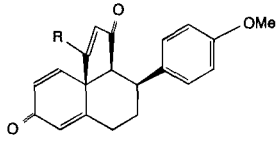
PARTIAL STEROID STRUCTURES AS INHIBITORS OF AROMATASE

There has been some effort in developing 'partial' steroids as inhibitors of aromatase. This presents a particular challenge to the medicinal chemist since they have to (a) maintain the tight binding achieved by some full steroid structures and (b) develop appropriate synthetic methodology. This approach has merit because appropriately designed inhibitors should have (a) a good chance of high selectivity towards the enzyme target (b) reduced potential for interception by metabolic enzymes *in vivo* and (c) a lower likelihood of being hormonal. Joint studies by ICI Pharmaceuticals (now Zeneca) and Whiting have developed a synthetic approach to tricyclospirodienones that mimic the A/B ring system and provide some recognition for the 17-oxo/17β-hydroxy position of the natural substrates [86]. Simple analogues (92) and (93), acting as the methodology specimen targets were

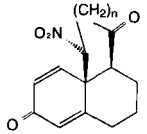
synthesized while further substituted analogues (94) to (96) were the probable final target molecules; compounds (94) to (96) are recorded as having far from ideal inhibitory action towards aromatase at micromolar levels. This was probably due to inappropriate substitution at the 19-position, the alkyl or aryl groups being somewhat large for the active site to tolerate. Later workers [87] introduced a nitro function at the equivalent '19-position' in (97) and (98) by spiroannulation of the precursor tetralins (99) and (100), respectively (no data). The paper does not disclose whether these nitro compounds were reduced or elaborated further.



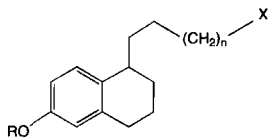
(92) R = Ph
(93) R = Pr



(94) R = Ph
(95) R = n-pentyl
(96) R = Pr



(97) n = 0
(98) n = 1



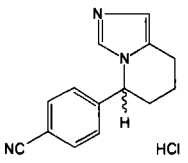
(99) R = H, X = NO_2, n = 1
(100) R = H, X = NO_2, n = 2

NON-STEROIDAL INHIBITORS OF AROMATASE

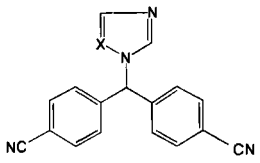
SUBSTITUTED FIVE-MEMBERED RING NITROGEN HETEROCYCLES

The azoles, both imidazole and triazole derivatives, have achieved highly respectable competitive reversible inhibition of aromatase and some with very tight binding. Their mechanism of action, as with other nitrogen based systems (see later), is no longer in dispute. Co-ordination of an appropriate unsubstituted heterocyclic nitrogen with the iron of the haem moiety of aromatase and occupancy of available active site space is well proven by spectroscopic studies and X-ray analysis of comparable enzyme systems such as $P450_{CAM}$.

Since the serendipidous discovery of CGS 16,949A (101), both industry and academia have pursued routes to enhanced inhibition and selectivity towards aromatase in these compounds along with improved pharmacological profiles in humans. Ciba Geigy have followed up (101) with related



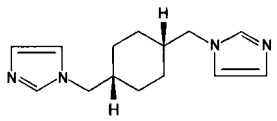
(101)



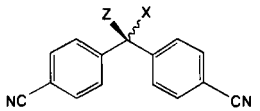
(102) X = CH . succinate
(103) X = N

systems possessing enhanced selectivity. CGS 18,320B (102), bis-(4-cyanophenyl)imidazol-1-ylmethane, as the hemi-succinate, has similar potency to (101) but reduced inhibition of the aldosterone pathways [88]. The triazole analogue (103, IC_{50} = 11.5 nM) has lower activity than CGS 18,320B (102, IC_{50} = 3.5 nM) towards aromatase but with a better profile towards progesterone and aldosterone biosynthetic pathways and with no significant effect on corticosterone production [89].

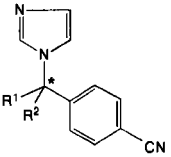
The X-ray structures of (101) and (102) have been determined and discussed along with those of an additional inhibitor (104) [90,91]; these three compounds were used to explore a SAR model with respect to the binding model of Banting *et al.* [92], using 4-hydroxyandrostenedione as a structural base for computer-aided molecular modelling (CAMM) [92]. This work agreed with the proposed model [92], but later work based on the potent (10*R*)-thiiranylandrost-4-ene-3,17-dione offered an alternative mode of binding of (101) and (102) within a putative active site for aromatase [93]. This work represents one of the best attempts to rationalize the active site



(104) CGS 14796E



(113) X = H, Z = various nitrogen heterocycles

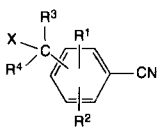


(105) $R^1 = R^2 = H$
(106) $R^1 = H$, $R^2 = Me$
(107) $R^1 = H$, $R^2 = Bu$
(108) $R^1 = H$, $R^2 = Nonyl$
(109) $R^1 = R^2 = Me$
(110) $R^1 = R^2 = -(CH_2)_4-$
(111) $R^1 = H$, R^2 = 3-pyridyl, CGS17342
(112) $R^1 = H$, R^2 = 4-cyanophenyl, CGS18320

* prochiral centre

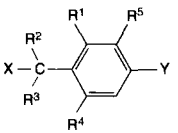
capacity of the enzyme using CAMM. For CGS 16949A, fadrazole, the model was extended to explain the enantiospecific inhibition. The (−)-enantiomer of (101) was assigned the (*S*)-configuration after X-ray analysis of its D-(−)-tartaric acid salt [94]. CAMM of the (*S*)-enantiomer confirmed the X-ray structure as the most likely conformation to be active within the active site. Binding modes of (101) and (102) were explored and rationalization of their potencies postulated [94] . This and other work [93] disclosed further structure-activity data on analogues of CGS 16,949A and CGS 18,320B. The strategy for the development of these compounds [95] was based on the well established methodology for the TXA-2 synthetase inhibitors [96]. Ring flexibility and size, plus substitution pattern on the aromatic nucleus for fadrazole was investigated. The extent of the SAR studies into analogues of (101), mainly benzonitriles, was revealed in some detail [93].

Substitution at the benzonitrile methylene led to (105) to (112) [93], and to (113), the nitrogen-containing moiety. Because of the nature of the substitution pattern on (113), the methine position is prone to biochemical oxidation and therefore fluoro substituted analogues were also synthesized and evaluated. Ciba Geigy's strategy led to CGS 47,465, CGS 45,688 and CGS 20,267 as the next generation compounds in the fadrazole programme [93]. Other structures investigated for inhibitory activity against aromatase include (114) [97] , (115) [98] and (116) [99]. CGS 20,267, the 1,2,4-triazole analogue of (102), was found to be an extremely potent and selective inhibitor of aromatase *in vivo* in humans.



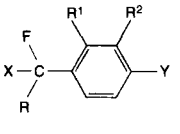
(114)

X = tetrazolyl,
R^1,R^2 = aryl, cycloalkyl, thioalkyl etc.,
R^3,R^4 = haloaryl, heterocyclic etc.,

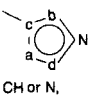


(115)

X = tetrazolyl, Y = CN or halogen etc.
R^1,R^2 = aryl, cycloalkyl, thioalkyl etc.,
R^3,R^4 = haloaryl, heterocyclic etc.,
R^5 = halogen etc.



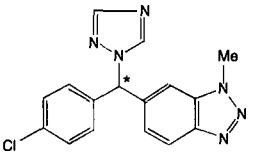
(116) X =



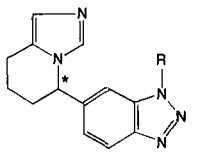
a,b,c,d either CH or N,
R^1, R^2, R^3, R^4 = as for (115)

Phase 1 trials confirmed a striking suppression of oestradiol, oestrone and oestrone sulphate levels soon after administration of the drug without compromising gluco- or mineralo-corticoid secretion [100]. The Janssen company has produced potent competitive reversible inhibitors of aromatase, R 76,713, the (+/−)-racemate (117, IC_{50} = 2.6 nM). This racemate has been resolved and the (−)-R 83,839 and the (+)-R 83,842 enantiomers were found to be effective the (+)-enantiomer being approximately 1.9 times

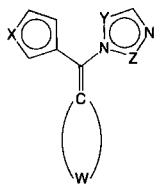
more active; R 76,713 (K_i = 1.3 nM), R 83,842 (K_i = 0.8 nM) and R 83,839 (K_i = 18 nM). Differential activities of the above inhibitors towards 17,20-lyase, 11β-, 7α- and 21-hydroxylase standard preparations have been recorded. With no effect against 7α- and 21-hydroxylases at 10μM, R 76,713 has a slight effect on 11β-hydroxylase which originates with R83,839. However,the observed 17,20-lyase inhibition is derived from R83,842 at $\geq$ 0.5μM, an IC_{50} = 1.8 μM, a concentration approximately 1300 times higher than that required to inhibit aromatase [101–103].



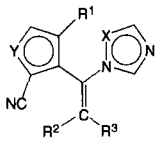
(117)



(118)
(119) R = OPr
(120) R = OCH_2CH_2OH



(121) Z =CH or N; Y = CH or N; X = CH or S etc., W = $(CH_2)_n$ etc.

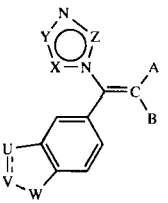


(122) X =CH or N, Y = S or CH=CH etc., R^1 = OCOR, NCOR etc., R^2, R^3 = cycloalkyl etc.

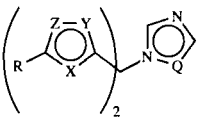
Further work on 'hybrid' structures between CGS16,949A and R76,713 has been undertaken (118) [104]. Both final products (118) and intermediates were tested and activities ranged from IC_{50} = 25 nM to IC_{50} > 1000 nM; an interesting comparison exists within these series between (119, IC_{50} = 37.5 nM) and (120, $IC_{50} \leq$ 1000 nM).

Schering AG have evaluated cycloalkylidene derivatives of type (121); one potent compound, 4-[1-cyclohexyliden-1-(1-imidazolyl)methyl]-benzonitrile, showed K_i = 0.19 nM compared with CGS 16,949A (K_i = 1.2 nM) [105]. Work was extended in a further patent with (122) (no data) [106].

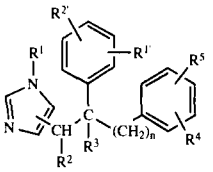
Later workers [107] included slightly extended systems (123) (no data). Deviating somewhat from the above systems, Schering AG also synthesized various bis-derivatives (124) as potential inhibitors of aromatase (no data) [108], exploring the halothiophenyl substitution pattern, Z = Y CH, X = S, R = Cl, Q = CH or N.



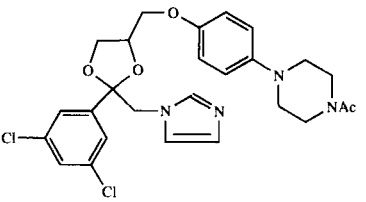
(123) X = Y = Z = CH or N; A, B = cycloalkyl etc., U = V = W = N, *N*-alkyl or CH etc.



(124) R = CN or halo etc., Z, Y = CH, CH=CH etc., X = N or S etc., Q = CH or N etc.



(125) $R^{1'}$ and $R^{2'}$= R^4 and R^5 = same or different and are Me, Et, , Halogen, OMe, NO_2 etc., R^1 = preferably H or alkyl, R^2, R^3 = H or OH etc. n = 0,1 or 2



(126)

Scientists at Orion-Yhtymä Oy have investigated compounds of type (125) [109] and have reported their aromatase and desmolase (20,21-lyase) activity for 4-(2,4-diphenylbutyl)-1H-imidazole, 4-[(2-(4-fluorophenyl)-4-phenylbutyl]-1H-imidazole, 4-[2,4-bis(4-fluorophenyl)butyl]-1H-imidazole and 4-[2-(4-cyanophenyl)-4-(4-fluorophenyl)butyl]-1H-imidazole. In a continuation of these studies, a pure enantiomer of 4-[1-(4-cyanophenyl)-4-(4-fluorophenyl)-1-butenyl]-1H-imidazole (stereochemistry not specified) was a good inhibitor of aromatase (IC_{50} = 190 nM) [110].

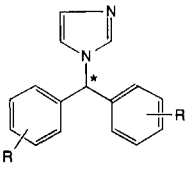
Further work by Syntex scientists on the Janssen compound, ketoconazole (126), has resolved the long-standing question about activity of the diastereoisomers toward aromatase. The (2*S*,4*S*)-diastereoisomer (IC_{50} = 3980 ± 0.24 nM) possessed the highest activity of the diastereoisomers as a competitive reversible inhibitor to aromatase, (2*S*,4*S*) ≫ (2*R*,4*R*)-(IC_{50} = 38300 ± 2.9 nM) = (2*R*,4*S*)-(IC_{50} = 39600 ± 2.5 nM) > (2*S*,4*R*)-(IC_{50} = 110400 ± 24.1 nM). In the same paper, there was an interesting discussion about selectivity towards a family of important P450 enzymes of the diastereoisomers and enantiomers of (126) [111].

Eli-Lilly workers have explored the activities of clotrimazole and fenarimol analogues of the general structures (127) and (128) and other six-membered ring systems (see later) against aromatase . As long suspected, their SAR study concludes that compounds most likely to exhibit high

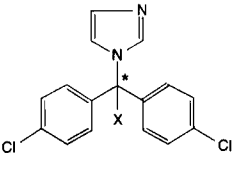
activity would have an organic moiety attached to the heterocyclic fragment at a position β to the nitrogen atom (129) [112].

A surprisingly high activity has been quoted for an atypical 1,2,4-triazole (130, IC_{50} = 0.03 nM) in a patent application by Yamanouchi Pharmaceuticals. This compound was one of seventy-five of general structure (131). The nitrogen in the triazole fragment β to the hydrophobic organic substituent R^1-Ph-N-CH_2Ph-R^2 is not often found adjacent to nitrogen in active compounds [113].

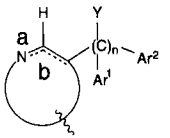
Organon recently reported that an imidazole-based aromatase inhibitor, (3αR)-*trans*-1-[(3α-ethyl-9-ethylthio-2,3,3α,4,5,6-hexahydro-1H-phenalen-2-yl)methyl]-1H-imidazole hydrochloride, displayed high potency (IC_{50} = 2.2 nM) and high selectivity, and was orally active in rats and beagles [114].



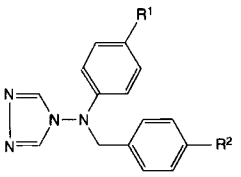
(127)



(128) X = various heterocycles

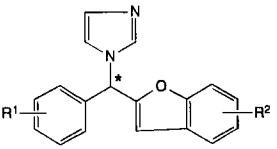


(129) Y = H, F, Cl, CH_3 or heterocycle
Ar = aromatic ring with electron withdrawing p-substituent, n = 1,3



(130) R^1 = NO_2, R^2 = Br

(131)

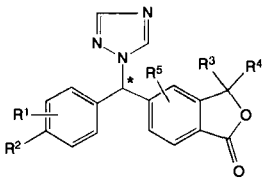


(132) R^1 = H, Me, OMe, halogen etc.
R^2 = H, Me, halogen etc.

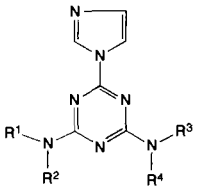
Originally synthesized as antifungal agents, a new class of aromatase inhibitor, substituted 1-[(benzofuran-2-yl)phenylmethyl]imidazoles (132) and have been shown to be active at IC_{50} < 10 nM and active *in vivo* using pregnant mare serum gonadotrophin-stimulated rats [115].

Some isobenzofuranones have also been synthesized and tested as potential inhibitors of aromatase by Glaxo [116]. Of general structure (133), 5-[(2,6-difluoro-4-nitrophenyl)-1,2,4-triazol-1-ylmethyl]-3,3-dimethyl-3H-isobenzofuran-1-one was found to be the most active (IC_{50} = 15 nM); it has been resolved (no data). Its analogues were selective and potent, submicromolar inhibitors of aromatase.

A slightly unconventional structural type has been patented by Zenyaku Kogyo Kabushiki Kaisha [117]; these s-triazine derivatives (134) are selective and potent.

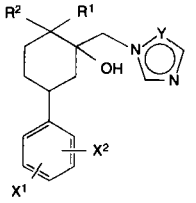


(133) R^1 = H or one or more halogen atoms
R^2 = CN or NO_2

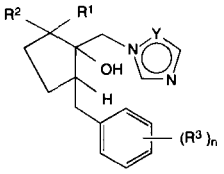


(134) R^1 to R^4 = lower alkyl or phenyl with or without halogen substitution

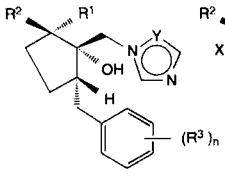
Kureha have investigated the activity of substituted cycloalkanols and related systems (135) and (136) against aromatase [118-120]. Cyclopen-



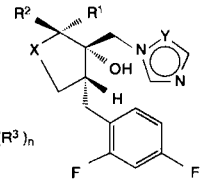
(135) R^1 and R^2 = alkyl
X^1 and X^2 = halogen
Y = CH or N



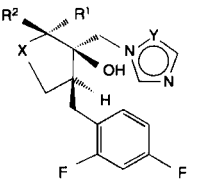
(136) R^1 and R^2 = alkyl
R^3 = various halogens, n = 1 or 2
Y = CH or N



(137) R^1 = R^2 = Me
R^3 = 4-Cl
n = 1, Y = CH



(138) R^1 = R^2 = Me
X = O
Y = CH

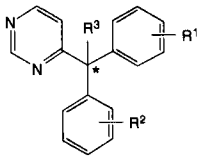


(139) R^1 = R^2 = Me
X = O
Y = CH

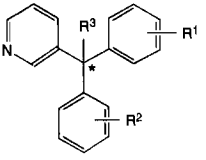
tanols (136) are shown to inhibit at $IC_{50} < 2 \times 10^{-5}$ nM. The most potent compound, racemic (137) had an IC_{50} = 300 nM with X = CH [120] while a mixture of type (138) and (139) had an IC_{50} = 270 nM [120].

SUBSTITUTED SIX-MEMBERED RING NITROGEN HETEROCYCLES

As with the imidazole and triazole class inhibitors of aromatase general SAR studies have confirmed previous deductions about requirements (see (131) and associated text), although in contrast some γ-nitrogen substitution patterns have shown fair activity.



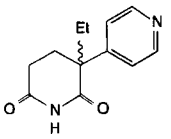
(140) R^1/R^2 = H, OH, Cl, Me or OMe
R^3 = H or OH



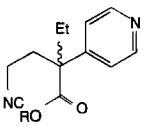
(141) $R^1 = R^2$ = halogen
R^3 = H or OH

Scientists at Eli Lilly, as part of the programme that produced the imidazoles (127) and (128), have studied (140) and (141) which have produced comparable activity [112].

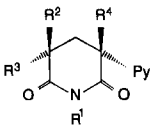
Further work on rogletimide (pyridoglutethimide, PG, 142), the 3-(4-pyridyl) analogue of aminoglutethimide (AG), has been done. Joint studies between the Cancer Research Campaign (Sutton) and Mann and co-workers (University of Reading) led to an improved synthesis of (143) and resolution of these enantiomeric intermediate cyanoesters and hence to enantiomers of (142). The inhibitory activity was found to be primarily in the (*R*)-(+)-pyridoglutethimide (IC_{50} = 10,000 nM), the (*S*)-(−)-enantiomer was 20-fold less inhibitory [121] towards aromatase. X-Ray analysis of the (*R*)-(+)-form of PG and its 3-n-butyl analogue (145) revealed a psuedo-axial orientation of the 3-(4-pyridyl) substituent in (146) and (147) [122]. A



(142)



(143)

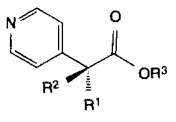


(144) $R^1 = R^2 = R^3$ = H, R^4 = Et
(145) $R^1 = R^2 = R^3$ = H, R^4 = n-Bu
(146) R^1 = H, R^2 = Me R^3 = H R^4 = Et
(147) R^1 = H, R^2 = H R^3 = Me R^4 = Et

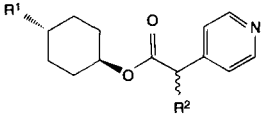
conformational flexibility study of the substituents on the glutarimide ring of (*R*)-(144) using CAMM with the MM2 force field [123] produced local minima for the ethyl side-chain of (*R*)-(144) but a more flexible situation was observed with (145). A psuedo-equatorial conformation of (*R*)-(144) was accessible although energy differences were not quoted. The rationalization of the SAR of these compounds was extended by overlaying (*R*)-(147), (148) and other *N*-alkyl derivatives with co-ordinates of androstenedione and exploring 'successful' binding modes. The psuedo-axial conformation of (*R*)-(144) seemed the most appropriate, with recognition of the A-ring of androstenedione by the glutarimide ring of the inhibitor, the carbonyl at the 6-position mimicking the 2-oxo functionality on the steroid. The psuedo-equatorial 3-(4-pyridyl) conformation of (*R*)-(144) in the same model was shown to be inappropriate considering previous SAR studies [92]. Alternative binding modes for (145) included its (*S*)-enantiomer with a psuedo-axial 3-(4-pyridyl) substituent; this alternative binding mode helped rationalise activities observed with an analogous *N*-alkyl PG series.

The above study had assumed that the solid-state conformations were appropriate in solution; an extensive high-field NMR analysis of (144), 5-methylated analogues (146) and (147), were carried out for verification of this model. The psuedo-axial conformation of (*R*)-(144) was confirmed in $CDCl_3$ solution. Further work on 5-alkyl diastereoisomers proved interesting. A 3,5-dioctyl analogue of (145) had less than expected activity. This was thought to be due to a competition of the alkyl chains for the hydrophobic binding site within the active site of aromatase [124].

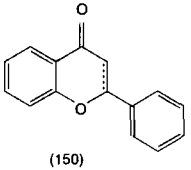
This work also helped explain SAR within esters of 4-pyridylacetic acid (148), and presented a rationalization of their differential activities against aromatase and rat 17,20-lyase [125] a range of activities between an IC_{50} = 89 nM and IC_{50} = 320 nM against aromatase and 280 to 131,000 nM against rat 17,20-lyase were noted. High activity against aromatase seemed



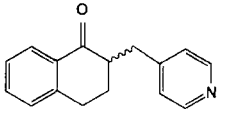
(148) R^1 to R^3 = alkyl



(149) R^1 to R^2 = alkyl



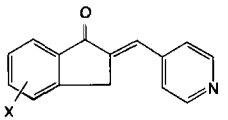
(150)



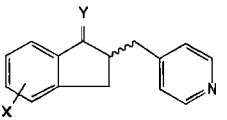
(151)

to be associated with cyclic substituents of high hydrophobicity. These studies were extended by CAMM and X-ray analysis of (149) and other analogues and clarified further structural requirements for activity between aromatase and rat 17,20-lyase [126].

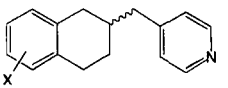
Hartmann and co-workers, have pursued modifications of the flavone system (150) to arrive at 2-substituted tetralones. Activity against aromatase was highest in (151, IC_{50} = 4300 nM) which was 8.6 times more potent than AG [127]. The nature of inhibition of (151) was explored further in (152) to (154). Variation of X from methoxy to hydroxy at various positions led to optimal activity in (154, X = 6-OMe; IC_{50} = 230 nM, K_i = 4 ± 2 nM) with no significant inhibition of 20,22-lyase. The *in vivo* activity of (154, X = 6-OMe) and (153, X = 5-OMe, Y = H,H; IC_{50} = 240 nM, K_i = 5 ± 2 nM) was disappointing, both having lower activity than AG in pregnant mare serum gonadotrophin-induced rats; *in vivo* metabolism was thought to be the problem as these compounds were demonstrated to lack oestrogenic activity [128, 129]. The hydrophobic nature of the tetralones was extended by synthesis of phenanthrenone analogues (155) to (158) which possess moderate activity but at a lower level than that of the parent system [130].



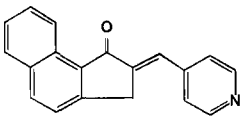
(152) X = halogen OH or O-alkyl



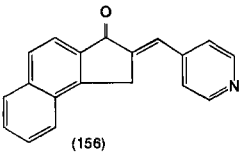
(153) X = halogen etc. Y = O, H and OH



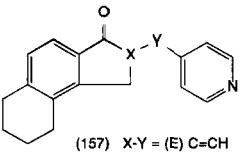
(154)



(155)



(156)



(157) X-Y = (E) C=CH
(158) X-Y = CH-CH_2

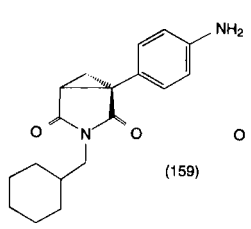
Coon and co-workers described some fundamental studies into the nature of the binding of various simple substituted pyridines to aromatase using visible spectroscopy (*ca.* 450 nm). Pyridine (K_i = 320000 nM), 3-phenylpyridine (K_i = 1480 nM) and 4-phenylpyridine (K_i = 360 nM) all displayed typical type II difference spectra demonstrating ligation to the iron of the haem moiety of aromatase, like the respective benzoylpyridines (see [1] for more detail of type of P450 spectra). Biphenyl, benzophenone and 2-benzoylpyridine all exhibit type I spectra, whereas 2-phenylpyridine had a

complex spectrum, a mixture of type I and II, not previously seen. The authors suggested that the plane of the steroid substrates within the active site of aromatase must be orthogonal to that of the haem rather than parallel, and that a good inhibitor must possess both a ligating moiety and one that has recognition of the hydrophobic region of the steroid in the correct arrangement [131].

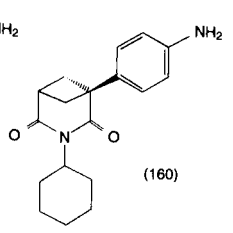
ANALOGUES OF AMINOGLUTETHIMIDE

The pursuit of enhanced activity and pharmacological profiles of analogues of AG continues. Most of the research was concerned with determination and 'fine-tuning' conformational states of the aminophenyl substituent in AG analogues along with some fundamental structure-activity work on AG. The activity of the pyridyl derivatives of AG and its analogues has been covered in earlier pages.

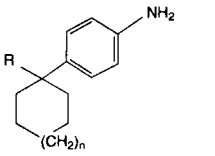
Early investigations suggested a psuedo-equatorial conformation for the 3-(4-aminophenyl) substituent in AG and the 3-ethyl group to have a direct affect on the rotational preferences of this aromatic substituent [132]. The solving of the X-ray structure of (±)-AG revealed, unusually, a preference for a psuedo-axial 3-(4-aminophenyl) substituent [133]. Detailed NMR studies, both for PG and AG in solution, confirmed this preference with a $\Delta G \approx 1.5$ kcal mol^{-1} determined for PG and assumed for AG [126]. The influence of the 3-alkyl group on the conformation of AG was investigated further by several groups. A continuation of the studies [134,135] into bicyclo AG analogues which included further 1-(4-aminophenyl)-3-azabicyclo[3.1.0]hexane and [3.1.1]heptane-2,4-diones revealed that the activity, as



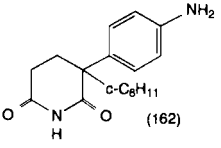
(159)



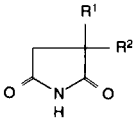
(160)



(161) R = alkyl
n = 1 or 2



(162)



(163) R^1 = alkyl or carboxyl
R^2 = prop-2-ynyl or prop-2-enyl

with AG itself, resides in the (*R*)-(+)-enantiomers of these systems. Potent and selective inhibitors emerged with (159, IC_{50} = 5 nM) and (160, IC_{50} = 13 nM) for the (+)-enantiomer.

Concurrent studies into the importance of the glutarimide ring function have proved interesting. Hartmann and Batzl found that *in vitro* potency could be maintained in simple cycloalkyl analogues of AG [136]. When R = H and n = 0, 1 or 2, aromatase inhibitory action remains as it does with (161). When R = Me, Et or n-Pr, n = 1, activity was up to approximately three times that of AG; *in vivo* activity, as compared with AG, was poorer. Returning to the glutarimides with this work in mind, these researchers developed a promising candidate compound, the (+)-enantiomer (162, IC_{50} = 150 nM) with increased potency over AG and good selectivity with respect to desmolase [137]. This compound was the subject of a theoretical analysis to determine the influence of the cyclohexyl moiety on the conformational preferences of the 3-(4-aminophenyl) group [138]. Racemic (162, K_i = 3.9 nM) was compared with the (+)-enantiomer (K_i = 2.0 nM) both of which were more potent binders to aromatase than AG. The (+)-enantiomer (162) of has been found to have no CNS depressive action and little supression of cortico- or mineralo-corticosteroid production [139], a comparable profile to R 76,713 and CGS 16,949A.

Thia analogues of AG have been investigated as potential inhibitors of AG. Somewhat unusually, mixtures of the 3-ethyl-[3-(4-thiophenyl)]- and 3-ethyl-[3-(2-thiophenyl)]-piperidine-2,6-diones, 3-ethyl-[3-(4-methylthiophenyl)]- and 3-ethyl-[3-(2-methylthiophenyl)]-piperidine-2,6-diones have been used to determine activities which were very low $IC_{50} \geq 174000$ nM against aromatase [140].

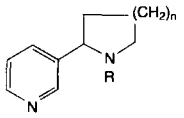
Nicholls and co-workers have continued studies into the pyrrolidine-2,5-diones as reduced ring analogues of AG [141, 142]. The synthesis of 3-alkyl-3-(prop-2-ynyl) and 3-alkyl-3-(prop-2-enyl)pyrrolidine-2,5-diones (163) has been described (no data) [142]. 1-Pentyl, 1-hexyl- and 1-heptyl-3-(4-aminophenyl)pyrrolidine-2,5-diones have been demonstrated to be potent selective inhibitors of aromatase both *in vitro* and *in vivo*, with better CNS profiles than AG along with less tendency to cause blood dyscrasias [141].

NON-STEROIDAL NATURAL PRODUCTS AND THEIR DERIVATIVES

Both routine random screening and 'informed' screening of natural products has led to the discovery of diverse structural types with aromatase inhibitory action. Following observations that cigarette smoke influenced the outcome of a sensitive assay for aromatase activity [143], a series of

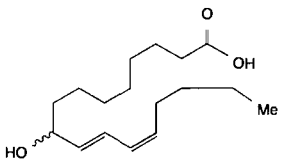
N-acylnornicotines (164) and *N*-acylanabasines (165) were investigated for their activity.

Systematic synthesis of analogues of (164) and (165) by increasing chain length of the *N*-acyl substituent revealed a dependency of activity that seemed to peak at C_{11}. These compounds were shown to have low toxicity in rats, LD_{50} = 367 mg kg^{-1} body weight for *N*-n-octanoylnornicotine, a naturally occurring basic component of the tobacco smoke that predominates. Evaluation of binding constants of these *N*-acyl systems [144] showed *N*-n-octanoylnornicotine to have a K_i = 650 nM comparable with AG; *N*-n-decanoyl- (K_i = 860 nM) and *N*-(4-hydroxyundecanoyl)-nornicotine (K_i = 240 nM) were also good competitive reversible inhibitors of aromatase [144].



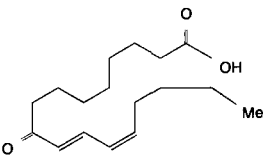
(164) n = 1, R = H or acyl
(165) n = 2, R = H or acyl

Natural extracts of *Urtica dioica* have been used to treat benign prostatic hyperplasia [145]. (10*E*,12*Z*)-9-Hydroxy-10,12-octadecanoic acid (166) was identified as the main component of the lipid fraction and the active principle [146]. Tomato lipoxygenase was used to synthesize (9*S*)-(166) which was also active against aromatase. Comparison of stereomodels of (166) and (167) with the natural substrates of aromatase suggested a structural similarity and hence the ketone (168) was also synthesized and was found to have a ten-fold increase in activity over (166) against aromatase. Further derivatives of (168) have been patented [147, 148] as have other related fatty acid esters [149].



(166)
(167)

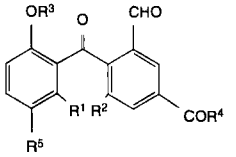
(9S) - (166)

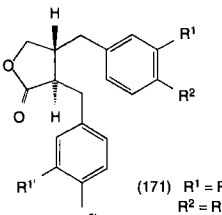


(168)

Workers at Takeda have isolated and characterized a microbial product TAN-931, obtained from *Penicillium funiculosum* N° 8974, as the carboxylic acid (169). Structure-activity studies on TAN-931 has identified the

3-formyl, 2′- and 6′-hydroxyl groups as important for activity. TAN-931 (IC_{50} = 17000 nM) was considerably modified by variation of R^1 to R^5 (170); 4-(2,6-dihydroxybenzoyl)-3-formyl-5-methoxy-*N*,*N*-dimethylbenzamide (IC_{50} = 15000 nM) was found to be a better inhibitor of aromatase *in vivo* in rats [150]. Both lignans and isoflavinoids are responsible for anti-oestrogenic effects in mammals [151]. Screening of the more common compounds in these classes has led to a mammalian lignan, enterolactone (171, IC_{50} = 14000 nM) *trans*-2,3-bis[(3-hydroxyphenyl)methyl]butyrolactone being identified, along with other examples, as a reversible competitive inhibitor of aromatase [151].





(169) R^1 to R^3 and R^5 = H, R^4 = OH
(170) R^1 = OH, OMe, NHR^6 = (R^6= alkyl) etc.
R^2 = OH, OMe, OAc etc.,
R^3 = H, Me, Ac etc.,
R^4 = OH or OMe
R^5 = H or Cl

(171) $R^1 = R^{1'}$ = OH
$R^2 = R^{2'}$ = H

CONCLUSIONS

Advances in molecular biology have had a significant impact on the understanding of the human placental and other aromatase enzymes. Comparison between sequences of known structure P450 enzymes and the aromatase primary sequence has led to a working computer model of aromatase that will aid research into the enzyme structure, function and inhibition. Further computer-aided rational design of new inhibitors, both steroidal and non-steroidal has had a high degree of success in providing the next generation of potent inhibitors, some of which, fortuitously, escape the metabolism problems of earlier analogues. Serendipity has also played a role, wide random and/or guided screening having revealed quite unusual structural types both in synthetic nitrogen-containing heterocyclic and natural non-steroidal inhibitors. Following the lead of known biological activity of herbal sources confirms the need for pharmacognosy as a discipline, as these have led to the isolation of reasonable inhibitors of aromatase with compounds whose mechanism of action have been rationalized readily by SAR approaches. Although new compounds have been discovered by a variety of approaches, the number of compounds entering clinical trials is somewhat low. This indicates the need for increased

effort. Investigations into the use of prophylactic treatment of high risk groups of women genetically predisposed to hormonal dependent breast cancer and the potential for treating female endometriosis requires medicinal chemists to continue the search for more potent highly specific inhibitors of this unique enzyme.

ACKNOWLEDGEMENTS

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5 Risperidone and related $5HT_2/D_2$ antagonists: A new type of antipsychotic agent?

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INTRODUCTION

Risperidone is a new antipsychotic with relatively pronounced effects on negative symptoms and low extrapyramidal side-effect (EPS) liability. The present overview describes the historical development (including syntheses) and pharmacological profile of risperidone and investigates to what extent its basic pharmacological properties (combined $5HT_2/D_2$ antagonism) occur among experimental and available neuroleptics. The medicinal chemistry programme that ultimately led to risperidone was initially based on the chemical structures of the neuroleptics lenperone and benperidol and on the pharmacological differences between haloperidol and pipamperone. Apart from risperidone, the programme generated a variety of pharmacologically distinct compounds, among others the neuroleptics declenperone, milenperone, pirenperone, setoperone, and ocaperidone; the antiemetic and gastrokinetic domperidone; the antihypertensive ketanserin; and the $5HT_2$ antagonists seganserin and ritanserin. In a series of benzisoxazole derivatives, risperidone was found to be different in pharmacological profile from haloperidol and comparable to pipamperone except for its higher potency for $5HT_2$ and D_2 antagonism. Combined $5HT_2/D_2$ antagonism is also frequently observed with other compounds, as evidenced by binding affinities for $5HT_2$ and D_2 receptors and ED_{50} values for antagonism of tryptamine- and apomorphine-induced behavioural effects in rats. Apart from risperidone, however, only four potent apomorphine antagonists ($ED_{50} < 0.63$ mg/kg) could be identified that display tryptamine antagonism at still lower doses: ORG-5222, tefludazine, zotepine, and olanzapine. To what extent these compounds are further comparable to risperidone is outside the scope of the present overview.

RISPERIDONE

Central dopamine D_2 antagonists with associated and predominant serotonin $5HT_2$ antagonism have been postulated as a new type of antipsychotic [1–4]. The benzisoxazole risperidone may be the prototype of this new class of antipsychotics [5–7]. Relative to conventional neuroleptics, risperidone has more pronounced effects on negative symptoms and lower EPS liability [8].

The present review describes the historical development and pharmacological profile of risperidone and investigates to what extent combined $5HT_2/D_2$ antagonism occurs among available and experimental neuroleptics. Structure-activity relationships for several chemical classes of neuroleptics are discussed on the basis of published binding affinities for $5HT_2$ and D_2 receptors and ED_{50} values for antagonism of tryptamine- and apomorphine-induced behavioural effects in rats. As a short introduction, the next two sections give an outline of the clinical problem of schizophrenia and the various options for therapeutic treatment.

THE PUZZLE OF SCHIZOPHRENIA

Schizophrenia has been described by Bleuler [9] as the splitting of the mind from reality. According to Kay [10], schizophrenia indeed represents a tragic split on many levels. It is a severe mental disorder, characterized by sensory, cognitive and motor disturbances. It significantly impairs the individual's capacity to function and radically transforms the patient's relationship to the environment. Schizophrenic patients often experience their environment with a strange feeling of unfamiliarity. They might see objects as well as persons change their colour, shape and brightness from minute to minute. They are affected by strange thoughts and ideas and uncontrolled feelings of anger, fear, suspicion and anxiety. During the florid period of schizophrenia, even simple activities, such as greeting a friend and sitting quietly at a dinner table, are disrupted. Probably the best-known feature of schizophrenia are the hallucinations, that is, sensory experiences or perceptions without corresponding external stimuli (for example, hearing voices or seeing persons who are actually not present). Schizophrenic patients may feel severely depressed as indicated by the high incidence of suicide [11, 12]. The disease often starts during the years of adolescence that determine the transition from child to adult and that are so decisive for the development of the individual's personality.

About one-third of schizophrenic patients recover successfully, one-third partially or temporarily, and the remaining one-third remain essentially unchanged [13]. Even for treatment-responsive patients, schizophrenia is regarded as a life-long illness for which there is no cure; like diabetes, it requires ongoing drug therapy to protect against relapse [14]. As the illness continues, normal interests, energy, and emotions seem to disappear, and the personality may undergo a gradual and possibly irreversible transformation. A life-time of disability may follow. In many cases, too, the family is highly affected by the emotional impact which is sometimes expressed as a misguided sense of guilt for the condition of their child and in other cases as

feelings of impotence as the patient's life is deformed by the psychosis. The treating clinician often experiences similar feelings of frustration and confusion.

The description of schizophrenia by Eugene Bleuler [9] and Emil Kraepelin [15] in the early part of the century led to an intensive search for the nature of this disease, its origin, its causes, its course and outcome, and most importantly, its treatment. Although our understanding of schizophrenia has advanced with painful slowness, the field has experienced remarkable growth in biological approaches to treatment and assessment. For instance, neuroleptic drugs, introduced in the 1950s, have become the drugs of choice for the treatment of schizophrenia. The therapeutic success of dopamine antagonists in improving psychotic symptoms has led to the dopamine hypothesis of schizophrenia, which relates the disorder to excessive dopaminergic neurotransmission in the brain. Neuroradiological and other methods of brain imaging have further advanced our knowledge of brain structure and chemistry. Data from these techniques have helped to characterize the impairments in brain anatomy and functioning of schizophrenic patients.

There are no objective psychological, physiological, or biochemical criteria for the diagnosis of schizophrenia. In milder cases, schizophrenia is difficult to differentiate from less severe emotional disorders. Even at post-mortem analysis, there is no distinguishing pathology by which the clinical diagnosis may be corroborated. It is sometimes argued that the clinical manifestations represent a multiple number of disorders of differing pathogenesis. Consequently, many theories have been propounded about its aetiology, ranging from purely psychosocial factors to a multiplicity of biological abnormalities. In recent years, several new diagnostic systems have been developed and older systems updated. These now widely accepted diagnostic criteria have immensely improved the reliability of the diagnosis but not necessarily its validity. The validity of a diagnosis depends on showing correspondences between diagnostic judgements and independent sources of confirmation. Schizophrenia, in fact, is known for its variability. The disorder takes on myriad forms, characterized by multiple symptoms that, in an untreated state, may fluctuate in intensity. As already mentioned, the clinical symptoms may be highly similar to those seen in other mental disorders. For instance, schizophrenics often show cardinal symptoms of a depressive illness. In addition to sad mood and increased suicide risk, they frequently exhibit lack of interest in their environment, loss of energy, demotivation, and disturbed sleep. It is obvious therefore that different diagnostic systems will select different samples of patients, which makes comparison between literature studies sometimes extremely difficult.

The diversity in symptoms, history, and course of illness raises serious doubt about the efficacy and even the logic behind our principal treatment, the neuroleptic drug. The use of dopamine receptor blockers, such as chlorpromazine and haloperidol, best fits a unitary or single-factor model of schizophrenia, one characterized by excessive central dopaminergic neurotransmission. However, the limitation of these drugs, and hence of the dopamine model as well, is revealed by their lack of efficacy for a large number of schizophrenic patients and for many symptoms. These observations, of course, do not contradict the beneficial role of dopamine antagonists in schizophrenia, which suggests that dopaminergic activity is in some way involved in the symptomatic expression of the illness.

The need for a revised and expanded view of schizophrenia has become increasingly evident. One of the major advances in recent times has come from Strauss and co-workers [16, 17], who differentiated at least two separate symptom profiles in schizophrenia. A positive or productive symptom was described as an abnormal feature, such as hallucinations, delusions, and disorganised thinking, that is present in the mental status. A negative or deficit symptom was defined as the absence of normal functions and, thus, includes features such as blunted affect, emotional withdrawal, and cognitive deficiency. The distinction between positive (florid) and negative (or defect) symptoms was originally proposed by Hughlings-Jackson [18] and resembles Bleuler's distinction between fundamental (negative) and accessory (positive) symptoms. Crow [19, 20] hypothesized that the positive-negative distinction may embody two different types of schizophrenia. Positive symptoms would prevail in the acute stage, probably corresponding to excessive dopaminergic neurotransmission in the brain. Patients of this type were thus characterized by a neurochemical abnormality that, logically, would be expected to respond well to neuroleptics and lead ultimately to a good outcome. Negative symptoms, by contrast, were thought to prevail mainly in the chronic stage and to signify a structural brain abnormality, such as indicated by enlarged ventricles in the brain, hence cell loss and cortical atrophy. These patients were expected to be neuroleptic-resistant and to carry a poor prognosis.

Whereas Crow's model viewed the positive and negative symptoms as unrelated facets of schizophrenia that may reflect separate pathological processes, Nancy Andreasen in Iowa described positive and negative symptoms as opposing features which characterize different subtypes [21, 22]. More recently, William Carpenter argued that negative symptoms do not form a stable and unitary construct, and therefore should be replaced by the concept of a deficit syndrome [23, 24].

If the positive-negative distinction is validated by empirical research, it

would constitute a milestone in clarifying systematic differences in the aetiology, pharmacotherapy, and prognosis of schizophrenia. Progress in schizophrenia research depends very much on identifying distinct subgroups of dimensions rather than on classifying together all who meet particular criteria for the diagnosis. Progress in treatment likewise demands a syndrome-specific approach rather than a general, 'one-size-fits-all' prescription. In seeking a solution to the schizophrenia puzzle, therefore, we must recognise it to be a puzzle, that is, a composite of numerous pieces that will make sense only once the interrelationships are understood.

PHARMACOLOGICAL TREATMENT OF SCHIZOPHRENIA

Pharmacological treatment of schizophrenia has been dominated by antipsychotic agents from two structural classes, the tricyclics represented by chlorpromazine and the butyrophenones with the prototype haloperidol. These drugs produced a revolution in the treatment of schizophrenia because of their efficacy in preventing the positive symptoms of the disease, such as hallucinations and thought disorders. However, these 'classical antipsychotics' have a few drawbacks; they have little or no effect on negative symptoms and they produce extrapyramidal side-effects (EPS) after overdose.

In an attempt to control the drawbacks of EPS and lack of effect on negative symptoms, various approaches have been proposed for the development of an improved type of antipsychotic, so-called 'atypical neuroleptics', that would have an extended therapeutic profile and reduced side-effect liability. The discovery of the various biogenic amines, their functional role as neurotransmitters in the brain, and the continuously expanding series of specific target receptors has stimulated the exploration of several hypotheses on the involvement of endogenous monoamines and neuropeptides in the pathophysiology of schizophrenia. Based on these hypotheses, various approaches for the development of an improved type of antipsychotic have been proposed. Some of these approaches are directed to interaction with non-dopaminergic systems, for example, with the noradrenergic system [25, 26], endorphin [27], phencyclidine and sigma receptors [28, 29], serotonin $5HT_{1C}$ receptors [2, 30, 31], serotonin $5HT_3$ receptors [32], serotonin $5HT_{1A}$ receptors [2], serotonin $5HT_6$ and $5HT_7$ receptors [33], CCK [34–36], glutamate [37, 38], GABA [39], and neurotensin [40, 41].

Other approaches still consider specific central dopamine antagonism to be crucial and aim for activity which specifically blocks one of these various dopamine receptor subtypes (D_1, D_2, D_3, D_4, or D_5) [42–47],

to dopamine receptors in particular brain regions (mesolimbic vs. striatal dopamine receptors) [48–50], or to dopamine receptors on different parts of the neuron (pre- vs. post-synaptic) [51, 52]. Inhibition of dopamine release by dopamine autoreceptor agonists may result in the same therapeutic effects as postsynaptic receptor blockers but without inducing EPS [53–57].

Another approach is followed by those who attempt to combine central dopamine antagonism with interactions with other neurotransmitter systems that would extend the therapeutic value and/or reduce the side-effects, for example, muscarinic receptors [58] and $5HT_{2A}$ receptors [1–4, 59]. Whereas most approaches have not yet resulted in a clinically useful antipsychotic, the latter approach has recently resulted in the introduction of risperidone. The chemistry and pharmacology of combined serotonin $5HT_{2A}$ and dopamine D_2 antagonists and in particular of risperidone will be further discussed below.

METHODS

The most important *in vitro* and *in vivo* methods that have been used to quantify the $5HT_2$ and D_2 antagonistic activity of the various compounds within the context of the present overview are summarized below.

IN VITRO RECEPTOR BINDING STUDIES

If not otherwise indicated, the receptor binding data were obtained from Leysen *et al.* [60]. Although some binding affinities were derived from other sources, they were all measured according to the same standard procedures [61], with the exception of most of the $5HT_2$ receptor-binding affinities listed in Table 5.1 that were measured using [^{3}H]-spiperone instead of [^{3}H]-ketanserin as a radioligand [62].

Binding affinity for $5HT_2$ receptors

Binding to serotonin $5HT_2$ receptors was studied with membranes of the heavy and light mitochondrial and microsomal fraction of rat frontal cortex homogenates (assay conditions: 10 mg tissue in 4.4 ml Tris-HCl buffer, pH 7.6, 15 min incubation at 37°C). The radioligand was [^{3}H]-ketanserin (1 nM) and specific binding was determined in the presence of methysergide (1 μM).

Binding affinity for D_2 receptors

Binding to dopamine D_2 receptors was studied using the total particulate membrane fraction of rat striatum homogenates (assay conditions: 12.5 mg tissue in 1.1 ml Tris-HCl buffer and salts, pH 7.6, 10 min incubation at 37°C). The radioligand was [^{3}H]-haloperidol (2 nM) and specific binding was determined in the presence of (+)-butaclamol (2 μM).

INTERACTION STUDIES IN ANIMALS

Apomorphine and tryptamine antagonism in rats

Apomorphine- and tryptamine-antagonism were evaluated in the apomorphine-, tryptamine-, and norepinephrine-interaction test in rats (ATN test) [63]. Male Wistar rats (240-300 g) were used.

Table 5.1. BINDING AFFINITIES (K_i; nM) FOR $5HT_2$ AND D_2 RECEPTORS, ED_{50}'s (mg/kg, s.c.) FOR ANTAGONISM OF TRYPTAMINE (TRY)- AND APOMORPHINE (APO)-INDUCED BEHAVIOURAL EFFECTS IN THE ATN TEST IN RATS, AND ED_{50}'s FOR ANTAGONISM OF APOMORPHINE-INDUCED EMESIS IN DOGS.

	K_i (nM)		*ED_{50} (mg/kg, s.c.)*		*ED_{50} (mg/kg)*			
	Receptor binding		*ATN test in rats*		*Apomorphine antagonism in dogs*			
Name	*$5HT_2$*	*D_2*	*TRY*	*APO*	*1 h, s.c.*	*1 h, p.o.*	*4 h, p.o.*	*16 h, p.o.*
Benperidol	6.6[a]	0.35[a]	0.29	0.0093	0.00059	0.0047	0.00099	0.046
Lenperone	NT[a]	4.6[a]	0.39	0.085	0.13	0.16	0.29	1.6
Declenperone	2.4[a]	9.3[a]	2.4	0.44	0.0088	0.13	0.28	2.4
Milenperone	9.2[a]	3.9[a]	0.51	0.025	0.0070	0.020	0.016	0.35
R034315	130[a]	3.7[a]	>40	>40	0.020	NT	0.057	NT
Domperidone	330[a]	0.90[a]	>40	>40	0.0077	0.031	0.040	0.20
Ketanserin	2.1[a]	220[a]	2.4	>40	NT	NT	39	NT
Pirenperone	2.1[a]	16[a]	0.11	0.098	0.040	0.091	0.48	4.7 (8 h)
Setoperone	2.3[a]	24[a]	0.11	0.22	0.011	0.022	0.031	0.12
Pimozide	33[a]	1.2[a]	9.3	0.049	0.025	0.085	0.016	0.043
Seganserin	2.1[a]	125[a]	0.68	>80	>2.5	16	24	NT
Ritanserin	1.2[a]	23[a]	0.26	>160	NT	12	13	NT
Risperidone	0.16	3.1	0.13	0.15	0.0057	0.0071	0.0079	0.013
9OH-risperidone	0.22[b]	4.1[b]	0.22	0.39	0.0094	0.011	0.0094	0.028
Ocaperidone	0.14[c]	0.75[c]	0.074	0.021	0.0024	0.0015	0.0012	0.0024

[a] [^{3}H]-spiperone instead of [^{3}H]-ketanserin as a radioligand [62]; [b] [88]; [c] [76]; NT = not tested

Apomorphine antagonism. Apomorphine-induced agitation was scored 0, 1, 2, or 3 every 5 min over the first hour after injection of apomorphine (1.25 mg/kg, i.v.) in rats that were pretreated with test compound (usually 0.5 h earlier). The criterion for drug-induced inhibition of agitation was less than 7 times score ≥ 2 (1.7% false positives in > 5000 controls) or less than 7 times score ≥ 1 (0.35% false positives).

Tryptamine antagonism. Sixty min after the apomorphine challenge, the same rats were injected with tryptamine (40.0 mg/kg, i.v.). Tryptamine-induced bilateral clonic seizures of the forepaws were scored 0, 1, 2, or 3 over 1 min after injection of tryptamine. The criterion for drug-induced blockade was a score < 2.

Apomorphine antagonism in dogs

Apomorphine (0.31 mg/kg, s.c.)-induced emesis was assessed up to 1 h after challenge in Beagle dogs of both sexes pretreated with test compound or solvent [64]. The criterion for drug-induced protection was complete absence of emesis (0% false positives; n > 1000).

DATA ANALYSIS AND PRESENTATION

IC_{50} values obtained by graphical methods were converted to K_i values and expressed in nM. ED_{50} values were calculated by probit analysis of categorical data [65] and expressed in mg/kg.

HISTORICAL DEVELOPMENT OF RISPERIDONE

RATIONALE FOR DEVELOPING A COMBINED $5HT_2/D_2$ ANTAGONIST

Ten years after the description of the neuroleptic activity of chlorpromazine by Delay *et al.* [66], Tedeschi *et al.* [67] reported that the phenothiazine neuroleptics were potent antagonists of the behavioural effects induced by the serotonin agonist tryptamine in rats. Together with the close resemblance between the chemical structures of serotonin and the hallucinogen LSD, this was a major reason for Woolley [68] to hypothesize that schizophrenia might be related to excessive serotonin in the brain.

In 1957, Paul Janssen discovered the butyrophenone family of neuroleptics, of which haloperidol became the archetype [69, 70]. Predicted from its pharmacological profile in animals, the postulated antipsychotic action of haloperidol was soon confirmed in clinical studies [71]. Numerous

pharmacological studies were undertaken to investigate whether it is possible to predict the clinical effects of neuroleptic drugs (major tranquillisers) from animal data [72–76]. Although devoid of relevant tryptamine antagonism, the butyrophenones appeared to be very effective antipsychotics. Tryptamine antagonism thus seemed not to be necessary for antipsychotic activity and the serotonin hypothesis of schizophrenia was rapidly discarded. The phenothiazines and the butyrophenones had one feature in common; low doses of compounds originating from both chemical classes antagonized the behavioural effects evoked by the dopamine agonists apomorphine and amphetamine. Primarily on the basis of this observation, the dopamine hypothesis of schizophrenia was born; it was thought that schizophrenia was related to excessive dopaminergic activity in the brain [77].

The involvement of central serotonin in schizophrenia was re-established because clozapine and pipamperone were the only two compounds out of a large number of neuroleptics that antagonized tryptamine-induced behaviour at lower doses than apomorphine-induced behavioural effects [78]. Both compounds could also be clinically differentiated from other neuroleptics by anti-autistic, disinhibiting and resocializing effects, low EPS liability, and regulatory effects on disturbed sleep rhythms [79–84]. Moreover, it was shown that the specific $5HT_2$ antagonist ritanserin could complement neuroleptic therapy by improving effects on negative symptoms and reducing EPS [85]. This notion stimulated the search for compounds that combined tryptamine and apomorphine antagonism at lower doses than pipamperone and clozapine.

CHEMICAL AND PHARMACOLOGICAL DEVELOPMENT

This section gives a historical overview of an almost fifteen-year period in the chemical and pharmacological search for potent tryptamine/apomorphine antagonists that eventually resulted in the discovery and development of risperidone. To allow a pharmacological comparison of the various compounds, Table 5.1 lists binding affinities for $5HT_2$ and D_2 receptors (nM) and ED_{50} values (mg/kg, s.c.) for antagonism of apomorphine- and tryptamine-induced behavioural effects in rats and for antagonism of apomorphine-induced emesis in dogs (at several time intervals after s.c. or p.o. administration). The experimental procedures have already been described in the Methods section.

Soon after the introduction of haloperidol, several analogues of the butyrophenone series were reported. The chemical structures of two of them, viz., benperidol (Janssen) and lenperone (Robins), are shown in

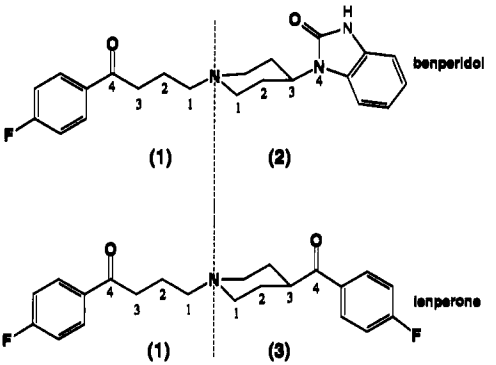
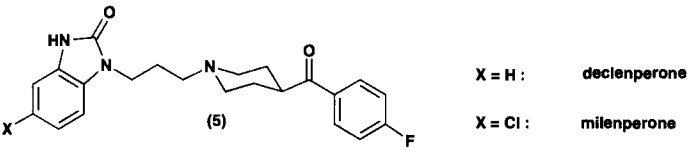


Figure 5.1. Chemical structures of benperidol and lenperone.

Figure 5.1. The butyrophenone moiety (1) is common to both structures. In lenperone, fragment (3) is almost symmetrical to the butyrophenone fragment (1): both fragments have the same distance of 4 atoms between the aromatic ring and the central nitrogen atom, the same keto function, and the same aromatic substitution. The cyclic form of the chain in fragment (3) (the piperidine ring) is the only difference between the two parts of the molecule. As both benperidol and lenperone bind to the D_2 receptor, it was hypothesized that fragment (2) has some pharmacophore similarity to fragment (3). In other words, the open form (4) (see Figure 5.2) of fragment (2) might have potential activity, just like the open form (1) of fragment (3) (that is, the butyrophenone moiety) in lenperone.

The possible combinations of the four different fragments (1), (2), (3), and (4) are shown in Figure 5.2. A chemical programme was started to evaluate the pharmacological activity profile of the unknown combinations (4)(2) and (4)(3). The synthesis of both series of compounds is illustrated in Schemes 5.1 and 5.2, respectively. Two members of each series are compared with lenperone and lenperone in Table 5.1. Declenperone and



milenperone resulted from combining fragments (4) and (3) to obtain (5). Both compounds were comparable to benperidol and lenperone: potent and centrally active dopamine and, to a less extent, serotonin antagonists;

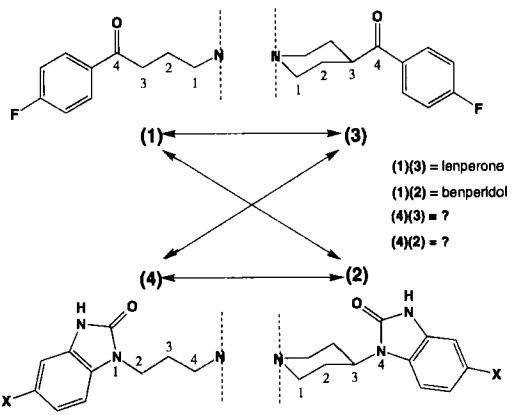
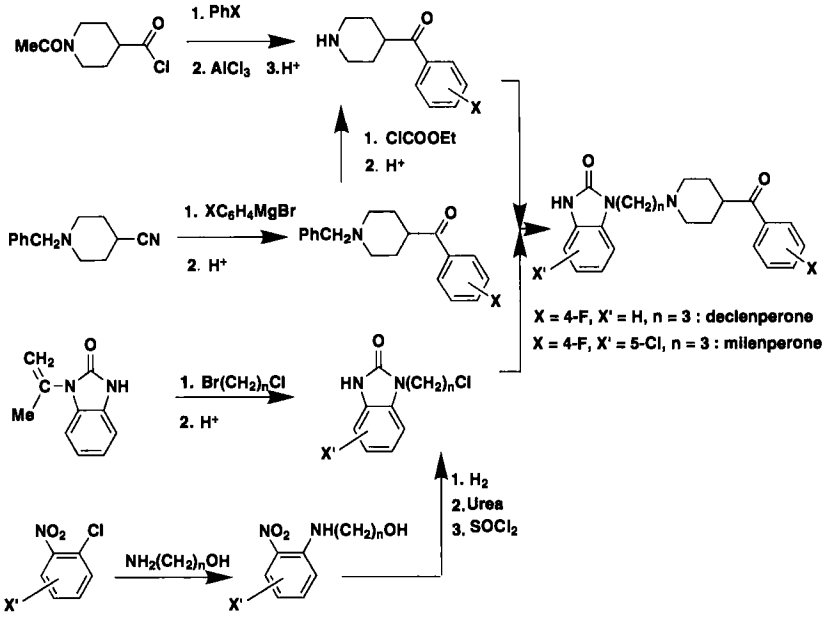
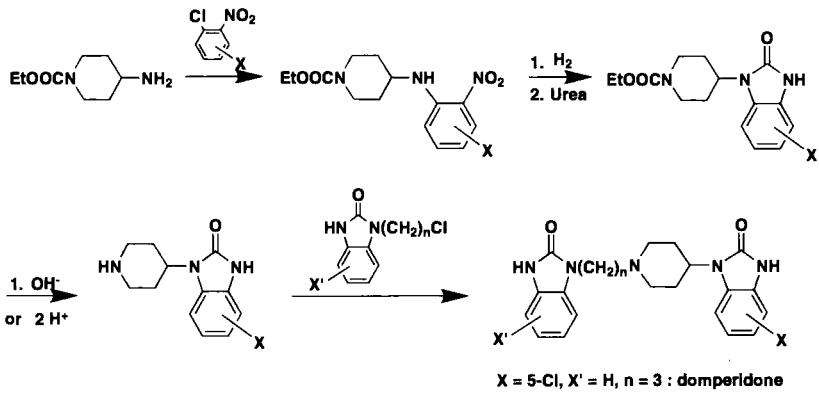


Figure 5.2. Various possibilities of combining fragments (1), (2), (3) and (4).

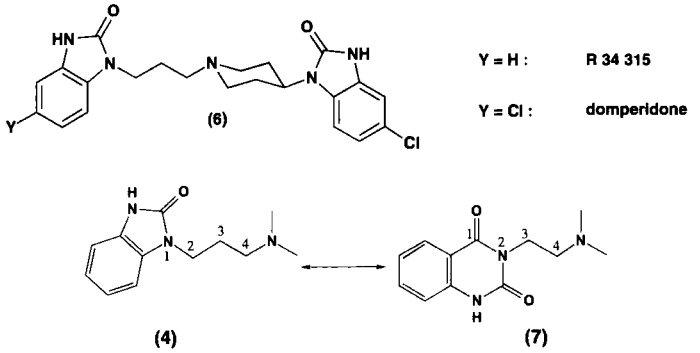


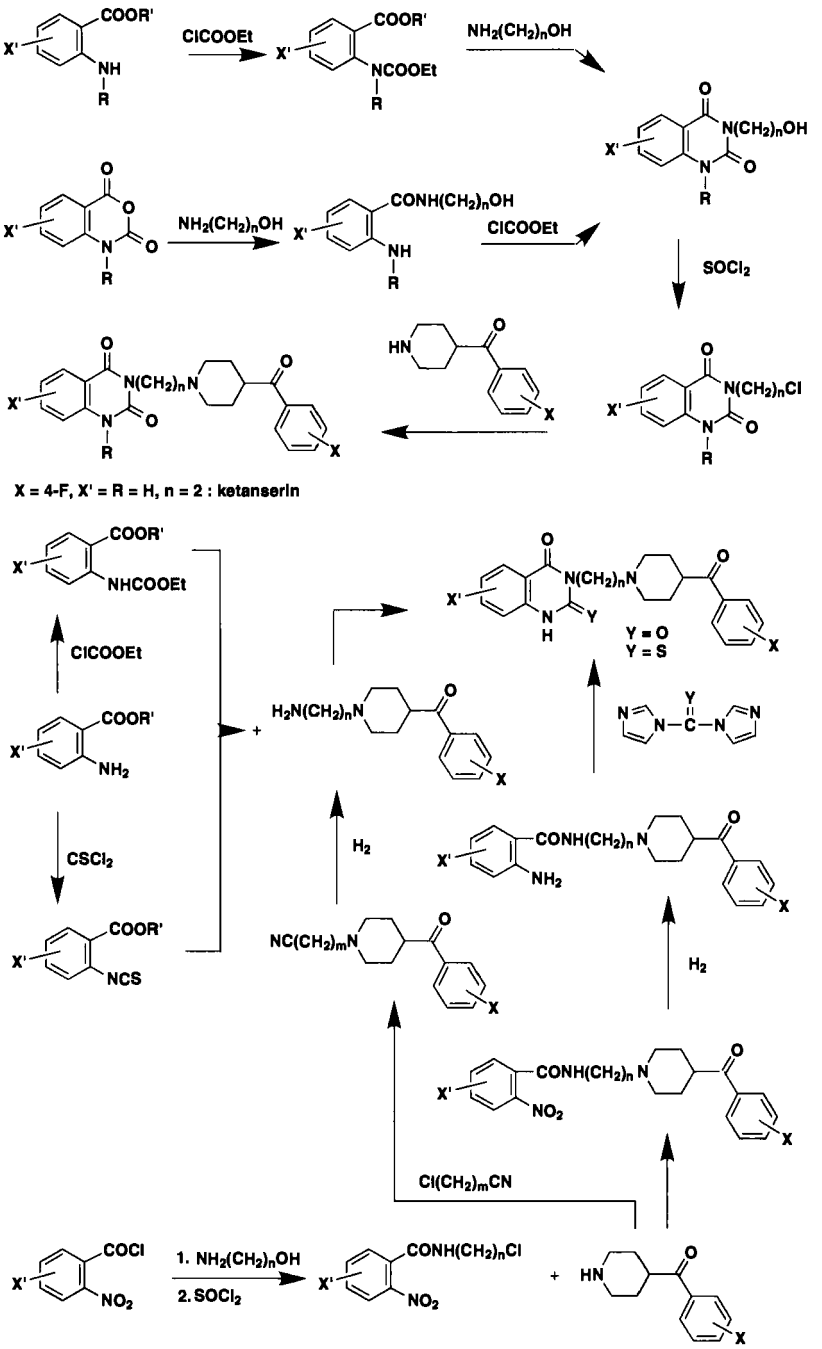
Scheme 5.1. Synthesis of N-*[(4-benzoyl)piperidinoalkyl]benzimidazolones.*



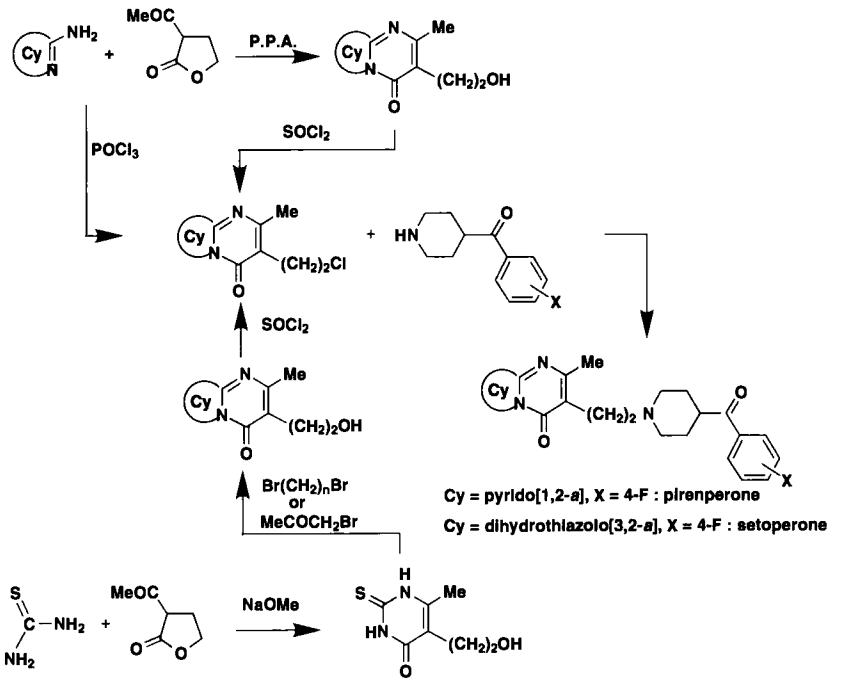
Scheme 5.2. Synthesis of N-*[(4-benzimidazolyl)piperidinoalkyl]-benzimidazolones.*

milenperone was several times more potent than declenperone, especially after oral administration in the apomorphine test in dogs. In clinical studies, however, milenperone showed no real advantage over haloperidol. Compound R 34 315 (6, Y = H) and domperidone (6, Y = Cl) were obtained from fragments (4) and (2). Both compounds showed much higher affinity for the D_2 than for the $5HT_2$ receptor and typically displayed a very large dissociation between central dopamine antagonism in the apomorphine test in rats and peripheral dopamine antagonism in the apomorphine test in dogs (Table 5.1). As peripheral dopamine antagonists, domperidone and R 34 315 block dopamine receptors in the gastrointestinal system and in the chemoreceptor trigger zone which are located outside the blood-brain barrier. Domperidone was further developed for the treatment of emesis and other gastrointestinal diseases such as dyspepsia.



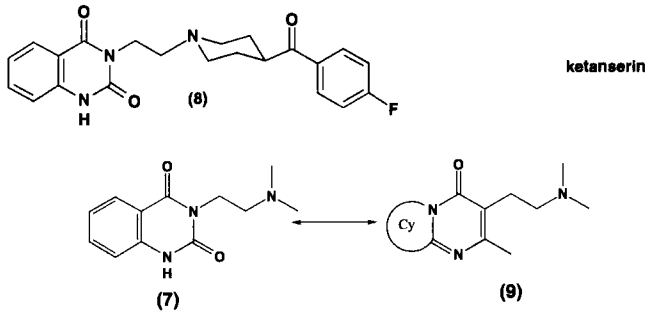


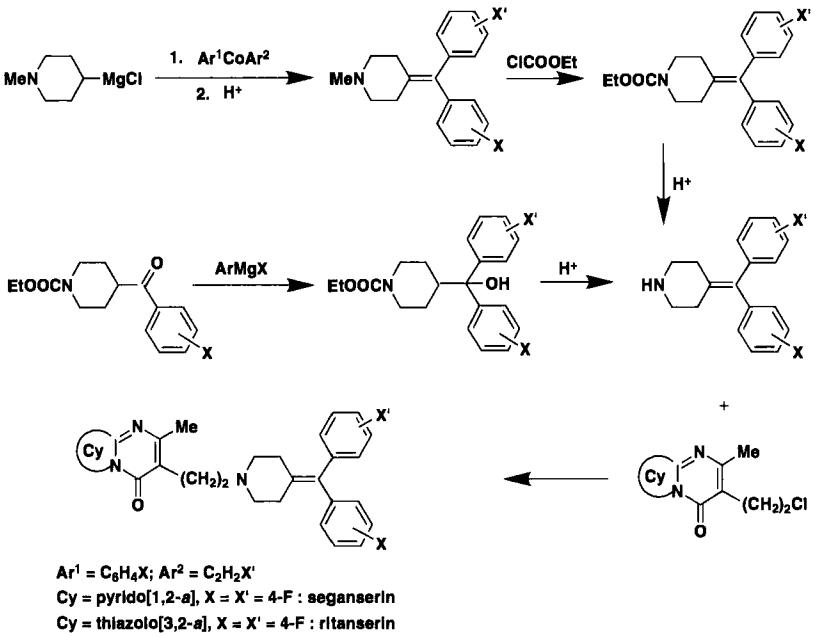
Schemes 5.3. and 5.4. Synthesis of N-*[(4-benzoyl)piperidinoalkyl]quinazolinones.*



Scheme 5.5. Synthesis of N-*[(4-benzoyl)piperidinoalkyl]-pyridopyrimidones and -thiazolopyrimidones.*

Another chemical programme was based on ring expansion of the five-membered imidazolone ring in fragment (4) with retention of the cyclic urea and the distance of four atoms between the aromatic ring and the central nitrogen atom, to obtain fragment (7). Combination of fragments (5) and (3) resulted in a chemical series of which the synthesis is illustrated in Schemes 5.3 and 5.4. Ketanserin (8), a representative of this series, showed

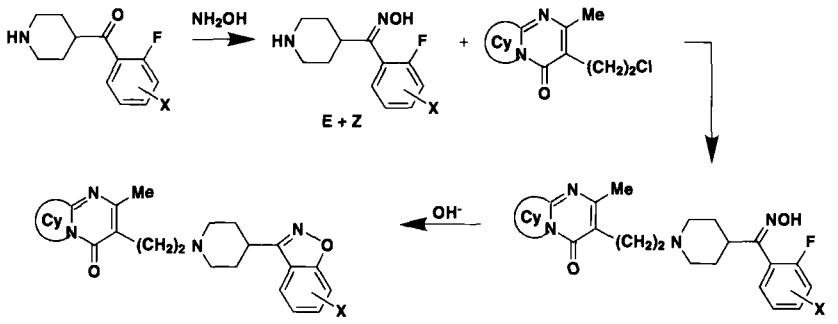




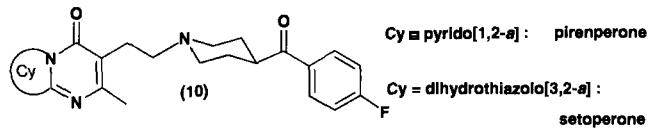
Scheme 5.6. Synthesis of N-*[(4-bisphenylmethylene)piperidinoalkyl]-pyridopyrimidones and -thiazolopyrimidones.*

high affinity for the $5HT_2$ receptor but 100-fold lower affinity for the D_2 receptor. The compound was devoid of relevant apomorphine antagonism in both rats and dogs. Being a relatively potent and peripherally-selective tryptamine antagonist (ED_{50}: 0.056 mg/kg for antagonism of tryptamine-induced cyanosis of the ears, an effect mediated via vascular $5HT_2$ receptors, versus 2.4 mg/kg for antagonism of the centrally-mediated tryptamine-induced convulsions), ketanserin was selected for clinical investigation. The compound is therapeutically active as an antihypertensive, presumably due to a synergistic action resulting from its peripheral serotonin $5HT_2$ antagonism combined with $alpha_1$-adrenoceptor blockade at higher dose levels.

Further chemical modification of the previous series resulted from replacing the benzene ring in fragment (7) by a five- or six-membered saturated or unsaturated ring to obtain the bicyclic and heterocyclic fragments (9). The synthesis of this series of compounds (10) is represented in Scheme 5.5. Two members of this chemical series were selected for further investigation: pirenperone (10, Cy = pyrido[1, 2-*a*]) and setoperone (10,

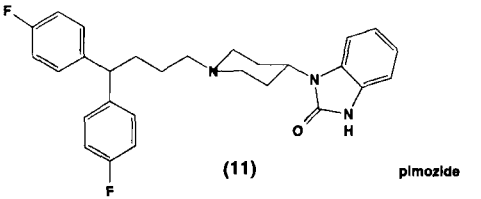


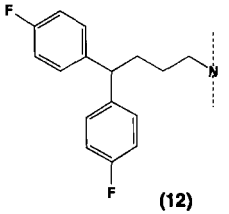
Scheme 5.7. Synthesis of N-*[(4-benzisoxazolyl)piperidinoalkyl)-pyridopyrimidones and -thiazolopyrimidones.*



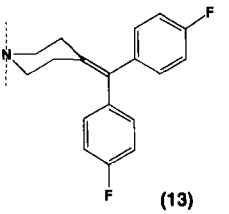
Cy = dihydrothiazole[3, 2-*a*]). The activity profile of both compounds is shown in Table 5.1. Like benperidol and milenperone, both compounds were potent and centrally active D_2 and $5HT_2$ antagonists but with a shift towards relatively more pronounced $5HT_2$ antagonism. Setoperone showed promising antipsychotic activity in preliminary clinical studies [86] but the compound was not further developed because of limited bioavailability. Enzymatic reduction of the keto function to the almost inactive alcohol, which proceeded with different rates in rapid and poor metabolizers, was found to be responsible for the bioavailability problems. This metabolic reduction was not observed in rats. These results stimulated chemists to design metabolism-resistant molecules with a prolonged duration of action in the apomorphine test in dogs.

One approach was based on a modification that had already shown to be successful in the synthesis of neuroleptics: comparison of benperidol (Figure 5.1) and pimozide (11) reveals that the butyrophenone chain can be replaced



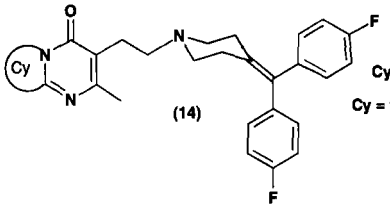


(12)



(13)

by a diphenylbutyl moiety with retention of neuroleptic activity. The diphenylbutyl fragment (12) can be transformed to fragment (13) by cyclization and partial desaturation of the butyl chain. Combination of fragments (13) and (9) generated a chemical series represented by (14) of



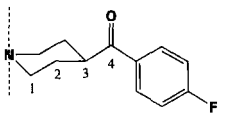
(14)

Cy = pyrido [1,2-*a*] : seganserin

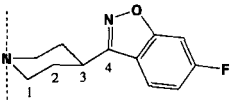
Cy = thiazolo [3,2-*a*] : ritanserin

which the synthesis is described in Scheme 5.6. Two members of this series, seganserin and ritanserin, showed high affinity for the $5HT_2$ receptor and were potent and specific tryptamine antagonists (Table 5.1). Both compounds, but in particular ritanserin, are often used as pharmacological tools to determine the specificity of pharmacological effects and to study the involvement of $5HT_2$ receptors in various pathologies.

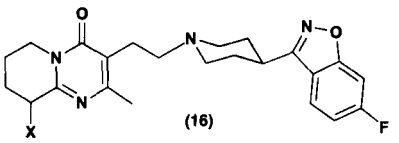
Another chemical modification was based on the observation in the chemical literature that the benzoyl moiety of compounds such as (3) can be replaced by a benzisoxazole ring system (15) with retention of pharmacological activity. It was hypothesized that the isosteric replacement of the benzoyl by the benzisoxazole fragment might avoid rapid metabolic inactivation. The synthesis of the resulting series of molecules is illustrated in Scheme 5.7. Risperidone (16, X = H) appeared to be a potent and centrally active antagonist at both D_2 and $5HT_2$ receptors and it showed a prolonged duration of D_2 antagonistic activity and excellent oral activity in



(3)



(15)

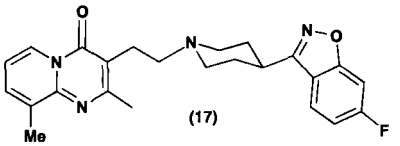


(16)

X = H risperidone

X = OH 9OH-risperidone

the apomorphine test in dogs (see the prolongation of duration of action in the series pirenperone, setoperone, risperidone; Table 5.1); the compound was therefore selected for clinical studies. Although pharmacokinetic studies in man revealed that risperidone is rather rapidly metabolized to 9-hydroxyrisperidone (16, X = OH), there is no real change in activity profile due to this metabolism: the metabolite is comparable to the parent compound both in receptor binding profile and *in vivo* pharmacological effects [7, 87, 88]. In fact, the close resemblance in pharmacological profile between risperidone and its 9-hydroxy derivative indicates that the carbon atom at position 9 of the pyrido[1, 2-a]pyrimidin-4-one moiety is not involved in the relevant receptor interactions. Ocaperidone (17) is another close analogue of risperidone. Like risperidone, ocaperidone appeared to be a potent and centrally active $5HT_2$ and D_2 antagonist with a prolonged duration of action (Table 5.1). Although ocaperidone was 10 times more potent than risperidone as a D_2 antagonist, the compound was not further developed since, in initial clinical studies, it showed no further benefit in therapeutic or side-effect profile above risperidone. The results obtained with risperidone and ocaperidone show that variation of the ring annelated to the common pyrimidinone moiety allows modification of the balance of serotonin and dopamine antagonism.



(17)

ocaperidone

PHARMACOLOGICAL PROFILE OF RISPERIDONE

IN VITRO RECEPTOR-BINDING PROFILE

The *in vitro* receptor-binding profile of risperidone has been extensively studied by Leysen *et al.* [6, 60, 61, 89]. Risperidone shows predominant affinity for rat frontal cortex $5HT_2$ receptors (K_i: 0.16 nM). Only at 10 times

higher concentrations, does it bind to rat striatal D_2 receptors (K_i: 1.4 nM), rat forebrain $alpha_1$-adrenoceptors (K_i: 0.81 nM), and guinea-pig cerebellum H_1 receptors (K_i: 2.1 nM); still higher concentrations bind to rat cortex $alpha_2$-adrenoceptors (K_i: 7.5 nM), cloned human D_4 and D_3 receptors (K_i: 7.0 and 13 nM). No additional receptor interactions are observed up to 200 times the K_i for the $5HT_2$ receptor. Comparative data for a large series of neuroleptics have been reported [60].

IN VIVO PHARMACOLOGICAL PROFILE

The high binding affinity of risperidone for $5HT_2$, D_2, H_1, $alpha_1$ and $alpha_2$ receptors is confirmed by functional interaction and receptor occupancy studies *in vivo*.

Serotonin $5HT_2$ antagonism

Risperidone dose-dependently inhibits $5HT_2$ receptor-mediated behavioural effects evoked by various serotonergic agonists such as tryptamine, mescaline and 5-hydroxytryptophan in rats (s.c. ED_{50} values at time of peak effect: 0.014 to 0.049 mg/kg) [5]. As a central $5HT_2$ antagonist, risperidone is up to 4.6 times more potent than the specific $5HT_2$ antagonist ritanserin [5]. The predominant activity of risperidone at central $5HT_2$ receptors is confirmed by 50% occupancy of rat frontal cortex $5HT_2$ receptors at doses of 0.037 to 0.12 mg/kg [90, 91]. At about 10 times lower doses, risperidone already shows effects characteristic for blockade of peripheral $5HT_2$ receptors: it reverses tryptamine- and compound 48/80-induced cyanosis (0.0011–0.0013 mg/kg, s.c.) and inhibits mast cell serotonin-induced gastric lesions (0.0053 mg/kg, s.c.) [5]. Blockade of peripheral $5HT_2$ receptors may antagonize some effects induced by endogenous serotonin (for example, caused by platelet aggregation) but is not known to result in any undesired effects.

Dopamine D_2 antagonism

Risperidone inhibits behavioural effects induced by dopaminergic agonists such as apomorphine, amphetamine and cocaine (s.c. ED_{50} values: 0.056 to 0.15 mg/kg); risperidone is 3.5 to 9.4 times less potent than haloperidol in this respect [5]. Risperidone occupies D_2 receptors in various rat brain regions by 50% at s.c. doses between 0.22 and 1.3 mg/kg [90, 91]. As a matter of fact, risperidone displays also the effects characteristic for blockade of peripheral D_2 receptors: it is a very potent antagonist of apomorphine-

induced emesis in dogs, irrespective the route of administration (i.v., s.c. and p.o. ED_{50} values: 5.0–7.1 μg/kg) [5], and it enhances prolactin release [92].

Histamine H_1 antagonism

Risperidone is a potent antagonist of compound 48/80-induced anaphylactic shock in rats (s.c. ED_{50}: 0.014 mg/kg) [5]. Compound 48/80 induces release of mast cell mediators, which in rats are predominantly histamine and serotonin. Accordingly, blockade of both peripheral histamine H_1 and serotonin $5HT_2$ receptors may be responsible for the protective action of risperidone. In guinea-pigs, risperidone is a potent antagonist of histamine-induced lethality (s.c. ED_{50}: 0.037 mg/kg) [7]. Ten-fold higher doses are required to occupy central H_1 receptors in the cerebellum of guinea-pigs (s.c. ED_{50}: 0.32–0.58 mg/kg) [91].

Alpha$_1$-adrenoceptor blockade

Risperidone blocks peripheral alpha$_1$-adrenoceptors at doses similar to those required for central D_2 antagonism as indicated by protection from norepinephrine-induced lethality (s.c. ED_{50}: 0.074 mg/kg) [5]. Blockade of peripheral alpha$_1$-adrenoceptors is likely to be responsible for the cardiovascular effects of risperidone, for example, hypotension, decreased arteriolar resistance, and reflex tachycardia. These cardiovascular effects show a pronounced tendency to tolerance and disappear with chronic treatment [7], as reported for prazosin [93]. Central alpha$_1$-adrenoceptors in the frontal cortex and the thalamus are occupied at 10-fold higher doses (s.c. ED_{50} values: 0.45–2.2 mg/kg) [60, 90, 91].

Alpha$_2$-adrenoceptor blockade

As an index of blockade of peripheral alpha$_2$-adrenoceptors, risperidone reverses the antidiarrhoeal effect of clonidine (s.c. ED_{50}: 0.67 mg/kg) [5]. At still higher doses, it occupies also central alpha$_2$-adrenoceptors in the entorhinal cortex/amygdaloid complex (s.c. ED_{50}: 2.0–5.4 mg/kg) [60, 91]. Blockade of central alpha$_2$-adrenoceptors is further evidenced by reversal of xylazine-induced loss of righting (s.c. ED_{50}: 2.4 mg/kg) [5]. As the interaction with alpha$_2$-adrenoceptors occurs at relatively high doses, it is not expected to play a role in the clinical effects of risperidone.

COMPARISON WITH HALOPERIDOL

LSD antagonism

The hallucinogenic 5-HT agonist LSD induces psychotic symptoms in man [68, 94]. Rats also seem to be sensitive to these stimulating effects of LSD as they can be trained to discriminate between LSD and saline; risperidone is a potent antagonist of the LSD-induced stimulus effects (s.c. ED_{50}: 0.024-0.028 mg/kg) [95–97]. As haloperidol is not active against the stimulus effects of LSD in rats, these results suggest that risperidone may be effective against some psychotic symptoms in man that are refractory to haloperidol.

Effects on slow wave sleep

In contrast to haloperidol, low doses of risperidone (0.01–0.16 mg/kg, i.p. or s.c.) increase deep slow-wave sleep at the expense of wakefulness, light slow-wave sleep and paradoxical sleep [98]. This effect is also observed with ritanserin and is probably related to $5HT_2$ antagonism. It may contribute to the therapeutic effect of risperidone in schizophrenic patients, in which deep slow-wave sleep is often impaired [99].

Fine-tuning of dopaminergic activity

The therapeutic effects of neuroleptics are thought to be related to appropriate inhibition and their EPS to excessive blockade of the central dopaminergic neurotransmission. The dose-dependent blockade of central D_2 receptors seems to proceed more gradually with risperidone than with haloperidol, as indicated by the relatively high doses required for catalepsy [5] and by the smooth dose-response curves for central D_2 receptor occupancy [60, 91], depression of spontaneous motor activity [100], antagonism of amphetamine-stimulated motor activity [101–103], and inhibition of apomorphine-induced agitation [104]. This gradual D_2 antagonism of risperidone may allow fine-tuning of central dopaminergic activity and, thereby, reduce EPS liability and enhance the margin for effects on negative symptoms.

Synergism of combined $5HT_2/D_2$ receptor blockade

The combined $5HT_2/D_2$ antagonism of risperidone seems to result in synergistic effects. Risperidone but not haloperidol is more potent against

the agitation elicited by a combined tryptamine-apomorphine challenge than against the agitation elicited by apomorphine alone [104]. Risperidone antagonizes LSD stimulus effects and completely blocks 5-HTP head twitches in rats at much lower doses (s.c. ED_{50}: 0.028 and 0.097 mg/kg, respectively) than haloperidol (s.c. ED_{50}'s: > 0.63 and 1.3 mg/kg, respectively) and ritanserin (s.c. ED_{50}'s: 11 and > 10 mg/kg, respectively) [97, 105]. This synergism of combined $5HT_2/D_2$ antagonism may result in a more efficient control of interacting serotonergic and dopaminergic stimuli than with conventional neuroleptics.

Effects on amphetamine-induced oxygen hyperconsumption

Amphetamine (2.5 mg/kg, s.c.) stimulates motor activity and increases oxygen consumption in rats [106]. Tricyclic antidepressants inhibit the oxygen hyperconsumption at lower doses than the motor stimulation, presumably by actions related to amine uptake inhibition. On the other hand, haloperidol and other neuroleptics reduce the motor stimulation and oxygen hyperconsumption in parallel, presumably as a consequence of central D_2 receptor blockade. Even though devoid of effects on amine uptake, risperidone inhibits amphetamine-induced oxygen hyperconsumption at lower doses than amphetamine-induced agitation (ED_{50}: 0.016 and 0.056 mg/kg, respectively) [5] and resembles tricyclic antidepressants rather than conventional neuroleptics in this respect (see Janssen and Awouters [76] for a more detailed discussion and a comparison of risperidone with other neuroleptics and antidepressants).

Effects on social interaction

In contrast to haloperidol and like clozapine, risperidone (0.062 mg/kg, i.p.) increases social interaction time between pairs of unfamiliar rats [107].

MECHANISM OF ACTION

A pharmacokinetic explanation

The levels of risperidone and its active metabolite in rat striatum increase less than dose-proportionally after s.c. administration of risperidone [88], which may be at least partially responsible for the only gradual increase in the blockade of central D_2 receptors observed with risperidone in receptor binding studies and behavioural interaction studies.

Differential interaction with dopamine receptor subtypes

Interaction with D_2 receptors in different brain regions. Risperidone does not show any regional selectivity for D_2 receptors from various rat brain regions (striatum, nucleus accumbens, tuberculum olfactorium, and substantia nigra), as indicated by receptor binding studies *in vitro* (K_i: 1.4–2.0 nM) [61], *in vivo* (s.c. ED_{50}: 0.5–1.2 mg/kg) [61, 91], and *ex vivo* (s.c. ED_{50}: 0.58–0.85 mg/kg) [60, 91]. Its exceptional therapeutic effects are thus not a consequence of preferential blockade of mesolimbic D_2 receptors [cf. 49, 50, 108].

Interaction with pre-synaptic and post-synaptic D_2 receptors. Risperidone inhibits at similar concentrations pre- and post-synaptic D_2 receptor-mediated responses [6]. Differential interaction with pre- and post-synaptic DA receptors seems thus not to be the basis for its therapeutic effects [51, 52].

Interaction with D_1, D_2, D_3, D_4, and D_5 receptors. The affinity of risperidone for D_4 and D_3 receptors is 5 and 9 times lower, respectively, than that for D_2 receptors; interactions with D_1 receptors occur only at high concentrations (see the section on receptor binding data). Studies on interactions with D_5 receptors are not yet available.

Interaction with muscarinic acetylcholine receptors

In contrast to clozapine for example, risperidone is completely devoid of the so-called 'built-in' anticholinergic activity [5, 6] that would reduce EPS liability [58].

Specific interactions with other types of receptors

Risperidone is devoid of relevant interaction with other types of receptors [61] that have been implicated in antipsychotic or antidepressant activity, for example, neurotensin [40], $5\text{-}HT_{1C}$ [2, 30, 31], $5\text{-}HT_{1A}$ [2], $5\text{-}HT_3$ [32], and sigma receptors [109].

Modulation of dopamine D_2 by serotonin $5HT_2$ antagonism

Central $5HT_2$ antagonism seems to potentiate the feed-back activation of the dopaminergic system in response to D_2 receptor blockade [110, 111] and to improve the signal-to-noise ratio of dopaminergic information transfer that is believed to be disturbed in schizophrenic patients [112]. In this way,

central $5HT_2$ antagonism may be expected to potentiate the behavioural disinhibitory effects that are normally observed with low doses of central D_2 antagonists, and to reduce the EPS that are normally observed at higher doses of central D_2 antagonists.

OCCURRENCE OF COMBINED $5HT_2/D_2$ ANTAGONISM AMONG NEUROLEPTIC DRUGS

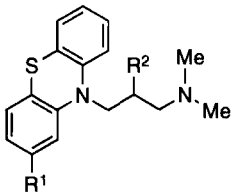
CHEMICAL CLASSIFICATION

A chemical classification of a large series of neuroleptics has been made. The various chemical classes have been listed in Tables 5.2 to 5.13 and are discussed separately below.

Table 5.2. CHEMISTRY AND PHARMACOLOGY OF DIMETHYLAMINOPROPYLPHENOTHIAZINES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Dimethylaminopropyl phenothiazines (class 1.1.1)



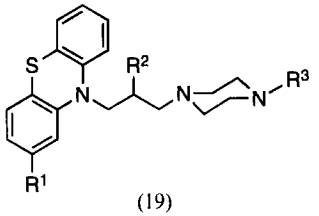
(18)

			K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	*Name*	$5HT_2$	D_2	*TRY*	*APO*
H	H	promazine	14	99	9.3	3.1
Cl	H	chlorpromazine	2.7	19	0.90	0.26
CF_3	H	trifluopromazine	1.4	2.7	0.78	0.074
OMe	H	methopromazine			2.3	1.8
COMe	H	acepromazine			1.4	0.45
COEt	H	propionylpromazine			2.7	0.51
H	Me	(±)-alimemazine	2.4	32	7.1	4.7
OMe	Me	(−)-levomepromazine	1.4	9.0	1.2	0.76
COBu	Me	(±)-M&B 18,706			>10	>10
H	NMe_2	(±)-aminopromazine			>10	>10

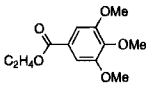
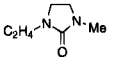
Table 5.3. CHEMISTRY AND PHARMACOLOGY OF PIPERAZINYL PHENOTHIAZINES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Piperazinyl phenothiazines (class 1.1.2)



(19)

				K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	*Name*	*5HT₂*	*D₂*	*TRY*	*APO*
H	H	Me	perazine	21	22	9.3	1.2
Cl	H	Me	prochlorperazine	7.5	3.1	3.6	0.26
CF_3	H	Me	trifluoperazine	8.7	4.3	1.8	0.037
COPr	H	Me	butaperazine	60	2.4	8.2	0.20
SEt	H	Me	thiethylperazine	50	4.5	12	0.17
SO_2NMe_2	H	Me	thioproperazine	200	1.4	> 40	0.15
H	Me	$C_2H_4OC_2H_4OH$	(±) dixyrazine	4.0	5.0	2.1	0.59
Cl	H	C_2H_4OH	perphenazine	4.3	6.5	0.51	0.037
Cl	H	C_2H_4OCOMe	thiopropazate	3.1	1.8	0.30	0.037
Cl	H		metophenazine			25	2.0
Cl	H		chlorimpiphenine			0.15	0.098
CF_3	H	C_2H_4OH	fluphenazine	3.5[a]	1.9[a]	0.59	0.056
CF_3	H	$C_2H_4OC_2H_4OH$	SKF-7261-A_2			0.22	0.085
CF_3	H		cyclophenazine			12	2.0
COMe	H	C_2H_4OH	acetophenazine			3.1	0.074
COEt	H	C_2H_4OH	carphenazine			2.7	0.13

[a] [76]

Tricyclics with 6, 6, 6-membered rings (class 1; Tables 5.2-5.5)

This series is made up of tricyclic molecules with 6, 6, 6-membered rings, of which the central ring contains one sulphur and/or one nitrogen atom. The series can be subdivided in the phenothiazines, thioxanthenes and acridines.

Phenothiazines (class 1.1; Tables 5.2-5.4).

Dimethylaminopropyl phenothiazines (class 1.1.1; Table 5.2)

This group of molecules is characterized by an aliphatic dimethylaminopropyl side-chain. It comprises the promazines (promazine, chlorpromazine, trifluopromazine, methopromazine, acepromazine and propionylpromazine), the mepromazines (alimemazine, levomepromazine, M&B18, 706), and the aminopromazines (aminopromazine) containing no substituent, a methyl substituent, or an amino substituent on the side-chain, respectively. Substitution on the side-chain as in the mepromazines and aminopromazines generates a chiral centre and results in stereoisomerism.

Piperazinyl phenothiazines (class 1.1.2; Table 5.3)

These molecules have a piperazinylpropyl side-chain. They can be further subdivided in the perazines with a methyl substituent on the nitrogen of the piperazine nucleus (perazine, prochlorperazine, trifluoperazine, butaperazine, thiethylperazine, and thioproperazine) and the phenazines that have larger substituents (dixyrazine, perphenazine, thiopropazate, metophenazin, chlorimpiphenine, fluphenazine, SKF- 7261-A_2, cyclophenazine, acetophenazine, and carphenazine). The methyl substituent on the propyl side-chain in dixyrazine generates a chiral centre, which gives rise to stereoisomerism.

Piperidinyl phenothiazines (class 1.1.3; Table 5.4)

These phenothiazines have a piperidine ring in the side-chain. Thioridazine and mesoridazine have an ethyl bridge between the nitrogen atom of the phenothiazine nucleus and carbon 2 of the piperidine ring. The substitution on carbon atom 2 of the piperidine creates a chiral centre in these molecules. The other compounds have a propyl bridge between the two nitrogen atoms of the phenothiazine and piperidine nuclei (propericiazine, perimethazine, duoperone, metopimazine, pipotiazine, piperacetazine, and pipamazine). The methyl substituent on the propyl side-chain in perimethazine generates a chiral centre.

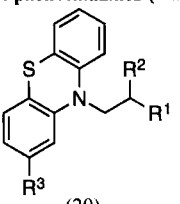
Thioxanthenes and acridines (class 1.2; Table 5.5).

The thioxanthenes and acridines contain only one heteroatom in the central ring, sulphur in case of the thioxanthenes and nitrogen in case of the acridines. The thioxanthenes are isosterics of the phenothiazines: the nitrogen in the central ring has been replaced by a carbon with sp^2

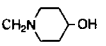
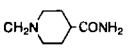
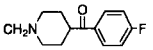
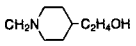
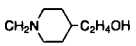
Table 5.4. CHEMISTRY AND PHARMACOLOGY OF PIPERIDINYL PHENOTHIAZINES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Piperidinyl phenothiazines (class 1.1.3)



(20)

				K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	H	SMe	(±) thioridazine	4.2	16	11	4.1
	H	SOMe	(±) mesoridazine			>80	>80
	H	CN	propericiazine	0.58	3.5	0.34	0.085
	Me	OMe	(±)-perimethazine			0.68	0.30
	H	SO_2Me	metopimazine	16[a]	1.5[a]	>160	>160
	H	Cl	pipamazine			1.3	0.50
	H	CF_3	duoperone			21[b]	2.0[b]
	H	SO_2NMe_2	pipotiazine	12	0.80	1.6	0.15
	H	COMe	piperacetazine	2.9	0.60	0.59	0.043

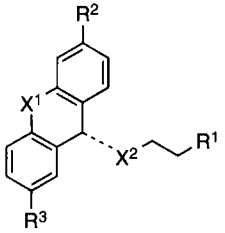
[a][76]; [b] ED_{50} at 4 h after p.o. administration

configuration. The substitution is isosteric: the π-electrons of the double bond replace the lone-pair electrons of the nitrogen. Chlorprothixene is thus the thioxanthene analogue of the phenothiazine chlorpromazine, SKF-10812A of trifluopromazine, thiothixene of thioproperazine, and flupenthixol of fluphenazine. The double bond gives rise to *E/Z* stereoisomerism. Teflutixol and the acridine clomacran are two exceptions since they contain

Table 5.5. CHEMISTRY AND PHARMACOLOGY OF THIOXANTHENES AND ACRIDINES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Thioxanthenes and acridines (class 1.2)



(21)

						K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	X^1	X^2	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	H	Cl	S	=CH	chlorprothixene (Z)	0.30	12	0.52	0.17
NMe_2	H	CF_3	S	=CH	SKF-10812A (E+Z)			0.15	0.074
	H	Cl	NH	$-CH_2$	(±) clomacran	12[a]	8.7[a]	0.90	0.13
	H	Cl	S	=CH	clopenthixol (E+Z)	2.4	6.3	0.59	0.098
					clopenthixol (Z)	1.6[a]	2.5[a]	0.15	0.049
	H	CF_3	S	=CH	flupenthixol (E+Z)	2.5	6.4	0.45	0.098
	F	CF_3	S	$-CH_2$	(±) teflutixol	2.9	4.7	1.4	0.20
	F	CF_3	S	=CH	pifluthixol (E+Z)	0.73	1.4	0.048 (4 h)	0.0070 (4 h)
	H	SO_2NMe_2	S	=CH	thiothixene (E+Z)	98	2.5	22	0.23
	H	Cl	S	=CH	clotixamide (Z)			9.3 (p.o.)	4.6 (p.o.)

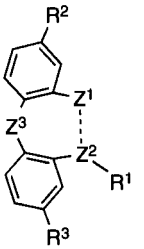
[a] [76]

a carbon with sp^3 instead of sp^2 configuration; the substitution on the central ring however creates a chiral centre resulting in optical antipodes. Like the phenothiazines, the thioxanthenes can be subdivided into those with a dimethylamino side-chain, those with piperidine in the side-chain, and those with piperazine in the side-chain.

Table 5.6. CHEMISTRY AND PHARMACOLOGY OF 6, 7, 6-MEMBERED TRICYCLICS SUBSTITUTED AT THE TWO-ATOM BRIDGE.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

6, 7, 6-Membered tricyclics substituted at the two-atom bridge (class 2.1)



(22)

						K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	Z^1-Z^2	Z^3	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	H	H	N=CR¹	CH_2	perlapine	16	462	2.7[a]	3.1[a]
	F	H	N=CR¹	CH_2	fluperlapine			2.4	8.1
	Cl	H	N=CR¹	NH	clozapine	3.3	152	1.2	11
	H	Cl	N=CR¹	S	clothiapine	0.98	16	0.39	0.098
	H	Cl	CH_2CHR^1	S	(±)-octoclothepine			0.049	0.043
	H	SMe	CH_2CHR^1	S	(±)-metitepine	0.5	3.9	0.098	0.065
	H	Cl	N=CR¹	O	loxapine	1.6	11	0.20	0.074
	H	H	N=CR¹	S	seroquel			≥10	6.2
	H	Cl	N=CR¹	O	amoxapine			1.8[a]	3.1[a]
$OC_2H_4NMe_2$	H	Cl	N=CR¹	S	zotepine	0.91	13	0.17	0.19

[a] ED_{50} after p.o. administration

Tricyclics with 6, 7, 6-membered rings (class 2; Tables 5.6-5.7)

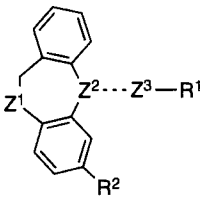
The series can be subdivided in tricyclic molecules that have a side-chain on the two-atom bridge and those that have a side-chain on the single-atom bridge of the 7-membered central ring. Each group can be further subdivided based on the heteroatom(s) in the central ring.

Compounds substituted at the two-atom bridge (class 2.1; Table 5.6).

The group is made up by apines (one nitrogen atom; perlapine, fluperlapine), azapines (two nitrogen atom; clozapine), thiapines (one nitrogen and one sulphur atom; clothiapine, seroquel), oxapines (one

Table 5.7. CHEMISTRY AND PHARMACOLOGY OF 6, 7, 6-MEMBERED TRICYCLICS SUBSTITUTED AT THE SINGLE-ATOM BRIDGE.
Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

6, 7, 6-Membered tricyclics substituted at the single-atom bridge (class 2.2)



(23)

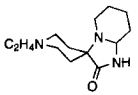
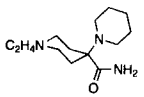
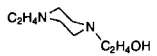
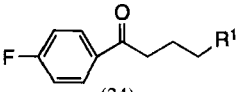
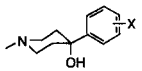
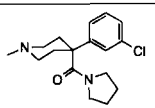
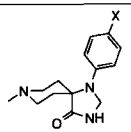
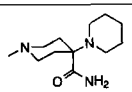
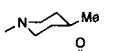
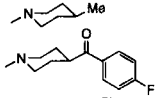
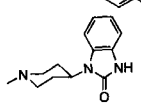
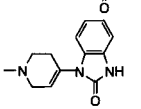
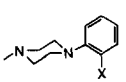
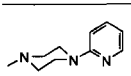
					K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	Z^1	Z^2-Z^3	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	H	CH_2	N-CH_2R^1	carpipramine	118	18	>10	>10
	Cl	CH_2	N-CH_2R^1	mosapramine			2.5	0.58
	Cl	CH_2	N-CH_2R^1	clocapramine			98	>160
	Cl	O	C=CHR^1	pinoxepine (Z)			0.59	0.17

Table 5.8. CHEMISTRY AND PHARMACOLOGY OF BUTYROPHENONES. Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Butyrophenones (class 3)



(24)

			K_i (nM)		ED_{50} (mg/kg)	
R^1	X	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	4-Cl	haloperidol	27	1.2	0.79	0.016
	4-Br	bromperidol	47	1.2	1.2	0.028
	4-Me	moperone	37	2.2	0.68	0.019
	3-CF_3	trifluperidol	3.8	1.5	0.34	0.016
	4-Cl, 3-CF_3	clofluperidol			1.2 (4 h)	0.025 (4 h)
		haloperidide	3.0	0.8	0.24	0.020
	H	spiperone	0.46	0.26	0.085	0.016
	F	fluspiperone			0.049	0.016
		pipamperone	1	98	0.58	3.1
		melperone	40	210	1.8	0.89
		lenperone			0.39	0.085
		benperidol	1.0	0.31	0.29	0.0094
		droperidol	1.1	0.8	0.51	0.012
	H	butropipazone	37	44	1.7	0.095
	OMe	fluanisone	26	4	1.2	0.049
		azaperone			0.45	0.34

nitrogen and one oxygen atom; amoxapine, loxapine), and tepines (one sulphur atom; zotepine, metitepine, octoclothepine). Zotepine has an aliphatic side-chain, the other compounds contain piperazine in the side-chain. Octoclothepine and metitepine contain a chiral centre and can exist as two different enantiomers.

Compounds substituted at the single-atom bridge (class 2.2; Table 5.7).

This group consists of the oxepine (1 oxygen atom) pinoxepine and the pramines carpipramine, clocapramine, and the recent mosapramine. The double bond in pinoxepine is responsible for *E/Z* isomerism.

Butyrophenones (class 3; Table 5.8)

Optimum activity is obtained with a fluoro atom in the para position of the phenyl in the butyrophenone group. The compounds can be subdivided in piperidinyl-, tetrahydropyridinyl-, and piperazinyl-substituted butyrophenones. Further subdivision of the piperidinyl butyrophenones is based on substitution at the 4 position of the piperidine ring: phenyl-substituted piperidinyl butyrophenones (haloperidol, bromperidol, moperone, trifluperidol, clofluperidol, haloperidide), spiperones (spiperone, fluspiperone), bipiperidinylbutyrophenones (pipamperone), and others (melperone, lenperone, and benperidol). Droperidol is an example of the tetrahydropiridinyl butyrophenones, and butropipazone, fluanisone, and azaperone are members of the piperazinyl butyrophenones.

Diphenylbutyl piperidines and piperazines (class 4; Table 5.9)

Pimozide, clopimozide, penfluridol and fluspirilene are members of the diphenylbutyl piperidines. The recent amperozide is a diphenylbutyl piperazine.

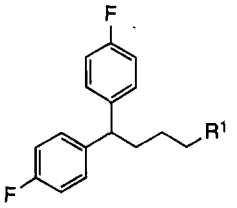
Benzamides (class 5; Table 5.10)

This series includes the diethylaminoethylbenzamides (metoclopramide, bromopride, and tiapride), the pyrrolidinyl benzamides (sulpiride, remoxipride, raclopride, and nemonapride), and the piperidinyl benzamides (clebopride).

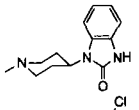
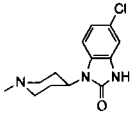
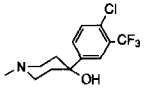
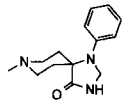
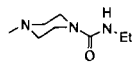
Table 5.9. CHEMISTRY AND PHARMACOLOGY OF DIPHENYLBUTYL-PIPERIDINES OR -PIPERAZINES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Diphenylbutyl-piperidines or -piperazines (class 4)



(25)

		K_i (nM)		ED_{50} (mg/kg)	
R^1	*Name*	*$5HT_2$*	*D_2*	*TRY*	*APO*
	pimozide	6.0	1.2	≥ 10 (4 h)	0.049 (4 h)
	clopimozide			≥ 40 (p.o., 16 h)	0.15 (p.o., 16 h)
	penfluridol	52	11	> 40 (p.o., 16 h)	0.23 (p.o., 16 h)
	fluspirilene	3.4	1.5	4.7 (4 h)	0.074 (4 h)
	amperozide	16	419	12	≥ 40

Spirones and related compounds (class 6; Table 5.11)

This series is composed of piperazinylbutyl azadiones: buspirone, tiospirone, SM-9018, gepirone, NAN-190, and tandospirone.

Pertines (class 7; Table 5.12)

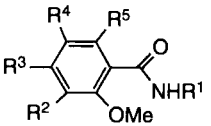
The pertines oxypertine, solypertine, milipertine, and alpertine are piperazinylethylindoles.

Miscellaneous compounds (class 8; Table 5.13)

A number of compounds that do not belong to any of the above chemical classes have been classified as a heterogeneous group of miscellaneous compounds. Some compounds are however to some extent related to one of the above series. Olanzapine and ORG-5222 are comparable to the 6, 7, 6-membered tricyclics substituted at the two-atom bridge but contain a 5, 7, 6-membered tricyclic and a tetracyclic ring system, respectively. Butaclamol and nelezaprine compare to 6, 7, 6-membered tricyclics substituted at the

Table 5.10. CHEMISTRY AND PHARMACOLOGY OF BENZAMIDES.
Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Benzamides (class 5)

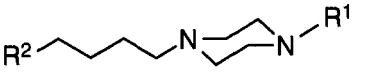


(26)

						K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	R^4	R^5	*Name*	$5HT_2$	D_2	*TRY*	*APO*
	H	NH_2	Cl	H	metoclopramide	2094	67	16	0.76
	H	NH_2	Br	H	bromopride			15	1.0
	H	H	SO_2Me	H	tiapride	>10000	400	> 40	12
	H	H	SO_2NH_2	H	sulpiride	1400	31	226	49
	H	H	Br	OMe	remoxipride	>10000	272	≥ 40	0.17
	Cl	H	Cl	OH	raclopride	10000	7	3.6	0.028
	H	NHMe	Cl	H	nemonapride			0.51	0.0071
	H	NH_2	Cl	H	clebopride	160	5.5	2.0	0.056

Table 5.11. CHEMISTRY AND PHARMACOLOGY OF SPIRONES.
Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Spirones (class 6)



(27)

			K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	Name	$5HT_2$	D_2	TRY	APO
		buspirone	460	48	4.1	0.45
		gepirone			>10	5
		tandospirone			5.4	5.4
		tiospirone	0.22	1.9	0.44	0.085
		SM-9018			0.037	0.016
		NAN-190			3.1	2.0

single-atom bridge but contain a pentacyclic and a 5, 7, 6-membered tricyclic ring system, respectively. Spiroxatrine contains the spiro moiety of spiperone but does not belong to the butyrophenones. Sertindole, tefludazine, molindone and ziprasidone are not directly related to one of the above chemical classes. Butaclamol, nelezaprine, tefludazine and molindone contain chiral centres.

PHARMACOLOGICAL EVALUATION

Tables 5.2 to 5.9 list binding affinities for $5HT_2$ and D_2 receptors and ED_{50}'s for tryptamine and apomorphine antagonism in rats for the above chemical classes. For each chemical class, the structure-activity relationships for $5HT_2$ and D_2 antagonism are discussed.

Tricyclics with 6, 6, 6-membered rings (class 1; Tables 5.2-5.5)

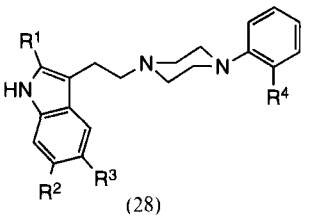
Phenothiazines (class 1.1; Tables 5.2-5.4). As is generally known, the potency of the phenothiazines at the D_2 receptor greatly depends on substitution at carbon 2 of the phenothiazine system. Variation in the side-chain does not produce a major shift in activity. Propericiazine is the most potent and promazine the least potent compound of the series.

Dimethylaminopropyl phenothiazines (class 1.1.1; Table 5.2)

Unsubstituted promazine shows 7 times higher *in vitro* binding affinity for the $5HT_2$ than for the D_2 receptor but is 3 times less potent *in vivo* for tryptamine than for apomorphine antagonism. Chlorpromazine is about 10 times more potent than promazine regarding D_2 antagonism, both *in vitro* and *in vivo*. The most pronounced increase in D_2 antagonistic activity is

Table 5.12. CHEMISTRY AND PHARMACOLOGY OF PERTINES.
Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

Pertines (class 7)



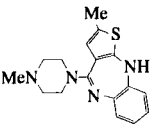
(28)

					K_i (nM)		ED_{50} (mg/kg)	
R^1	R^2	R^3	R^4	*Name*	$5HT_2$	D_2	*TRY*	*APO*
Me	OMe	OMe	H	oxypertine	8.6	30	2.7	1.3
H	OCH_2O		OMe	solypertine			5.4	2.1
Me	OMe	OMe	OMe	milipertine			7.1	1.0
COOEt	OMe	OMe	H	alpertine			≥ 10	≥ 10

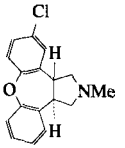
Table 5.13. CHEMISTRY AND PHARMACOLOGY OF SOME MISCELLANEOUS STRUCTURES.

Listed are binding affinities (K_i; nM) for $5HT_2$ and D_2 receptors and ED_{50}'s (mg/kg, s.c.) for antagonism of tryptamine (TRY)- and apomorphine (APO)-induced behavioural effects in rats.

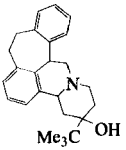
Miscellaneous structures (class 8)



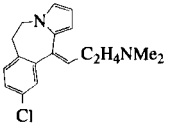
olanzapine (29)



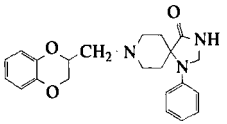
ORG-5222 (30)



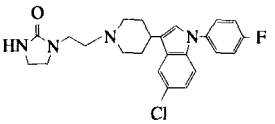
butaclamol(31)



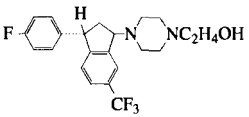
nelezaprine (32)



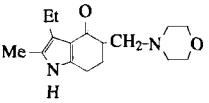
spiroxatrine (33)



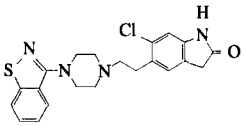
sertindole (34)



tefludazine (35)



molindone (36)



ziprasidone (37)

Name	*K_i (nM)* $5HT_2$	D_2	*ED_{50} (mg/kg)* *TRY*	*APO*
Olanzapine			0.22	0.34
ORG-5222 (trans)			0.011	0.018
Butaclamol	1.9 (+)	3.6 (+)	0.68 (±)	0.098 (±)
(E)-Nelezaprine			0.34	0.17
Spiroxatrine	86	1.4	> 2.5	0.016
Sertindole			0.59	6.2
(±) Tefludazine (trans)	0.86	5.6	0.042	0.085
(±) Molindone	2350	25	7.1	0.13
Ziprasidone			0.59	0.29

obtained with the trifluoromethyl substituent at carbon 2 of the phenothiazine nucleus. Substitution at carbon 2 of the propyl side-chain does not produce a major shift in activity. Preferential tryptamine over apomorphine antagonism is not obtained with any of the compounds; methopromazine,

alimemazine, and levomepromazine show both activities at comparable doses.

Piperazinyl phenothiazines (class 1.1.2; Table 5.3)

Relative to the corresponding promazines, the perazines are at least as potent as D_2 antagonists but slightly less potent as $5HT_2$ antagonists. The phenazines are also relatively specific apomorphine antagonists, with the exception of chlorimpiphenine and SKF-7261-A_2 which display tryptamine and apomorphine antagonism at closely related doses and are also the most potent tryptamine antagonists of the whole series.

Piperidinyl phenothiazines (class 1.1.3; Table 5.4)

These phenothiazines have a piperidine ring in the side-chain. Thioridazine and mesoridazine show low *in vivo* activity. The other compounds of the series are considerably more potent with exception of metopimazine which is completely devoid of *in vivo* activity up to 160 mg/kg. None of the compounds is a preferential tryptamine antagonist but thioridazine, perimethazine, and pipamazine show almost concomitant tryptamine and apomorphine antagonism. Piperacetazine is the most potent D_2 antagonist of the listed compounds.

Thioxanthenes and acridines (class 1.2; Table 5.5). The thioxanthenes are comparable in activity to their isosteric phenothiazine analogues. Piflu-thixol shows slight preference for the $5HT_2$ receptor *in vitro* but not *in vivo* 4 h after s.c. administration. The acridine clomacran closely resembles its corresponding thioxanthene analogue chlorprothixene except for its lower affinity for the $5HT_2$ receptor.

Tricyclics with 6, 7, 6-membered rings (class 2; Tables 5.6-5.7)

6, 7, 6-Membered tricyclics substituted at the 2-atom bridge (class 2.1; Table 5.6). All these compounds show markedly higher affinity for the $5HT_2$ than for the D_2 receptor. Preferential $5HT_2$ antagonism *in vivo* is obtained only with clozapine and, to a less extent, with fluperlapine. The replacement of the nitrogen atom bridge in clozapine by an oxygen or sulphur atom and a concomitant shift of the chloro substituent to the opposite ring to obtain loxapine and clothiapine, respectively, results in enhanced D_2 antagonistic activity but also in a decreased ratio of $5HT_2$ over D_2 antagonism. When comparing seroquel with clothiapine, it is evident that the removal of the chloro substituent and the elongation of the side-chain markedly decrease potency. Amoxapine, perlapine, zotepine, octoclothepine and metitepine show $5HT_2$ and D_2 antagonism at closely related doses. The latter two compounds are the most potent ones of the series.

6, 7, 6-Membered tricyclics substituted at the single-atom bridge (class 2.2; Table 5.7). Pinoxepine antagonizes apomorphine at a relatively low dose but tryptamine antagonism requires somewhat higher doses. The pramines also show predominant D_2 antagonism; the recent mosapramine is the most potent member of the series.

Butyrophenones (class 3; Table 5.8)

The butyrophenones are in general rather potent and specific D_2 antagonists. The phenylpiperidinyl butyrophenones and the spiperones are very comparable regarding their apomorphine antagonistic activity (ED_{50} about 0.02 mg/kg). Preferential $5HT_2$ antagonism is obtained with pipamperone both *in vitro* and *in vivo* and with melperone *in vitro*.

Diphenylbutyl piperidines and piperazines (class 4; Table 5.9)

The diphenylbutyl piperidines are in general relatively potent, long acting, and specific apomorphine antagonists. The recent diphenylbutyl piperazine amperozide shows preferential $5HT_2$ antagonism but low *in vivo* activity.

Benzamides (class 5; Table 5.10)

All benzamides are specific D_2 antagonists but they show a large variation in *in vivo* activity. The ED_{50}'s for apomorphine antagonism vary from 49 mg/kg for the least potent compound sulpiride to 0.0071 mg/kg for the recent nemonapride.

Spirones and related compounds (class 6; Table 5.11)

Tiospirone and SM-9018 are potent apomorphine antagonists. Conversion of the spiro ring system of tiospirone to the annelated ring system of SM-9018 results in a potency increase with factors of 12 and 5 for tryptamine and apomorphine antagonism, respectively. The other compounds of the series are less potent apomorphine antagonists and better known for their interaction with $5HT_{1A}$ receptors. Interaction with $5HT_{1A}$ receptors may also contribute to the biological activity of tiospirone and SM-9018. None of the compounds is a preferential tryptamine antagonist; SM-9018, NAN-190, and tandospirone are about equipotent as apomorphine and tryptamine antagonist.

Pertines (class 7; Table 5.12)

This series shows a low potency as tryptamine and apomorphine antagonists. Doses of 2.7 and 1.3 mg/kg are required for the most potent compound, oxypertine, to antagonize tryptamine and apomorphine, respectively. Alpertine is the least potent compound of the series. Oxypertine and solypertine show apomorphine and tryptamine antagonism at closely related doses.

Miscellaneous compounds (class 8; Table 5.13)

Apart from spiroxatrine, all listed compounds antagonize the behavioural effects of both apomorphine and tryptamine. The *N*-phenylindolylpiperidine sertindole is the only predominant tryptamine antagonist. The chloro substitution at the 5-position of the indole has been reported to result in the highest affinity for both D_2 and $5HT_2$ receptors and optimal association of both activities [113]. However, sertindole is also a very potent $alpha_1$-adrenoceptor blocker: norepinephrine-induced lethality in rats is antagonized at 0.15 mg/kg, that is, at 40 times below the dose required for apomorphine antagonism (Megens, unpublished data). Olanzapine, ORG-5222, nelezaprine, tefludazine and ziprasidone are about equipotent as tryptamine and as apomorphine antagonist. ORG–5222 and spiroxatrine are the most potent apomorphine antagonists.

CONCLUSIONS

Tables 5.1 to 5.13 list ED_{50} values for tryptamine and apomorphine antagonism. These ED_{50}'s were obtained under standard conditions in the ATN test in rats. In this test, apomorphine and tryptamine are successively injected in the same rat at 30 and 90 min, respectively, after test compound administration (see Methods section). Different ED_{50} values may be obtained when the compound is tested at time of peak effect in the separate apomorphine and tryptamine tests. For instance, the ED_{50} of risperidone for inhibition (score < 3) of tryptamine-induced bilateral convulsions is 0.014 mg/kg at time of peak effect in the separate tryptamine test [5] but as high as 0.13 mg/kg for blockade (score < 2) of tryptamine-induced convulsions in the ATN test (Table 5.1). Nevertheless, it seems justified to compare for all compounds the ratio's of the listed ED_{50} values for apomorphine and tryptamine antagonism obtained under standard conditions in the ATN test.

Table 5.14 shows the compounds that antagonize the tryptamine-induced

behavioural effects at lower doses than the apomorphine-induced effects. These preferential tryptamine antagonists originate from several chemical classes: five 6-7-6 membered tricyclics (clozapine, amoxapine, clocapramine, perlapine, zotepine) and two close analogues (olanzapine, ORG-5222); one butyrophenone (pipamperone); one biphenylbutylpiperazine (amperozide); four pyrimidones (ritanserin, seganserin, setoperone, risperidone, 9OH- risperidone); and two miscellaneous compounds (tefludazine, sertindole).

Ritanserin, seganserin, amperozide, and clocapramine do not belong to the $5HT_2/D_2$ antagonists since they lack apomorphine antagonistic activity. Ritanserin and seganserin are potent and specific tryptamine antagonists (ED_{50}'s; 0.26 and 0.68 mg/kg, respectively) that, up to 100 times higher doses, are devoid of apomorphine antagonism. Amperozide and clocapramine are considerably less potent as tryptamine antagonists (ED_{50}'s: 12 and 98 mg/kg, respectively). Setoperone is an experimental compound of which the development has been stopped because of its rapid metabolism in man (see the section on the historical development of risperidone). 9OH-Risperidone is the active metabolite of risperidone having similar pharmacological

Table 5.14. ED50'S (MG/KG, S.C.) OF FOR ANTAGONISM OF TRYPTAMINE (TRY)-AND APOMORPHINE (APO)-INDUCED BEHAVIOURAL EFFECTS IN RATS

Name	*Chemical class*	*ED_{50} (mg/kg, s.c.)* *TRY*	*APO*	*Ratio TRY/APO*
Ritanserin	9[a]	0.26	> 160	< 0.002
Seganserin	9[a]	0.68	> 80	< 0.009
Amperozide	4	12	≥ 40	≤ 0.30
Clocapramine	2.2	98	> 160	< 0.61
Sertindole	8	0.59	6.2	0.10
Clozapine	2.1	1.2	11	0.11
Pipamperone	3	0.58	3.1	0.19
Fluperlapine	2.1	2.4	8.1	0.30
Tefludazine	8	0.042	0.085	0.49
Setoperone	9[a]	0.11	0.22	0.50
9OH-risperidone	9[a]	0.22	0.39	0.56
Amoxapine	2.1	1.8 (p.o.)	3.1 (p.o.)	0.58
ORG-5222	8	0.011	0.018	0.61
Olanzapine	8	0.22	0.34	0.65
Risperidone	9[a]	0.13	0.15	0.87
Perlapine	2.1	2.7 (p.o.)	3.1 (p.o.)	0.87
Zotepine	2.1	0.17	0.19	0.89

[a] Chemical class 9 refers to the pyrimidones

properties (see the section on the historical development of risperidone). Apart from risperidone, only four of the remaining compounds can be considered as potent $5HT_2/D_2$ antagonists (ED_{50} for apomorphine antagonism: < 0.63 mg/kg): ORG-5222 (0.018 mg/kg), tefludazine (0.085 mg/kg), zotepine (0.19 mg/kg), and olanzapine (0.34 mg/kg). Further comparison of the pharmacological properties of these compounds with those of risperidone is outside the scope of the present review. It should be noted, however, that the pharmacology of these compounds is not restricted to $5HT_2$ and D_2 antagonism and that associated pharmacological interactions may interfere with the beneficial effects of combined $5HT_2/D_2$ antagonism. Furthermore, their practical usefulness is dependent on pharmacokinetic properties such as oral absorption, metabolism, and duration of action.

GENERAL CONCLUSION

The present overview shows that combined $5HT_2/D_2$ antagonism is found within a variety of chemical classes. Optimal association of serotonin $5HT_2$ and dopamine D_2 antagonism in the absence of disturbing effects might be achieved with risperidone. Risperidone can indeed be clearly differentiated from conventional neuroleptics: by its ability to antagonize LSD-induced stimulus effects; its effects on deep slow-wave sleep; its smooth dose-response curves for effects related to blockade of central D_2 receptors; its efficient control of interacting serotonergic and dopaminergic stimuli; its ability to inhibit amphetamine-induced oxygen consumption at lower doses than amphetamine-induced agitation; and, its effects on social interaction. Optimal association of central D_2 and $5HT_2$ antagonism may, at least partially, be responsible for these differences relative to conventional neuroleptics.

Finally, it can be stated that blockade of central D_2 receptors remains the cornerstone of antipsychotic therapy. Co-ordinated $5HT_2$ and D_2 antagonism might act synergistically in decreasing psychotic symptoms and at the same time reduce the risk of neurological imbalances. $5HT_2$ antagonism may potentiate the behavioural disinhibitory effects that are sometimes reported after low doses of D_2 antagonists, and it may at the same time reduce the EPS that are usually observed at higher doses.

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6 Protein Tyrosine Kinase Inhibitors

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INTRODUCTION

The finding in 1979 that tyrosine residues could be phosphorylated in mammalian cells [1] stimulated the search for the enzymes which catalyse

this process and for pathways in which tyrosine phosphorylation is involved. Although phosphorylation of the tyrosine residues in proteins is a rare cellular event, accounting for less than 0.1% of cellular phosphoproteins [2], it soon became clear that the protein tyrosine kinases (PTKs*), enzymes which catalyse the transfer of the γ-phosphate from a donor such as ATP or GTP to the acceptor tyrosine residue of a protein substrate, play a pivotal rôle as mediators of the processes involved in cell proliferation. Further investigations have shown that these enzymes are over-expressed in certain cancers, prompting the search for selective inhibitors of PTKs as potential chemotherapeutic agents.

Over the past fifteen years, a vast amount of research has been carried out to try to identify the rôle of PTKs in the general functioning and development of normal mammalian cells, and in the uncontrolled proliferation of malignant cells. Although this work is primarily concerned with PTK inhibitors, it is important to understand the structure and function of the target enzymes in order to appreciate the rationale behind the search for inhibitors. The following sections attempt, therefore, to briefly describe the classification and structure of PTKs, their mode of action, their cellular targets, and the processes in which they are involved.

BACKGROUND

CLASSIFICATION OF PROTEIN TYROSINE KINASES

The protein tyrosine kinase family of enzymes can be broadly classified into two groups; receptor protein tyrosine kinases and non-receptor protein tyrosine kinases, depending upon whether or not they possess extracellular ligand-binding domains.

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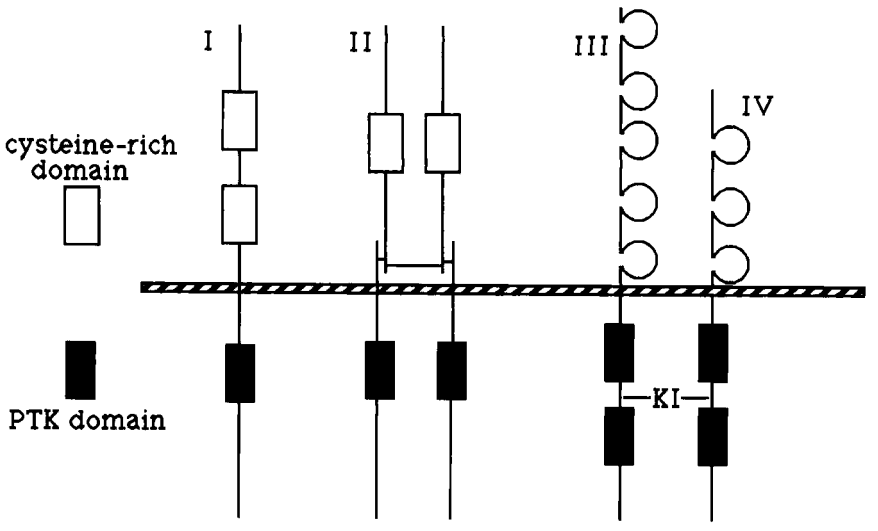
*The following abbreviations are used in this review: ATP, adenosine triphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; CSF1R, colonstimulating factor-1 receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; GTP, guanosine triphosphate; HER, human EGFR; HGF, hepatocyte growth factor; IGF1R, insulinlike growth factor receptor IR, insulin receptor; MGF, macrophage growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PIP_2, phosphatidylinositol-4,5-biphosphate; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; TGF-α, transforming growth factor-α.



	Subclass I	*Subclass II*	*Subclass III*	*Subclass IV*
Growth factor associated	EGFR TGFαR	IR IGF1R NGFR HGFR	PDGFR-A PDGFR-B CSF1R MGFR	FGFR
Oncogenic	HER2/*erb*B2 HER3/*erb*B3	*ltk/tyk-1* *ros* *trk* *met*	*kit* *fms* *flt* *ret*	*flg* *bek*

Figure 6.1. Schematic representation of the four subclasses of receptor PTKs (adapted from [4, 6]). KI = Kinase Insert

Receptor protein tyrosine kinases

Growth factor receptor PTKs have previously been reviewed in depth by Yarden and Ullrich [3] and Ullrich and Schlessinger [4]. All receptor PTKs share common structural features which are vital for their biological rôle. This homology includes a large glycosylated, extracellular ligand-binding domain, a hydrophobic single transmembrane region, and a cytoplasmic region which incorporates the kinase catalytic domain [3, 5]. Structural differences between receptor PTKs have become apparent and this has led to an extension of the classification of the receptor PTKs into four sub-classes (*Figure 6.1*) [4, 6]. It is important to note that some of the

receptor PTKs are associated with growth factors, whilst others are protein products derived from retroviral oncogenes.

The most important region, in terms of this review, is the PTK catalytic domain. This is the most highly conserved region of all the receptor PTKs, and contains a consensus sequence [3, 5, 7] that functions as part of the ATP binding site. In order to explain the multiple activities of PTKs, it has also been postulated that there are several distinct sites of interaction with substrates, regulatory factors, and cellular components involved in protein transport [4].

The catalytic activity of the receptor PTKs is initiated by the binding of the appropriate ligand to the extracellular domain. This leads to receptor oligomerisation [7] which enables interaction between the adjacent cytoplasmic domains. As a result of this interaction, autophosphorylation takes place in which the adjacent cytosolic domains of the receptor oligomer cross-phosphorylate each other on tyrosine [4 and references therein] thereby enhancing the PTK activity towards exogenous substrates.

Non-receptor protein tyrosine kinases

Non-receptor PTKs have been reviewed by Cooper [8]. These enzymes have no extracellular domain, and are contained wholly within the cell but some are associated with cell membranes and have been implicated in the signal transduction pathway as amplifiers of the signal from the receptor PTKs [9, 10].

The non-receptor PTKs, like the receptor PTKs may be sub-divided into smaller groups (*Table 6.1*) [6], of which the *src* family represents the best characterised and most extensively studied [11]. The non-receptor PTKs have a 300-amino acid sequence which shows a high degree of homology and corresponds to the kinase domain [5]. In addition, the *src* family, which includes $pp60^{c\text{-}src}$ and its retroviral precursor $pp60^{v\text{-}src}$, has other homologous regions which are not found in receptor PTKs;

(i) short amino-terminal sequences required for myristylation. This acylation is required for membrane localisation [12–14], and

Table 6.1 NON-RECEPTOR PROTEIN TYROSINE KINASES AND REPRESENTATIVES OF EACH SUBFAMILY [6]

Subfamily	*Proto-oncogenes*
src	*blk, fgr, fyn, hck, lck, lyn, src, yes*
fes	*fer/tyk-3, fes/fps*
abl	*abl, arg*

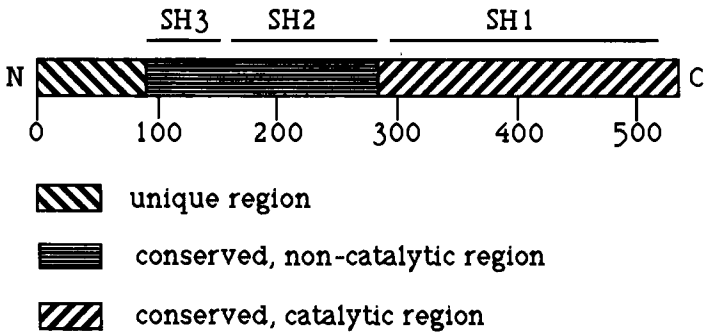


Figure 6.2. Schematic representation of non-receptor tyrosine kinases [15, 16]

(ii) two additional domains named *src* homology 2 and 3 (SH2 and SH3) (*Figure 6.2*) [15, 16], the function of which is not fully understood as yet. These domains are non-catalytic but the evidence suggests that they are involved in the regulation of both the PTK and transforming activity [17].

The rôle of the SH2 domain is more clearly understood than that of the SH3 domain. It is believed that the SH2 domain has multiple functions which include regulation of the kinase activity and the recruitment of specific substrates to the cell membrane. These effects may well be associated with the ability of the SH2 domain to recognise phosphorylated tyrosine residues [15, 16, 18].

It has been proposed that a number of protein kinases undergo self-regulation *via* a mechanism whereby a small polypeptide sequence, from a regulatory region of the protein kinase itself, inhibits the catalytic domain by acting as a competitive inhibitor of exogenous substrates [19]. Such an inhibitory rôle has been suggested for the amino-terminal sequences of $pp60^{c\text{-}src}$ (SH3 region) where it is proposed that the amino-terminal substrate-like sequences bind to the carboxy terminal catalytic domain [20].

CELLULAR SUBSTRATES OF PROTEIN TYROSINE KINASES

Despite the substantial differences between the receptor and non-receptor PTKs, the nature of their cellular substrates appears to be relatively consistent. The process of autophosphorylation distinguishes PTKs from the activities of serine/threonine kinases, and is particularly notable as, initially, substrates other than PTKs themselves proved to be elusive. However, the fact that phosphotyrosine residues may act as binding sites for cytosolic proteins containing SH2 domains gives a clue as to the structural

requirements of PTK substrates. Four major substrates with potential regulatory activity have been identified as substrates of both types of PTKs [17 and references therein]; phosphatidylinositol 3-kinase (ptdIns-3K), phospholipase C-γ (PLCγ), *ras* GTPase activating protein (*ras*GAP), and pp74$^{c\text{-}raf}$ (a serine/threonine kinase). PtIns-3K, PLCγ and *ras*GAP all contain SH2 domains, whilst it is possible that pp74$^{c\text{-}raf}$ is associated with another protein that contains SH2 domains. Many other proteins have been shown to be phosphorylated on tyrosine in cells transformed by *src* and related oncogenes. These do not contain SH2 domains and do not appear to associate closely with PTKs. Further substrates with SH2 domains have also been elucidated [21].

Growth factors induce the phosphorylation of tyrosine in numerous cellular substrates and this allows for the possible formation of further SH2 domain-phosphotyrosine complexes, thereby creating the potential for a cascade of cytoplasmic tyrosine kinases which amplify the initial growth factor signal and ultimately lead to the activation of enzymes such as protein kinase C (PKC). PKC, a serine/threonine kinase, appears to play a pivotal rôle in the convergence of the PTK signalling pathways with the serine/threonine pathway but the interaction of these multiple pathways is still far from understood.

CELLULAR RESPONSES TO PROTEIN TYROSINE KINASE ACTIVITY

It is clear that PTKs play a pivotal rôle in the general functioning and development of human cells. However, in a number of diseases including certain cancers, atherosclerosis, hypertension and psoriasis, the cells have lost their ability to regulate the activity of PTKs. This loss of regulatory ability may be a result of the mutation of proto-oncogenes to give oncogenes, inducing conformational changes and thereby relaxing the negative constraints usually exerted on the kinase domain. The PTKs thus become continuously activated, resulting in the uncontrolled proliferation characteristic of these diseases. However, the most common cause of proliferative diseases appears to involve autocrine activation in conjunction with receptor over-expression. Thus, many tumours and tumour cell-lines have been shown to co-express growth factors and their specific receptors [4].

The irregular expression of PTKs in human cancers has been extensively observed (*Table 6.2*) [6]. As seen from this list, the over-expression of PTKs appears to be widespread amongst some of the most common types of malignancy, and it would seem likely that investigations of other neoplasms will divulge associated PTKs.

Table 6.2 PROTEIN TYROSINE KINASES AND HUMAN CANCER (ADAPTED FROM [6])

cancer	*associated PTKs*
breast	EGF-R/TGFα-R, IGF1-R, CSF1-R/*fms*, FGF-R/*bek*/*flg*, HER-2/*neu*/*erb*B2
lung	EGF-R/TGFα-R, IGF1-R, HER-2/*neu*/*erb*B2, *kit*
ovarian	EGF-R/TGFα-R, IGF1-R, HER-2/*neu*/*erb*B2
brain	EGF-R/TGFα-R, FGF-R/*bek*/*flg*, *bcr-abl*, *ros*
leukaemias	CSF1-R/*fms*, *bcr-abl*, *lck*
colon	NGF-R/*trk*, *lck*, *src*
stomach	HER-2/*neu*/*erb*B2
pancreas	EGF-R/TGFα-R, IGF1-R, PDGF-R
bladder	EGF-R/TGFα-R, *src*
kidney	EGF-R/TGFα-R, *src*
prostate	FGF-R/*bek*/*flg*
oesophagus	EGF-R/TGFα-R, IGF1-R
uterus	IGF1-R
colorectum	EGF-R/TGFα-R
midgut	PDGF-R
squamous cell carcinoma	EGF-R/TGFα-R
neuroblastoma	NGF-R/*trk*, *ret*
soft tissue	PDGF-R
thyroid	*ret*
nose	EGF-R/TGFα-R

The significance of the PTKs in the regulation of cell growth makes them important targets for the development of specific inhibitors as potential chemotherapeutic agents. The inhibition of specific signal-transduction processes activated by oncogenes could lead to the reversal of cell transformation, without destroying the cell. In view of the side-effects observed with current anti-cancer treatments, drugs which preserve cell function are highly desirable and should be the future of anti-proliferative therapy.

ASSAYS FOR DETERMINING THE ACTIVITY OF PROTEIN TYROSINE KINASE INHIBITORS

The discovery of new PTK inhibitors requires a screening system which is both rational and convenient. The screening system used usually belongs to one of three methodologies; *in vitro*, *in situ*, or *in vivo* studies.

In vitro studies are generally used to determine the potency and specificity

of inhibitors. This involves the isolation of a PTK as the purified or partially purified (an immunocomplex) enzyme, and the inhibitor is assayed against the enzyme to determine its ability to prevent either the phosphorylation of a specific peptide substrate or the autophosphorylation of the PTK itself. The inhibition is assessed by monitoring the rate of transfer of [γ-^{32}P]ATP to the tyrosine residue of the substrate or the PTK. Both of these methods provide a measure of the potency of an inhibitor towards a particular PTK, but the former method has the additional advantage of allowing kinetic measurements to be made in order to determine the mechanism of inhibition, that is, does the inhibitor compete at the substrate or ATP binding site of the kinase domain, or does it exert its effect by an allosteric mechanism? A problem with the former method, however, is one of substrate specificity; phosphorylation of different peptide substrates by the same enzyme may be inhibited to different degrees by the same compound. It is not practicable, therefore, to compare the results from different assays unless the same protocol is used. *Table 6.3* [22] shows the diversity of these *in vitro* assays. Ideally, *in vitro* studies should employ more than one PTK as some potent inhibitors are specific towards certain PTKs and inactive against others. It is also useful to test PTK inhibitors against other protein kinases, including PKA, PKC and other serine/threonine kinases, in order

Table 6.3 EXAMPLES OF THE *IN VITRO* ASSAYS USED TO MEASURE PTK ACTIVITY [22]

PTK	*Substrates*	*Source*
EGFR	autophosphorylation; GAT; angiotensin II; RR-*SRC*	A431 cells (human squamous carcinoma)
PDGFR	autophosphorylation	NIH3T3 cells (normal mouse fibroblasts)
IR	autophosphorylation	placenta; liver
pp60^{src}	i) autophosphorylation; enolase; α-casein	RSV-transformed NIH3T3 cells
	ii) autophosphorylation; α-casein	tsNRK cells (mutant RSV transformed normal rat kidney cells)
	iii) autophosphorylation; enolase	TMK-1 cells (human gastric carcinoma)
p185^{erbB2}	i) autophosphorylation	TMK-1
	ii) autophosphorylation	NIH3T3 cells transfected with HER1-2 chimeric protein
p56^{lck}	autophosphorylation; enolase; angiotensin I	bovine thymus
p210$^{bcr\text{-}abl}$	autophosphorylation; GAT	K562 human myeloid leukaemia
p60$^{v\text{-}abl}$	angiotensin I; α-casein	recombinant v-*abl* expressed in *E. coli*

to demonstrate their specificity. Such studies have been rare but Barret *et al.* have shown the way forward with their study of the inhibition of 6 protein kinases by 14 inhibitors, in which they employ 6 well-described and at least partially purified protein kinases, including serine/threonine kinases and receptor and non-receptor PTKs [23]. The substrate used for all the PTKs was the same, poly(E,Y)1-4, a polymeric mixture of peptide chains containing glutamic acid and tyrosine in a 4:1 ratio [24].

In addition to observing the potency of an inhibitor *in vitro*, it is necessary to consider its activity in intact cells. These studies give useful information as to whether there is a correlation between the inhibition of the isolated enzyme and the enzyme *in situ*. This may be considered by assessing specific phosphotyrosine levels in whole cells, or more usually, by measuring the inhibition of cell proliferation (by assaying the amount of [^{3}H]-thymidine incorporated during DNA synthesis). As well as giving information about PTK inhibition, *in situ* studies indicate possible cytotoxic effects and the pharmacokinetic profile of the inhibitor; potent *in vitro* inhibitors often have lower activity *in situ* due either to their inability to permeate the cell membrane, or to metabolic degradation.

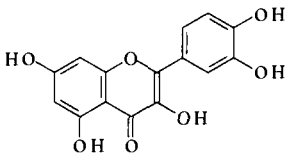
If potent and selective activity is observed *in vitro* and/or *in situ*, it is appropriate to test the inhibitors *in vivo*. For PTK inhibitors, it is usual to assess their anti-proliferative effects in cancer cell-lines which are known to over-express PTK activity. These studies, however, cannot confirm that any arrest of cell growth is due exclusively to PTK inhibition.

In the following sections, the term IC_{50} is used as a measure of the potency of an inhibitor and is defined as the concentration that inhibits the activity of the enzyme (or cell proliferation) by 50% compared to a control.

PROTEIN TYROSINE KINASE INHIBITORS

FLAVONOIDS AND RELATED COMPOUNDS

The discovery that the Rous sarcoma virus *src*-gene product, $pp60^{v\text{-}src}$, displays PTK activity stimulated the search for PTK inhibitors [25–27]. Graziani and co-workers had observed that the cyclic-AMP (cAMP) independent protein kinase activity of Ehrlich ascites tumour cells was inhibited by the flavone quercetin (1), whilst cAMP-dependent protein kinase activity was unaffected. In pursuing these findings, Graziani *et al.* found that quercetin inhibited the tyrosine phosphorylation activity of $pp60^{v\text{-}src}$ both in *in vitro* and *in vivo* (K_i = 6–11 μM) [28]. In addition, this inhibition was found to be competitive with respect to ATP or GTP, but



(1)

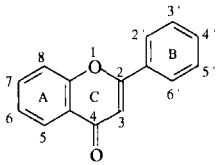
non-competitive with respect to the protein substrate. As with the earlier findings, cAMP-dependent protein kinase activity was unaffected.

Unfortunately, this competitive inhibition with respect to ATP means that quercetin also has the potential to interact with nucleotide binding sites in protein serine/threonine kinases [28–33] and quercetin was thus thought to have little value as a therapeutic candidate. Later studies, however, have shown that inhibition of PTKs by competition with ATP does not necessarily mean that an inhibitor will be non-selective.

Quercetin had previously been found to form a strong fluorescent complex with ATP [34], and it is possible that the mechanism of inhibition of protein kinases by quercetin may be associated with a type of hydrogen-bonded complex, although the fact that quercetin does not inhibit ATP in all protein kinases detracts from this theory somewhat. Ferrel *et al.* have suggested that the similarity between the charge distribution in the pyranone ring of flavones and the pyrimidine ring of ATP may account for the inhibitory activity of flavonoids in many ATP-binding enzymes [35].

Flavones

The flavones have been one of the most extensively studied group of PTK inhibitors, and these studies have enabled detailed structure-activity relationships to be made. The data in *Table 6.4* show the inhibition of three PTKs by 20 naturally occurring flavones [36–38] listed in descending order of the number of hydroxyl substituents. A number of trends become apparent immediately, the most notable being that inhibitory activity is



(2)

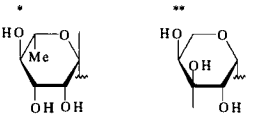
generally improved by increasing the number of hydroxyl functions. Hydroxyl groups at the 5- and 7-positions as well as the 4′-position seem to be important for inhibitory activity, although two out of these three generally confer as much activity as all three. The derivation of many natural flavones from phloroglucinol often gives this useful 5,7-dihydroxy relationship.

It is noticeable that those flavones substituted only with hydroxyl residues show reasonable activity irrespective of their position, except for morin

Table 6.4 INHIBITION OF PTKS BY NATURALLY OCCURRING FLAVONES [36-38]

										$IC_{50}(\mu M)$		
Flavone	*3*	*5*	*6*	*7*	*8*	*2′*	*3′*	*4′*	*5′*	*EGFR*	*p40*	*p56^{lck}*
Myricetin (2–1)	OH	OH	–	OH	–	–	OH	OH	OH	–	35	31
Quercetin (1)	OH	OH	–	OH	–	–	OH	OH	–	17	36	26
Robinetin (2–2)	OH	–	–	OH	–	–	OH	OH	OH	–	23	–
Morin (2–3)	OH	OH	–	OH	–	OH	–	OH	–	–	–	166
Kaempferol (2–4)	OH	OH	–	OH	–	–	–	OH	–	11	–	28
Fisetin (2–5)	OH	–	–	OH	–	–	OH	OH	–	–	122	–
Myricitrin (2–6)	*	OH	–	–	–	–	OH	OH	OH	–	>1150	–
Datiscetin (2–7)	OH	OH	–	OH	–	OH	–	–	–	–	–	210
Luteolin (2–8)	–	OH	–	OH	–	–	OH	OH	–	–	–	14
Apigenin (2–9)	–	OH	–	OH	–	–	–	OH	–	93	332	15
Galangin (2–10)	OH	OH	–	OH	–	–	–	–	–	–	–	296
Resokaempferol (2–11)	OH	–	–	OH	–	–	OH	–	–	–	–	19
Kaempferide (2–12)	OH	OH	–	OH	–	–	–	OMe	–	–	–	100
Kaempferol-O^3-α-rhamnoside (2–13)	*	OH	–	OH	–	–	–	OH	–	–	–	>950
Kaempferol-O^3-α-arabinoside (2–14)	**	OH	–	OH	–	–	–	OH	–	–	–	>500
Chrysin (2–15)	–	OH	–	OH	–	–	–	–	–	–	–	20
Gonkwanin (2–16)	–	OH	–	OMe	–	–	–	OH	–	–	–	292
Nevadensin (2–17)	–	OH	OMe	OH	OMe	–	–	OMe	–	–	140	–
Acacetin (2–18)	–	OH	–	OH	–	–	–	OMe	–	146	–	–
Flavone (2–19)	–	–	–	–	–	–	–	–	–	225	–	–

Protein tyrosine kinases – (i) EGFR (epidermal growth factor receptor) was isolated from A431 cell membranes; (ii) p40 was purified from bovine thymocytes; (iii) p56^{lck} was isolated from bovine thymus.



(2-3) and datiscetin (2-7), which possess a hydroxyl at the 2′-position, and galangin (2-9). Indeed, galangin appears to be an anomaly as it is 16-fold less potent than chrysin (2-15), the only difference being a hydroxyl at the 3-position. Conversely, the addition of the 3-hydroxyl residue to resokaempferol (2-11) increases the potency by one order of magnitude compared with 4′,7-dihydroxyflavone (*Table 6.5*, 2-35).

Glycosylation of the 3-hydroxyl group of (2-6), (2-13) and (2-14) markedly reduces inhibitory activity, suggesting that a bulky group at this position prevents the interaction of the flavone at the active site of the enzyme. In addition, the replacement of hydroxyl substituents with methoxy groups generally reduces activity, suggesting that hydrogen-bonded interactions are important in the inhibition of PTKs by flavonoids.

The results in *Table 6.5* show data for the inhibition of the lymphoid restricted PTK, $p56^{lck}$, by 20 synthetic flavones containing only hydroxyl or methoxy groups [39, 40]. These results tend to confirm the trends seen with the naturally occurring flavones. A single hydroxyl group confers little activity (2-20) and (2-36), whereas a second hydroxyl substituent may give potent activity (2-30), (2-31), and (3-33). The activity of (2-31) is particularly surprising as none of the naturally occurring flavones possess the 3′,

Table 6.5 INHIBITION OF THE $p56^{lck}$ PTK BY SYNTHETIC FLAVONES [39,40]

compound	*3*	*5*	*6*	*7*	*8*	*3′*	*4′*	*5′*	$IC_{50}(\mu M)$
(2-20)	–	–	–	–	–	–	OH	–	504
(2-21)	–	–	–	–	–	OMe	OH	OMe	26
(2-22)	–	–	–	OH	OH	OMe	OH	OMe	121
(2-23)	–	–	OH	–	–	OMe	OH	OMe	131
(2-24)	–	–	–	OH	–	OMe	OH	OMe	>1000
(2-25)	OH	OH	–	OH	–	OMe	OH	OMe	69
(2-26)	–	–	–	–	–	OMe	OMe	OMe	>2000
(2-27)	–	–	–	OH	–	OMe	OMe	OMe	61
(2-28)	–	–	–	OH	OH	OMe	OMe	OMe	395
(2-29)	–	OH	–	–	–	OH	–	–	315
(2-30)	–	–	OH	–	–	OH	–	–	16
(2-31)	–	–	–	OH	–	OH	–	–	39
(2-32)	–	–	–	OH	OH	OH	–	–	178
(2-33)	–	OH	–	–	–	–	OH	–	16
(2-34)	–	–	OH	–	–	–	OH	–	118
(2-35)	–	–	–	OH	–	–	OH	–	169
(2-36)	–	–	–	OH	–	–	–	–	336
(2-37)	–	–	–	OH	OH	–	–	–	315
(2-38)	OH	–	–	OH	OH	–	–	–	296
(2-39)	OH	–	–	OH	–	OH	OH	–	14

6-dihydroxy relationship. The lack of substituents on the B-ring reduces activity [except for chrysin (2-15), *Table 6.4*], and two adjacent hydroxyls on the A-ring similarly reduces inhibition [(2-27) *vs.* (2-28) and (2-31) *vs.* (2-32)].

As with the naturally occurring compounds most flavones possessing a 4′-hydroxyl and any other group are relatively active. A surprising observation was the activity of (2-21) with two methoxy groups surrounding the 4′-hydroxyl. The extrapolation of this relationship to include compounds with substituents in the A-ring reduced activity (2-22)-(2-25). Even more remarkable was the reasonable inhibitory activity shown by the trimethoxy derivative (2-27).

Cushman *et al.* have also prepared a series of 29 flavones with a methoxycarbonyl or carboxyl substituent at the 3-position [39]. The IC_{50} values were greater than 2 mM for all of these compounds against the $p56^{lck}$ PTK, irrespective of the substituents on the A- and B-rings. This correlates well with the fact that bulky substituents at this position reduce the inhibitory activity of the natural flavones. It may thus be assumed that PTK inhibition by flavones may only be achieved with either no substituent, or a hydroxyl group at the 3-position.

The substitution of groups other than hydroxyls has generally been unsuccessful in preparing potent PTK inhibitors. However, Cushman *et al.* [39] synthesized four flavones incorporating an amino residue at the 4′-position, which appears to be an important position for potent flavone inhibitors (*Table 6.6*). Compound (2-40) was more potent than its hydroxyl equivalent (2-20), whilst (2-41) was equipotent with its counterpart (2-35). Compounds (2-42) and (2-43) are the most potent flavone inhibitors of $p56^{lck}$ to date, (2-42) being 100 times more potent than its hydroxyl analogue (2-34). A subsequent study on hydroxylated and/or nitrated amino flavones [41], although failing to produce more potent inhibitors, showed that substitution of amino by nitro, or relocation of the amino group to the 3′-position, resulted in a decrease in activity. Thus, it was demonstrated that

Table 6.6 INHIBITION OF THE $p56^{lck}$ PTK BY AMINO-SUBSTITUTED FLAVONES [39]

compound	*5*	*6*	*7*	*4′*	*IC_{50} (μM)*
(2-40)	–	–	–	NH_2	210
(2-41)	–	–	OH	NH_2	138
(2-42)	–	OH	–	NH_2	1.2
(2-43)	OH	–	OH	NH_2	7.4

substituents other than hydroxyl could be used in the preparation of potent flavone PTK inhibitors, and the activity of inhibitors containing amino groups seems to reinforce the proposition that hydrogen-bonding plays an important role in the mechanism of action of these compounds within the catalytic domain.

Cunningham *et al.* prepared a number of flavones with substituents only on the B-ring [42]. In agreement with the above results, these workers found that an amino residue at the 3′- or 4′-position produced the most active compounds (*Table 6.7*) in terms of the inhibition of cell proliferation of normal 3T3 murine fibroblasts and ANN-1 cells (3T3 fibroblasts transformed by the Abelson murine leukaemia virus). The most active inhibitor, 3′-amino-4′-methoxyflavone (2-46), was inactive against the PTK pt*abl*50 from the Abelson virus, but inhibited the PTK activity of the EGF receptor derived from A431 cells by 42% at a concentration of 50 μM. It seems likely, therefore, that the cytotoxic effect of these compounds is mediated by a process other than the inhibition of PTKs, particularly as there is little selectivity of cytotoxic effect on the ANN-1 over the 3T3 cells.

Certain generalizations can thus be made with regards to the structural features required for PTK inhibition by the flavones. Most studies on the flavones have, however, centred around the pursuit of potency, rather than investigating their selectivity or mechanism of action. Hagiwara *et al.* [32] attempted to redress this balance by investigating the biological activities of seven flavones with zero to six hydroxyl substituents [flavone (2-19), 7-hydroxyflavone (2-36), chrysin (2-15), apigenin (2-9), fisetin (2-5), quercetin (1) and myricetin (2-1)], and found that inhibitory potencies for the pp130^{fps} and IR PTKs correlated well with the number of hydroxyl

Table 6.7 ACTIVITIES OF SUBSTITUTED FLAVONES AGAINST PROLIFERATION OF ANN-1 AND 3T3 CELLS [42]

			$IC_{50}(\mu M)$	
compound	*3′*	*4′*	*ANN-1*	*3T3*
(1)			22–43	29–50
(2-40)		NH_2	10–20	18
(2-44)	NH_2	–	24–32	20
(2-45)	NH_2	Cl	8–27	12–25
(2-46)	NH_2	MeO	0.8–2.5	8
(2-47)	MeO	NH_2	20–35	38–54
(2-48)	OH	OH	8–16	19–26
(2-49)	Cl	Cl	5–20	58
(2-50)	MeO	MeO	23–35	23–36

residues. They also found that these compounds inhibited several serine/threonine kinases but that here the extent of hydroxylation had less influence.

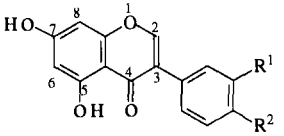
Myricetin (2-1) was found to inhibit the IR PTK (K_i = 2.6 μM) and pp130^{fps} (K_i = 1.8 μM), but was inactive against the PTK of the particulate fraction of human platelets. Kinetic analysis indicated that myricetin was competitive with respect to ATP in the oncogenic PTK pp130^{fps} but non-competitive with respect to ATP in the insulin receptor. These workers, therefore, classified three types of PTK:

(i) Susceptibility to flavonoids, ATP competitive (pp130^{fps});
(ii) Susceptibility to flavonoids, ATP non-competitive (IR);
(iii) Resistance to flavonoids (human platelet PTK).

These workers have attempted to explain these observations in terms of a flavonoid binding site at, or near, the ATP binding site of the oncogene-encoded PTKs but remote from the ATP binding site of the insulin receptor. Kinetic analysis of the mode of inhibition of other PTK inhibitors seems to confirm the existence of inhibitor binding sites separate from the ATP and peptide substrate binding sites (see erbstatin and lavendustin A).

Isoflavonoids

The isolation of orobol (3), from *Streptomyces neyagawaensis* var. *orobolere*, constituted the first report of an isoflavone PTK inhibitor [43]; this compound inhibited the EGFR PTK activity of membranes from A431 cells, with an IC_{50} of 11 μM. It was, however, the isolation of the *Pseudomonas* product genistein (4) [44] (a structural isomer of orobol) that provided evidence that flavonoid and/or isoflavonoid compounds could indeed be considered as potential therapeutic agents. Whereas quercetin also inhibited serine/threonine kinases, genistein showed a surprising specificity

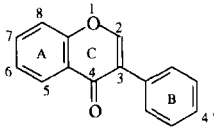


(3) R^1=OH, R^2=H
(4) R^1=H, R^2=OH

as a PTK inhibitor. Thus, Akiyama *et al.* [45] showed that genistein (4) inhibited pp60$^{v\text{-}src}$, pp110$^{gag\text{-}fes}$ and the purified EGFR PTK, with IC_{50} values of 26, 24 and 22 μM, respectively, but that it failed to inhibit the

serine/threonine phosphorylation of five other enzymes at levels in excess of 350 μM. In addition, it was found that inhibition of the EGFR PTK activity was competitive with ATP but, unlike quercetin, genistein was able to discriminate between the catalytic sites for ATP in the protein kinases tested. *In situ* studies in EGF-stimulated A431 cells indicated that genistein lowered the levels of phosphotyrosine. These results, probably more so than the earlier findings with quercetin, led to increased interest in flavonoids and/or isoflavonoids as PTK inhibitors, as for the first time a compound with high specificity for PTKs had been found.

Subsequently, the biological effects of genistein have probably been investigated more thoroughly than for any other PTK inhibitor. However, little effort has been applied to the preparation of synthetic analogues, the single report being by Ogawara *et al.* who prepared 23 isoflavone derivatives [36]. The rationale behind the synthesis of these isoflavone derivatives was based on comparisons between genistein (4) and other natural isoflavonoids (*Table 6.8*). Hydroxyl groups at the 5- and 4′-positions seem to be essential. A bulky group at the 7-position (5d) abolishes activity, whilst a hydroxyl group would appear to be preferable. In this case, compounds were generally prepared in such a way as to conserve the basic



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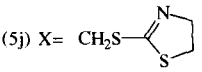


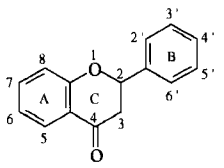
Table 6.8 INHIBITION OF THE AUTOPHOSPHORYLATION OF THE EGFR BY ISOFLAVONOIDS [36]

compound	*2*	*5*	*7*	*4′*	$IC_{50}(\mu M)$
Genistein (4)	–	OH	OH	OH	2.6
Prunetin (5a)	–	OH	OMe	OH	15
Diadzin (5b)	–	–	OH	OH	>400
Biochanin A (5c)	–	OH	OH	OMe	92
Genistin (5d)	–	OH	OGlu	OH	>400
(5e)	CO_2Et	OH	OH	$OCOCO_2Et$	2.3
(5f)	CO_2H	OH	OH	OH	6.5
(5g)	CO_2Et	OH	OH	OH	5.8
(5h)	CO_2Me	OH	OH	OH	6.1
(5i)	Me	OH	OH	OH	7.0
(5j)	X	OH	OH	OH	12.5

structure of genistein but with a modification at the 2-position. When the 4′-, 5- and 7-hydroxy groups were present, reasonable activity was obtained (6–12 μM), irrespective of the 2-substituent, although the compounds (5f-i) with relatively small substituents were the most active. Bulkier groups, as in (5j), were less active, but the major difference in activity occurred when the 4′-hydroxyl group was replaced. Generally, activity was abolished (compounds not shown), but in one case, (5e), activity was maintained at a similar level to genistein. The inhibitory profile of these synthetic isoflavonoids was similar to genistein with a high specificity for PTKs.

In addition, Geahlen *et al.* demonstrated that genistein and biochanin A failed to inhibit the PTK p40 at levels greater than 1800 μM [37]. Thus, it is apparent that the isoflavonoid PTK inhibitors show selectivity between PTKs.

Genistein has proved to be a useful molecular tool for probing the biochemical pathways and biological processes in which PTKs may be implicated. Dean *et al.* [46] showed that genistein inhibited mitogenesis induced by PDGF and EGF in mouse 10T1/2 fibroblasts and rat liver TS113 epithelial cells, but not responses induced by angiotensin or ATP, suggesting that the effects of genistein are due to the inhibition of tyrosine phosphorylation. These effects were shown to be associated with inhibition of phosphatidylinositol turnover and calcium mobilization in response to ligand activation of the growth factor receptor. Similar observations were reported for the inhibition of agonist-stimulated human platelets [47], thrombin-induced platelet aggregation [48–50], EGF-stimulated A431 cells [51] and rat hepatocytes [52]. Linassier *et al.* [53], however, showed that the cytostatic effect of genistein on NIH3T3 cells did not appear to be mediated by the inhibition of the EGFR PTK, but possibly through inhibition of the ribosomal S6 kinase. Similarly, Abler *et al.* [54] found that genistein inhibited responses to insulin in rat adipocytes without blocking insulin's stimulation of receptor autophosphorylation or substrate tyrosine phosphorylation by the receptor kinase, indicating that genistein blocks the effects of insulin downstream from the insulin receptor and that the effects cannot necessarily be attributed to inhibition of PTK activity.



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Further evidence suggests that mitogenic responses inhibited by genistein may be due to inhibition of both topoisomerase I and II [55, 56]. The mode of action of this inhibition is different from other topoisomerase inhibitors which tend to be more cytotoxic [55]. Additionally, genistein was shown to induce topoisomerase II-dependent DNA cleavage *in vitro* [57, 58] without acting as an intercalating agent, but possibly through interaction at the ATP binding site. It has been suggested that inhibition of topoisomerase II may account for the apparent inducement of apoptosis by genistein in human thymocyte cultures, rather than through inhibition of PTKs [59].

Genistein has also been used to demonstrate the involvement of PTK activity in the stimulation of eicosanoid biosynthesis (leukotrienes and prostaglandins), possibly by inhibition of interleukins [60–62].

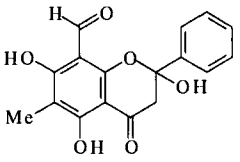
Flavanones

The inhibition of PTKs by flavanones has had limited attention compared with other flavonoids. Geahlen *et al.* investigated the activity of a series of six flavanones on the bovine thymocyte PTK p40 (*Table 6.9*) [37]. Clearly, these compounds appear relatively inactive compared to their corresponding flavones, indicating that the planar nature of the C-ring is an important feature for inhibitory activity. However, it is also apparent that several known PTK inhibitors, for example, genistein (4), have little effect on p40; therefore, p40 may not be the best enzyme to assess the potency of certain compounds, and these results reinforce the need for the wider screening of potential inhibitors.

To underline this, a recent report has shown that a flavanone, desmal (7), from the tropical plant *Desmos chinensis* [63], inhibited EGF-stimulated A431 membrane fractions (EGFR PTK) with an IC_{50} of 8 μM (similar to the most potent polyhydroxylated flavones). Surprisingly, desmal was competitive with the peptide substrate and was non-competitive with ATP. It did not inhibit PKC or phosphatidylinositol kinase at 300 μM, but did inhibit

Table 6.9 INHIBITION OF THE p40 PTK BY FLAVANONES [37]

compound	*3*	*5*	*7*	*3′*	*4′*	IC_{50} *(μM)*
Fustin (6a)	OH	–	OH	OH	–	>1000
Taxifolin (6b)	OH	OH	OH	OH	OH	888
Pinocembrin (6c)	–	OH	OH	–	H	>1000
Naringenin (6d)	–	OH	OH	–	OH	294
(6e)	–	OH	OH	–	OMe	>1000
(6f)	–	–	OH	–	–	>1000



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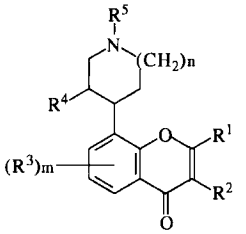
morphological changes induced by EGF and intracellullar tyrosine phosphorylation in NIH3T3 cells at 80–160 μM. These unexpected results suggest that desmal may act as a lead compound for further investigation into flavanones as PTK inhibitors.

Benzopyranones

Naik *et al.* have patented a series of 4*H*-1-benzopyran-4-one tyrosine kinase inhibitors, of general structure (8), as antitumour agents [64].



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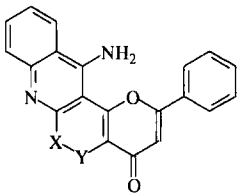
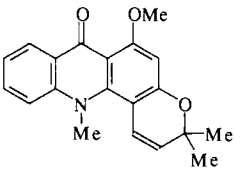


(9a) X,Y= CH=CH
(9b) X,Y= CH_2CH_2

Pyranoacridinones and benzophenanthrolines

In an attempt to prepare novel PTK inhibitors we have synthesized a number of pyranoacridinones, for example, (9), which incorporate structural elements of both the phosphate donor and the flavones [65]. It was hoped that these species would bind specifically at the flavone and/or ATP binding site of the oncogene-encoded PTKs, proposed by Hagiwara *et al.* [32], and so be specific inhibitors of these PTKs.

12-Amino-2-phenylpyrano[2,3-*a*]acridin-4-one (APPA) (9a) and its dihydro derivative (9b) were tested in a biological assay [66] for inhibition of growth factor-mediated cell proliferation along with two naturally occurring pyranoacridines, acronycine (10) and norisoacronycine (11) and the



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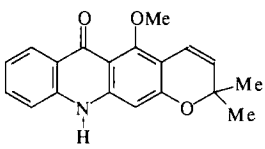

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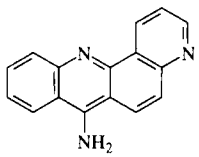

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results are shown in *Table 6.10*. DHER cells, NIH3T3 cells which over-express the EGF receptor, can be stimulated to proliferate by either EGF or calf-serum. The inhibition of this proliferation can be assayed by monitoring of (^{3}H-Me) thymidine uptake by cells treated with the inhibitor and comparing this with that of the control cells. The data in *Table 6.10* shows that APPA (9a) is a selective inhibitor of the EGF-dependent proliferation of these cells with an IC_{50} of 1.9 μM compared with that for inhibition of the calf-serum-dependent proliferation of >> 10μM. None of the pyranoacridines tested inhibited EGFR autophosphorylation or the phosphorylation of the peptide substrate polyGAT by the EGFR.

In another study, a series of pyranoacridines, for example, (9), and benzo[*b*][1,7]phenanthrolines, for example, (12), were tested as inhibitors of the spontaneous proliferation — cell growth not specifically induced by the stimulation of EGF or foetal calf serum (FCS) — of a human derived gastric carcinoma cell-line MKN 45 [67]. APPA inhibited the proliferation of these cells with an IC_{50} of approximately 105 nM but was non-cytotoxic at concentrations below 100mM. 7-Aminobenzo[*b*][1,7]phenanthroline (12) was also a potent inhibitor of the proliferation of MKN45 cells with an IC_{50} below 1 μM. Once again, cell viability studies showed that these species were

Table 6.10 ANTI-PROLIFERATIVE ACTIVITY OF PYRANOACRIDINONES AND BENZOPHENANTHROLINES [65,67]

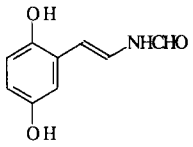
	$IC_{50}(\mu M)$			
	DHER cells		*MKN45 cells*	*% cell viability*
compound	*EGF-dependent*	*CS-dependent*	*spontaneous*	*MKN 45 cells*
(9a)	1.9	>>10	0.105	>95
(9b)	5.8	nd	0.5	>95
(10)	3.6	>>32	nd	nd
(11)	6.5	nd	nd	nd
(12)	nd	nd	<1	>90

non-cytotoxic. In both the pyranoacridine and benzophenanthroline series the fully aromatic compounds were more active than their dihydro derivatives (*cf.* flavones and flavanones).

STYRENE AND STILBENE DERIVATIVES

Erbstatin and its analogues

Erbstatin (13) was isolated in 1986 from a strain of *Streptomyces* by Umezawa *et al.* [43] who found that this compound inhibited the PTK activity of A431 cell membrane fractions (EGFR autophosphorylation) with an IC_{50} of 3 μM and the growth of A431 cells *in situ* with an IC_{50} of 20 μM. The structure of erbstatin was determined by X-ray crystallography and was shown to consist of two planar components — the benzene ring and the *N*-formylaminoethenyl side-chain — with both groups being almost co-planar [68]. This corresponds well with other PTK inhibitors which require planarity for activity.



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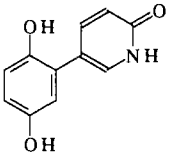


(14)

The structure of erbstatin (13) may be considered to resemble tyrosine, or more specifically the conformation of tyrosine within a peptide. It was not surprising, therefore, when it was reported that erbstatin was competitive with respect to the peptide substrate and non-competitive with respect to ATP for the inhibition of the EGFR PTK purified from A431 cells [69]. Erbstatin also appeared to be specific for tyrosine kinases as it was reported that it did not inhibit PKC [69] and its inhibitory action against cAMP-dependent protein kinase was much weaker (IC_{50} ~ 550 μM) [43]. These results, however, were contradicted by Bishop *et al.* [70] who demonstrated that erbstatin was a relatively potent inhibitor of PKC (IC_{50} = 19.5 μM), and that this inhibition was competitive with respect to ATP and non-competitive with respect to the phosphate acceptor. Similar potency was observed against three PKC isozymes (α, β and γ) and other serine/threonine kinases. Further contrary kinetic information has recently been reported by Hsu *et al.* [71] who observed that erbstatin inhibited the EGFR PTK as a partial competitive inhibitor with respect to both ATP and

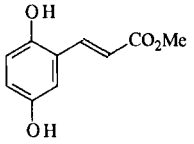
the peptide substrate, suggesting that it binds at a site distinct from the ATP and peptide binding sites of the enzyme, and thus lowers the affinities of the enzyme for both substrates. A structurally-related compound, RG 14921 (14), was a non-competitive inhibitor with respect to both substrates. These distinct modes of action by similar compounds suggest a dynamic and possibly extended structure of the catalytic centre of the kinase domain of the receptor, which is subject to various subtle changes on the initial interaction with an individual effector, subsequently forming a stable enzyme-inhibitor complex.

In addition, an early finding had shown that erbstatin inhibited autophosphorylation and internalisation of the EGFR in cultured cells, but that the inhibition of EGFR activity required much higher concentrations than were necessary to arrest cell growth [72]. Thus, the specificity of erbstatin for PTKs has been challenged, and with it the potential of erbstatin as a therapeutic agent. The action of erbstatin in *in vivo* studies is further complicated by its inherent instability to iron-catalysed oxidative decomposition in animal serum [73]. Indeed, it was shown that erbstatin completely disappeared after 30 min in calf serum [74]. To overcome this problem, erbstatin may be given with an iron-chelating agent (foroxymithine) when assessing *in vivo* activity; when this combination was used, erbstatin displayed anti-tumour activity in mice bearing the L1210 leukaemia [73] and inhibited the growth of MCF-7 human breast carcinoma cells in nude mice [75], as well as arresting the growth of four oesophageal tumours. The implication is that the anti-neoplastic activity is due to inhibition of PTK activity, but this may be disputed by the lack of effect on the Br-10 human breast carcinoma cell-line [75].

Takekura *et al.* demonstrated that erbstatin inhibited both EGF-induced and serum-stimulated cell growth in six human gastric carcinoma cell-lines in a dose-dependent manner [76]. In one cell-line, TMK-1, erbstatin inhibited the PTK activity of EGFR, $p185^{erbB2}$ and $pp60^{c\text{-}src}$ *in vitro*, indicating that the inhibition of growth of these cells may be due to the inhibition of more than one PTK. However, as erbstatin was given alone, the activity shown may be due to a decomposition product.

To overcome the chemical instability of erbstatin, Umezawa *et al.* prepared methyl 2,5-dihydroxycinnamate (15), which showed similar activity to erbstatin but was more stable in calf serum [74]. Subsequently, these workers have demonstrated the use of erbstatin and methyl 2, 5-dihydroxycinnamate in delaying S-phase (DNA synthesis) induction by EGF in quiescent NRK cells [74] and ER12 cells [77], without showing irreversible cytotoxicity. Thus, it is possible that PTK inhibitors may modify malignant phenotypes in EGFR over-expressing phenotypes. Erbstatin has

also been shown to inhibit serum-induced M-phase progression in NRK, NIH3T3 and RSV-NRK cells, thereby delaying proliferation [78]. In RSV-^{ts}NRK cells, erbstatin and methyl 2, 5-dihydroxycinnamate induced morphological and cytoskeletal change, bringing them closer to the morphology of their normal counterparts [79]. Umezawa *et al.* have patented a series of erbstatin analogues (16) possessing antitumour, antimicrobial and tyrosine-specific protein-kinase inhibitory activities [80].



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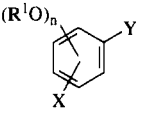
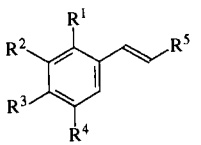


(16) Y= CH=CHNC, CH=CHNHR^2, $CH_2CH_2NHR^2$
X= H, halo

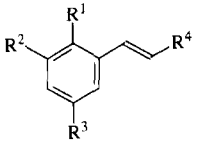
Erbstatin has also been used as a pharmacological probe into other areas of cell function where PTKs may be implicated, including the association of PTK activity with the activation of PLC in A431 cells [81], the signalling pathways of platelet activating factor and thrombin in platelets [82], and angiogenic endothelial cell proliferation [83].

The early findings that erbstatin appeared to be competitive with the peptide substrate of PTKs promoted much interest in analogues or similar compounds, for example, cinnamamides, tyrphostins, piceatannol and its analogues, and lavendustin and its analogues. These are discussed later, but first, it is worth considering the synthesis of analogues where the essential structural features of erbstatin were maintained. Isshiki *et al.* [84, 85] demonstrated the requirement for activity of two hydroxyl groups on the phenyl ring either *ortho* or *para* to each other (17g-k), whilst the replacement of the formamide moiety with a carboxylic acid or acetamide group had little effect (*Table 6.11*, 17h and 17i *vs.* 13).



(17a-k)

The finding that methyl 2, 5-dihydroxycinnamate (15) was more stable than erbstatin and of similar potency prompted investigations into whether more stable and more potent inhibitors could be prepared by elongating the



(18a-h)

ester chain (*Table 6.12*) [86]. Lengthening the chain (18a-d) increased stability, but decreased the inhibition of cell growth, although PTK inhibitory activity was more or less conserved. The inhibitory activity on A431 cell membrane fractions in this assay showed erbstatin to be more potent than previously observed; this was probably due to the use of a different peptide substrate (RR-*SRC*).

Tyrphostins and related compounds

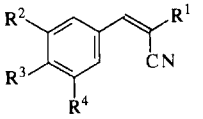
The most successful and productive lead from erbstatin has arisen from the work carried out by Levitzki and co-workers in the preparation and biological evaluation of the so-called 'tyrphostins'. The discovery that compounds with a benzylidenemalononitrile moiety are potent and selective inhibitors of the EGFR PTK initiated a vigorous research programme to prepare even more potent and selective inhibitors. To date, over 1000 tyrphostins have been prepared including second generation tyrphostins which, paradoxically use earlier tyrphostins as lead compounds. Tyrphostins have previously been reviewed in depth [87–89], so only those

Table 6.11 INHIBITION OF PTK ACTIVITY OF A431 CELL MEMBRANE FRACTIONS [85]

compound	R^1	R^2	R^3	R^4	R^5	$IC_{50}(\mu M)$
(13)	OH	–	–	OH	NHCHO	3.3
(17a)	OH	–	–	–	NHCHO	>600
(17b)	–	OH	–	–	NHCHO	>60
(17c)	–	–	OH	–	NHCHO	>60
(17d)	OH	–	–	Br	NHCHO	>26
(17e)	OH	–	–	OMe	NHCHO	>33
(17f)	OH	–	OH	–	NHCHO	>140
(17g)	OH	OH	OH	–	NHCHO	4.1
(17h)	OH	–	–	OH	NHAc	15.5
(17i)	OH	–	–	OH	COOH	4.4
(17j)	OH	OH	–	–	NHCHO	4.5
(17k)		OH	OH	–	NHCHO	7.3

compounds that have prompted deeper biological evaluation will be discussed here.

The first reported tyrphostins utilized the *cis*-cinnamonitrile nucleus as the main structural motif [90]. These compounds were prepared with erbstatin as the model compound, but had the advantages of being easily prepared (by Knoevenagel condensations) and also allowed for numerous structural modifications.



(19a-q)

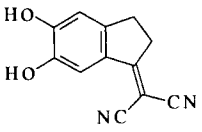
As with other PTK inhibitors, the number of hydroxyl residues on the benzene ring appears to be an important criterion, with a 3,4-dihydroxy relationship appearing to be the minimum requirement for potent activity (*Table 6.13*) [91]. The nature of the *trans* group, however, dictates the potency of these compounds, with major enhancement of activity being conferred by the presence of amide (19k), thioamide (19l) or aminodicyanoethylene (19n) groups. In some respects, these groups could be regarded as being analogous to the *N*-formylaminoethenyl chain of erbstatin.

Substituents other than hydroxyl residues on the benzene ring of benzylidenemalononitriles significantly reduce activity [91]. The same

Table 6.12 INHIBITON OF PTK ACTIVITY OF A431 CELL MEMBRANE FRACTIONS AND A431 CELL GROWTH [86]

					$IC_{50}(\mu M)$	
compound	R^1	R^2	R^3	R^4	EGFR	cell growth
(13)	OH	–	OH	NHCHO	0.5	21
(15)	OH	–	OH	CO_2Me	0.77	1.8
(18a)	OH	–	OH	CO_2Et	0.82	1.3
(18b)	OH	–	OH	CO_2Pr	0.99	9.0
(18c)	OH	–	OH	CO_2Bu	1.44	16
(18d)	OH	–	OH	CO_2He*	2.72	30
(18e)	OH	–	OH	CO_2Bz*	1.21	23
(18f)	OH	OH	–	CO_2Bu	1.27	6.4
(18g)	OH	OH	–	CO_2He*	5.68	1.1
(18h)	OH	–	OH	CHO	3.05	6.1

*Bz = $PhCH_2$; He = n-hexyl



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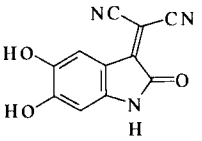

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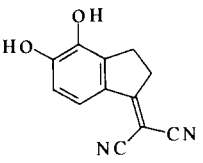

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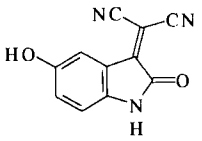

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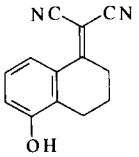


(24)

applies for replacement of the benzene ring with other heteroaromatic ring systems (not shown) [92].

In order to establish the required conformation of the polyhydroxy-*cis*-cinnamonitrile moiety, a series of conformationally-constrained tyrphostins were prepared (*Table 6.14*) [92]. The isatins (21) and (23) are 14–30 times more potent inhibitors than their corresponding 'open' tyrphostins (19d) and (19b). Instability of these compounds, due to the heterocyclic

Table 6.13 INHIBITION OF EGFR PTK BY *CIS*-CINNAMONITRILE TYRPHOSTINS [91]

compound	R^1	R^2	R^3	R^4	$IC_{50}(\mu M)$
(19a)	CN	–	OH	–	560
(19b)	CN	OH	–	–	375
(19c)	CN	OH	–	OH	37
(19d)	CN	OH	OH	–	35
(19e)	CN	OH	OH	OMe	6
(19f)	CN	OH	OH	Me	6
(19g)	CN	OH	OH	OH	3
(19h)	CO_2H	–	OH	–	308
(19i)	CO_2H	OH	OH	–	70
(19j)	$CONH_2$	–	OH	–	800
(19k)	$CONH_2$	OH	OH	–	10
(19l)	$CSNH_2$	OH	OH	–	2.4
(19m)	$C(NH_2)=C(CN)_2$	–	OH	–	0.125
(19n)	$C(NH_2)=C(CN)_2$	OH	OH	–	2.5
(19o)	$C(NH_2)=C(CN)_2$	OH	OH	OH	0.8
(19p)	$C(NH_2)=C(CN)_2$	OH	OH	OMe	1.2
(22q)	$C(NH_2)=C(CN)_2$	OH	OH	Br	0.5

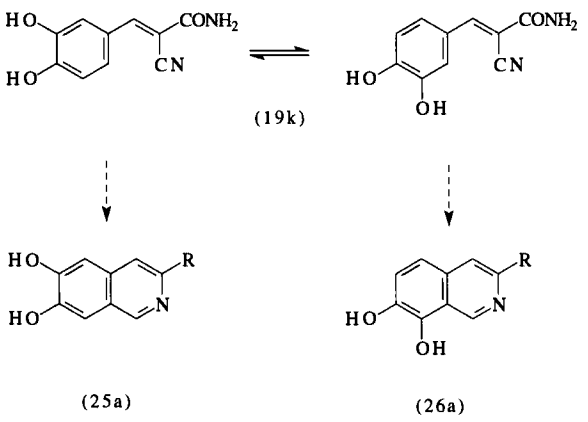


Figure 6.3. Rationale behind the preparation of 6,7- and 7,8-dihydroxyisoquinoline-3-carboxamides [94–96]

ring-opening reaction of isatins, prompted the preparation of their more stable and more potent carbocyclic analogues (20) and (22). A slight deviation from planarity, for example, in (24), appears to reduce inhibitory activity to a large degree. These results suggest that optimal potency is achieved with tyrphostins that have a coplanar catecholic ring and a biscyanoalkene group. The difference in activity between (20) and (22) indicates that the configuration of (20) is required for optimal activity, and possibly reflects the type of conformation taken up by the 'open' tyrophostins within the kinase domain.

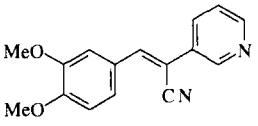
Smyth *et al.* had previously investigated the possibility of preparing conformationally constrained inhibitors of lavendustin A to extend planarity and improve interaction at a putative cleft-like catalytic centre [93]. These workers used compound (19k) as a model for the design of constrained tyrphostins (*Figure 6.3*) [94–96].

As a result of this rationale, the isomeric 6,7- and 7,8-dihydroxyisoquin-

Table 6.14 INHIBITION OF EGFR PTK ACTIVITY BY CONFORMATIONALLY CONSTRAINED TYRPHOSTINS [92]

compound	$IC_{50}(\mu M)$
(20)	0.5
(21)	2.5
(22)	7
(23)	12.5
(24)	1200

oline-3-carboxamides (25a) and (26a) were prepared to represent the two 180° rotational conformers of (19k) (*Table 6.15*) [94, 95]. Neither of these compounds inhibited EGFR PTK activity, but (26a) was a more potent inhibitor of $p56^{lck}$ PTK activity than (19k). Subsequent alterations to the amide substituent (26b-e) confirm the greater potency of the 7,8-dihydroxyisoquinoline-3-carboxamides as inhibitors of PTKs [95, 96], indicating that this may be a useful structural motif for the development of future selective PTK inhibitors. Interestingly, variations of the amide substituent on the 6,7-dihydroxyisoquinoline-3-carboxamides, (25b) and (25c), show selective inhibition of the EGFR PTK over $p56^{lck}$. Naphthalene, quinoline and 2-iminochromene ring systems have also been considered, but these are relatively inactive [96].



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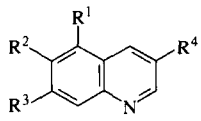


(28)

Maguire *et al.* [97] have synthesized a series of quinolines (28) as conformationally restricted analogues of the 2,3-diarylacrylonitrile (27). A number of these compounds are potent inhibitors of the autophosphorylation of the human PDGFR, with IC_{50} values in the nanomolar range (*Table 6.16* and others not shown).

Using 3,4-dihydroxy-*cis*-cinnamonitrile (19d) as a template, a number of keto or amide analogues substituted at the α-position were prepared and

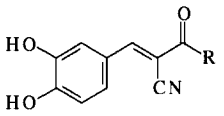
Table 6.15 INHIBITION OF PTKS BY DIHYDROXYISOQUINOLINE-3-CARBOXAMIDES AND RELATED COMPOUNDS [94,95]

		$IC_{50}(\mu M)$	
compound	*R*	*EGFR*	*$p56^{lck}$*
(19k)		10	22
(25a)	$CONH_2$	>100	1900
(25b)	CONHPh	5.6	600
(25c)	$CONHCH_2Ph$	3.1	>1000
(26a)	$CONH_2$	>100	0.5
(26b)	CONHPh	7.7	1
(26c)	$CONHCH_2Ph$	1.4	1
(26d)	$CONH(CH_2)_2Ph$	1.4	40
(26e)	CO_2Me	–	0.2

Table 6.16 INHIBITION OF THE PHOSPHORYLATION OF HUMAN PDGFR BY QUINOLINES [97]

compound	R^1	R^2	R^3	R^4	$IC_{50}(nM)$
(28a)	H	OMe	OMe	3-thienyl	1-2
(28b)	H	OMe	OMe	4-$MeOC_6H_4$	1-15
(28c)	H	OMe	OMe	*trans*-β-styryl	1-5
(28d)	H	OMe	OMe	5-Cl-2-thienyl	7-30
(28e)	H	H	OMe	3-thienyl	2-60
(28f)	H	H	F	3-thienyl	4-25
(28g)	Me	H	Me	3-thienyl	1-5
(28h)	H	OMe	OMe	3-F-4-$MeOC_6H_3$	3-5

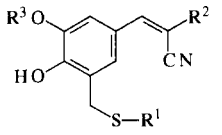
produced some very potent inhibitors (*Table 6.17*) [98]. Aromatic ketones enhance activity, but substitutions in the benzoyl ring as in (29a-c) have little effect on inhibitory activity. In the amide series, the distance of the phenyl group from the amide link (29d-i) is insignificant for PTK inhibition, but may be important in terms of anti-proliferative activity (29h and 29i *vs.* 19l). This may be associated with pharmacokinetic properties such as cell membrane permeability and biochemical stability.



(29a-i)

Having extensively investigated the permutations for substitutions at the α-position, Gazit *et al.* have recently reported the synthesis of tyrphostins based on substitution at the 5-position [98]. 3,4,5-Trihydroxybenzylidene-malononitrile was an active PTK inhibitor, but unstable. However, 3-methoxy-4,5-dihydroxy- (19e) or 3-methyl-4,5-dihydroxybenzylidene-malononitrile (19f) are almost as potent and more stable. Shiraishi *et al.* had shown that the introduction of a (phenylthio)methyl group at the 5-position and the replacement of the α-cyano group by an amide gave highly potent α-cyanocinnamamide inhibitors [99] (see later section on cinnam-amides).

A series of *S*-aryl substituted 5-thiomethyltyrphostins based on the α-cyanocinnamamides was therefore prepared (*Table 6.18*) [98]. Replace-ment of the phenyl ring with neutral aromatic or heteroaromatic rings (30g, 30i, and 30k) causes a decrease in inhibitory activity, but increasing the



(30a-k)

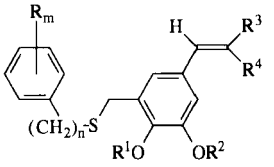
distance between the two aromatic rings (30d) has little effect. Substituents on the aromatic rings at the 5-position (30e, 30f and 30h) do not seem to influence activity to a great extent, but can be detrimental (30j). Replacement of the α-amide with α-cyano (30a) decreases activity against the purified EGFR PTK but has little effect on the anti-proliferative activity against HER-14 cells; a dihydroxybenzoyl group (30b) at the α-position improves inhibitory activity against EGFR PTK 3-fold. The replacement of the methoxy group in the 3-position with a hydroxyl group (30c) has little effect on the 5-*S*-aryl-substituted tyrphostins.

Shiraishi *et al.* have patented a series of 3-phenylthiomethylstyrene derivatives (31) as anti-allergic and tyrosine kinase inhibiting agents [100] whilst Salari has patented a series of tyrphostins (32) for the treatment of allergic, inflammatory and cardiovascular diseases [101].

Tyrphostins were initially designed to compete with the substrate binding site, as had been assumed for the mode of inhibition for erbstatin at that time. The mode of action of the various tyrphostins is, however, very

Table 6.17 INHIBITION OF PTK ACTIVITY AND DHER CELL PROLIFERATION BY AMIDE AND KETO-SUBSTITUTED TYRPHOSTINS [98]

		$IC_{50}(\mu M)$				
		EGFR	*autophosphorylation*		*proliferation*	
compound	*R*	*PTK*	*EGFR*	*HER1-2*	*EGF-dep.*	*serum*
(29a)	3,4-$(HO)_2C_6H_3$	0.37	5	18	8	>50
(29b)	4-FC_6H_4	0.56	5.1	13.6	20	80
(29c)	4-$NO_2C_6H_4$	2.3	15	10	20	<<20
(29d)	NHPh	0.7	1.25	42	15	>50
(29e)	$NHCH_2Ph$	2.0	0.1	13.5	3.5	25
(29f)	$NHCH_2CH_2Ph$	0.93	<0.625	23	nd	nd
(29g)	$NH(CH_2)_3Ph$	0.7	0.7	35	2.5	25
(29h)	$NH(CH_2)_4Ph$	1.1	5	22	3	10
(29i)	NHCHMePh	0.4	2.5	37	3.5	10
(19d)		35	31	nd	30	60
(19l)		2.4	7.5	nd	10	>50



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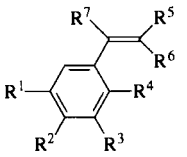
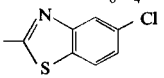
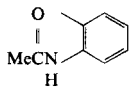
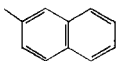
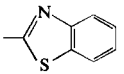


(32)

complicated and has been sub-divided into four classes: (i) competitive inhibitors with respect to both substrate and ATP; (ii) mixed competitive with respect to both substrate and ATP; (iii) competitive with respect to ATP and mixed competitive with respect to the substrate; (iv) competitive with respect to the substrate and mixed competitive or non-competitive with

Table 6.18 INHIBITION OF EGFR PTK, AUTOPHOSPHORYLATION AND PROLIFERATION OF HER14 CELLS BY 5-*S*-ARYL-SUBSTITUTED TYRPHOSTINS [98]

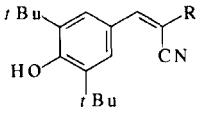
					$IC_{50}(\mu M)$		
				EGFR	*autophosph.*		*prolifn*
compound	R^1	R^2	R^3	*PTK*	*EGFR*	*HER 1-2*	*HER-1*
(35f)	Ph	$CONH_2$	Me	2.9	0.4	0.13	12.5
(30a)	Ph	CN	Me	370	6.7	2.5	6
(30b)	$PhCH_2$	COC_6H_3-3,4-$(OH)_2$	Me	0.75	4.5	6.2	2
(30c)	$PhCH_2$	$CONH_2$	H	0.94	0.64	1.0	3
(30d)	$PhCH_2$	$CONH_2$	Me	1.95	0.90	0.2	12.5
(30e)	2-$ClC_6H_4CH_2$	$CONH_2$	Me	2.5	1.0	0.25	10
(30f)	4-HOC_6H_4	$CONH_2$	Me	2.5	2.5	1.6	2
(30g)		$CONH_2$	Me	4.4	>83	15	4.5
(30h)		$CONH_2$	Me	5.6	22	3.3	9
(30i)		$CONH_2$	Me	6.6	1.7	4.6	10
(30j)		$CONH_2$	Me	8.7	1.75	1.65	12
(30k)	H	$CONH_2$	Me	18.6	19	0.35	35

respect to ATP [89]. However, most tyrphostins show excellent specificity for PTKs over serine/threonine kinases, irrespective of their mode of inhibition.

The HER2 PTK is implicated in malignant forms of breast and ovarian cancers [102]. It is possible to discriminate between this kinase and the closely related EGFR PTK (also known as HER1) by considering inhibition of the chimeric PTK, EGF/*neu* (HER1-2). Only one compound (30k) differentiates dramatically between HER1 and HER1-2 kinases in favour of the HER1-2 kinase, although this differential is not seen in terms of anti-proliferative potential [98]. This may be explained by the fact that such compounds act at a position downstream in the tyrosine kinase cascade, a fact that may explain the similar anti-proliferative potentials of the compounds in *Table 6.18*, despite their disparate activity against the isolated PTKs.

Similarly, for compounds that are selective for HER1 over HER1-2 (29e-g) *in vitro*, the blocking of mitogenic signalling induced by EGF in NIH 3T3 cells over-expressing either HER1 or HER1-2 was identical. HER1 and HER2 selective blockers were all competitive with respect to ATP, indicating that high intracellular ATP levels may prevent the inhibitors from binding to the receptor [102].

The ability to distinguish between closely related PTKs is an important feature of the tyrphostins. Early results indicated that potent inhibitors of the EGFR PTK were poor inhibitors of the IR PTK [90, 91], in spite of its high homology to the EGFR in the kinase domain. This led to the proposition that it may be possible to synthesize selective tyrphostins for each of the known PTKs involved in cell proliferation and transformation. Indeed, inhibitors that are selective for the PDGFR over the EGFR PTK have been found, for example, (33) (IC_{50} = 0.5 μM {PDGFR}, 460 μM {EGFR}) [88]. A similar compound, (34), was found to be the only one of twelve various tyrphostins to inhibit the NGF receptor tyrosine kinase, but did not inhibit EGF or PDGF receptor PTKs [103].



(33) R=CN
(34) R=$CSNH_2$

Levitzki and co-workers [104] claim that tyrphostins can discriminate between transforming *abl* and normal *abl* PTKs, suggesting that the transforming potency of the *abl* PTKs may result from their higher affinities

toward intracellular signal transducers and that oncogene products can differ from their homologous proto-oncogene products in substrate specificity. This discrimination may have implications for the design of inhibitors to counter *abl*-associated human leukaemias.

Conflicting evidence exists for the action of tyrphostins on EGFR PTK activity in different intact cells. Levitzki and co-workers [105] found that (19d) and (19l) inhibited EGF-stimulated receptor autophosphorylation and tyrosine phosphorylation of endogenous substrates in HER14 cells at doses which correlated with the IC_{50} for cell proliferation, but required long-term exposure. Faaland *et al.* [106], however, found that (19l) was rapidly taken up by A431 cells and was a non-competitive inhibitor of the EGF receptor tyrosine kinase, but partially inhibited EGF-stimulated EGFR PTK activity, suggesting an indirect mechanism of action.

The tyrphostins have probably been used more than any other class of PTK inhibitors to investigate the role of PTKs in numerous biological and biochemical systems. PDGF is believed to be prominently involved in disorders of vascular smooth muscle cells (VSMC) such as atherosclerosis, hypertension and restenosis. Tyrphostins, particularly (33), reversibly inhibited PDGF-dependent growth of VSMC as well as PDGF-dependent DNA synthesis, by inhibiting PDGFR autophosphorylation and the subsequent signalling cascade [107]. Sauro and Thomas [108, 109] have used tyrphostins to demonstrate a link between vasoconstriction and VSMC proliferation, suggesting that PTKs may be involved in the development of hypertension. Further evidence for this association was shown by the ability of tyrphostins to block the calcium channel current in VSMC, a process probably modulated by a PTK [110].

The interaction of PTK pathways with other cellular signal transduction mechanisms has also been studied by utilizing tyrphostins. Levitzki and co-workers [111] showed that potent EGFR PTK inhibitors were also potent blockers of the EGF-induced production of phosphoinositides, but not the production induced by the Ca^{2+} ionophore A23187, thereby supporting a proposal that phospholipase C is activated through its phosphorylation by the EGFR. Further evidence linking the tyrosine kinase activity of the EGFR to the PIP_2 hydrolysis pathway was supplied by Margolis *et al.* [112], who demonstrated that the tyrphostin (19l) blocked EGF-induced tyrosine phosphorylation of PLC-II, its association with the EGFR and EGF-induced Ca^{2+} release. Thus, the tyrosine phosphorylation of PLC-II plays a key part in molecular events initiated on the cell surface and leading to cell proliferation. Similar observations were noted after the activation of the B cell antigen receptor [113].

Further work by Levitzki and co-workers [114] demonstrated that

tyrphostins dose-dependently inhibited thrombin-induced aggregation and phosphorylation of p43, the main substrate for PKC, thereby suggesting a role for PTK activity in platelet signal transduction in conjunction with the PKC pathway. These workers later suggested that PTK activity may be involved after PKC activation, and that thrombin-induced aggregation and serotonin release correlated with the potency of inhibition of tyrosine phosphorylation [115].

Tyrphostins have also proved to be useful agents in characterising the essential proteins and biochemical pathways that regulate neutrophil activation [116] and the involvement of PTK activity in the bradykinin-mediated signal transduction processes in human fibroblasts [117]. Experimental evidence has suggested that growth factors promote cell survival by suppressing apoptosis; tyrphostin (19g) induced apoptosis in two leukaemia cell-lines, probably by inhibiting PTKs induced by interleukin-3 and granulocyte macrophage colony stimulating hormone [118].

The potential use of tyrphostins as anti-proliferative agents has been widely investigated. Reddy *et al.* [119] showed that tyrphostins inhibited cell growth in two breast cancer cell-lines with abundant EGFR content in a reversible dose-dependent manner (0.1–10 μM), as well as inhibiting a number of oestrogen receptor-positive and receptor-negative cell-lines induced by insulin, IGF-1, IGF-2 or TGF-α. Total inhibition of oestrogen-stimulated phosphorylation of the EGFR as well as oestrogen-induced cell proliferation was observed, indicating that functioning PTK pathways are required for oestrogen activity.

Tyrphostins have been shown to induce terminal erythroid differentiation of mouse erythroleukaemia (MEL) cells [120] and K562 cells [121], the latter being concomitant with inhibition of $p210^{bcr\text{-}abl}$ tyrosine phosphorylation in intact cells. The authors suggest a therapeutic potential for these compounds to purge Philadelphia chromosome-positive cells in preparation for autologous bone marrow transplantation in chronic myelogenous leukaemia [121].

EGF, TGFα and IGF-1 have all been implicated in the proliferation of pancreatic cancer cells. Certain tyrphostins reversibly arrest the growth of three human pancreatic cell-lines stimulated by EGF or serum, and may have potential as useful agents in the treatment of pancreatic cancer [122].

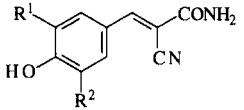
There is evidence that there is an over-expression of the TGFα gene in psoriatic keratinocytes, and accumulation of TGFα itself in psoriatic skin. TGFα is also known to activate the EGFR of keratinocytes, which implies that persistent autocrine activation of the EGFR may play a major role in cell hyperproliferation in human psoriatic epidermis. Tyrphostins were shown to inhibit EGF-stimulated keratinocyte proliferation with similar

potency to that observed with DHER cells [123]. They also inhibited EGF-independent cell growth (probably TGFα-induced) with similar potency. Inhibition was reversible and apparently non-toxic, so the tyrphostins have potential as agents to treat hyperproliferative conditions in human skin.

A number of growth factors with PTK activity have been associated with spermatogenesis. In view of this, tyrphostins (19d) and (19k) were shown to inhibit Sertoli cell-secreted growth-factor stimulation of A431 cell growth [124]. Thus, there is a potential for PTK inhibitors to be used as male contraceptives if a specific inhibitor of growth factor-induced spermatogenesis can be found.

Cinnamamides

Shiraishi *et al.* [99, 125] have demonstrated that 4-hydroxycinnamamide derivatives (35) are potent inhibitors of the autophosphorylation of



(35a-k)

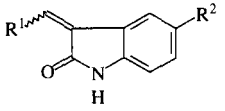
the EGFR PTK from A431 cell membrane fractions (*Tables 6.19* and *6.20*).

These compounds also appear to be specific inhibitors of PTKs as they do

Table 6.19 INHIBITION OF EGFR PTK ACTIVITY BY 4-HYDROXYCINNAMIDE DERIVATIVES [99,125]

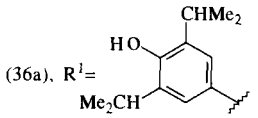
compound	R^1	R^2	$IC_{50}(\mu M)$
ST638 (35a)	EtO	$PhSCH_2$	0.4
(35b)	EtO	$4'\text{-}MeC_6H_4SCH_2$	20
(35c)	EtO	$4'\text{-}MeOC_6H_4SCH_2$	4
(35d)	EtO	$C_6F_5SCH_2$	3
(35e)	EtO	$4'\text{-}MeC_6H_4SCH_2$	3
(35f)	MeO	$PhSCH_2$	0.5
(35g)	$PhCH_2O$	$PhSCH_2$	0.8
(35h)	$PhCH_2$	$PhCH_2$	0.6
(35i)	Ph	Ph	0.4
(35j)	*iso*-Pr	*iso*-Pr	1
(35k)	*tert*-Bu	*tert*-Bu	50

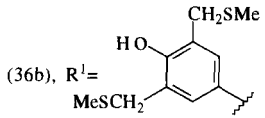
not inhibit the activity of PKA, PKC or several other serine/threonine kinases [99]. The most potent of these compounds, ST638 (35a), was also found to inhibit the activity of EGFR, $pp60^{v\text{-}src}$ and $p70^{gag\text{-}actin\text{-}v\text{-}fgr}$ PTKs in intact cells [126]. The nature of inhibition by ST638 was shown to be by competition with respect to the exogenous peptide substrate in the EGFR and $p130^{gag\text{-}v\text{-}fps}$ PTKs, but the effect on EGFR autophosphorylation in intact A431 cells was negligible [127].



(36a-h)

Varying the substituents at the *meta*-positions has little effect on activity, although substituents on the *S*-aryl rings as in (35b-e) reduce activity about 10-fold. The *tert*-butyl disubstituted compound (35k) also has reduced activity, which may be due to steric effects. The use of an oxindole





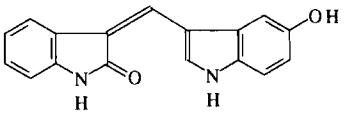
substituent instead of the amide gave relatively potent inhibitors, (36a) and (36b) (*Table 6.20*).

Levitzki and co-workers [98] used ST638 (35a) as a model compound for the preparation of some potent inhibitors (see tyrphostins). Buzzetti *et al.* [128] used (35a) as a lead compound for the preparation of 3-arylidene-2-

Table 6.20 INHIBITION OF PTKS BY 3-ARYLIDENE-2-OXINDOLES [128]

			$IC_{50}(\mu M)$	
compound	R^1	R^2	*EGFR*	*v*-abl
(36a)	see above	H	0.44	0.5
(36b)	see above	H	0.44	–
(36c)	1-HO-2-naphthyl	H	–	194
(36d)	1,2-$(HO)_2$-2-naphthyl	H	–	5
(36e)	5-quinolyl	H	–	3
(36f)	8-HO-5-quinolinyl	H	–	6
(36g)	3-indolyl	H	–	5
(36h)	3-indolyl	OH	–	0.4

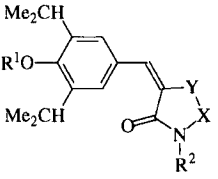
oxindoles, (36c-h) (*Table 6.20*). Structure-activity analysis of these compounds revealed that replacement of the benzene ring by a naphthalene as in (36c), afforded a less active compound, although partial saturation of the naphthalene as in (36d), enhances inhibitory activity. Replacement with a quinoline moiety had a variable effect depending on the attachment position and substituents. Compounds (36e) and (36f) were reasonably active, whilst others (not shown) were inactive. The indolylmethylene derivatives, (36g) and (36h), displayed good activity, with (36h) being the most potent compound in this series tested against the *v-abl* PTK; once again a hydroxyl substituent on this compound influences its potency. Buzzetti has patented methyleneoxindoles, for example, (37), as tyrosine kinase inhibitors [129].



(37)

The pretext behind the preparation of these oxindoles is presumably to mimic the purine moiety of ATP. These results, however, offer little information in terms of structural features for inhibitors of PTKs. Kinetic analysis of their inhibitory activity may reveal these to be compounds of future interest.

Imai *et al.* [130] and Buzzetti *et al.* [131] have patented a series of di-isopropylbenzylidene-substituted heterocycles (38) and arylidene-heterocyclic derivatives (39) respectively for their use as tyrosine kinase inhibitors.



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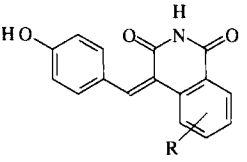
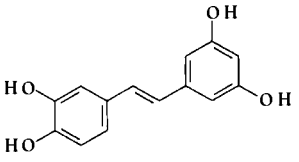


(39)

Piceatannol and its analogues

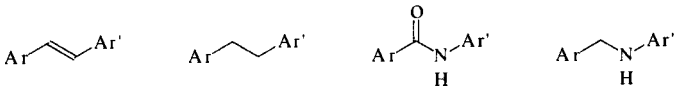
Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) (40) is a plant secondary metabolite isolated from the seeds of *Euphorbia lagascae* which has been shown to have *in vivo* antitumour properties [132]. Geahlen and



(40)

McLaughlin demonstrated that piceatannol may exert this activity by inhibition of PTKs [133]. They found that piceatannol was a competitive inhibitor of the p40 PTK with respect to the peptide substrate (K_i = 16 μM), and also inhibited the $p56^{lck}$ PTK both *in vitro* (IC_{50} = 82 μM) and *in situ*. In addition, piceatannol had little effect on PKA activity.

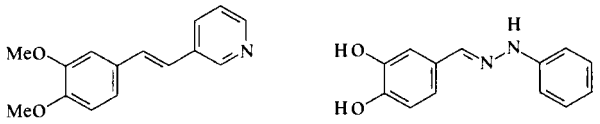
Thus, once again a natural product had been found with a certain similarity in structure to tyrosine, which apparently inhibited PTKs specifically and by competition with respect to the peptide substrate. Piceatannol, however, is not a particularly potent inhibitor, so efforts were concentrated on increasing the inhibitory activity of this type of compound whilst retaining its selectivity. Cushman *et al.* [134] synthesized a series of pyridine-containing stilbene and amine derivatives based on the structure of piceatannol (41-44), but none of these compounds was as potent as



(41) (42) (43) (44)

piceatannol for the inhibition of $p56^{lck}$. The most potent of these inhibitors was 1-(3,4-dimethoxyphenyl)-2-(3-pyridyl)ethene (45) (IC_{50} = 158 μM) and kinetic studies indicated that the inhibition by this compound was due to competition with respect to ATP and not with the peptide substrate.

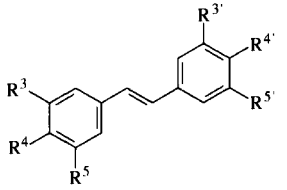
A series of 33 phenylhydrazone derivatives were prepared by the same workers [135]. Only one compound (46) (IC_{50} = 70 μM), was as active as



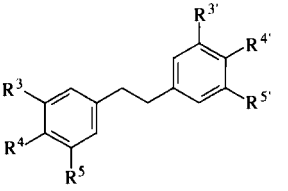
(45) (46)

piceatannol and kinetic analysis indicated that, like the lead compound, it was a competitive inhibitor of $p56^{lck}$ PTK activity with respect to the peptide substrate and non-competitive with respect to ATP.

Cushman and co-workers also reported the preparation of a number of polyhydroxylated stilbene analogues of piceatannol and their testing for inhibition of the $p56^{lck}$ PTK [136]. As indicated in *Table 6.21*, five of these compounds (47d-g and 47j) showed enhanced enzyme inhibitory activity in



(47a-j)



(48a, b)

relation to the natural product. The benzylated precursors of these compounds (not shown) were inactive, indicating that free phenolic hydroxyl groups are likely to be required for activity. As with the flavonoid inhibitors described earlier, increasing the number of hydroxyl groups generally increases activity. Certain anomalies arise in the positioning of hydroxyl substituents in the tri- and tetraphenols: when the *para*-hydroxyl of piceatannol is moved to the *meta*-position, (47j), inhibitory activity is increased four-fold; a similar increase in potency is observed with the same

Table 6.21 INHIBITION OF $p56^{lck}$ PTK ACTIVITY BY POLYHYDROXYLATED STILBENE DERIVATIVES OF PICEATANNOL [136]

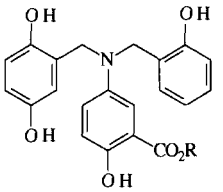
compound	R^3	R^4	R^5	$R^{3'}$	$R^{4'}$	$R^{5'}$	$IC_{50}(\mu M)$
(47a)	OH	OH	–	–	–	–	203
(47b)	OH	–	OH	–	–	–	193
(47c)	OH	–	–	OH	–	–	236
(47d)	OH	–	–	–	OH	–	38
(47e)	OH	OH	–	OH	–	–	57
(47f)	OH	OH	–	–	OH	–	18
(47g)	OH	–	OH	OH	–	–	18
(47h)	OH	–	OH	–	OH	–	114
(47i)	OH	OH	–	OH	OH	–	74
(47j)	OH	–	OH	OH	–	OH	16
(48a)	OH	–	OH	OH	–	OH	480
(48b)	OH	OH	–	OH	–	OH	1520
(40)	OH	OH	–	OH	–	OH	64

change from (47e) to (47g), but the converse occurs with (47h) to (47f). Reduction of the central double bond (48a and 48b) resulted in a significant decrease in activity.

Whether piceatannol or its derivatives have a future in the investigation of PTKs and their inhibition remains to be seen as they appear to lack the required potency to be of use. In addition, the selectivity of these compounds remains uncertain as insufficient work has been done on this problem.

LAVENDUSTIN A AND ITS ANALOGUES

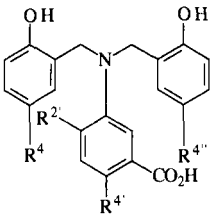
The screening of bacterial culture filtrates has proved a fruitful exercise in eliciting potent PTK inhibitors, none more so than lavendustin A (49a), from *Streptomyces griseolavendus*, which inhibits the EGFR PTK phosphorylation of the peptide substrate, RR-*SRC*, with an IC_{50} of 11.5 nM [137], more than 90 times more potent than erbstatin (13). Lavendustin A did not inhibit PKC or PKA activity, but weakly inhibited phosphatidylinositol kinase; it did not inhibit autophosphorylation of the EGFR or its PTK activity *in situ* [138]. In view of this, Onoda *et al.* prepared the methyl ester of lavendustin A (49b) [138], which inhibits the EGFR PTK activity with an IC_{50} of 2 μM, but does inhibit autophosphorylation of the EGFR (IC_{50} = 130–260 μM). Lavendustin A (49a) inhibited the growth of A431 cells, NIH3T3 cells, RSV-NIH3T3 cells, P388 leukaemia cells and L1210 leukaemia cells with IC_{50} values of between 50 and 80 μM [137], whereas (49b) inhibited these cells with IC_{50} values of between 9 and 21 μM [138].

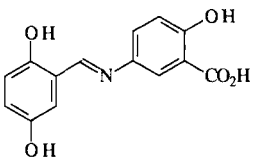


(49a) R=H
(49b) R=Me

These results demonstrated that the inactivity of lavendustin A *in situ* is probably due to poor cell penetration, but that the carboxylic acid residue is a requirement for potent inhibition of kinase activity.

The total synthesis of lavendustin A and various derivatives was achieved and these were assessed for PTK inhibitory activity along with various





(53)

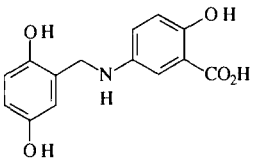
(50) $R^{4'}$=OH, R^4=$R^{2'}$=$R^{4''}$=H
(51) R^4=$R^{4'}$=$R^{4''}$=OH, $R^{2'}$=H
(52) R^4=$R^{2'}$=OH, $R^{4'}$=$R^{4''}$=H

synthetic intermediates (*Table 6.22*). The addition of one hydroxyl group increased the potency of lavendustin A over lavendustin B (50) by two orders of magnitude. The addition of a further hydroxyl, (51), however, did not increase potency. The intermediate, (54), was also relatively potent and it was suggested that this may constitute the core structure responsible for inhibition of PTK activity.

Kinetic studies indicated that lavendustin A was a competitive inhibitor of the EGFR PTK with respect to ATP and non-competitive with respect to the peptide substrate [137]. More detailed analysis [139] indicated that inhibition occurred by partial competition with respect to both ATP and the peptide substrate, suggesting that lavendustin A binds at a site in the kinase domain which is distinct from the binding sites for ATP and the peptide, but interacts with the kinase in such a way as to lower the binding affinities of the enzyme for its substrates. An analogue of lavendustin A, RG14467 (56), was also considered and found to be partially competitive with respect to

Table 6.22 INHIBITION OF EGFR PTK ACTIVITY FROM MEMBRANE FRACTION OF A431 CELLS [137]

compound	IC_{50}(μM)
(49a)	0.012
(50)	1.34
(51)	0.028
(52)	0.43
(53)	2.78
(54)	0.044
(55)	17.7
(13)	1.12



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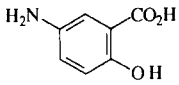
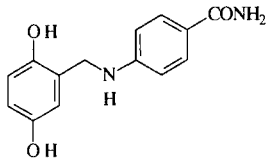


(55)

ATP and partially non-competitive with respect to the peptide substrate. This suggests the possibility that the two compounds actually bind at different sites within the kinase domain.

The active pharmacophore of lavendustin A appears to reside in the benzylamine salicylate (54), and has been the basis for the synthesis of analogues, particularly in view of its similarity to the 2,5-dihydroxyphenyl ring of erbstatin and the diaryl pattern of piceatannol. Smyth *et al.* [140] attempted the hybridization of these features by preparing compounds with the stilbene nucleus of piceatannol, but retaining the salicylate arrangement which is almost unique to lavendustin A amongst PTK inhibitors (Table *6.23*). The salicylate (57) which incorporates the 2,5-dihydroxyphenyl ring and the stilbene nucleus was consistently more potent than the other compounds tested, particularly in its inhibition of the $p56^{lck}$ PTK. Replacing the imine nitrogen of compound (53) with a carbon increased the potency 1400-fold. In contrast, this same alteration in the reduced forms (54 and 59) had little influence on activity.

These results indicate that the nitrogen is not an essential part of the lavendustin A pharmacophore. More surprising, though, is the observation that the styryl nucleus is not essential [compounds (59) and (60) are



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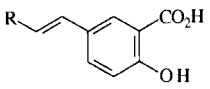
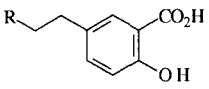


(57) R=2,5-$(HO)_2C_6H_3$
(58) R=3,4-$(HO)_2C_6H_3$

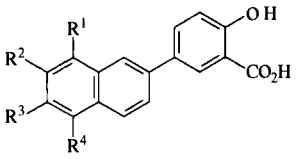


(59) R=2,5-$(HO)_2C_6H_3$
(60) R=3,4-$(HO)_2C_6H_3$

Table 6.23 INHIBITION OF IMMUNOPRECIPITATED PTK AUTOPHOSPHORYLATION [140]

	$IC_{50}(\mu M)$		
compound	*p56*^{lck}	*EGFR*	*c*-erb *B2*
(49a)	8	7	9
(53)	85	9	70
(54)	10	4	0.9
(57)	0.06	2	0.6
(58)	nd	5	0.2
(59)	1	20	4
(60)	1	12	0.6

relatively potent] for activity, which contrasts with the inactivity seen with piceatannol derivatives of this kind. This reflects the importance of the salicylate moiety as an integral part of the active pharmacophore of lavendustin A.



(61)

Conformationally constrained mimetics (61a-d) of the two most potent compounds (57) and (58) were subsequently prepared to test the hypothesis that extended planarity may improve the interaction of PTK inhibitors at a putative cleft-like catalytic centre (a theory based on the supposed similarity between the actual X-ray structure of the catalytic subunit of PKA and the expected active site of PTKs) [93]. Despite the fact that many PTK inhibitors prefer to adopt a planar conformation, these compounds were relatively inactive compared with their parent compounds (*Table 6.24*), implying that a degree of flexibility is necessary for activity within the active site. The ability of these types of compounds to adopt the correct conformational arrangement is further supported in a study of [(hydroxybenzylidene)amino]salicylates and [(hydroxybenzyl)amino]salicylates (*Table 6.25*) based upon the lavendustin A pharmacophore (54) [141].

In each case, the amino derivatives are more potent than their more constrained *anti*-imino counterparts (not shown), showing the importance

Table 6.24 INHIBITION OF THE AUTOPHOSPHORYLATION OF $p56^{lck}$ PTK [93]

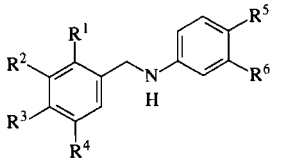
compound	R^1	R^2	R^3	R^4	$IC_{50}(\mu M)$
(61a)	OH	H	H	OH	64
(61b)	H	OH	OH	H	149
(61c)	H	H	OH	OH	41
(61d)	H	H	H	H	>450

of the reduced bond linking the two phenyl rings which allows for correct positioning, possibly in a *cis*-like conformational arrangement. Structural modifications indicate that the 2,5-dihydroxyphenyl moiety (62e-i) is necessary for activity, as is the salicylate group [(54) *vs.* (62e), (62a) *vs.* (62b)]. Activity against EGF-dependent cell growth was enhanced by the methyl esters, probably because the acids encountered problems in entering the cells due to their polarity. The methyl ester (62e) of the active lavendustin A pharmacophore was found to be a competitive inhibitor with respect to ATP and non-competitive with respect to the peptide substrate.

The role of the salicylate group was investigated in greater depth by Chen *et al.* [142] and the most potent compounds (comparable to lavendustin A) are shown in *Table 6.26*. The imine precursors were much less active than the amine series, confirming previous results. Once again, these compounds were generally much more potent *in vitro* than in the cell-based assay, probably due to poor cellular penetration, except for those with extended

Table 6.25 INHIBITION OF THE PHOSPHORYLATION OF RR-*SRC* BY THE EGFR FROM ER22 CELL MEMBRANE FRACTIONS AND EGF-STIMULATED DNA SYNTHESIS IN ER22 CELLS [141]

							$IC_{50}(\mu M)$	
compound	R^1	R^2	R^3	R^4	R^5	R^6	*EGFR*	*prolifn.*
(62a)	OH	–	–	–	OH	CO_2H	25	>20
(62b)	OH	–	–	–	OH	CO_2Me	45	20
(62c)	–	OH	–	–	OH	CO_2Me	>100	21
(62d)	–	–	OH	–	OH	CO_2Me	>10	>10
(54)	OH	–	–	OH	OH	CO_2H	0.03	92
(62e)	OH	–	–	OH	OH	CO_2Me	0.6	9
(62f)	OH	–	–	OH	CO_2Me	OH	0.2	10
(62g)	OH	–	–	OH	–	CO_2H	2.4	>20
(62h)	OH	–	–	OH	OMe	CO_2Me	5	9.5
(62i)	OH	–		OH	–	CO_2Me	1.6	9
(62j)	OMe	–	–	OMe	OH	CO_2Me	>100	>20



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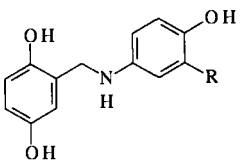


(63)

(hydrophobic) side-chains (63g-i). A hydrophobic aromatic chain [as in (63d)], appears to greatly enhance inhibitory potency in the ester series, and obviously improves cell penetration, as indicated by its inhibition of cell proliferation. It was suggested that these compounds may target a hydrophobic subsite within the kinase domain, distinct from the ATP and substrate binding sites, inducing a conformational change which reduces the binding affinities for the two substrates. In addition, these compounds also block EGFR autophosphorylation and are generally selective for PTKs over serine/threonine kinases.

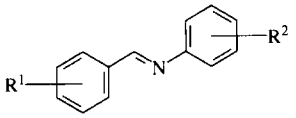
These workers also propose that the salicylate moiety may be involved in the chelation of Mg^{2+}, an important co-factor in the phosphorylation reaction. The decrease in chelating power of alkyl salicylates, for example, (63j) compared with (54), appears to be supported by the lower inhibitory

Table 6.26 INHIBITION OF THE PHOSPHORYLATION OF RR-*SRC* BY THE EGFR FROM ER22 CELL MEMBRANE FRACTIONS AND EGF-STIMULATED DNA SYNTHESIS IN ER22 CELLS [142]

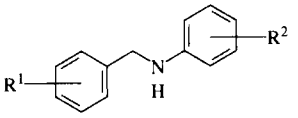
		$IC_{50}(\mu M)$	
compound	*R*	*EGFR*	*prolifn.*
(63a)	$CONHOCH_2Ph$	0.05	>10
(54)	CO_2H	0.03	92
(63b)	CO_2Ph	0.07	10
(63c)	CO_2CH_2Ph	0.08	2.5
(63d)	$CO_2(CH_2)_3Ph$	0.03	1.5
(63e)	$CO_2C_6H_4$-3-OH	0.09	>>5
(63f)	CO_2-2-naphthyl	0.09	>5
(63g)	$CO_2CH_2CHMeCH_2Bu^t$	~4	0.8
(63h)	$CO_2(CH_2)_2CHMeCH_2Bu^t$	>>1	0.8
(63i)	$CO_2(CH_2)_2CHMe(CH_2)_3Pr^i$	7	0.8
(63j)	CH_2CO_2H	6	38

potency of these compounds. Notably, other compounds (63a-f) containing the salicylate moiety retain very potent *in vitro* activity.

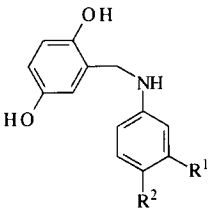
We have prepared analogues of the intermediates (53) and (54) which lack the carboxylic acid group in an attempt to investigate the possibility that the spacing between the hydroxyl groups is the critical factor in inhibition of



(64) R^1=3-OH, R^2=4-OH



(65) R^1=4-OH, R^2=4-OH



(66)

PTKs by lavendustin A derivatives [unpublished results]. These compounds, for example, (64) and (65), inhibit the PTK activity of the EGFR from A431 cells, with IC_{50}s of 33μM, but, unlike lavendustin A, these compounds should be able to penetrate the cell membrane easily.

Boiziau *et al.* have patented a series of dihydroxybenzylamine derivatives (66) as drugs and inhibitors of EGFR-associated tyrosine kinases [143].

INHIBITORS OF MARINE ORIGIN

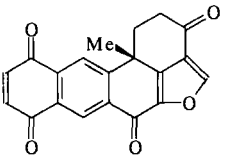
Many potent PTK inhibitors have been isolated from microbial or plant products. It is only in recent years, however, that inhibitors have been isolated from marine sources, thus expanding the diversity of PTK inhibitors. The following sections describe some of these marine products and their activities.

Polyketides

Halenaquinone (67) and related pentacyclic polyketides (68-72) from the

marine sponge *Xestospongia* cf. *carbonaria* were tested against the $pp60^{v\text{-}src}$ PTK [144]. Halenaquinone (67) was found to be an irreversible inhibitor of the $pp60^{v\text{-}src}$ PTK and also inhibited the PTK activity of the EGFR from A431 cell lysates (IC_{50} = 1.5 μM and 19 μM respectively). These compounds may react *via* a Michael addition of a reactive thiol within a cellular protein thus resulting in irreversible inhibition *via* covalent binding of the inhibitor to the PTK, as has been postulated for herbimycin A [145] (see later section). This hypothesis is supported, to some extent, by an increase in the apparent IC_{50} of these compounds when 2-mercaptoethanol was added to the enzyme reaction mixture.

The absence of at least one of the four enone sites in compounds (69-72) may explain their low inhibitory activity as compared to halenaquinone (67) [*Table 6.27*]. The activity of halenaquinol (68) may be due to its conversion to halenaquinone during testing, a phenomenon which could also apply to the 2,5-dihydroxyphenyl moiety of other potent inhibitors such as erbstatin and lavendustin. Another metabolite, 14-methoxyhalenaquinone (73) from



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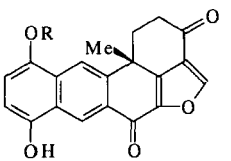
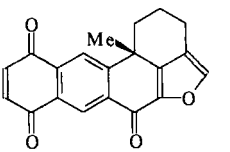


(68) R=H
(69) R=$SO_3^-Na^+$



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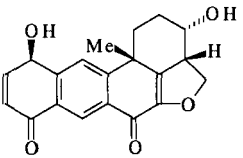

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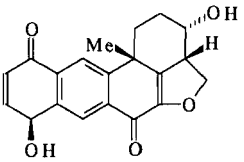

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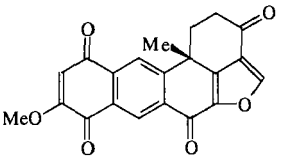

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Table 6.27 INHIBITION OF $pp60^{v\text{-}src}$ PTK ACTIVITY BY MARINE POLYKETIDES [144]

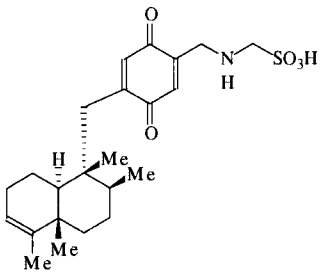
compound	$IC_{50}(\mu M)$
(67)	1.5
(68)	0.55
(69)	28
(70)	60
(71)	>>200
(72)	>>200

the same sponge also showed reasonable activity in a similar assay (IC_{50} = 5μM) [146]. Other synthetic variants and natural analogues were less potent than halenaquinone against $pp60^{v\text{-}src}$ [144–146].

Limited structure-activity relationship studies indicate that the minimum requirements for strong PTK inhibitory activity of halenaquinone derivatives is the presence of a pentacyclic skeleton with electrophilic sites at each end of the molecule. Additionally, a planar polyunsaturated framework with a quinone end ring is a necessary, but insufficient condition for activity [146].

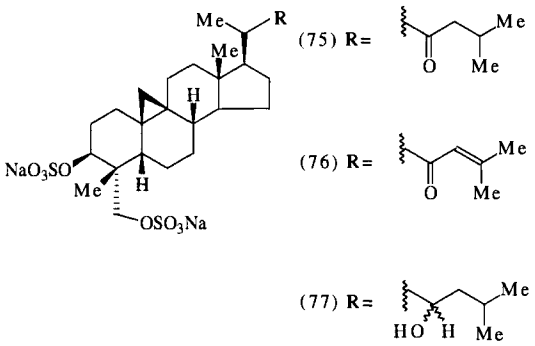
In other biological testing, halenaquinone (67) was found to be inactive against PKC [146]. In terms of its potential anti-proliferative activity, halenaquinone (67) inhibited cell growth in five cell-lines which express oncogenic PTK activity with an IC_{50} of 1–10 μM [144]. Further investigation showed that halenaquinone (67) and xestoquinone (70) were potent growth inhibitors but ineffective as cytotoxins in assays against over 50 types of tumour cells including leukaemia, non-small cell lung, small cell lung, colon, CNS, melanoma, ovarian and renal cancers [146].

Five metabolites of the sponge *Dysidea avara*, each bearing a 4, 9-friedodrimane array linked to a quinone moiety containing additional



(74)

heteroatoms, were also investigated [147]. Only melemeleone B (74) showed moderate activity against $pp60^{v\text{-}src}$ (IC_{50} = 28μM), although the inclusion of 2-mercaptoethanol in the assay mixture may have affected the results, indicating that a quinone group plus additional functionalities are required for effective activity.



Cycloartanol disulphates

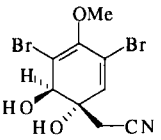
Three novel cycloartanol disulphates (75-77) were isolated from the green alga *Tydemania expeditiones* as moderate inhibitors of $pp60^{v\text{-}src}$ (*Table 6.28*) [148]. Conversion to the corresponding alcohols abolishes this activity.

(+)-Aeroplysinin-1

(+)-Aeroplysinin-1 (78) is a naturally occurring tyrosine metabolite from the marine sponge *Verongia aerophoba*. Kreuter *et al.* [149] demonstrated that during treatment with (+)-aeroplysinin-1, MCF-7 breast carcinoma cells lost their ability to respond to EGF stimulation, leading to cell death. Almost total inhibition of autophosphorylation of the EGFR purified from MCF-7 cells was observed at concentrations of (+)- aeroplysinin-1 as low as 0.5 μM. These results and a previous observation [150] that (+)-aeroplysinin-1 displayed high anti-tumour activity in the L5178y mouse

Table 6.28 INHIBITION OF $pp60^{v\text{-}src}$ BY CYCLOARTANOL SULPHATES [148]

compound	*IC_{50}(μM)*
(75)	20
(76)	25
(77)	63



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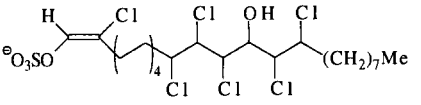


(79)

ascites tumour model *in vivo*, indicate that this compound has potential as a chemotherapeutic agent. However, further investigations into the inhibition of PTKs by (+)-aeroplysinin-1 have been scarce.

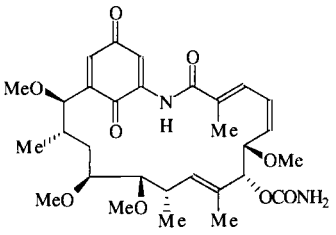
Malhamensilipin A

A chlorosulpholipid, malhamensilipin A (79) has been isolated from a cultured chrysophyte alga, *Poterioochromonas malhamensis*, and is a modest inhibitor of $pp60^{v\text{-}src}$ (IC_{50} 35 μM) [151]. Further investigations are required to establish selectivity.

MISCELLANEOUS INHIBITORS

Herbimycin A

Herbimycin A (80), from *Streptomyces hygroscopicus,* is a representative of the benzoquinoid ansamycin group of antibiotics. This compound was found to reverse the morphology of Rous sarcoma virus-infected rat kidney cells [152]. Other members of this group containing a benzoquinone moiety were also found to induce the phenotypic change from transformed to normal morphologies, accompanied by a loss of activity of the PTK $pp60^{v\text{-}src}$ and a reduction in the level of *in vivo* tyrosine phosphorylation [153]. Thus,



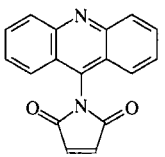
(80)

it seemed likely that the inactivation of the PTK activity of $pp60^{v\text{-}src}$ by these compounds caused the reversal of the transformed phenotype [154]. Further investigations showed that herbimycin A caused reversal of phenotype in several other cell-lines transformed by oncogenes whose protein products are PTKs, for example, *src, yes, fps, ros, abl,* and *erb*B, but was unable to reverse the transformed morphologies induced by non-PTK oncogenes, such as *ras, raf* and *myc* [155]. It has been demonstrated that the return to normal phenotype correlates with a decrease in the levels of proteins phosphorylated on tyrosine residues in those cells transformed by *src, ros* and *erb*B.

In vitro studies with herbimycin A have shown that it inhibits non-receptor PTKs such as $p120^{v\text{-}abl}$, $p130^{v\text{-}fps}$, $p210^{bcr\text{-}abl}$, $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ with IC_{50} values of about 10 μM [156]. The addition of compounds containing sulphydryl groups completely abolishes the inhibitory activity of herbimycin A toward $pp60^{v\text{-}src}$ as well as preventing reversal of cell morphology, suggesting the possibility that sulphydryl groups of $pp60^{v\text{-}src}$ are involved in the inactivation of its PTK activity [157]. Fukazawa *et al.* [145] considered the effects of several thiol reagents on $p60^{v\text{-}src}$; some were potent inactivators of the enzyme, others showed no effect. However, pre-treatment with the ineffectual reagents protected the kinase from inactivation by herbimycin A, suggesting that the thiol group to which the inhibitor binds is not essential for kinase activity, but may be located close to the active site.

Herbimycin A does not inhibit the activities of PKA or PKC, although the thiol reagent *N*-(9-acridinyl)maleimide (81) (an inactivator of $p60^{v\text{-}src}$) does inhibit these enzymes [158]. Thus, herbimycin A appears to bind specifically to reactive thiol groups of non-receptor PTKs, thereby conferring a biochemical basis for its selectivity in reversing transformation of cells.

Like other PTK inhibitors, herbimycin A has also been shown to have other cellular effects, for example, the inhibition of substrate tyrosine phosphorylation, inositol phospholipid turnover and calcium elevation in T cells [159]. However, much interest has evolved around the anti-cancer



(81)

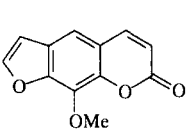
potential of this compound, particularly in the Philadelphia chromosome (*bcr-abl* fused gene product) associated leukaemias. Herbimycin A has been shown to induce erythroid differentiation of human chronic myelogenous leukaemia K562 cells [160, 161] and inhibits the growth of acute limphoblastic leukaemia cells [162], both of which express high levels of the *bcr-abl* tyrosine kinase. Herbimycin A did not inhibit the growth of Philadelphia chromosome-negative leukaemia cells [162], indicating that specific inhibitors of the *bcr-abl* gene product offer possible antitumour therapy in Philadelphia chromosome-positive leukaemias.

Herbimycin A inhibited the growth of seven colon tumour cell-lines to a greater extent than it inhibited normal colonic mucosal cells [163]. It also dose-dependently inactivated $pp60^{v\text{-}src}$, correlating well with inhibition of cell growth. These effects were reversible on removal of the inhibitor. In addition, herbimycin A at about 20 ng/ml caused 50% inhibition of growth of a mouse leukaemia cell-line which expresses *v-abl* at high levels; administration of herbimycin A to nude mice inoculated with these cells enhanced their survival rate [164].

These observations show that herbimycin A should be a valuable lead compound for the future development of PTK inhibitors with therapeutic potential in these type of malignancies.

Psoralens and UV light (PUVA)

The combination of psoralens, for example, 8-methoxypsoralen (82) and ultra-violet A light (320–400 nm wavelength) may be used in the treatment of skin diseases such as psoriasis [165]. Mermelstein *et al.* [166] found that



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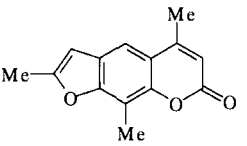
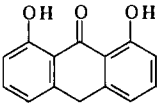


(83)

in A431 cells exposed to PUVA- 4,5',8-trimethylpsoralen (83) and UVA -the EGF-stimulated EGFR activity was dramatically reduced. This event was associated with increased serine phosphorylation of the EGFR, indicating that PUVA-induced serine phosphorylation may mediate EGFR activity, and that the biological actions of the psoralens may be associated with alterations in PTK signal transduction pathways.

Hypericin

The activity of PUVA as an inhibitor of the EGFR PTK in A431 cells could be relevant to the treatment of psoriasis. Bearing this in mind, it was observed that dithranol (84), a widely-used anti-psoriatic drug, shares a structural similarity with many known PTK inhibitors. De Witte *et al.* therefore examined whether this compound and related structures showed specific inhibition of PTKs and chose the naphthodianthrone, hypericin (85), for further investigation [167]. Hypericin (85) from the genus



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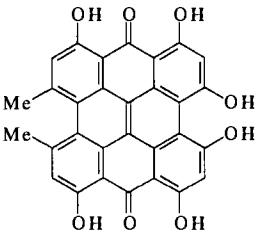


(85)

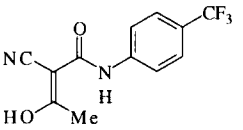
Hypericum [168] causes a dose-, time- and temperature-dependent inhibition of EGF-stimulated autophosphorylation of the EGFR PTK from membrane fractions of A431 cells, with IC_{50} values ranging from 0.37 to 8.7 μM [167]. In comparison, quercetin (1) (IC_{50} = 21 μM) was not time or temperature-dependent. Time-dependent inhibition may be explained by metabolism of hypericin to a more potent inhibitor, but pre-incubation in the phosphorylation buffer before adding the membranes did not generate a more active inhibitory species, indicating that the inhibition depends on the contact time between the inhibitor and the enzyme. Photosensitisation of hypericin by irradiating the assay samples with fluorescent light decreases the IC_{50} value to 44 nM, probably due to the formation of hypericin radicals.

Hypericin at high concentrations appears to be an irreversible inhibitor and shows non-competitive inhibition with respect to both ATP and the peptide substrate (lower concentrations, however, suggest mixed competi-

tion). Irreversible inhibition may be inferred to be by covalent binding to the enzyme, possibly through a Michael addition as postulated for other PTK inhibitors. Hypericin also appears to be fairly specific for PTKs, being relatively inactive against several other serine/threonine kinases, although it does inhibit PKC activity ($IC_{50} = 3.9$ μM) [169]. Hypericin has anti-retroviral effects, including human immunodeficiency virus (HIV), *in vitro* and *in vivo*, and lacks toxicity in mice and humans [170]. This compound, therefore, may serve as a therapeutic tool to investigate the links between PTK activity and the intracellular transmission of HIV-induced cytopathic signals.

Leflunomide

The active metabolite of leflunomide, A77 1726 (86), inhibits the proliferation of a variety of mammalian cell-lines in culture. This proliferation is likely to be mediated by the inhibition of PTK activity, as A77 1726 inhibits the EGF-dependent proliferation of the human foreskin fibroblast (HFF) cell-line reversibly at 30–40 μM, and inhibits the autophosphorylation of the EGFR PTK (20–30 μM) and the purified enzyme (40 μM) [171]. The failure to inhibit PKC at >150 μM shows a certain specificity for PTK inhibition. Leflunomide was developed for its potential as an immunomodulatory drug. These results may link this activity with PTK signalling pathways.



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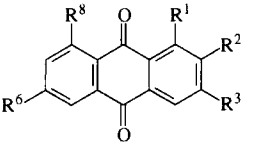


(87a-f)

Emodin

Jayasuriya *et al.* [172] isolated the anthraquinone emodin (87a) from the Chinese medicinal plant *Polygonum cuspidatum*, and found it to have potent inhibitory activity against the PTK $p56^{lck}$ by competition with respect to ATP. Emodin has previously been shown to possess vasorelaxant and immunosuppressive properties [173, 174] and these results tend to confirm the link between these attributes and PTK activity suggested by Huang *et al.* [175].

Limited structure-activity studies have been carried out with analogues of emodin (*Table 6.29*) [176]. A hydroxyl group at the β-position, which cannot

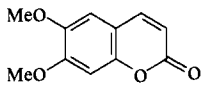
Table 6.29 INHIBITION OF $p56^{lck}$ PTK ACTIVITY BY EMODIN AND ITS ANALOGUES [6,176]

compound	R^1	R^2	R^3	R^6	R^8	$IC_{50}(\mu M)$
(87a)	OH	–	Me	OH	OH	18.5
(87b)	OH	–	Me	OMe	OH	>2500
(87c)	OH	–	Me	OGlu	OH	>2500
(87d)	OH	–	–	–	–	>2500
(87e)	–	OH	–	–	–	89
(87f)	–	OAc	–	–	–	>2500

hydrogen bond intramolecularly, would seem necessary for activity [*cf.* (87a) and (87e) *vs.* (87d)] whilst replacement with methoxy (87b) or acetyloxy (87f) groups totally abolishes activity. Once again, the quinone moiety is present in these inhibitors, and this together with a hydroxyl group at the β-position may well be the minimum requirements for activity.

Scoparone

Scoparone (88), a coumarin isolated from the Chinese herb *Artemisia scoparia*, exhibits immunosuppressive and vasodilator activity, and reduces plasma lipoprotein levels in hyperlipidaemic rabbits. These observations contrast with the immunosuppressive drug cyclosporin A (89) which is associated with development of hypertension and hyperlipidaemia.

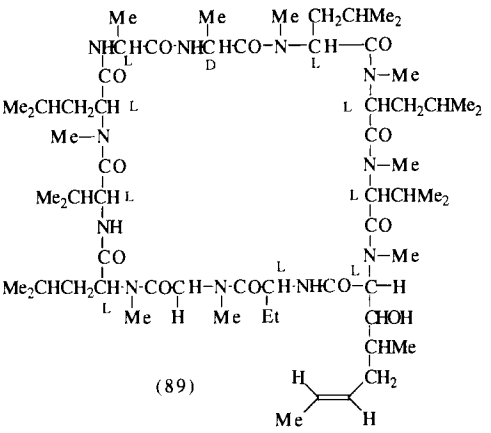


(88)

Certain PTK inhibitors, including genistein, have been associated with the immunosuppressive response. Thus, cross-desensitisation and non-additivity responses of scoparone and genistein to phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells, indicates that the immunosuppressive scoparone may act in part through inhibition of PTKs [175]. In contrast, no cross-desensitisation occurred between scoparone and cyclosporin, indicating different mechanisms of action.

BE-23372M

BE-23372 (90), isolated from the fungus *Rhizoctonia solari*, is one of the



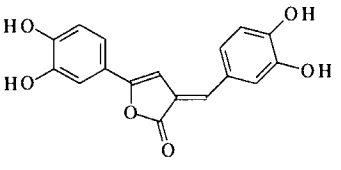
(89)

most potent and selective inhibitors of EGFR PTK from A431 cells (IC_{50} = 0.02–0.03 μM) [177–179]. It also inhibits other tyrosine kinases, that is, *erb*B-2, $p43^{v\text{-}abl}$, IRK and $pp60^{c\text{-}src}$ with IC_{50} values of 0.42, 1.0, 3.3 and 4.5 μM respectively [179]. It is 200 to 1000-fold less potent against PKC and PKA. In addition, BE-23372M inhibits the growth of A431 human epidermoid carcinoma and MKN-7 stomach cancer cell-lines with IC_{50} values of 8 and 24 μM respectively.

A kinetic study revealed that it was competitive with respect to both ATP and the peptide substrate [178]. The presence of structural elements found in other PTK inhibitors, such as 3, 4-dihydroxyphenyl rings and the incorporation of two styrene moieties into the same compound, is noteworthy. Further investigations should demonstrate the potential of this natural product as a clinically useful PTK inhibitor.

Epiderstatin

Epiderstatin (91), a piperidinedione isolated from *Streptomyces pulveraceus*



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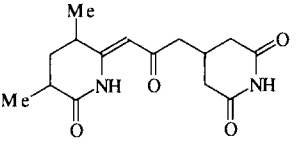


(91)

subsp. *epiderstagenes* [180] was shown to be a potent inhibitor of the proliferation of quiescent mouse epidermal keratinocytes stimulated by EGF [181]. However, epiderstatin failed to inhibit the *in vitro* activity of the EGFR PTK. The mechanism of action, therefore, remains unknown, but it would seem possible that its activity may be due to the inhibition of other PTKs downstream from the EGFR.

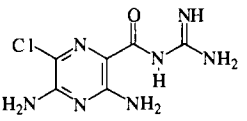
Angelmicins

Angelmicins A and B from the actinomycete *Microbispora* have been found to inhibit oncogenic signal transduction. Reduction in the autophosphorylation of $p60^{v\text{-}src}$ tyrosine kinase was observed in a dose-dependent manner with these antibiotics [182].

Angelmicin B inhibited the growth of *abl* and *src* transformed cells with IC_{50} values ranging from 0.5 to 5.0 μg/mol, in a defined serum-free medium. The IC_{50} values increased 7- and 10-fold respectively for *src* and *abl* transformed cells in serum-containing medium. Angelmicin B has the ability to revert the oncogene-transformed cell phenotype of low serum requirement to the normal cell phenotype. Moreover, no inhibitory effect on PKC and PKA was observed with this compound. Exact structures for the angelmicins have not yet been established. The presence of an anthraquinone and six sugar moieties have been postulated on the basis of structural analysis [182].

Amiloride

The diuretic amiloride (92) was one of the first compounds to be identified as an inhibitor of EGFR autophosphorylation in A431 cells (IC_{50} = 300 μM) [183]. It was also found to inhibit the PTK activity of both the PDGFR and IR. This inhibition is due to competition with ATP. The lack of



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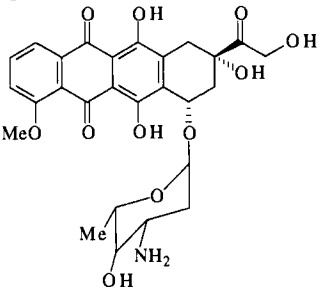


(93)

potency, and numerous other effects, including the inhibition of serine/threonine kinases, made this a poor lead compound for further investigation, despite its ability to inhibit tumour growth *in vivo* [184].

Adriamycin

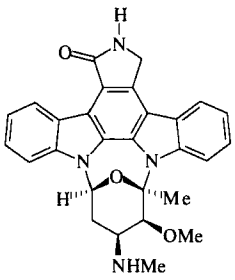
The anti-neoplastic agent adriamycin (93) has been shown to inhibit the activity of several PTKs by competition with respect to ATP, but not the activity of serine/threonine kinases, except PKC [185]. Whether the pharmacological properties of adriamycin can be attributed to its effects on either PTKs or PKC is not clear, but evidence that adriamycin may exert its cytotoxic activity by interaction at the cell surface [186], without entering the cell, suggests a link with the inhibition of cell membrane receptors.

Staurosporine

Staurosporine (94), a member of the indolecarbazole group of antibiotics was isolated from *Streptomyces staurosporeus* [187] and has proved to be a very potent but non-specific inhibitor of several kinases including EGFR, IR, v-*src*, c-*src*, *lck*, HPK40, PKA and PKC. IC_{50}s at nM level have been observed in all cases [188 and references therein]. Staurosporine exerts its effect by competition with ATP [189], although other ATP-competitive protein kinase inhibitors did not compete with staurosporine, suggesting the putative existence of a distinct, specific binding site for staurosporine on these enzymes. It has been shown that staurosporine (10nM) caused transformed *trk*-3T3 cells (NIH3T3 cells transformed with the *trk* oncogene) to revert to the normal phenotype [190], and inhibited NGF dependent-tyrosine phosphorylation by the $pp140^{c\text{-}trk}$ NGF receptor and $pp70^{trk}$ PTKs at 10–100nM. Staurosporine can thus be considered as a potential lead compound for the possible development of specific PTK inhibitors, although it gained interest as a PKC inhibitor due to slightly higher potency toward PKC. Another bisindole alkaloid K252a (95), which shows little selectivity for PKC, inhibits the PTK activity of *trk* selectively over other tyrosine kinases such as EGFR and *src* [191]. A number of K252a derivatives have been claimed to be inhibitors of the *trk* family of oncogenes [192].

Dianilinophthalimides (DAPHs)

During an attempt to synthesize selective protein kinase inhibitors based on the staurosporine structure, Trinks *et al.* [193] observed that DAPHs



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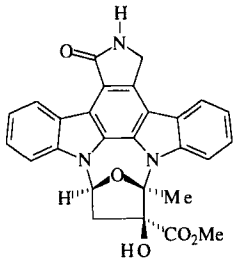
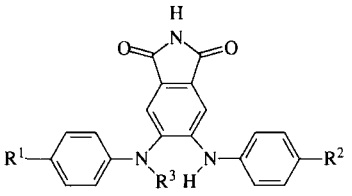


(95)

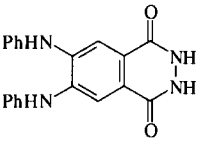
inhibited the EGFR PTK selectively and competitively with respect to ATP. They had little effect against PKC, PKA and other PTKs (*Table 6.30*). Compounds (96a-c) showed greater than 40-fold selectivity for inhibition of the EGFR PTK over the closely related c-*src* PTK, and even greater selectivity over the v-*abl* PTK, PKC and PKA. The hydroxy-substituted derivative (96d), showed little selectivity, whilst (96f) displayed selectivity for inhibition of the c-*src* PTK over the EGFR PTK. Compound (96e) indicates that inhibition can be achieved with unsymmetrical compounds. Other compounds were prepared (not shown) that illustrated certain structural features required for inhibitory activity. One of these is the importance of a free phthalimide nitrogen, as substitution at this position completely abolishes activity. Additionally, small substituents at positions R^1 and R^2 are required; bulkier substituents, such as methoxy, diethylamino and iodo completely abolish activity, as do cyano groups. A free diphenylamine nitrogen is also required. DAPH 1 (96a) was tested *in vitro* against a panel of protein kinases and found to be very selective (*Table 6.31*) [194]. It has been postulated that an asymmetric propeller-shaped

Table 6.30 INHIBITION OF PTKS, PKC, AND PKA BY 4,5-DIANILINOPHTHALIMIDE DERIVATIVES [193]

				$IC_{50}(\mu M)$				
compound	R^1	R^2	R^3	*EGFR*	*v*-abl	*c*-src	*PKC*	*PKA*
(96a)	H	H	H	0.3	>50	16	80	>500
(96b)	F	F	H	0.7	>50	50	32	>500
(96c)	Me	Me	H	2.5	>100	>100	>100	>500
(96d)	OH	OH	H	2.5	12	8	75	>500
(96e)	OMe	H	H	2.0	>100	64	>100	>500
(96f)	H	H	Me	8.7	>50	1.0	>500	>500



(96a-f)



(97)

conformation is responsible for the selectivity in contrast to the planar staurosporine aglycone. The degree of inhibition of the EGFR PTK was reflected in similar results obtained for inhibition of autophosphorylation of EGFR from A431 cells. Potent inhibitors of the EGFR PTK at the enzyme level also showed potent anti-proliferative activity against Balb/MK cells (mouse epidermal keratinocytes which are dependent on EGF). DAPH 1 (96a) is reported to inhibit the growth of xenografts of the A431 and SK-OV-3 tumours, which over-express the EGFR and p185$^{c\text{-}erbB2}$ [194].

These results demonstrate once again the potential for ATP-competitive compounds to be potent and selective inhibitors of protein kinases. Indeed, dianilinophthalimides may prove to be useful candidates for future investigation. Closely related phthalazinone derivatives such as compound (97), are also claimed to be PTK inhibitors [195].

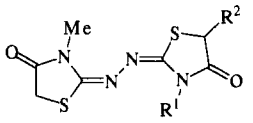
Thiazolidinediones

Thiazolidinediones represent a class of synthetic compounds that have been

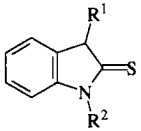
Table 6.31 *IN VITRO* SELECTIVITY OF KINASE INHIBITION BY DAPH1 (96a) [194]

enzyme	$IC_{50}(\mu M)$
EGFR	0.84
EGFR ICD	0.3
v-*abl*	>50
c-*src*	16
c-*lyn*; c-*fgr*; TPK IIB	>150
CK-1, CK-2	>200
PKCα	6.0
PKCβ-1	30
PKCβ-2	4.8
PKCε,ζ,η; PPK; PKA	>500

discovered somewhat serendipitously, but nevertheless provide an alternative model structure for the design of PTK inhibitors. The three compounds (98a-c) shown in *Table 6.32* inhibit both EGFR autophosphorylation (*in*



(98a-c)



(99a-i)

vitro and *in situ*) and c-*src* PTK activity relatively potently, compared with genistein, but do not inhibit v-*abl* PTK activity [196]. In addition, these compounds do not inhibit PKC or PKA. They show mixed type inhibition and may differentiate betwen members of the PTK family with different substrate preferences. The substituent R^2 seems to dictate the activity of these compounds as activity is completely lost when R^2 is changed to H, Me, OH, CH_2OH, CH_2COOH or OSO_3H (not shown) [196].

These compounds also inhibit the proliferation of EGF-dependent and EGF-independent cell-lines; the ability to inhibit both EGFR and c-*src* PTK activity may be a reflection of this broad spectrum of activity.

Indoles

Although not directly related to thiazolidinediones, the 2, 3-dihydro-2-thiono-1*H*-indole-3-alkanoic acids and their amides (*Table 6.33*) and the 2, 2′-dithiobis(1*H*-indole-3-alkanamides) and their carboxy derivatives (*Tables 6.34-6.37*) bear several similarities in that they are based upon heterocyclic ring systems, some of which are joined by two adjacent hetero atoms, and have been discovered as PTK inhibitors independently of a natural lead compound [197, 199–205].

Table 6.32 INHIBITION OF EGFR, V-*ABL*, AND C-*SRC* PTK ACTIVITY BY THIAZOLIDINE-DIONES [196]

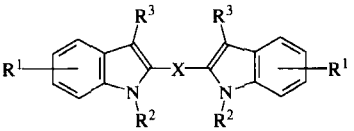
			$IC_{50}(\mu M)$		
compound	R^1	R^2	*EGFR*	*v*-abl	c-*src*
(98a)	$CH_2CH{=}CH_2$	$=CH_2$	6	>100	4
(98b)	$CH_2C(Me){=}CH_2$	$=CH_2$	1	~100	3
(98c)	$CH_2C(Me){=}CH_2$	CH_2NMe_2	2	>100	7
(4)			1	39	>50

The alkanoic acids and derivatives (99a-f) (Table *6.33*) appear to be non-competitive inhibitors of the activity of the native EGFR complex shed from A431 cell membranes [198] with respect to the peptide substrate [199]. A structure-activity analysis reveals that for both thiones and disulfides (*Tables 6.33* and *6.34*), the propanoic acid derivatives (99c), (99e) and (100e-i) appear to possess the optimal length for a side-chain. *N*-Methylation of indolinethiones (99b), (99e), and (100b) generally decreases activity, possibly due to steric or electronic effects. For the corresponding methyl esters, (99d) and (100c), the inhibitory activity is diminished. These observations probably reflect the requirement for these compounds to hydrogen bond within the active site of the enzyme. The amide derivatives (99g-i) are more potent inhibitors of PTK $pp60^{v\text{-}src}$, once again illustrating the point that minor structural changes allows differential recognition between closely related enzymes. The ability of thiones to tautomerise to the corresponding thiol, enabling them to rapidly oxidize to the disulphide, together with the greater inhibitory potency of the disulphides, indicates that the activity of the monomeric thiones is probably due to conversion to the disulphide during the assay [197].

Substitution on the indole ring of the disulphides (100g-i) is tolerated but reduces the ability to inhibit proliferation of Swiss 3T3 mouse fibroblasts. For these compounds, enzyme inhibition does not generally correlate with inhibition of cell growth. In contrast to the *in vitro* results, the esters, (99d) and (100c), are considerably more active than the corresponding acids, (99c) and (100b), reflecting their greater lipophilicity and consequent ability to cross the cell membrane.

Table 6.33 INHIBITORY EFFECTS OF 2,3-DIHYDRO-2-THIOXO-1*H*-INDOLE-3-ALKANOIC ACIDS AND DERIVATIVES ON THE EGFR AND $pp60^{v\text{-}src}$ PTKS AND THE PROLIFERATION OF SWISS 3T3 CELLS [197]

			$IC_{50}(\mu M)$		
compound	R^1	R^2	*EGFR*	$pp60^{v\text{-}src}$	3T3
(99a)	CH_2CO_2H	H	14.9	–	–
(99b)	CH_2CO_2H	Me	>100	–	–
(99c)	$(CH_2)_2CO_2H$	H	1.6	–	64
(99d)	$(CH_2)_2CO_2Me$	H	>100	–	16
(99e)	$(CH_2)_2CO_2H$	Me	6.7	–	–
(99f)	$(CH_2)_3CO_2H$	H	6.7	–	–
(99g)	$CH_2CONHCH_2Ph$	H	~100	3.2	–
(99h)	$(CH_2)_2CONH_2$	H	22	2.0	–
(99i)	$(CH_2)_2CONHCH_2Ph$	H	~100	9.3	–

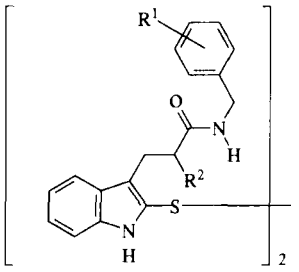


(100a-l)

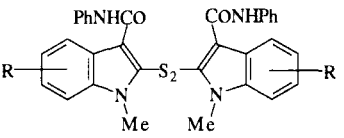
In response to these observations with the alkanoic acids and their methyl esters, Denny and co-workers prepared a series of amide analogues to assess the inhibitory properties of such neutral, hydrogenbond donor groups [200]. The effect of the amide group on the disulphides was generally inconsequential, except for the *N*-benzylamide (100e) which is a more potent inhibitor of the EGFR PTK than any of the free acids (100a), (100b), and (100d-i) although once again they are all potent inhibitors of $pp60^{v\text{-}src}$. The potency of (100e) prompted the preparation of similar compounds (*Table 6.35*), investigating the effect of further substitution on the benzyl ring or the α-position of the propanamide side-chain. None of these compounds are as active as the parent compound for the inhibition of EGFR PTK, but activity for inhibition of $pp60^{v\text{-}src}$ is increased. Indeed, several of these compounds (101b), (101e), and (101f) show a remarkable difference in potency for the two closely related PTKs, leading to the conclusion that the nature of the

Table 6.34 INHIBITORY EFFECTS OF 2,2′-DITHIOBIS(1*H*-INDOLE-3-ALKANOIC ACIDS) AND DERIVATIVES ON THE EGFR AND $pp60^{v\text{-}src}$ PTKS AND THE PROLIFERATION OF SWISS 3T3 CELLS [197,200]

					$IC_{50}(\mu M)$		
compound	R^1	R^2	R^3	*X*	*EGFR*	$pp60^{v\text{-}src}$	3T3
(100a)	H	H	CH_2CO_2H	S_2	10.2	–	>50
(100b)	H	Me	CH_2CO_2H	S_2	53	–	36
(100c)	H	H	CH_2CO_2Me	S_2	18	–	2.3
(100d)	H	H	CH_2CO_2H	S_1	9.3	–	8
(100e)	H	H	$(CH_2)_2CO_2H$	S_2	4.2	51	59
(100f)	H	Me	$(CH_2)_2CO_2H$	S_2	3.1	–	–
(100g)	5-Me	H	$(CH_2)_2CO_2H$	S_2	8.4	–	>50
(100h)	6-Me	H	$(CH_2)_2CO_2H$	S_2	2.9	–	–
(100i)	7-Me	H	$(CH_2)_2CO_2H$	S_2	1.5	–	–
(100j)	H	H	$CH_2CONHMe$	S_2	9.4	0.75	–
(100k)	H	H	CH_2CONMe_2	S_2	12	1.2	–
(100l)	H	H	$(CH_2)_2CONHCH_2Ph$	S_2	0.85	2.9	5.9



(101a-g)



(102a-p)

side-chain is an important determinant for the inhibitory specificity of these compounds on different kinases.

The effect of some of the amides on intracellular tyrosine phosphorylation in Swiss 3T3 fibroblasts does not correlate with that observed in isolated enzymes. The compounds (100e) and (101g) are inactive against EGFR autophosphorylation, whereas they are potent inhibitors of FGF-mediated tyrosine phosphorylation. The compound (101g) is also effective against PDGFR autophosphorylation [200].

Rewcastle *et al.* [201] observed that 3-*N*-phenylcarboxamides (*Table 6.36*) are also effective inhibitors of EGFR and pp60$^{v\text{-}src}$, but that there is no clear correlation with indole ring substitution and potency, although there is some specificity between EGFR and pp60$^{v\text{-}src}$ by some analogues. Compounds (102f) and (102g) are specific inhibitors of EGFR and compounds (102d), (102e), (102h), and (102n) have some specificity for pp60$^{v\text{-}src}$. The opposite specificities of compounds (102g), and (102h) show

Table 6.35 INHIBITORY EFFECTS OF 2,2′-DITHIOBIS[*N*-(PHENYLMETHYL)-1*H*-INDOLE-3-PROPANAMIDES] AND DERIVATIVES ON THE EGFR AND pp60$^{v\text{-}src}$ PTKS AND THE PROLIFERATION OF SWISS 3T3 CELLS [200]

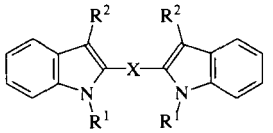
			$IC_{50}(\mu M)$		
compound	R^1	R^2	*EGFR*	*pp60$^{v\text{-}src}$*	3T3
(101a)	4-CO_2H	H	7.4	1.5	–
(101b)	4-CO_2Me	H	44	1.8	–
(101c)	3-OH-4-CO_2H	H	8.5	nd	–
(101d)	3-OH-4-CO_2Me	H	~100	~100	–
(101e)	H	NHCOMe	51	0.5	–
(101f)	H	$NHCOCF_3$	>100	0.7	–
(101g)	H	NH_2	7.6	1.5	5.3

Table 6.36 INHIBITORY EFFECTS OF 2,2′-DITHIOBIS(1-METHYL-*N*-PHENYL-1*H*-INDOLE-3-CARBOXAMIDES) ON THE EGFR AND pp60$^{v\text{-}src}$ PTKS AND THE PROLIFERATION OF SWISS 3T3 CELLS [201]

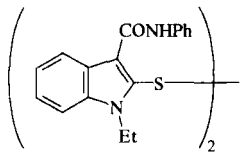
		$IC_{50}(\mu M)$		
compound	*R*	*EGFR*	*pp60$^{v\text{-}src}$*	3T3
(102a)	H	10	3.2	12
(102b)	4-Me	>100	>100	4.0
(102c)	4-OMe	>100	>100	1.9
(102d)	4-OAc	20	0.65	nd
(102e)	5-F	>100	6.0	nd
(102f)	5-Cl	4.3	>100	9.3
(102g)	5-Br	11.4	~100	nd
(102h)	5-CF_3	>100	5.8	nd
(102i)	5-Me	>100	>100	22
(102j)	5-OMe	>100	>100	20
(102k)	5-OAc	5.3	7.7	nd
(102l)	5-OH	40.5	3.9	nd
(102m)	6-OMe	3.6	3.6	17
(102n)	6-OAc	>100	3.5	nd
(102o)	7-OMe	6.5	3.6	24
(102p)	7-aza	22.3	5.2	10

that size, lipophilicity and electronic properties have little effect on specific activities. Like other amide derivatives, these compounds, for example, (102a), (102m), and (102o) also inhibit bFGF-mediated tyrosine phosphorylation in Swiss 3T3 fibroblasts and have no effect on EGFR autophosphorylation [201].

In contrast to the enzyme inhibition data, 4-substituted derivatives (*Table 6.36*) are more effective as inhibitors of the growth of Swiss 3T3 mouse fibroblasts [201]. To explore the effect of different substituents with different electronic properties at the 3-position, Palmer *et al.* [202] prepared a number of compounds and found that a wide variation in the nature of the side-chain at the 3-position is permissible (*Table 6.37*). Hydrogen bonding capability and lipophilicity of the substituent seem to be beneficial for inhibition of EGFR activity in the *N*-alkyl-3-carboxamide series, as shown by the compounds (103a), (103b), (103g), (103h), and (103l). Anionic character of the side-chain, (103j) and (103t), and disubstitution (103i) are more effective for pp60$^{v\text{-}src}$ inhibition. *N*-Aryl-3-carboxamide derivatives (103c-f) (Table *6.37*) are much more effective for pp60$^{v\text{-}src}$ inhibition. The ketones (103n), (103p), and (103q), but not (103o) which is inactive against EGFR, show moderate activity against both kinases. The 3-cyano



(103a-s)



(104)

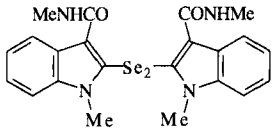
derivative (103s) displays some specificity towards EGFR whereas sulphone (103r) is more effective for $pp60^{v\text{-}src}$. Interestingly, higher alkyl groups on the indole nitrogen would seem to decrease activity since the N-ethyl derivative (104) is inactive against both the $pp60^{v\text{-}src}$ and EGFR.

Kinetic studies indicate non-competitive inhibition at both peptide substrate and ATP by the compounds of this class [203]. Reversal of inhibition by sulphydryl reagents suggests that thiol-disulphide exchange with an SH-group in the catalytic site of the enzyme is one possible mechanism of inhibition [204].

Table 6.37 INHIBITORY EFFECTS OF 3-SUBSTITUTED 2,2′-DITHIOBIS(1*H*-INDOLES) ON THE EGFR AND $pp60^{v\text{-}src}$ PTKS AND THE PROLIFERATION OF SWISS 3T3 CELLS [202]

		(X = S-S)	$IC_{50}(\mu M)$		
compound	R^1	R^2	*EGFR*	$pp60^{v\text{-}src}$	3T3
(103a)	H	CONHMe	6.1	7.9	–
(103b)	H	$CONHCH_2Ph$	~15	16.3	–
(103c)	H	CONHPh	~100	7.6	–
(103d)	Me	$CONHC_6H_4CO_2Me$-3	>100	5.4	–
(103e)	Me	$CONHC_6H_4CO_2Me$-4	>100	4.2	–
(103f)	Me	$CONHC_6H_4CO_2H$-4	16.9	4.3	–
(103g)	Me	$CONH_2$	4.7	0.8	–
(103h)	Me	CONHMe	1.8	6.7	1.8
(103i)	Me	$CONMe_2$	21.2	0.5	–
(103j)	Me	$CONHCH_2CO_2H$	10.0	1.01	–
(103k)	Me	$CONH(CH_2)_2NMe_2$	17.5	15.2	>50
(103l)	Me	$CONHCH_2Ph$	1.7	8.0	8.5
(103m)	Me	CONMePh	>100	5.5	–
(103n)	Me	COMe	5.6	11.5	–
(103o)	Me	COPh	>100	2.6	–
(103p)	Me	$COC_6H_4CO_2H$-4	5.5	4.0	–
(103q)	Me	$CONHC_6H_4CO_2Me$-4	6.1	7.1	–
(103r)	Me	$SO_2C_6H_4Me$-4	28.7	5.5	–
(103s)	Me	CN	6.9	28	–
(103t)	Me	$CONHCH_2C_6H_4CO_2H$-4	5.3	0.8	–

As diselenides are much less sensitive to sulphydryl reagents a series of indoline-2-selenone dimers, for example, (105), have been synthesized and found to be 10-times more potent than the compounds of the disulphide series [205], although similar kinetics have been observed. Reversal of inhibitory effects by sulphydryl reagents, although to a lesser extent than for the disulphides, suggests that a common mechanism is involved in inhibition by both disulphides and diselenides [204]. These indole derivatives are potent PTK inhibitors, although evidence for their selectivity over serine/theorine kinases remains to be seen.



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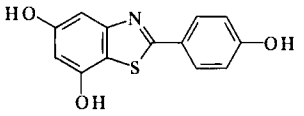

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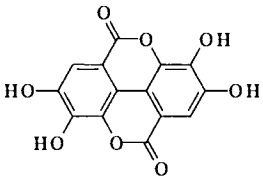
Benzothiazoles

Stevens *et al.* [206] have prepared a number of polyhydroxylated 2-phenylbenzothiazoles. A marginal inhibitory activity towards the EGFR PTK (from A431 cells) is reported for these compounds, 4, 6-dihydroxy-2-(4-hydroxyphenyl)benzothiazole (106) being the most active with an IC_{50} of ~ 150 μM.

Ellagic acid and analogues

The discovery that ellagic acid (107) is a potent but non-specific PTK inhibitor ($pp60^{v\text{-}src}$, IC_{50} = 0.3 μM; PKA, IC_{50} = 0.6 μM) prompted Dow *et al.* [207] to synthesize analogues of the acid possessing better selectivity towards $pp60^{c\text{-}src}$. A number of tricyclic ellagic acid analogues, (108) and (109), have been prepared and bioassayed. Although, these analogues are



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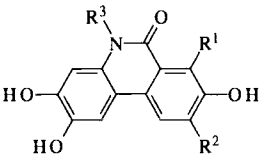

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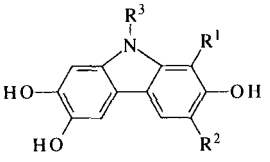

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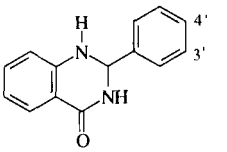


(110a-d)

less potent than ellagic acid, selectivity is observed in both series (*Table 6.38* and *6.39*). The unsymmetrical hydroxylation pattern is found to be more effective for selectivity and potency in both the phenanthridine (108) (*Table 6.38*) and carbazole (109) series (*Table 6.39*). *N*-Substitution in the carbazole series seems to play an important part both in selectivity and potency (*Table 6.39* and others not shown).

A partial mixed non-competitve inhibition for ellagic acid and phenanthridines and a competitive inhibition for carbazoles (with respect to ATP) has been reported [207]. Further investigations are required both at enzymatic (for other kinases) and cellular level before clinical trials.

Quinazolines and quinazolinones

As previously stated, molecular orbital calculations by Ferrel *et al.* indicated that the charge distribution of the pyranone ring in flavonoids is similar to that of the pyrimidine ring of ATP [35]. Thus, a number of 2-aryl-1,2,3,4-tetrahydroquinazolin-4-ones, (110a-d), were prepared on the basis that the ring system of these compounds might bear a charge distribution more similar to the pyrimidine ring than the flavonoids, and

Table 6.38 INHIBITION OF KINASES BY PHENANTHRIDINES [207]

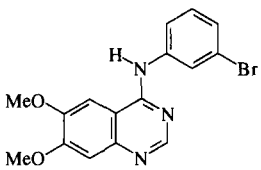
				$IC_{50}(\mu M)$	
compound	R^1	R^2	R^3	*c-src*	*PKA*
(107)				0.3	0.6
(108a)	H	OH	H	1.0	>800
(108b)	OH	H	H	0.9	92
(108c)	H	OH	Et	4.1	>2000
(108d)	OH	H	Et	11	541
(108e)	H	OH	CH_2Ph	5.4	390
(108f)	OH	H	CH_2Ph	21	284
(108g)	H	OH	$CH_2C_6H_4NO_2$-4	0.9	8.4
(108h)	OH	H	$CH_2C_6H_4NO_2$-4	61	309

Table 6.39 INHIBITION OF KINASES BY CARBAZOLES [207]

				$IC_{50}(\mu M)$	
compound	R^1	R^2	R^3	*c-src*	*PKA*
(107)				0.3	0.6
(109a)	H	OH	H	1.5	58
(109b)	OH	H	H	0.8	47
(109c)	H	OH	CH_2Ph	7.0	115
(109d)	OH	H	CH_2Ph	2.0	210
(109e)	H	OH	COPh	4.0	415
(109f)	OH	H	COPh	0.9	240
(109g)	H	OH	SO_2Ph	24	>600
(109h)	OH	H	SO_2Ph	15	250
(109i)	OH	H	$CH_2C_6H_4CN$-4	0.4	275
(109j)	OH	H	$CH_2C_6H_3Cl_2$-2,6	0.9	435
(109k)	OH	H	$CH_2C_6H_4SO_2Ph$-4	0.6	317

may, therefore, produce more potent inhibitors (*Table 6.40*) [208]. The 3′-and 4′-amino derivatives (110c) and (110d) were found to be more cytotoxic than quercetin (1), but neither of these compounds were inhibitors of the pt*abl*50 PTK or showed selective toxicity against the transformed cell-line, ANN-1. Interestingly, morphological studies showed that these compounds appeared to bring about cell death in the same manner as quercetin.

4-(3′-Bromoanilino)-6,7-dimethoxyquinazoline, PD153035 (111), is one of the most potent inhibitors of the EGFR PTK (IC_{50} = 29 ± 5.1pM). Little effect on other PTKs, for example, PDGFR, FGFR, IR, CSF-I-R, and *src* has been observed, except for $p185^{erbB2}$ (IC_{50} = 2.3 μM). Other biological effects, including inhibition of the autophosphorylation of the EGFR in human epidermal carcinoma and Swiss 3T3 fibroblasts at nanomolar concentration (14-15 nM) compared with >10 μM for PDGF and bFGF, blockage of EGF-mediated cellular processes such as mitogenesis, early



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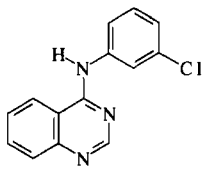

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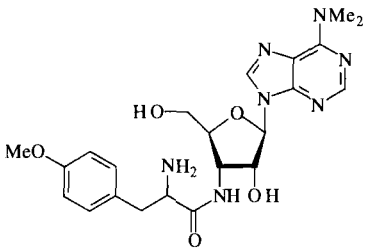
Table 6.40 INHIBITION OF THE PROLIFERATION OF ANN-1 AND 3T3 CELLS BY QUINAZOLINONES [208]

			$IC_{50}(\mu M)$	
compound	*3'*	*4'*	ANN-1	3T3
(110a)	–	NO_2	58–69	71
(110b)	NO_2	–	46–48	57
(110c)	–	NH_2	10–29	28
(110d)	NH_2	–	7–21	18–24

gene expression and oncogenic transformation, and reversal of the transformed morphology of cells, make it one of the most valuable candidates for clinical trials [209]. A similar compound 4-(3'-chloroanilino)quinazoline (112) is also reported to be a very potent (IC_{50} = 40 nM) and selective inhibitor of the EGFR PTK (from A431 cells and human placenta) and the EGF-stimulated growth of KB (human oral carcinoma) and normal rat kidney cell-lines with an IC_{50} of 1μM [210]. Competitive kinetics with respect to ATP and non-competitive with respect to the peptide substrate imply that it acts as an ATP analogue but with much higher affinity.

The synthesis of similar quinazoline derivatives as tyrosine kinase inhibitors has already been claimed by Barker [211].



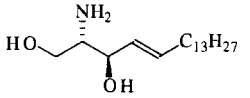
(113)

Puromycin

Puromycin (113) can inhibit tyrosine kinase activity in HL-60 leukaemia cells at micromolar level, without affecting PKC. It has been postulated that a metabolite of puromycin, PMP (complex with a peptide) is the active species rather than puromycin itself [212].

Sphingosines

The association of many PTKs with lipid membranes makes them potential targets for agents which modulate this interaction [213]. Sphingosines are known inhibitors of PKC activity but it has been shown that certain well defined sphingosines, for example, (114), inhibit the activity of *src* PTKs [214]. Paradoxically, some *N*-substituted sphingosines enhance the activity of these PTKs, whilst maintaining their inhibition of PKC. Inhibition of the autophosphorylation of the IR PTK both *in vitro* and *in vivo* has been observed by Arnold and Newton [215]. The mechanism by which these compounds affect PTKs is not understood.



(114)

$Me(CH_2)_4(CH{=}CHCH_2)_4CH_2CH_2CO_2H$

(115)

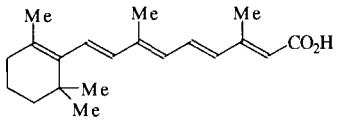
Fatty acids

Unsaturated fatty acids demonstrate different effects on different kinases. Arachidonic acid (115) (200 μM) stimulates PKC activity 3-fold [216], significantly inhibits cAMP-dependent PTK activity [217], inhibits strongly (IC_{50} 56 μM) the PDGFR PTK both *in vitro* and *in vivo* [218], and has no affect on the EGFR PTK *in vivo*. The effect of other fatty acids has also been studied [218, 219]. Inhibition seems to depend on the degree of unsaturation and the availability of the free OH group. Inhibition of the PDGFR PTK by the unsaturated fatty acids decreased as the extent of unsaturation decreased (arachidonic acid > linolenic acid > linoleic acid > oleic acid), while saturated fatty acids and phospholids were found to be inactive. A mechanism for inhibition has been postulated which involves the binding of the inhibitor to domain(s) of the PDGFR responsible for either dimerisation or catalytic activation.

Inhibition of certain PTKs by amino acid derivatives of polyunsaturated fatty acids has been claimed by Literati *et al.* [220].

Retinoic Acid

The oxidation product of vitamin A, retinoic acid (116) has been shown to inhibit EGF-stimulated cell proliferation, and this corresponds to its inhibition of the EGFR PTK. The possible mechanism involves alterations in the EGFR [221].



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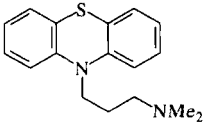


(117)

Chlorpromazine

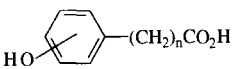
Chlorpromazine (117) is a non-specific inhibitor of *src* PTKs. The inhibition of phosphorylation of different phospholipids to different extents by chlorpromazine suggests that this inhibition of kinase activity involves the interaction with phospholipids [222].

Hydroxyphenylacetic acid

Shechter *et al.* [223] demonstrated that hydroxyphenylacetic acids (118, n = 1) displayed inhibitory activity in NB_2 rat lymphoma cells at millimolar level. An inhibitory order of $o > p > m$ has been reported. Despite the lack of potency, *o*-hydroxyphenylacetic acid exhibited a lower index of cytotoxicity and was specific for tyrosine kinases over serine kinases. The molecular distance between the phenol and the carboxylic acid group in hydroxyphenylacetic acid seems optimum as introduction of an extra methylene group (118, n = 2) resulted in a decrease in potency.

Hydroxybenzoic acid

At the same time that genistein was isolated from a *Pseudomonas* fermentation broth, *p*-hydroxybenzoic acid (119) was also found to inhibit



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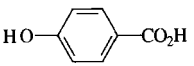
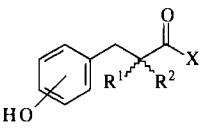


(119)

the EGFR PTK activity of A431 cell membranes [224]. Gazit *et al.*, however, found that it was inactive against the purified EGFR PTK [91].

Inhibition of PTKs with derivatives of 3-(hydroxyphenyl)lactic (120) and -pyruvic (121) acids has been claimed by Goto *et al.* [225].

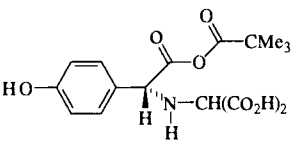
t-Boc-tyr-aminomalonic acids (122) and other analogues have been shown to inhibit the IR PTK, competitively with respect to the peptide substrate [226].



(120) R^1=H, R^2=OH
(121) R^1R^2= =O
X = OH, NH_2, OR

Phenazocine

(+)-Phenazocine (123) was found to be a potent inhibitor of the EGFR PTK, whilst other opiates were found to be inactive during an investigation of the association of the anti-neoplastic effects of opioid compounds with PTK inhibition [227].



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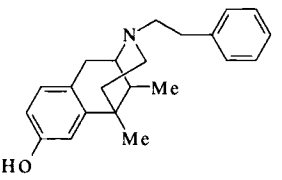
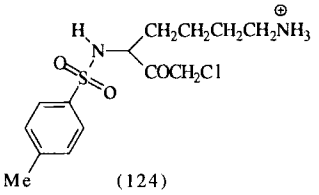


(123)

Halomethyl ketones

Some halomethyl ketone derivatives have been shown to possess the ability to inhibit tyrosine kinase activity. Richert *et al.* [228] observed that TLCK (124), a protease inhibitor, also inhibited $pp60^{v\text{-}src}$ kinase activity and reverted avian sarcoma virus transformed cells to normal phenotype.

Navarro *et al.* [229] have reported a number of amino acid and peptide derivatives of halomethyl ketones as inhibitors of tyrosine kinase activity in membranes from A431 epidermal carcinoma cells. *N*-α-*t*-Butoxycarbonyl-



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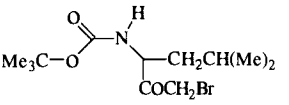
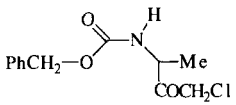


(125)

leucyl bromomethyl ketone (125) and *N*-α-benzyloxycarbonylalaninyl chloromethyl ketone (126) were found to be most potent. Although



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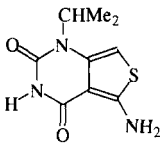


(127)

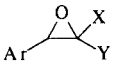
bromomethyl ketone (125) showed little discrimination between EGFR, $pp60^{src}$ and $p130^{fps}$, inhibition was selective versus serine/threonine kinases.

*5-Amino-1-isopropylthieno[3, 4-*d*]pyrimidine-2, 4-dione*

Zhang *et al.* [230] have reported the inhibition of human peripheral blood lymphocyte membrane PTK activity by 5-amino-1-isopropylthieno[3, 4-*d*]pyrimidine-2, 4-dione (127). They observed that inhibition was competitive with respect to the synthetic peptide substrate polyGAT.

Oxiranes

A large number of 3-aryloxiranes (128) have been claimed to be potent tyrosine kinase inhibitors with low toxicity [231].



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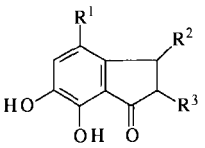


(129)

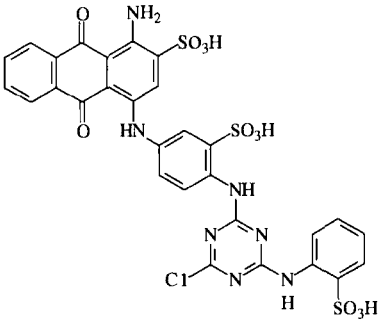
Dihydroxyindanones

Dow [232] has claimed that a number of dihydroxyindanone derivatives (129) can inhibit PTK activity.

Cibracron blue

Cibracron blue (130) is a dye which has ability to inhibit enzymes such as kinases, for example, PKA [233], dehydrogenases, ATPases, quinone reductase, and phosphodiesterase [234 and ref. therein]. When tested against

HPK40 from the human promyelocytic cell-line HL60, it was found to be very potent (IC_{50}, 2.5 μM) but it was more than 40-times less potent against EGFR, $pp60^{c\text{-}src}$, $p56^{lck}$ and PKC [188].



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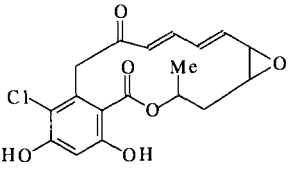


(131)

Radicicol

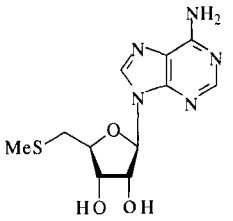
Radicicol (131) is a microbial agent, that has the ability to revert the v-*src* transformed rat fibroblast cell 3Y1 to normal morphology [235, 236] and promote differentiation in Friend Leukaemia cells [235]. It was found to be a potent (IC_{50} = 0.3 μM) and specific inhibitor of *src* kinase.

Vanadium

Inhibition or activation of PTKs by vanadium depends on the oxidation state of vanadium and the nature of the tyrosine kinase [237]. Vanadyl (4+) has inhibitory activity against the IR PTK (IC_{50} = 23μM) and IR-like GF-1 receptor (IC_{50} = 19μM). Cytoplasmic PTKs were poorly inhibited. It was observed that inhibition was non-competitive with respect to ATP, metal ion and substrate concentration. Autophosphorylation of both receptors was also inhibited in a concentration-dependent manner. Vanadyl compounds can act as a lead for the development of other inorganic PTK inhibitors which could be useful clinical agents.

ATP analogues

5-Methylthioadenosine (132), a methyl transferase inhibitor was found to be a specific inhibitor of the FGFR PTK in intact cells in a dose-dependent manner [238]. It was also observed that inhibition was rapid and reversible.



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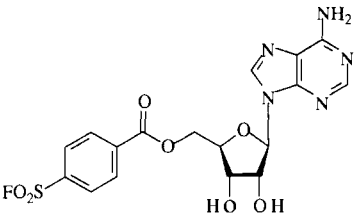


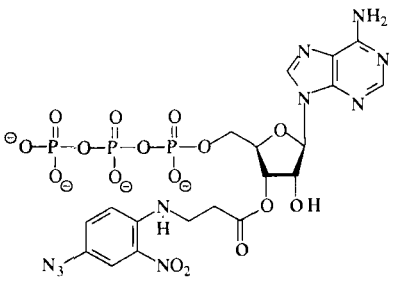
(133)

Although inhibition of NGF-induced phosphorylation was also reported [239], no effect was observed on PDGF- and EGF-induced phosphorylation [238].

Analogues of adenosine nucleotides incorporating reactive substituents such as benzoyl, azido and aldehyde groups have been used to irreversibly modify the nucleotide binding of certain proteins [240]. One such analogue, 5′-[4-(fluorosulphonyl)benzoyl]adenosine (5′-FSBA) (133) inhibits the PTK activity of $p60^{v\text{-}src}$ at millimolar levels by forming a covalent adduct with a tyrosine residue at the ATP binding site [241]. A similar mode of inhibition was observed for the EGFR PTK [242]. Unsurprisingly, the phosphorylation of serine and threonine residues was also affected.

3′-*O*-{3-[*N*-(4-Azido-2-nitrophenyl)amino]propionyl}-5′-triphosphate (134) was also found to inhibit the EGFR PTK from A431 cells [243].

Diadenosine 5′-oligophosphates $A(P)_nA$ (135, n = 2–6) also inhibit $pp60^{v\text{-}src}$ [244]. $A(P)_nA$ (135, n = 4) was found to inhibit $pp60^{v\text{-}src}$ kinase (IC_{50} = 1μM) selectively with respect to $pp60^{c\text{-}src}$ (IC_{50} = 46μM) and such results support the idea that the selective inhibition of closely related enzymes is possible.



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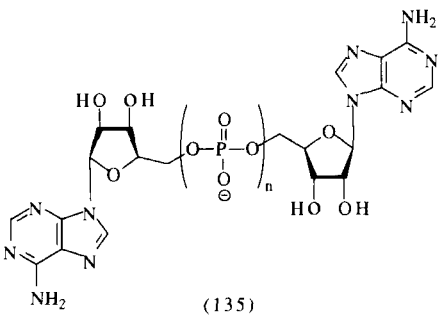


(135)

Oligodeoxynucleotides (ODNs)

5′-GTCCACCATGGCGCGGCCGGC-3′ (136) ODN1

A number of adenosine (α-nucleotide)-based inhibitors have been reported in the literature [241–244, 246, 248-252], but with few exceptions, all lack specificity for tyrosine kinases. Recently, a new class of nucleoside-based inhibitors has been described by Bergan *et al.* [245]. The synthetic oligodeoxynucleotides (ODNs) inhibit tyrosine kinases, aptamerically and non-competitively with respect to ATP. ODN1 (136), a potent and specific inhibitor of *abl* tyrosine kinase [IC_{50} ($p210^{bcr\text{-}abl}$) = 0.52 μM: $IC_{50}(p145^{abl})$ = 0.62μM] seems to interact with the *abl* portion of p210 during inhibition, as it inhibits almost equally the bcr-*abl* and *abl* kinases. Although it inhibits the PDGFR with an IC_{50} value of 4.4 μM, it was found to be inactive against *src*, *lck*, and EGFR PTKs, and against *cdc*-2 and MAP serine/threonine kinases at concentrations less than 50 μM. In addition, ODN1 (136) demonstrated a potent inhibitory activity against $p210^{bcr\text{-}abl}$ autophosphorylation *in vivo* and *in vitro*. A closely-spaced GGC sequence was found to be critical for inhibition. An enhancing effect on inhibition by a phosphorothioate linkage in the backbone was also observed.

MULTISUBSTRATE INHIBITORS

PTKs catalyse the direct transfer of the γ-phosphate from ATP to a tyrosine residue in a substrate molecule. Assuming that the PTK reaction is independent of a phosphorylated enzyme intermediate, and involves the direct nucleophilic attack of the hydroxy group of tyrosine on the γ-phosphate of ATP, a transition state can be postulated incorporating a penta-coordinated γ-phosphorus atom with the β- and γ- phosphate groups forming a complex with divalent metal ions (Mg^{2+} or Mn^{2+}) (*Figure 6.4*) [246].

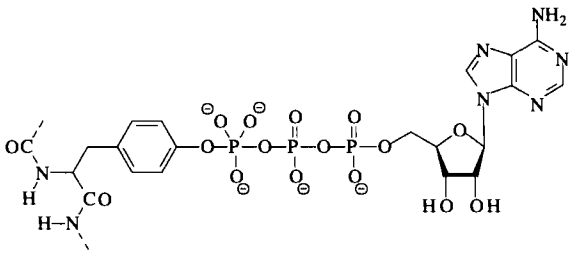


Figure 6.4. Transition state for the phosphorylation of a tyrosine residue of a protein [246]

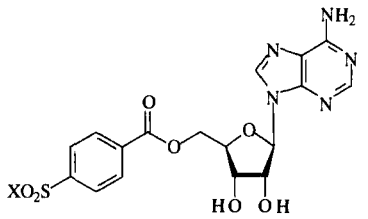
A number of attempts have been made to prepare PTK inhibitors by synthesizing multisubstrate transition state inhibitors which covalently link a tyrosine mimic to a triphosphate mimic with or without a spacer unit. In reality, transition states are intrinsically unstable and very short-lived, so these types of compounds are more correctly described as intermediate analogues [247]. Multisubstrate inhibitors, however, offer opportunities for specificity not always available to single substrate analogue inhibitors, since if both portions are recognised by a particular enzyme, the probability of a different enzyme showing the same degree of recognition for both components is likely to be reduced. This rationale is further supported by observations that even when the enzyme substrate complex is imperfectly mimicked, simultaneous occupancy of two binding sites by a single molecular entity offers a substantial gain in binding energy [248]. A further consideration in the design of multisubstrate inhibitors is the fact that conformational changes in enzymes are frequently required in multisubstrate interactions, especially when ordered binding is observed, as is the case for several PTKs.

In preparing multisubstrate inhibitors of PTKs, it has become apparent that a crucial requirement is to obtain the correct structure to imitate the tyrosine substrate portion. It can be assumed that the nucleoside component of the inhibitor should provide sufficient affinity by binding at the ATP binding site, whilst selectivity could be derived from the element of the inhibitor binding to the tyrosine site. Most attempts at preparing multisubstrate inhibitors have, therefore, focused on covalently linking an adenosine moiety with a tyrosine mimic or analogue connected by a spacer group, which may mimic the phosphate groups.

Sulphonylbenzoyladenosine analogues and related compounds

Kruse *et al.* [248] recognised the need to prepare membrane-permeable inhibitors, which necessitated the replacement of the highly charged triphosphate moiety with a more hydrophobic component. Since the *p*-sulphonylbenzoyl moiety may be capable of imitating the phosphate chain, the known inhibitor 5′-FSBA (133) was used as a model for the ATP analogue. A series of inhibitors derived from this compound were prepared and tested (*Table 6.41*).

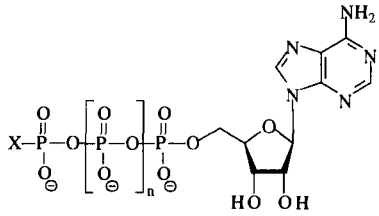
The sulphonamides (137a-e) were prepared to probe the distance between adenosine and tyrosine binding sites and to examine the necessity of the amide NH for enzyme recognition in place of the phenolic hydrogen of tyrosine. The amide NH would appear to improve potency, although sulphone (137h) showed similar inhibitory activity. The effect of chain



(137a-i)

length appears negligible, indicating that these compounds bind only at the ATP binding site, a supposition that is supported by the similar potency of these compounds for a serine kinase (phosphorylase kinase), and the lack of activity exhibited by the phenol and tyrosine derivatives (137f) and (137g).

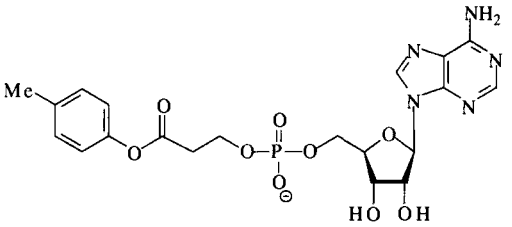
These workers also prepared compounds (138), where X = tyrosine



(138)

Table 6.41 INHIBITION OF $p60^{v\text{-}abl}$ AND PHOSPHORYLASE KINASE BY SULPHONYL-BENZOYL ADENOSINE ANALOGUES [248]

		$IC_{50}(\mu M)$	
compound	*X*	$p60^{v\text{-}abl}$	*phosphorylase kinase*
(133)	F	150	250
(137a)	NH_2	120	60
(137b)	NHPh	19	25
(137c)	$NHCH_2Ph$	45	–
(137d)	$NH(CH_2)_2Ph$	50	–
(137e)	$NH(CH_2)_3Ph$	56	50
(137f)	OPh	170	80
(137g)	4-$OC_6H_4CH_2CHCONH_2$ (NHAc)	120	–
(137h)	Me	63	–



(139)

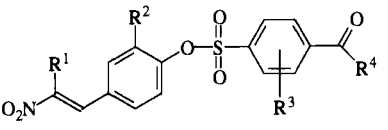
mimetic, whereby the adenosine moiety was connected to similar mimetics *via* triphosphate (n = 1) or tetraphosphate (n = 2) linkers [249]. Once again, none of these compounds showed particularly potent inhibitor activity, indicating that binding was restricted to the ATP binding site.

Baginski *et al.* [250] also considered this type of bisubstrate intermediate, concentrating their efforts on one compound (139), which was found to be a competitive inhibitor of the $pp60^{v\text{-}src}$ PTK (IC_{50} = 22μM, K_i = 7–11μM) with respect to ATP but non-competitive with respect to the phosphate acceptor. Thus, inhibition was due to binding only at the ATP site but, surprisingly, the inhibition of PKC was much less apparent (IC_{50} = 1 mM) indicating a degree of specificity.

Nitrostyrene analogues

A common feature of PTK inhibitors which may interfere with substrate binding is the presence of a styrene nucleus, which has been postulated to act as a conformationally constrained analogue of tyrosine. Traxler *et al.* [246] found that nitrostyrenes were moderately potent and selective inhibitors of the EGFR PTK, with comparable activity to erbstatin, suggesting that they may be useful as tyrosine mimetics. These nitrostyrenes were, therefore, combined with a sulphonylbenzoyl moiety (derived from 5′-FSBA) to prepare multisubstrate inhibitors of various protein kinases (*Table 6.42*).

These sulphonylbenzoyl-nitrostyrenes inhibited the EGFR PTK from A431 cell membranes at the low micromolar level and showed specificity over v-*abl* PTK and PKC. The most potent inibitors are (140c) and (140d) which possess hydroxy groups *ortho* and *meta* to the carboxylic acid group, and this once again emphasises the requirement for these substituents for enzyme recognition. Indeed, substituents in these positions may enhance the complexation of bivalent cations. Computer-aided molecular modelling studies support the hypothesis that one of the oxygen atoms of the



(140a-q)

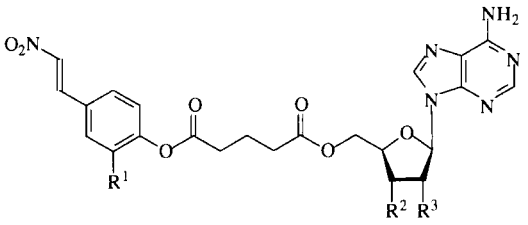
carboxylic acid group together with the *ortho*-hydroxyl group can be superimposed on the oxygen atoms of the γ- and β-phosphoryl groups of ATP, which are necessary for complexing with bivalent cation (M^{2+}) in the transition state (*Figure 6.4*). All the carboxy derivatives (140a-e) show specificity for the EGFR PTK but generally lack anti-proliferative activity, indicating that their acidic nature confers a lack of cellular penetration on these compounds. Conversion to the esters, (140f) and (140g), and the amides (140j-n) enhances the anti-proliferative activity whilst maintaining relatively good potency and selectivity for the EGFR PTK. The adenosine derivatives (140h) and (140i) retain high potency for the EGFR PTK but have lost selectivity, denoting that they principally interact at the nucleotide

Table 6.42 INHIBITION OF PROTEIN KINASES AND MK CELL PROLIFERATION BY SULPHONYLBENZOYL-NITROSTYRENES [246]

					$IC_{50}(\mu M)$			
compound	R^1	R^2	R^3	R^4	*EGFR*	*v-abl*	*PKC*	*prolfn.*
(140a)	H	H	H	OH	1.0	~100	>500	>50
(140b)	H	OMe	H	OH	2.8	40	>500	35
(140c)	H	H	2-OH	OH	0.05	27	500	>50
(140d)	H	H	3-OH	OH	0.31	~100	>500	>50
(140e)	Me	OMe	H	OH	2.5	nd	nd	13
(140f)	H	H	H	OMe	1.5	>100	>500	12
(140g)	H	H	2-OH	OMe	1.8	>100	nd	5.7
(140h)	H	H	H	adenosine	0.6	>100	13	61
(140i)	H	OMe	H	adenosine	0.5	~100	10	45
(140j)	H	H	H	NHMe	0.4	35	290	5.2
(140k)	H	H	H	NHBu	4.8	>100	>500	3.8
(140l)	H	H	H	NHBu^t	1.3	>100	340	2.1
(140m)	Me	OMe	H	NHMe	4.0	nd	>500	2.1
(140n)	H	H	H	$NH(CH_2)_2NHCO_2Bu^t$	1.4	>100	320	4.8
(140o)	H	H	H	$NH(CH_2)_2NH_2.HCl$	5.4	5	60	32
(140p)	H	H	H	*L*-ala-*L*-ala-OH	7.0	35	>100	>50
(140q)	H	H	H	*L*-ala-*L*-ala-$NH(CH_2)_2NH_2.HCl$	17	15	250	>50

binding regions. The incorporation of dipeptide residues in compounds, (140p) and (140q), decreases both inhibitory and anti-proliferative activity.

In vivo studies with (140j) show that it exhibits antitumour activity against the A431 human epidermoid carcinoma, but that it also displays cumulative toxicity and is metabolically unstable. However, the high potency and selectivity of these sulphonylbenzoyl-nitrostyrenes implies that they act as multisubstrate complex inhibitors binding to both the ATP and substrate binding sites of the EGFR PTK.



(141a) R^1=H, R^2,R^3=$OCMe_2$
(141b) R^1=OMe, R^2,R^3=$OCMe_2O$
(141c) R^1=H, R^2=R^3=OH

Further studies were carried out by Peterli *et al.* [251] combining a nitrostyrene (as a tyrosine mimetic) with adenosine, using glutaric acid as the triphosphate substitute (*Table 6.43*). These adenosine-nitrostyryl-glutarates (141a-141c) were found to be 10-times more potent than their nitrostyryl-glutarate precursors (not shown). This inhibition suggests that these compounds act as bisubstrate inhibitors of EGFR PTK, although their moderate activity against PKC, v-*abl* and c-*src* suggests that they may interact primarily with the ATP binding sites of these protein kinases. These compounds are reasonable inhibitors of the proliferation of mouse

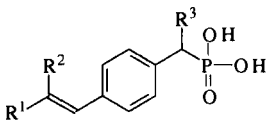
Table 6.43 EFFECTS OF ADENOSINE NITROSTYRYL GLUTARATES ON THE INHIBITION OF VARIOUS PROTEIN KINASES AND PROLIFERATION OF BALB/MK CELLS [251]

	$IC_{50}(\mu M)$				
compound	*EGFR*	*v*-abl	*c*-src	*PKC*	*Balb/MK*
(141a)	0.66	>100	>100	95	15.6
(141b)	0.6	>100	>100	78	4.4
(141c)	1.5	50	24	43	22.2

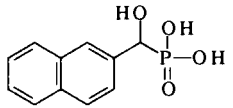
epidermal keratinocytes (Balb/MK cells), which are strongly dependent on EGF for cell growth. Incorporation of alkyl spacers between the β-nitrostyrene and the glutaryl moiety resulted in the loss of potency [252].

Phosphonate Inhibitors

Burke *et al.* [253] prepared methylphosphonate and (hydroxymethyl)phosphonate derivatives (142) of various styrenes as hydrolytically stable analogues which incorporate additional recognition features contained by phosphotyrosine itself. None of these, however, inhibited EGFR autophosphorylation or the PTK activity of $p56^{lck}$.

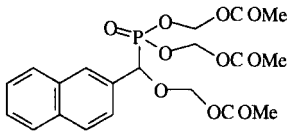


(142)

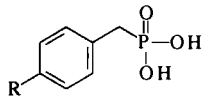


(143)

A similar approach was utilized by Saperstein *et al.* [254] in which naphthalene was used to mimic the styrene nucleus. The most potent inhibitor of this series (143), which inhibited the IR PTK with an IC_{50} of 200 μM, was found to be specific for PTK over serine kinase. The inhibition was



(144)



(145)

competitive with respect to the substrate and non-competitive with respect to ATP. Conversion to the prodrug (144) by esterifying the acid and hydroxyl groups allowed penetration into intact cells. Despite being inactive *in vitro*, this ester inhibited insulin-stimulated autophosphorylation and physiological responses in intact Chinese hamster ovary (CHO) cells.

The synthesis of benzylphosphoric acid derivatives (145) as tyrosine kinase inhibitors has also been reported by Dow and Goldstein [255].

PEPTIDES

The use of peptides as PTK inhibitors is an area of research predominantly based upon the replacement of tyrosine residues in peptide substrates (sequences surrounding the PTK phosphorylation site or other unrelated peptides) that are unable to be phosphorylated. This approach was demonstrated by Wong and Goldberg [256] who found that a dehydrophenylalanine analogue (ΔPhe) (146) of [Val^5]angiotensin II (147) inhibited the phosphorylation of [Val^5]angiotensin II by pp60$^{c\text{-}src}$ PTK with a K_i of 0.7 μM.

AspArgVal(ΔPhe)ValHisProPhe (146)

AspArgValTyrValHisProPhe (147)

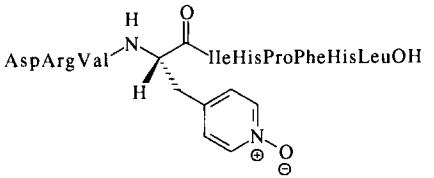
The observations that an analogue of human gastric undecapeptide (residues 22–30) (148) acts as a substrate for EGFR [257] and IR [258] PTKs, led Yuan *et al.* [259] to prepare inhibitors of these PTKs. Two analogues (149) containing a D- and L-tetrafluorotyrosyl residue were prepared. The L-form was found to inhibit the IR PTK by competition with both ATP and the peptide substrate ($K_i = 4\mu M$) while the D-form was competitive with the substrate only ($K_i = 20\ \mu M$).

ArgArgLeu(Glu)$_5$Ala-X-Gly (148) X = Tyr

(149) X = D- or L-F_4-Tyr

The rationale behind this work was to increase the acidity of the phenol so as to produce ionic species at physiological pH to promote binding and also to reduce the nucleophilicity of the phenolic residue so as to block enzymatic phosphorylation.

Lysine in the vicinity of the ATP binding site of the PTK takes part in the transfer of γ-phosphate of ATP to the tyrosine residue in the substrate [260]. The covalent bonding of an inhibitor with the PTK through these lysine residues may result in very potent inhibition. In view of this consideration, Cushman *et al.* [261] prepared an analogue (150) of angiotensin I (151) by replacing the tyrosine residue with β-(4-pyridyl-1-oxide)-L-alanine. This analogue demonstrated a reversible and weak competitive inhibition against p56^{lck} in contrast to the expected suicide substrate. Recently, the same group



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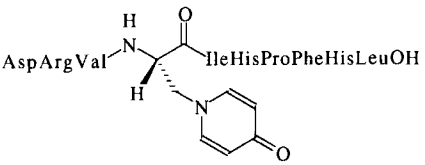


(152)

has reported the preparation of another analogue (152) of angiotensin I [262].

AspArgValTyrIleHisProPheHisLeuOH (151)

Somatastatin is a native peptide hormone which inhibits growth hormone and other endocrine secretion as well as cellular functions [263, 264]. The inhibitory activity of various analogues of somatastatin has been studied. TT-232 (153) exhibited the highest PTK inhibitory activity against HT-29 human colon carcinoma cells [265, 266]. Good antitumor activity against a Lewis lung metastasis model in mice was also observed. No effect was noticed on growth hormone secretion.

D-PheCysTyrD-TrpLysCysThrNH_2 (153)

The peptides (154-156) based on the region surrounding residue 1151 of the insulin receptor (1142-1153 region) have been shown to inhibit the autophosphorylation of the insulin receptor, EGFR and $pp60^{c\text{-}src}$ [267].

ArgAspIleTyrGluThrAspTyrTyrArgLysNH_2 (154)

ArgAspIleMpaGluThrAspMpaMpaArgLysNH_2 (155)

ArgAspIlePheGluThrAspPhePheArgLysNH_2 (156)

(Mpa = 4-methoxyphenylalanine)

Inhibition of the IR PTK by peptides based upon the (1143-1155) region of the IR PTK, phosphorylated or non-phosphorylated, has also been reported [268]. A peptide (157) corresponding to the region surrounding residue 419 in $pp60^{v\text{-}src}$ (415-424) has been shown to inhibit the PTK activity of $pp60^{v\text{-}src}$ [269]. PLC-γ^1 is one of the more studied *in situ* substrates for the EGFR PTK, in which the phosphorylation sites are known with certainty [270, 271]. Fry *et al.* [272] have reported the synthesis of a very potent peptide inhibitor (158) (K_i = 10μM) of the EGFR PTK, which is based upon the sequence surrounding tyrosine 472 in PLC-γ^1.

H_2NGluAspAsnGluTyrThrAlaArgGlnGlyOH (157)

LeuAlaGluGlySerAla-X-TyrGluGluOH (158) X = D-F_4-Tyr

Recent observations linking the SH2 domain of non-receptor PTKs to the regulation of enzymatic activity has made this an attractive target for pharmaceutical investigation. Sato *et al.* [273] synthesized a 21-amino acid peptide (159) corresponding to residues (137-157) in the SH2 domain of $pp60^{v\text{-}src}$, which inhibited $pp60^{v\text{-}src}$ PTK activity (IL_{50}, 7.5 μM) and EGFR autophosphorylation without affecting serine and threonine kinases. This inhibition was non-competitive with respect to both ATP and the substrate. A phosphorylated peptide (160) corresponding to the carboxylic terminus of $pp60^{c\text{-}src}$ has also been shown to inhibit tyrosine kinase activity of $pp60^{v\text{-}src}$ by binding to the SH2 domain [274].

VAPSDSIQAEEWYFGKITRRE (159)

ThrSerThrGluProGlnTyr(P)GlnProGlyGluAsnLeu (160) P = OPO_3H_2

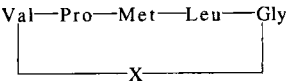
Nomizu *et al.* [275] have recently described the synthesis of hexameric linear (161-163) and cyclic peptides, (164) and (165), based on the sequence corresponding to the autophosphorylation segment around tyrosine 751 of the PDGFR β-unit. In the binding assay to SH2 domains of target proteins, the linear and cyclic L-isomers, (161), (162), (164), and (165), showed a good binding activity whilst the D-isomers were less active.

H_2NGly-X-ValProMetLeuOH

(161) X = L-Tyr(OPO_3H_2)

(162) X = L-Pma

(163) X = D-Pma



(164) X = L-Tyr(OPO_3H_2)

(165) X = Pma

Some synthetic random peptides containing tyrosine, glutamic acid, alanine and lysine have been shown to act both as substrates and inhibitors of different PTKs, depending upon the amino acid ratio [276]. Recently, it has been reported that inhibition by polylysine depends on the chain length (>20.5 KDa) [277].

CONCLUDING REMARKS

There is now a vast array of compounds which act as PTK inhibitors. The majority of the structural types known to be PTK inhibitors are planar but a number of non-planar and/or chiral species which inhibit these enzymes are known. As the range of compounds increases, it should become possible to investigate the potential of PTK inhibitors and to address some of the key questions pertaining to their use as chemotherapeutic agents. One particularly important question is whether the ideal compound is one which specifically inhibits one protein kinase or one which inhibits a range of protein kinases (including both tyrosine and serine/threonine kinases). There is little doubt that the future biological testing of PTK inhibitors is dependent upon the answer to this question. It is also clear that the most useful studies are those in which new compounds are tested against a panel of kinases which includes receptor PTKs, non-receptor PTKs, and serine/threonine kinases.

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7 Squalene Synthase Inhibitors: Their Potential as Hypocholesterolaemic Agents

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INTRODUCTION

Atherosclerosis is a progressive disease of the major coronary arteries and coronary heart disease is the major cause of death in Western countries. The disease is asymptomatic for many years as the lesion matures but the onset of symptoms can be rapid and fatal. Hypercholesterolaemia is well recognized as a major independent risk factor for coronary disease [1] and a number of studies have shown that reducing elevated levels of serum cholesterol in man leads to a reduction in the incidence of coronary-related deaths [2, 3].

In humans, 70% of cholesterol is derived from *de novo* synthesis in the liver and developments in the past few years have shown that one of the most effective approaches to lowering serum cholesterol levels is by inhibiting sterol biosynthesis [4]. In addition to 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) which is a major rate-limiting step, the reaction catalyzed by squalene synthase (SQS) is also an important control point in the pathway (*Figure 7.1*) [5–9]. A number of therapeutic agents are available which inhibit HMGR; in clinical studies such inhibitors effectively reduce serum cholesterol levels in a dose-related manner [4] and a recent study has demonstrated that lowering raised cholesterol levels in patients with coronary heart disease improves survival [10].

Major toxic effects are not commonly associated with this class of inhibitor; however, mevalonic acid, the product of the enzymic action of HMGR, is a common precursor of all isoprene derivatives including ubiquinone and dolichol. SQS is the first committed step in sterol biosynthesis. Thus, agents which inhibit this enzyme are particularly

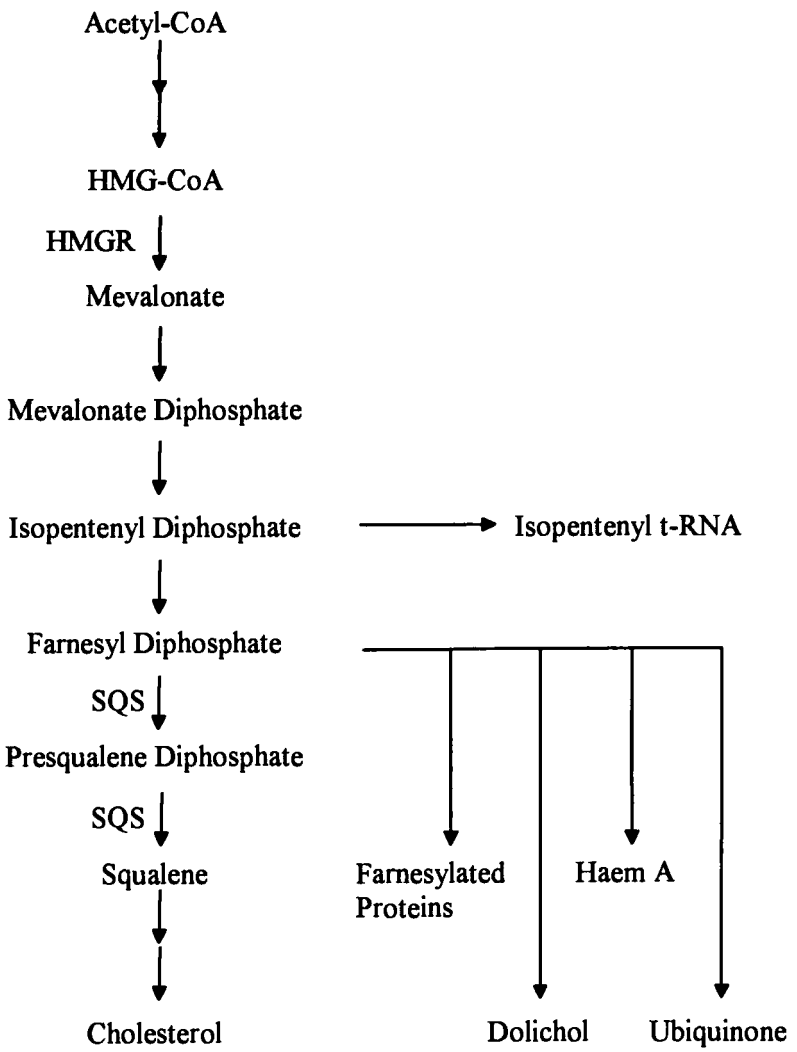


Figure 7.1. Cholesterol biosynthetic pathway.

attractive as non-steroidal pathways should be minimally affected and represent potential new therapies for elevated serum cholesterol levels. This review focuses on recently isolated series of natural product inhibitors of SQS and their evaluation as potential hypocholesterolaemic agents; their

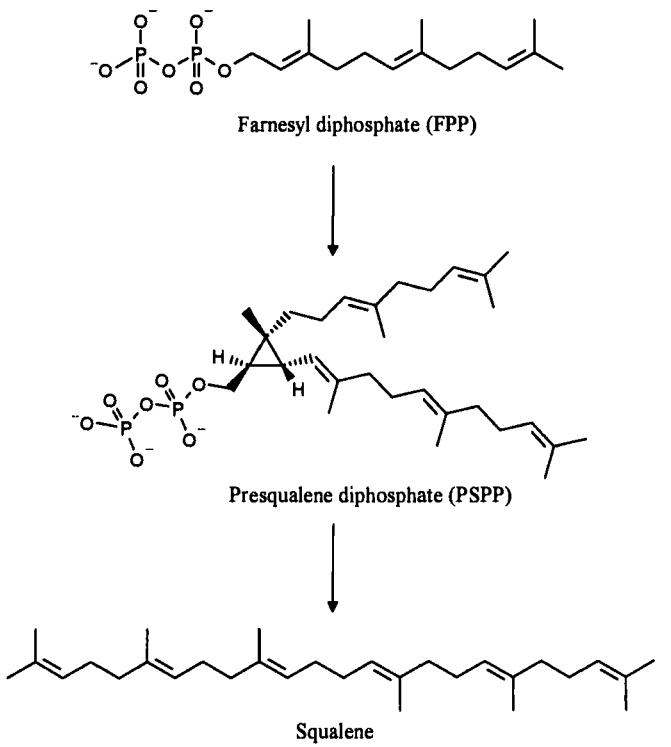
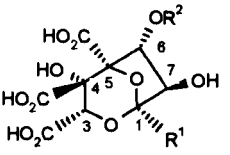


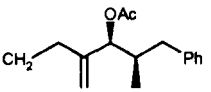
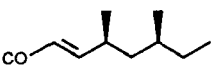
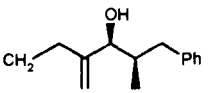
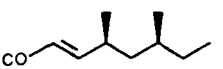
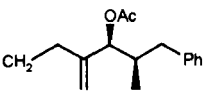
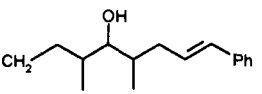
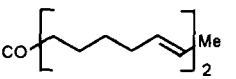
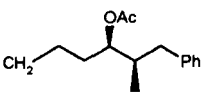
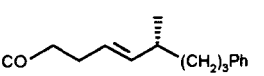
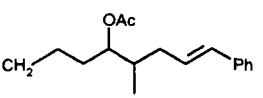
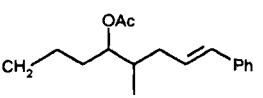
Figure 7.2. Transformation of farnesyl diphosphate into squalene.

antifungal properties form a second aspect of this review which covers the scientific literature up to December 1994.

ENZYMOLOGY

SQS (farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) is a microsomal enzyme which catalyzes the formation of squalene from farnesyl diphosphate (FPP) in two distinct steps (*Figure 7.2*) the mechanism of which has been studied extensively [11–16]. In summary, two molecules of FPP are condensed head to head to form presqualene diphosphate (PSPP) which is then converted into squalene by a reductive rearrangement. The enzyme requires Mg^{2+} for both synthesis and metabolism of PSPP. NADPH serves as a reductant for squalene synthesis.



Compound	R1	R2
squalestatin S1 zaragozic acid A (1)		
squalestatin S2 (2)		
squalestatin H1 (3)		H
zaragozic acid B (4)		
zaragozic acid C (5)		
zaragozic acid D (6)		CO(CH2)6Me
zaragozic acid D2 (7)		CO(CH2)8Me

A truncated, soluble, active fragment of rat hepatic microsomal SQS has been purified to homogeneity [17]; cDNA sequences for rat [18] and human enzymes [19–21] have been published. The yeast-derived enzyme has been purified to homogeneity [22, 23] and the molecular cloning of the gene reported [19, 24–26]; truncated, soluble and active recombinant proteins

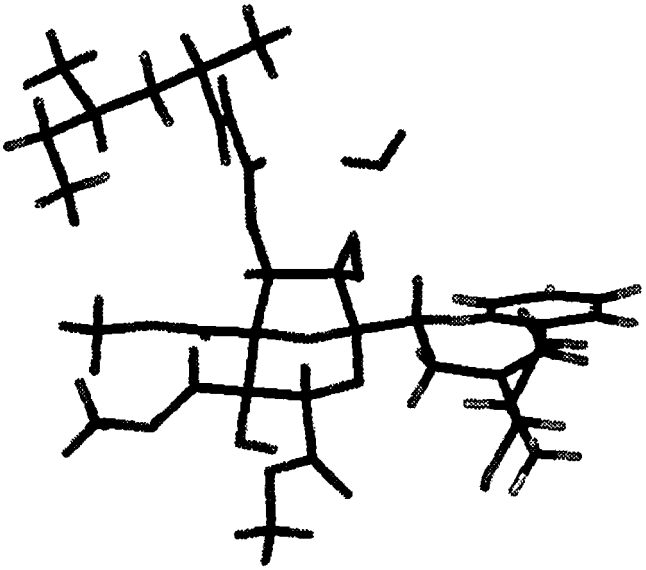


Figure 7.3. Single crystal X-ray diffraction study of the trimethyl ester of S2.

have been purified to homogeneity [27, 28]. Significant homology exists between the mammalian and fungal enzymes [19].

NATURAL PRODUCTS

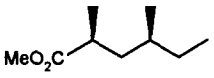
In the absence of a high affinity inhibitor lead from substrate analogues of FPP and in view of the lability associated with the diphosphate moiety in such compounds, the challenge, at the outset of the programme at Glaxo, was to identify inhibitors without a phosphorus-containing diphosphate mimetic. A high-throughput screening programme found potent SQS inhibitory activity in fermentation broths of a newly isolated species of *Phoma* C2932 (coelomycetes) [29]. Subsequent investigations revealed that inhibition was due to a novel family of compounds termed the squalestatins possessing the polar and highly substituted core unit 4,6,7-trihydroxy-2, 8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic acid [30]. Three squalestatins were isolated initially and these were named S1 (1), S2 (2), and H1 (3), S1 being the major metabolite. These differ structurally from one another in terms of their C-1 and C-6 side-chains.

A group at Merck has also reported the isolation of S1, which they have named zaragozic acid A, from an unidentified fungus, ATCC 20986 [31].

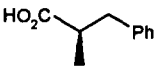
Throughout this review this compound will be referred to as S1 for clarity. Other fungal cultures produced novel metabolites possessing the same 2, 8-dioxabicyclo[3.2.1]octane system but different side-chains. *Sporormiella intermedia*, ATCC 20985, produced zaragozic acid B (4) [31]. *Leptodontium elatius*, ATCC 70411, produced zaragozic acid C (5) [31, 32], whereas *Amauroascus niger*, ATCC 74156, produced zaragozic acids D (6) and D_2 (7) [33]. A group at Tokyo Noko University - Mitsubishi has also reported the isolation of S1 from yet another organism, *Setosphaeria khartoumensis* L1685 [34].

STRUCTURE ELUCIDATION

The structure of S1 was determined at Glaxo by extensive NMR studies on the parent tricarboxylic acid, the derived trimethyl ester and the trimethyl ester of the minor metabolite H1 [30]. The relative stereochemistry was established from single crystal X-ray diffraction studies on the trimethyl ester of the minor metabolite S2 (*Figure 7.3*). The absolute stereochemistry of S1 was obtained by degradation of the C-6 ester side-chain to the known (2*S*, 4*S*) methyl 2,4-dimethylhexanoate ester (8).



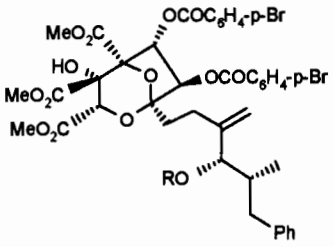
(8)



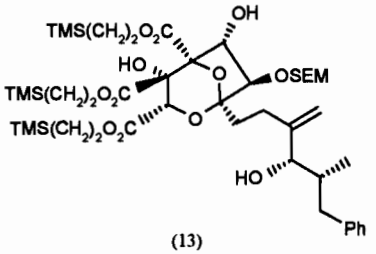
(9)

The group at Merck established the configuration of the homobenzylic asymmetric centre of zaragozic acid A (S1) by degrading S1 to (*R*)-(−)-2-methyl-3-phenylpropanoic acid (9) [35]. The derivation of the absolute stereochemistry of the C-6 and C-7 centres was based on CD measurements on the 6,7-bis(4-bromobenzoate) (10), while the configuration of the allylic acetoxy group was established from NMR considerations of the derived (*R*)- and (*S*)-*O*-methyl mandelates (11) and (12). Finally, X-ray diffraction studies on the two crystalline derivatives (13) and (14) established the configuration of the remaining asymmetric centres and provided independent structural confirmation.

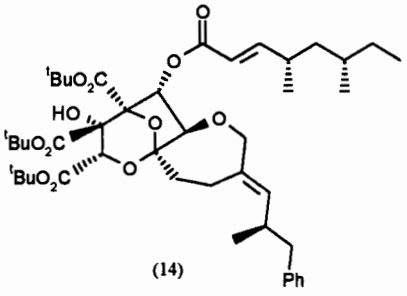
The structures of zaragozic acids B (4) and C (5) were determined predominantly on the basis of NMR experiments [32, 36]. The relative stereochemistry of zaragozic acid C (5) was established from X-ray crystallography on the tri-t-butyl ester of the derived triol (15) [37]. By analogy with zaragozic acid A, the absolute stereochemistry of the 6,7-diol



Compound	R
(10)	Ac
(11)	
(12)	

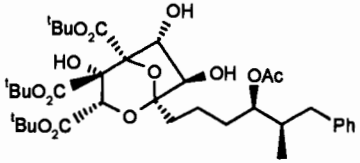


(13)

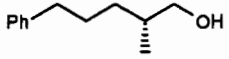


(14)

TMS = Me_3Si; SEM = $Me_3Si(CH_2)_2OCH_2$



(15)



(16)

moiety was established by conversion of (15) to the 6, 7-bis(4-bromobenzoate) analogue, and examination of its CD spectra. The absolute stereochemistry of the ester side-chain of zaragozic acid C was determined by degradation to (+)-(*R*)-2-methyl-5-phenyl-1-pentanol (16) and total synthesis of (16) and the ester side-chain [37]. Finally, the structures of zaragozic acids D and D_2 were determined by spectroscopic means [33].

BIOLOGICAL EFFECTS *IN VITRO*

S1 is a potent, selective inhibitor of SQS from both rat and marmoset liver with an IC_{50} of 12 nM [38]. Similar inhibitory activity was seen against SQS present in microsomes isolated from Hep G2 cells, a human hepatoma line. Against SQS from *Candida albicans* S1 had an IC_{50} of 5 nM [29]. Furthermore, S1 exhibited potent broad spectrum antifungal activity *in vitro* [29]. The tricarboxylic acid moiety of S1 resembles citric acid and so the effects of S1 on the early citrate-utilizing steps in the biosynthetic pathway were examined. S1 had no effect on citrate efflux from mitochondria at concentrations up to 1 mM and no effect on ATP:citrate lyase activity at concentrations up to 100 μM. S1 does not inhibit HMGR at concentrations up to 5 μM [38].

The group at Merck has reported S1 to be a potent competitive inhibitor of rat liver SQS with an apparent K_i value of 78 pM, and an IC_{50} value of 0.5 nM under their assay conditions [31, 33].

Independent studies by the group at Tokyo Noko University-Mitsubishi have shown that the inhibition of SQS by S1 is competitive with respect to [^{3}H]FPP with a K_i value of 1.6 nM and an IC_{50} of 3 nM [34]. S1 inhibited both partial reactions effected by SQS. The first partial reaction was assayed by measuring $^3H^+$ released from [^{3}H]FPP in the absence of NADPH and was inhibited 50% by S1 at 14 nM. The second partial reaction, determined by measuring squalene formation from [^{3}H]PSPP in the presence of NADPH, was >90% inhibited at 33.4 nM.

The *in vitro* activities of squalestatins S1, S2 and H1 [38–40] are summarized in *Table 7.1* and the SQS inhibitory activities of zaragozic acids A (S1), B, C, D, and D_2 [31, 33] in *Table 7.2*.

The *ras* oncogene has been implicated in a number of human cancers. Farnesylation of Ras protein by a farnesyl-protein transferase (FPTase) appears to be required for Ras membrane localization and cell-transforming activity. It is possible that inhibition of Ras farnesylation might lead to the development of new cancer treatments. S1 was found by Merck scientists to inhibit Ras FPTase (IC_{50} 250 nM) [41]; zaragozic acids B, C, D, and D_2 possess IC_{50} values of 1000, 150, 100 and 100 nM, respectively [33].

Table 7.1. SQS INHIBITORY ACTIVITIES AND ANTIFUNGAL DATA FOR SQUALESTATINS S1, S2 and H1

Compound	*IC_{50} (nM)*		*MIC[a] (μg/ml)*		
	Rat SQS	*Candida*	*Candida*	*Asper.*	*Crypt.*
S1	12	5	8	16	0.5
S2	5	7	125	16	1
H1	26	30	>125	>125	>125

[a] Minimum inhibitory concentrations against *Candida albicans* C316, *Aspergillus fumigatus* 48238 and *Cryptococcus neoformans* 2867E

EFFECTS OF S1 ON FPP LEVELS IN ISOLATED HEPATOCYTES

S1 inhibited the synthesis of cholesterol from [2-^{14}C]acetate in isolated rat hepatocytes, with an IC_{50} of 39 nM [38]. The concentration of [^{3}H]FPP in isolated rat hepatocytes was increased while that of squalene decreased following a 30 min incubation of [^{3}H]mevalonolactone in the presence of S1 at concentrations of 5, 50 and 500 nM. The concentration of [^{3}H]FPP decreased slowly over the next hour and other isoprenoid intermediates could not be detected. In the presence of 50 or 500 nM S1, the amount of radiolabel in FPP increased approximately 6-fold at all time points, as would be expected from the inhibition of SQS. In control cells, labelling of cholesterol from [^{3}H]mevalonate increased linearly over 90 min. Radiolabelled squalene was detected at low but measurable concentrations in control cells but was not detectable in the presence of S1. This provided strong evidence that SQS is the site of action of S1 and underlined the selectivity of this compound.

Table 7.2. SQS INHIBITORY ACTIVITIES FOR ZARAGOZIC ACIDS A, B, C, D AND D_2

	Rat SQS	
Compound	*IC_{50} (nM)*	*K_i (pM)*
zaragozic acid A	0.5	78
zaragozic acid B	0.2	29
zaragozic acid C	0.4	45
zaragozic acid D	6	NR
zaragozic acid D_2	2	NR

NR = not reported

INHIBITION OF CHOLESTEROL SYNTHESIS IN HEP G2 CELLS

Zaragozic acids A (S1), B and C inhibited the incorporation of [^{3}H]mevalonate into cholesterol in Hep G2 cells with IC_{50} values of 6, 0.6, and 4 μM, respectively [31]. Inhibition of cholesterol biosynthesis was paralleled by a dose-dependent increase in incorporation of label into the FPP and carboxylic acid fractions and a dramatic decrease in labelling of cholesterol, squalene, and lanosterol when compared with control. Analysis of the carboxylic acid fractions revealed [^{3}H]farnesoic acid and the related 15-carbon dicarboxylic acid derivative [31, 42] which suggested that the diversion of mevalonate into the farnesol-farnesoic acid-dicarboxylic acid pathway is one of the major metabolic consequences of inhibition of SQS by the zaragozic acids.

BIOLOGICAL EFFECTS OF S1 *IN VIVO*

The above *in vitro* data for accumulation of label into the FPP and organic acid fractions were supported at Merck by similar findings from *in vivo* studies with S1 in mice [31]. Furthermore, in studies at Glaxo, when rats were dosed with S1, a novel isoprenoid-derived acidic compound was excreted in the urine. This product could be labelled by [^{14}C]acetate or [^{3}H]mevalonolactone and had identical t.l.c. properties to the C15 dicarboxylic acid metabolite of FPP [unpublished results].

We have shown that cholesterol biosynthesis in rat liver was inhibited by 50% after dosing S1 intravenously (i.v.) at 0.1 mg/kg [38]. Similar findings in mice have been reported by the group at Merck; S1 inhibited hepatic cholesterol synthesis when administered subcutaneously (s.c.) with an ED_{50} value of 0.2 mg/kg [31]. However, it was only weakly active in blocking cholesterol biosynthesis when administered orally (p.o.) in this species (ED_{50} 100 mg/kg).

Inhibitors of HMGR are reported to have minimal effect on serum cholesterol levels in rodents, although other mammalian species including rabbits and primates respond to these agents. Marmosets have a similar lipoprotein metabolism to that of man, and at Glaxo we have shown that this species is sensitive to inhibitors of HMGR. Serum cholesterol levels in adult marmosets were significantly reduced when S1 was dosed p.o. at 10 mg/kg/d for 7 days, and cholesterol lowering of up to 75% could be achieved at an oral dose of 100 mg/kg/d over the same period. Cholesterol lowering was apparent within 24 h and could be maintained for at least 8 weeks on prolonged dosing with no attenuation of the response (*Figure 7.4*) [38].

At an oral dose of 10 mg/kg/d for 7 days, apolipoprotein B levels were

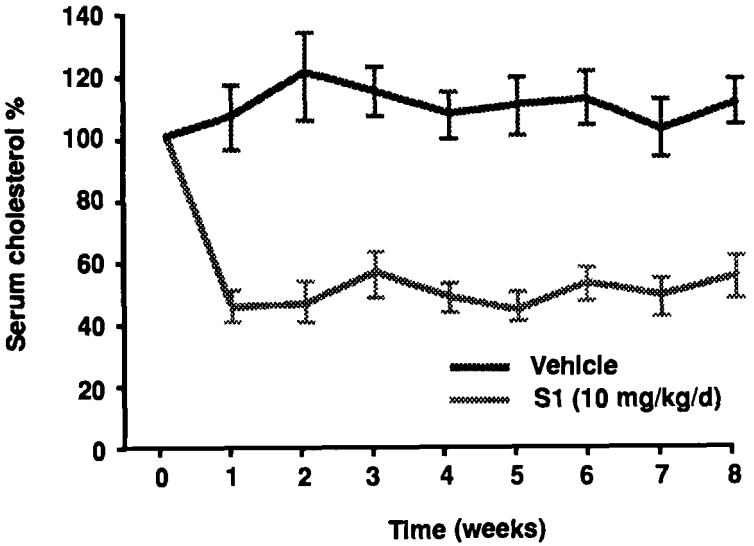


Figure 7.4. Cholesterol lowering in marmosets: effect of prolonged dosing.

reduced by 45%, whereas apolipoprotein A1 levels were unchanged [38] (*Table 7.3*). Our observations with S1 in marmosets provided the first demonstration that a potent inhibitor of SQS could affect serum cholesterol *in vivo*. Furthermore, it is important that only the apolipoprotein B levels, characteristic of the atherogenic low density lipoprotein, are reduced by a potential therapeutic agent.

BIOSYNTHESIS

The highly complex structure of the squalestatins stimulated both the Glaxo

Table 7.3. EFFECT OF S1 ON APOLIPOPROTEIN LEVELS IN MARMOSETS

Serum parameter	*% Change in level*
cholesterol	51 ± 4
apolipoprotein B	45 ± 9
apolipoprotein A1	0 ± 4

Marmosets were dosed orally with S1 as described under screening procedures. Results are expressed as the mean change in levels compared with the pre-dose values (± S.E.). Apolipoprotein levels were measured immunoturbidometrically.

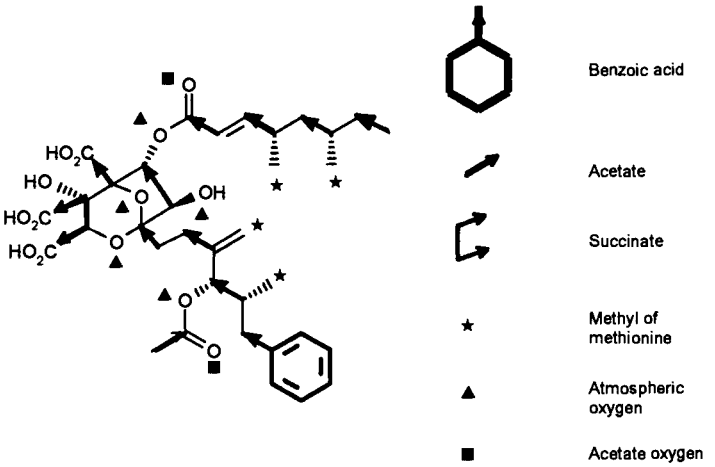


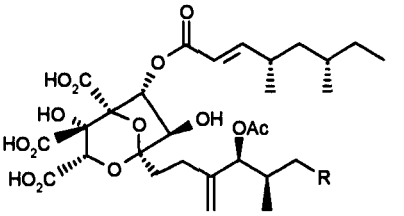
Figure 7.5. Labelling patterns of S1 from [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C, $^{18}O_2$]acetate, L-[methyl-^{13}C]methionine, DL-[3-^{13}C]phenylalanine, [carboxy-^{13}C]-, [$^{13}C_6$]benzoic acid, [2,3-^{13}C]succinic acid and $^{18}O_2$ (gas).

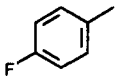
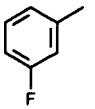
and Merck groups to study their biosynthesis. The biosynthetic origin of the carbon atoms of S1 has been examined by incorporation studies with single and multiple labelled ^{13}C precursors; these studies indicated that the major portion of the molecule was derived from two polyketide chains made up of acetate units [43]. Importantly, one of these chains had benzoic acid, which can be derived from phenylalanine, as a starter unit. The C-3, C-4 atoms and attached carboxylic acids were derived from a four-carbon unit related to succinate whilst branched chain methyl groups were derived from methionine. Studies with [1-^{13}C, $^{18}O_2$]acetate and $^{18}O_2$ indicated that five of the oxygen atoms, including both of the ketal oxygens, were derived from atmospheric oxygen whilst the oxygen atoms at the two ester carbonyls were derived from acetate. The results of these studies are summarized in *Figure 7.5.* Similar findings were also reported by the group at Merck [44].

DIRECTED BIOSYNTHESIS

Having established the biosynthetic origin of S1 and, in particular, that the starter unit of one of the polyketide chains appeared to be benzoic acid, feeding experiments of the producing organism utilizing a broad range of benzoic acid analogues were conducted with the aim of generating novel squalestatins. The structural features of those entities incorporated were narrowly defined and the *in vitro* activities of analogues isolated are

Table 7.4. SQUALESTATINS DERIVED BY DIRECTED BIOSYNTHESIS AND THEIR SQS INHIBITORY ACTIVITIES



		IC_{50} (nM)	
Compound	*R*	*rat*	*Candida*
(17)		32	13
(18)		38	4
(19)		43	8
(20)		32	7
(21)		25	4
(22)		a	NT

[a] IC_{50} of derivative (22) was reported by Merck to be 0.1 ng/ml, whereas that of S1 was 0.2 ng/ml.

summarized in *Table 7.4.* Thus, feeding of the squalestatin producer *Phoma* sp. C2932 with monofluorobenzoic acids produced the novel fluorinated squalestatins (17) - (19) [45], whereas feeding with thiophenecarboxylic acids produced the thienyl analogues (20) and (21) [46]. In addition, the group at

Merck has recently published the preparation of the 2-furyl analogue (22) by feeding ATCC 20986 with 2-furoic acid [47]. This analogue was found to be equipotent with S1.

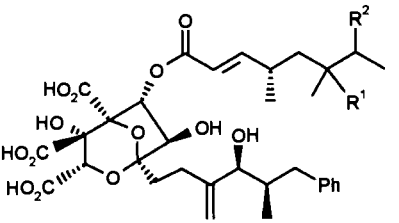
BIOTRANSFORMATIONS

As part of a programme to explore the effect of structural modifications on the biological activities of the squalestatins, micro-organisms were screened for their ability to convert S1 to novel derivatives [40]. Biotransformation of S1 by two actinomycetes, S15106 and S15138, yielded three S2 products hydroxylated in the C-6 side-chain (23), (24) and (25) which retained potent SQS inhibitory activity (*Table 7.5*). Many strains were found to hydrolyze the 4,6-dimethyloctenoate or acetate esters to yield H1 or S2; the C-3-methyl ester of S1 (see *in vitro* Structure-Activity Relationships section) was obtained using *Fusarium* sp F13945.

MINOR METABOLITES

Large scale reverse phase HPLC of acidified extracts of the crude calcium salts obtained from 5000-litre fermentations of *Phoma* sp. C2932 gave 24

Table 7.5. SQUALESTATINS DERIVED BY BIOTRANSFORMATION OF S1 AND THEIR SQS INHIBITORY ACTIVITIES

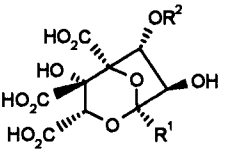


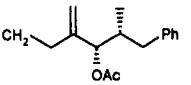
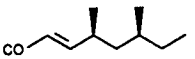
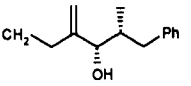
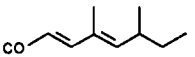
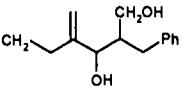
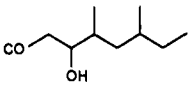
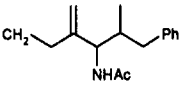
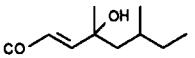
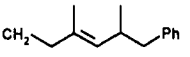
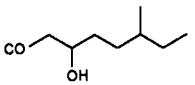
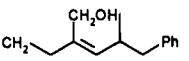
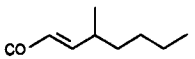
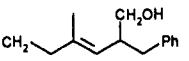
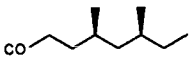
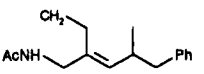
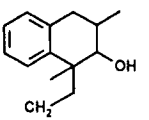
			IC_{50} (nM)	
Compound	R^1	R^2	*rat*	*Candida*
(23)	β-H	OH	ND	3
(24)	β-H	OH	12	ND
(23) + (24)[a]	β-H	OH	6	6
(25)	OH	H	9	10

ND = not determined

[a] 78:22 diastereoisomeric mixture of (23):(24)

Table 7.6. SQUALESTATIN NOMENCLATURE



R^2	*Letter*	R^1	*number*
H	H		1
	S		2
	T		3
	U		4
	V		5
	W		6
	X		7
	Y		8
			9

novel squalestatins in addition to the 3 principal metabolites S1, S2 and H1 [48]. The nomenclature applied to the squalestatins is illustrated in *Table 7.6* which shows a generalized structure for the squalestatin nucleus which is named squalestatin R^2R^1. Substitution of a letter for group R^2, followed by a number for group R^1, both drawn from *Table 7.6* then describes a particular squalestatin. The *in vitro* activities of selected minor metabolites are presented in *Table 7.7* [unpublished results].

SCREENING PROCEDURES

MEASUREMENT OF SQUALENE SYNTHASE ACTIVITY

SQS activity was monitored by the formation of [^{14}C]squalene from

Table 7.7. SQS INHIBITORY ACTIVITIES AND ANTIFUNGAL DATA FOR MINOR METABOLITES

	IC_{50} (nM)		*MIC (μg/ml)*		
Compound	*Rat SQS*	*Candida*	*Candida*	*Asper.*	*Crypt.*
S3	5	NT	31	31	31
S4	5	NT	NT	NT	NT
S5	32	NT	31	16	4
S8	9	NT	62	62	31
7-deoxy S1	35	NT	16	1	0.1
H2	252	NT	62	62	62
H5	200	>200	NT	NT	NT
H6	>500	>200	NT	NT	NT
H7	290	>200	NT	NT	NT
H9	400	>200	NT	NT	NT
6-deoxy H1	35	NT	NT	NT	NT
6-deoxy H5	>500	NT	NT	NT	NT
7-deoxy H5	>200	NT	NT	NT	NT
T1	25	NT	62	8	1
U1	11	NT	62	31	2
U2	4	NT	31	31	8
V1a	5	2	62	62	31
V1b	5	2	62	62	8
V2	19	2	62	62	31
X1	19	NT	NT	NT	NT
Y1	13	NT	8	1	0.1

a,b refer to diastereoisomers at the centre to which the hydroxy group is attached in the C-6 side-chain

[^{14}C]FPP using male rat liver or *Candida albicans* 2005E (at Glaxo) or MY1055 (at Merck) microsomes as enzymes source. In the Glaxo assays [38, 49], 50 mM ascorbate and 20 units/ml ascorbate oxidase were used as an endogenous oxygen consumption system to prevent further metabolism of squalene. In the Merck assay [31], a squalene epoxidase inhibitor was included at 1 μg/ml and hence the reactions were run aerobically. The concentration of rat liver homogenate protein employed by the Glaxo group was 2.2 mg/ml whereas the concentration used by the Merck group was 2.2 μg/ml. As a consequence of the differences in the assays used by the two groups the IC_{50} values reported by Glaxo for identical compounds are numerically higher than those reported by Merck. Thus, the IC_{50} of S1 quoted by Glaxo is 12 nM (range 4–22 nM) whereas that quoted by Merck is 0.3 nM (range 0.09–0.75 nM).

IN VITRO ANTIFUNGAL ASSAY

Minimum inhibitory concentrations (MICs) were determined in whole-cell assays against a selected panel of clinically relevant fungi utilizing broth microdilution methodology. MICs were defined as the lowest concentration of drug completely inhibiting visible growth, and are expressed in μg/ml [39, 50].

MEASUREMENT OF CHOLESTEROL BIOSYNTHESIS IN RAT HEPATOCYTES

Rat hepatocytes were isolated by collagenase perfusion, and incubated with [^{14}C]acetate for 30 min in the presence and absence of SQS inhibitors. Further reaction was stopped by saponification. The mixture was extracted and [^{14}C]cholesterol was separated by HPLC and quantified [38].

INHIBITION OF CHOLESTEROL BIOSYNTHESIS IN HEP G2 CELLS

Hep G2 cells were maintained in lipoprotein-depleted foetal bovine serum for 2 days to stimulate cholesterol synthesis. The cells were incubated in serum-free medium with SQS inhibitors for 2 h and then with [5-^{3}H]-mevalonate for 1 h. The cells were then washed and saponified. The products were separated by solvent extractions and acid-base washings, and the radioactivities measured [31, 50].

INHIBITION OF HEPATIC LIPID SYNTHESIS IN MICE

Mice were dosed s.c. with inhibitor dissolved in 0.09% NaCl, or p.o. (by

gavage) with inhibitor suspended in 5% emulphor. Thirty minutes post-dose D,L-[5-^{3}H]mevalonolactone was administered by the s.c. route. Fifteen minutes later, the animals were sacrificed and the livers removed and saponified. The saponified livers were extracted and examined by HPLC. The assay measured the incorporation of [5-^{3}H]mevalonolactone into cholesterol [31, 50].

MEASUREMENT OF CHOLESTEROL BIOSYNTHESIS *IN VIVO* IN RATS

Squalestatins were dosed i.v. to rats followed immediately by intraperitoneal (i.p.) administration of [1-^{14}C]acetate. The rats were sacrificed after 1 h and the livers removed and saponified. After extraction, [^{14}C]cholesterol was separated by HPLC and quantified [38].

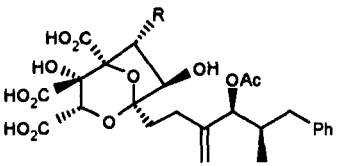
EFFECT ON SERUM CHOLESTEROL LEVELS IN MARMOSETS

Marmosets of either sex were fasted overnight and blood samples (0.5 ml) were taken from the femoral vein. Serum was obtained by centrifugation and serum cholesterol concentrations were determined. The control group animals were dosed with vehicle. Compounds were either administered daily for 7 days and serum cholesterol levels determined at the end of this dosing regime or dosed once and serum cholesterol levels determined at days 2, 3, 5 and 7 post-dosing. The results were expressed as the means of the change in serum cholesterol levels from pre-dose values [38, 39].

IN VITRO STRUCTURE-ACTIVITY RELATIONSHIPS

The results of studies with substrate analogues of FPP have been reviewed recently [51, 52]. Publications with natural product inhibitors have centred primarily on S1 which incorporates the highly functionalized 2,8-dioxabicyclo[3.2.1]octane ring system possessing carboxylic acid groups at C-3, C-4 & C-5, hydroxyl groups at C-4 and C-7, and two lipophilic side-chains at C-1 and C-6. The 1,3-dioxane ring adopts a chair conformation substituted equatorially by the three carboxylic acids and the C-1 phenylhexyl side-chain. The axial C-4 tertiary hydroxyl group and ethano bridge substituted with a secondary hydroxyl group and the lipophilic ester side-chain complete the gross structural features (see *Figure 7.3*). Following the structure elucidation of this novel class of compounds and the emerging highly interesting biological profile, chemical programmes were established to identify the key structural features essential for SQS inhibitory activity.

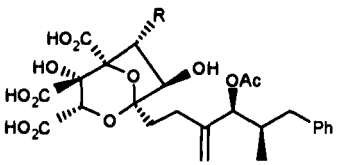
Table 7.8. SQS INHIBITORY ACTIVITIES FOR C-6 MODIFIED SQUALESTATINS



		Squalene Synthase IC_{50} (nM)	
Compound	*R*	*Rat*	*C. albicans*
(26)	MeCOO	2	NT
(27)	$Me(CH_2)_3COO$	6	26
(28)	$Me(CH_2)_8COO$	8	NT
(29)	$Ph(CH_2)_6COO$	9	7
(30)	MeOCOO	3	NT
(31)	$Me(CH_2)_3OCOO$	4	8
(32)	$Me(CH_2)_8OCOO$	7	2

NT = not tested.

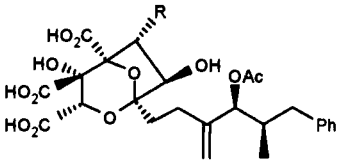
Table 7.9. SQS INHIBITORY ACTIVITIES FOR C-6 LONG-CHAIN ESTER, CARBAMATE, CARBONATE AND ETHER ANALOGUES OF S1



		Squalene Synthase Assays	
Compound	*R*	*Rat - RA*[a]	*Candida - RA*[a]
(33)	$Me(CH_2)_{10}COO$	0.29	3.13
(34)	$Me(CH_2)_9NHCOO$	0.14	0.9
(35)	$Me(CH_2)_9OCOO$	3.0	4.35
(36)	$Me(CH_2)_{11}O$	2.5	9.26
(37)	$PhO(CH_2)_{11}OCOO$	3.0	6.07

[a] RA = relative activity of the test compound expressed as the quotient of the IC_{50} determined for S1 divided by the IC_{50} of the test compound.

Table 7.10. WHOLE-CELL ANTIFUNGAL ACTIVITY OF C-6 MODIFIED SQUALESTATINS



(38) R = $OCO(CH_2)_8Me$
(39) R = $OCO_2(CH_2)_8Me$

	MIC (μg/ml)		
Fungus	*S1*	*(38)*	*(39)*
C. albicans C316	8	16	1
A. fumigatus 48238	16	2	0.25
C. neoformans 2867E	0.5	2	0.06

Methodology has been established for the selective manipulation of this polyfunctionalized system and detailed structure-activity relationships derived for inhibition of this enzyme have been published.

MODIFICATIONS AT C-6 AND C-7

A wide range of substitutive modifications at C-6 has been reported. At Glaxo both long- and short-chain esters and carbonates [for example (26) -(32), *Table 7.8*] have been shown to possess enzyme inhibitory activities closely similar to that of S1 [53].

Merck have reported that long-chain esters, carbonates, ethers, and carbamates are well tolerated [50]. Representative examples from these series, (33) - (37), are shown in *Table 7.9*. They found that the related short-chain derivatives were significantly less active (2–15% that of S1).

The antifungal activities of C-6 modified analogues have been reported by both groups. Whilst S1 is a potent antifungal agent, H1, which we have reported to be a potent inhibitor of both mammalian and fungal enzymes [40], is essentially without whole-cell antifungal activity [53]. Within a series of C-6 esters we provided evidence that lipophilicity was an important feature in fungal cell wall penetration of these agents with the C-6 decanoate derivative (38) showing increased antifungal activity against *Aspergillus*

Table 7.11. WHOLE-CELL ANTIFUNGAL ACTIVITIES OF C-6 MODIFIED SQUALESTATINS (ZARAGOZIC ACIDS)

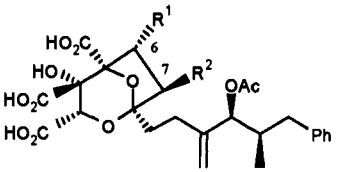
	MFC[a] *(μg/ml)*					
Fungus	*S1*	*(33)*	*(34)*	*(35)*	*(36)*	*(37)*
C. albicans MY 1055	16	4	8	8	4	>128
C. tropicalis MY 1012	2	8	16	2	64	0.5
C. parapsilosis MY 1010	8	8	8	2	8	4
Cry. neoformans MY 2061	1	0.12	0.5	0.25	0.12	16
A. fumigatus MF 4839	32	8	32	2	0.5	0.15

[a] Minimum fungicidal concentrations (MFC, μg/ml) against *Candida albicans* MY1055, *Candida tropicalis* MY 1012, *Candida parapsilosis* MY 1010, *Cryptococcus neoformans* MY 2061 and *Aspergillus fumigatus* MF 4839

fumigatus. The nonyl carbonate (39) was identified as the most potent antifungal squalestatin reported in this series [53] (*Table 7.10*).

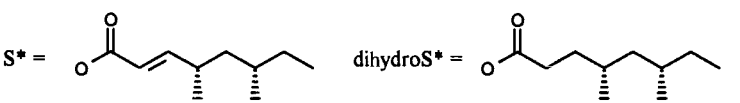
Complementary findings have been reported by the group at Merck [50] who disclosed data for a series of five compounds with different linkages at

Table 7.12. INFLUENCE OF C-6 AND C-7 SUBSTITUENTS ON SQS INHIBITORY ACTIVITY



Compound	*R*[1]	*R*[2]	*IC*$_{50}$ *(nM)*
H1 (3)	OH	OH	26
(40)	H	OH	35
(41)	OH	H	73
(42)	H	H	57
(43)	OH	OCO_2Me	380
(44)	S*[a]	OCO_2Me	15

[a] throughout this document:



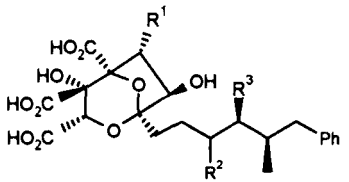
C-6, (33) - (37) (*Table 7.9*). With the exception of the C-6 carbamate (34) these analogues were shown to be 3-9 fold more active than S1 in a yeast SQS assay. All were relatively more effective inhibitors in the yeast than in the rat SQS assay. Their whole-cell antifungal activity (*Table 7.11*) compares favourably with S1.

As H1 retains potent rat SQS inhibitory activity, further studies at Glaxo have centred on establishing the contribution to binding made by each of the hydroxyl groups at C-6 and C-7 (*Table 7.12*). C-6-Deoxy H1 (40), isolated as a minor metabolite, retains good enzyme inhibitory activity as do the related C-7-deoxy (41) [unpublished results] and the C-6, C-7-dideoxy (42) analogues [54]. However, the nature of the C-6 substituent can critically influence whether modifications made at C-7 are tolerated. Thus for example in analogues possessing a C-7-methoxycarbonyloxy substituent, we observed poor activity in the compound possessing a C-6-hydroxy group (43) whereas incorporation of the C-6 dimethyloctenoate ester as in (44) conferred on the system enzyme inhibitory activity closely similar to that of S1 [53].

C-1 PHENYLHEXYL UNIT

Natural products possessing at C-6 either a 4,6-dimethyloctenoate (e.g., S1) or 4,6-dimethyloctanoate ester [squalestatin Y1 (45) *Table 7.13*] possess

Table 7.13. SQS INHIBITORY ACTIVITIES FOR C-1 MODIFIED SQUALESTATINS



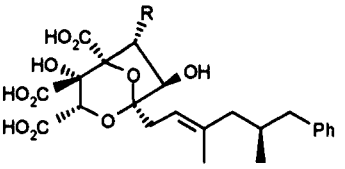
				IC_{50} (nM)	
Compound	R^1	R^2	R^3	*rat*	*Candida*
(45)	dihydroS*	$=CH_2$	OAc	13	NT
(46)	dihydroS*	Me	OAc	3	7
(47)	dihydroS*	Me	H	13	14
(48)	dihydroS*	=O	OH	5	NT
(49)	dihydroS*	OH	OH	4	13

NT = not tested

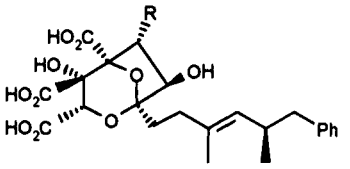
potent SQS inhibitory activity and we have reported that a number of substitutive modifications to the C-1 side-chain are well tolerated in both series [29, 39, 55]. Thus the C-4′-acetoxy group and the C-3′-*exo* double bond are not essential; potent activity is retained in both the natural product allylic alcohol S2 [29] and the semi-synthetic analogues, (46) and (47) possessing a C-3′ methyl group with or without the presence of the C-4′-acetoxy group [39]. Furthermore, analogues in which the C-3′-methylene group is replaced by a C-3′-oxo (48) or C-3′-hydroxyl (49) group are equipotent with S1 [55] whilst good enzyme inhibitory activity is retained in analogues in which the C-3′ methylene group is isomerized towards C-2′, C-3′ (50) or C-3′, C-4′ (51) with loss of the acetoxy group [39] (*Table 7.14*).

The group at Merck have made similar observations; potent enzyme inhibitory activity is retained in the analogue in which the C-4′-acetoxy group is absent (53) [50] and in zaragozic acids B (4) and C (5) which possess a C-3′ methyl group [31] (*Table 7.15*).

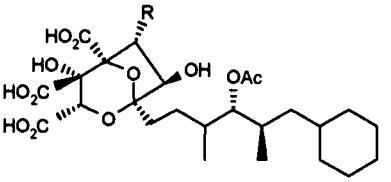
Table 7.14. SQS INHIBITORY ACTIVITIES FOR C-1 MODIFIED SQUALESTATINS



(50)



(51)



(52)

		IC_{50} (nM)	
Compound	*R*	*rat*	*Candida*
(50)	S*	65	NT
(51)	S*	32	NT
(52)	dihydroS*	165	93

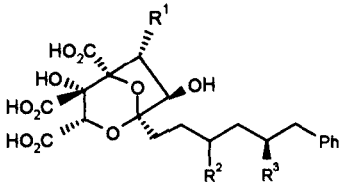
NT = not tested

The terminal aryl ring is an important feature and we have reported a significant loss of activity in the cyclohexyl analogue (52) [39] (*Table 7.14*). Chain truncated analogues were shown by both groups to possess substantially reduced activity [50, 55] and in studies to define the chain-length requirements for securing potent SQS inhibitory activity in a series derived from dihydro S1 (45), we established that an appropriately substituted phenylhexyl unit was optimal [55]. Studies at Merck established that the analogue without substituents along the hexyl backbone (54) [50] possesses reduced activity (*Table 7.15*).

Activities of C-1 modified analogues of S1 were closely similar against the rat and fungal enzymes; we have reported that compounds with comparable enzyme inhibitory activities to S1 against the fungal enzyme possessed similar whole-cell antifungal activity [50].

In a series derived from S1, we have established that the preferred C-1 phenylhexyl substitution pattern is dependent on the nature of substituents at C-6 and C-7. Thus, for example, the C-1-modified S1 analogue (47) [39] is a potent inhibitor of SQS but this modification to the C-1 side-chain is increasingly less favoured on progressive simplification of the substituents at C-6 and C-7 (*Table 7.16*). Thus the corresponding C-6, C-7 diol (55) [39] possesses reduced inhibitory activity and the C-6, C-7-dideoxy analogue (56) [unpublished results] is without significant activity. Similarly, in contrast to the finding that S2 possessed similar potency to S1 [29], the corresponding deacetylated natural product H2 (57) was significantly less active than H1 (*Table 7.7*) and our studies showed there was limited scope

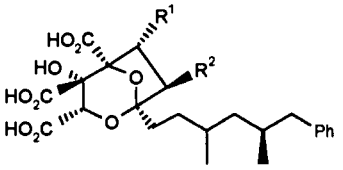
Table 7.15. RAT SQS INHIBITORY ACTIVITIES FOR C-1 MODIFIED SQUALESTATINS



Compound	R^1	R^2	R^3	*SQS Assay RA*[a]
(53)	S*	$=CH_2$	Me	1.67
(54)	dihydroS*	H	H	0.144

[a] RA = relative activity of the test compound expressed as the quotient of the IC_{50} determined for S1 divided by the IC_{50} of the test compound.

Table 7.16. THE INFLUENCE OF SUBSTITUENTS AT C-6 AND C-7 ON SAR FOR MODIFICATIONS TO THE C-1 SIDE-CHAIN OF SQUALESTATINS



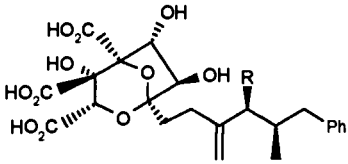
Compound	R^1	R^2	*Rat SQS* IC_{50} *(nM)*
(47)	S*	OH	13
(55)	OH	OH	415
(56)	H	H	>500

for replacing the C-4′ acetoxy group in this series (*Table 7.17*) [56]. Potent SQS inhibitory activity was retained in analogues incorporating certain small ethers; thus the allyl ether (58) is equipotent with H1. In contrast, sterically bulky ethers and esters were not tolerated and the poor activity shown by the acetamido analogue (59) suggested the need for a small lipophilic group at this position devoid of hydrogen bond donor properties.

ROLE OF THE TRICARBOXYLIC ACID MOIETY

In a series of methyl esters of S1 (*Table 7.18*) we reported that the C-3- and C-4-monomethyl esters, (60) and (61), retained potent SQS inhibitory

Table 7.17. RAT SQS INHIBITORY ACTIVITIES FOR H1 ANALOGUES MODIFIED AT THE ALLYLIC CENTRE IN THE C-1 SIDE-CHAIN



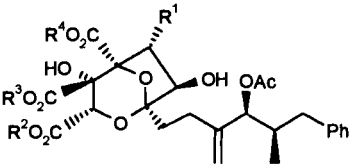
Compound	*R*	*IC₅₀ (nM)*
H1 (3)	MeCOO	26
H2 (57)	HO	252
(58)	CH_2=$CHCH_2O$	27
(59)	MeCONH	2225

activity and these esters were shown to be stable under the assay conditions [57]; the C-3, C-4-dimethyl ester (63) retained reduced enzyme inhibitory activity. In contrast, the C-5-monomethyl ester (62) was without significant activity. These data strongly suggested that the C-5-carboxylic acid was crucial for enzyme inhibitory activity and indicate that free carboxylic acid groups are not required at C-3 and/or C-4 for potent SQS inhibitory activity to be retained.

Related studies by the group at Merck supported the view that the C-3 carboxylic acid was not required for enzyme activity while a C-5 acid was essential for SQS inhibitory activity. In contrast to our findings, they reported that the C-4 monomethyl ester (61) was about 10-fold less active than the parent tricarboxylic acid [58].

We have shown that the nature of the C-6 substituent again critically influences SAR for modifications made to the carboxylic acids (*Table 7.18*); thus while the lack of activity shown by the C-5-monomethyl ester of H1 (66) is again consistent with the C-5-carboxylic acid being of critical importance, the C-3 and C-4 monomethyl esters, (64) and (65), show a significant reduction in activity when compared with that for H1; from these data we suggested that a trianionic species may be required for maximal activity in this series [57].

Table 7.18. THE ROLE OF THE TRICARBOXYLIC ACID MOIETY OF SQUALESTATINS IN PROMOTING RAT SQS INHIBITORY ACTIVITY



Compound	R^1	R^2	R^3	R^4	IC_{50} (nM)
S1 (1)	S*	H	H	H	12
(60)	S*	Me	H	H	7
(61)	S*	H	Me	H	4
(62)	S*	H	H	Me	>500
(63)	S*	Me	Me	H	134
H1 (3)	OH	H	H	H	26
(64)	OH	Me	H	H	220
(65)	OH	H	Me	H	355
(66)	OH	H	H	Me	>500

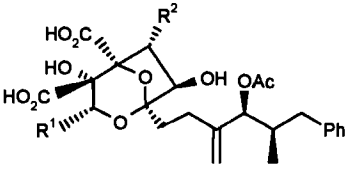
Further modifications to the tricarboxylic acid moiety have been reported which support these conclusions and are summarized below.

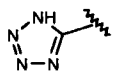
C-3 MODIFICATIONS

A wide range of S1 analogues has been prepared incorporating neutral, acidic and basic groups at this position. Thus, for example, we have reported that the hydroxymethyl (67) [59] and decarboxylated (68) [60] analogues are potent inhibitors of SQS (*Table 7.19*). Replacement of the carboxylic acid by the bioisosteric and acidic tetrazol-5-yl group (69) was well tolerated and a range of C-3 heterocyclic analogues retained good SQS inhibitory activity [61]. Interestingly, reversal of the charge at C-3 is well tolerated; the aminomethyl analogue (70) retained potent SQS inhibitory activity [62].

Again different SAR were established for C-3 modified H1 analogues. The hydroxymethyl analogue (71) [59] showed reduced activity compared with the parent tricarboxylic acid H1, and decarboxy (72) [60] and aminomethyl (74) [62] analogues are without significant activity (*Table 7.19*). A more detailed examination within the C-3-hydroxymethyl series of the structural requirements for potent SQS inhibitory activity revealed that such activity was retained only in those analogues which possessed C-1 and C-6 substituents closely similar to those in the natural product S1 [59]. In a

Table 7.19. RAT SQS INHIBITORY ACTIVITIES FOR C-3 MODIFIED SQUALESTATINS



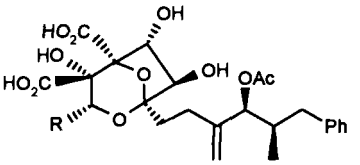
	$R^2 = S^*$		$R^2 = OH$	
R^1	*Compound*	IC_{50} *(nM)*	*Compound*	IC_{50} *(nM)*
$HOCH_2$	(67)	15	(71)	395
H	(68)	23	(72)	25000
	(69)	4	(73)	25
H_2NCH_2	(70)	4	(74)	>500

series of C-3 heterocyclic H1 analogues, there was a strong dependence of activity on the nature of the C-3 substituent. Incorporation of the acidic tetrazole group (73) provided a compound with potent enzyme inhibitory activity (*Table 7.19*). Reduced activity was observed for all other analogues tested although SQS inhibitory activity similar to that of the C-3 methyl ester (64) (IC_{50} 220 nM) was retained in those compounds, (75) - (78) (see *Table 7.20*), for which the electrostatic potential map of the C-3 substituent was similar to that of the methyl ester (64) [61].

C-4 MODIFICATIONS

The C-4-hydroxyl group is not essential for inhibitory activity. We have reported that the C-4-deoxy analogue of S1 (79) is a potent inhibitor of SQS and the *epi*deoxy analogue (80) retains significant enzyme inhibitory activity [63] (*Table 7.21*). Merck have reported that whilst etherification of the C-4

Table 7.20. RAT SQS INHIBITORY ACTIVITIES FOR C-3 HETEROCYCLIC ANALOGUES OF H1



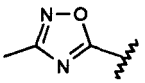
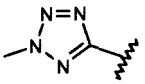
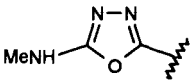
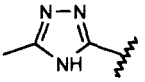
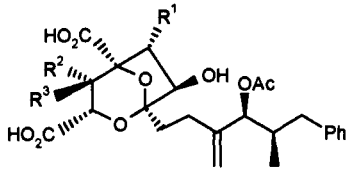
Compound	*R*	*IC_{50} (nM)*
(64)	MeO_2C-	220
(75)		310
(76)		156
(77)		147
(78)		312

Table 7.21. RAT SQS INHIBITORY ACTIVITIES FOR C-4 MODIFIED SQUALESTATINS



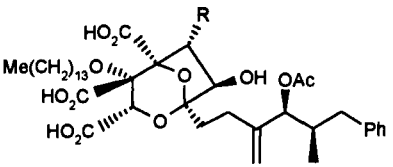
R^2	R^3	Compound	$R^1 = S^*$ RA	Compound	$R^1 = OH$ RA
H	HO_2C	(79)	1	(83)	0.04
HO_2C	H	(80)	0.1	(84)	0.02
H	HO	(81)	0.2	np	np
HO	H	(82)	0.2	np	np

np = not prepared

[a] RA = relative activity of the test compound expressed as the quotient of the IC_{50} determined for S1 divided by the IC_{50} of the test compound.

hydroxyl group in a series of potent C-6 ether analogues [e.g. (85)] was poorly tolerated [50], C-4 long-chain ether analogues of H1 retain potent SQS inhibitory activity (*Table 7.22*). Thus, for example, the tetradecyl analogue (86) has 17% of the enzyme inhibitory activity of S1 [50]; the

Table 7.22. INFLUENCE OF C-6 SUBSTITUENT OF SQUALESTATINS ON RAT SQS INHIBITORY ACTIVITY IN C-4 ETHERS



Compound	R	RA[a]
(85)	$O(CH_2)_{13}Me$	†
(86)	OH	0.17

[a] RA = relative activity of the test compound expressed as the quotient of the IC_{50} determined for S1 divided by the IC_{50} of the test compound.

† not active at 161 nM.

authors suggested that in such analogues the C-4 substituent could subserve the function of the C-6 side-chain. C-4 Short-chain ether derivatives of H1 [50] and the corresponding C-4-deoxy analogues (83, 84) [63] show reduced enzyme inhibitory activity (*Table 7.21*).

We have established routes to the corresponding epimeric C-4-decarboxylated analogues of S1, (81) and (82); these compounds retain good SQS inhibitory activity (*Table 7.21*) supporting the earlier conclusion that the C-4 carboxylic acid is not essential for enzyme inhibitory activity [63].

MONOCYCLIC ANALOGUES

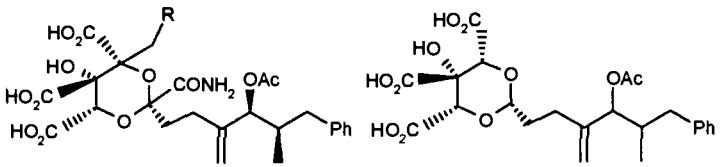
We have reported the conversion of S1 into the monocyclic 1,3-dioxane analogue (87) [64]. An IC_{50} value of 11 nM for (87) clearly indicated that the bicyclic core was not essential for potent SQS inhibitory activity (*Table 7.23*). However, a significant reduction in activity was observed in the related acetoxy analogue (88) and, in a separate study at Glaxo [65], the totally synthetic analogue (89), without substituents at C-2 or C-4, possessed only moderate activity (rat liver IC_{50} 8.0 μM; *C. albicans* IC_{50} 1.2 μM). These latter findings suggested that substituents at these positions were important for optimal enzyme binding.

ACYCLIC ANALOGUES

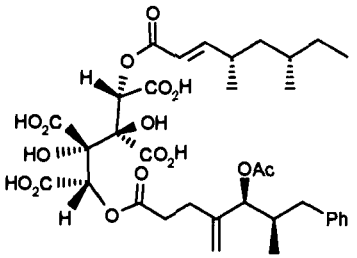
Methodology for the conversion of the squalestatins into acyclic analogues has been reported; the tetracarboxylic acid (90) [66] possesses reduced but significant enzyme inhibitory activity with an IC_{50} value of 500 nM.

Workers at Merck investigated simplified acyclic structures incorporating

Table 7.23. RAT SQS INHIBITORY ACTIVITIES FOR MONOCYCLIC SQUALESTATINS



Compound	*R*	*IC_{50} (nM)*	*Compound*	*IC_{50} (nM)*
(87)	S*	11	(89)	8000
(88)	CH_2COO	270		



(90)

potency enhancing C-1 and C-6 side-chains identified from SARs established for series derived from their studies with the squalestatins. A dicarboxylic acid unit was found to be essential for enzyme inhibitory activity [*Table 7.24*, (91) - (94), IC_{50} values in the range 200–470 nM] although the structure of this acidic moiety did not critically affect potency and some flexibility in the spatial relationship between esters at C-6 and C-4′ in the natural products was observed [compare (91) and (94)] [67].

SAR SUMMARY

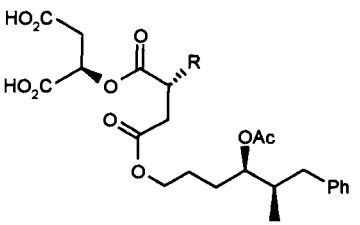
In a series derived from S1, the C-4-hydroxyl group is not essential for enzyme inhibitory activity. The C-5-carboxylic acid is of crucial importance but potent activity can be retained in molecules with reduced ionic character. Thus, free carboxylate groups are not required at C-3 or C-4; this indicates that an appropriately substituted dicarboxylic acid can act as a novel dianionic replacement for the metabolically and chemically unstable diphosphate group in inhibitors of SQS (*vide infra*). Derivatization of the C-7 hydroxyl group in S1 is well tolerated. Furthermore, in series possessing at C-6 either a 4, 6-dimethyloctenoate or dimethyloctanoate ester group, a wide range of substitutive modifications to the C-1 side-chain is well tolerated. These studies established that the terminal aryl ring is an important feature and that within the S1 series, maximal activity is associated with analogues incorporating an appropriately substituted phenylhexyl group. The bicyclic ring is not essential as potent enzyme inhibitory activity is retained in a single ring 1, 3-dioxane analogue.

The structurally simplified natural product C-6-deacyl S1 (H1) is a potent SQS inhibitor, and in series derived from this compound, potent activity can be retained in molecules with reduced molecular size. Thus the hydroxyl groups at C-6 and C-7 are not essential and the C-6, C-7-dideoxy analogue retains good SQS inhibitory activity. In series possessing a free hydroxyl

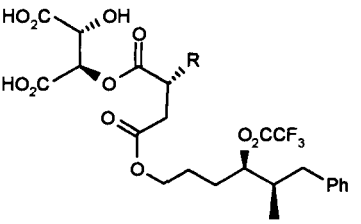
group at C-6, the C-5-carboxylic acid is of crucial importance; however, in contrast to findings in the S1 series, modifications made elsewhere in the molecule have led to a reduction in activity.

The different SAR established for analogues of S1 and its 6-deacyl analogue H1 clearly reflect different structural requirements for effective inhibition of SQS by each series of compounds. At the outset of this programme, the close structural similarities of S1 and H1 with presqualene diphosphate and farnesyl diphosphate, respectively, were well recognized; both squalestatins possess polar trianionic head groups to which is attached either two or one lipophilic group, respectively. The differences in SAR observed between the two series could reflect whether they exert their inhibitory effect as either PSPP or FPP mimetics.

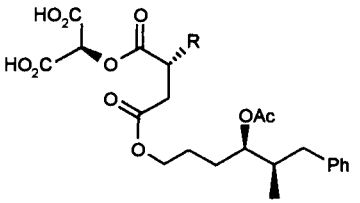
Table 7.24. RAT SQS INHIBITORY ACTIVITIES FOR TOTALLY SYNTHETIC ACYCLIC ANALOGUES OF SQUALESTATINS



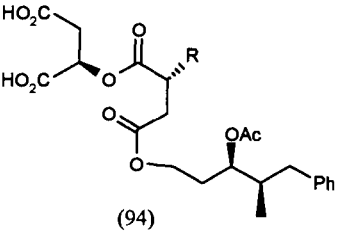
(91)



(92)



(93)



(94)

Compound	*R*	*IC_{50}(nM)*[a]
(91)	S*	470
(92)	S*	300
(93)	$PhO(CH_2)_{10}CO_2$	200
(94)	$PhO(CH_2)_{10}CO_2$	200

[a] IC_{50} value for S1 = 0.35 nM

LIPID-LOWERING STUDIES AND CHOLESTEROL BIOSYNTHESIS INHIBITION

STUDIES WITH S1

We have reported that S1 is orally active in marmosets, achieving 50% reduction in serum cholesterol levels at a dose of 10 mg/kg/d [38]. However, S1 has low bioavailability in this species due to poor oral absorption [unpublished results]; serum cholesterol levels are reduced by 50% and 86% when S1 is dosed i.v. for 7 days at 0.1 mg/kg/d [unpublished results] and 1 mg/kg/d [39], respectively. Furthermore, we have shown that S1 possesses good lipid-lowering properties (ED_{50} 0.15 mg/kg/d i.v.) in cynomolgus monkeys [unpublished results]. However, there was evidence of liver toxicity in both marmosets and cynomolgus monkeys at threshold lipid-lowering doses which precluded its further progression [unpublished results].

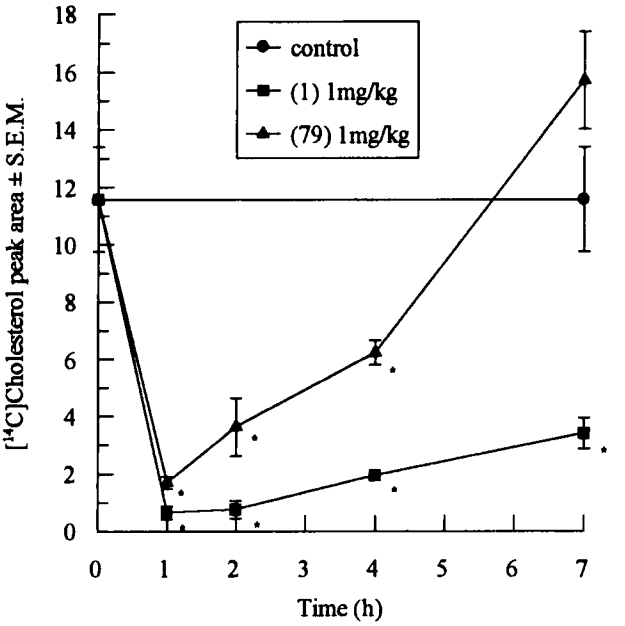
In our initial 7 day p.o. studies with S1, lipid lowering in marmosets was observed within 24 h and the effects of a single dose of 1 mg/kg of S1 revealed that this compound has a profound and extended effect on serum cholesterol levels in marmosets. Thus, a maximum effect was seen after 3 days, and serum cholesterol was still reduced by 50% at 7 days post-dose [39].

These findings were paralleled by an observed extended inhibition of cholesterol biosynthesis in marmoset liver slices after dosing with S1 at 1 mg/kg i.v.; cholesterol biosynthesis was completely inhibited at times up to 24 h and was still reduced by about 75% four days after dosing [unpublished results]. These data suggested that the extended effect on serum cholesterol levels was due to slow clearance from marmoset liver which has been supported by autoradiographic studies in these animals where measurement of liver levels of radioactivity up to 48 h after a single i.v. dose again showed slow clearance from the liver [unpublished results].

Structural features affecting the *in vivo* profile of S1 have been investigated and data have been reported for analogues which retain SQS inhibitory activity and which possess altered physicochemical properties, molecular size and ionic character.

CORE-MODIFIED ANALOGUES

To eliminate potential differences in oral absorption, lipid lowering studies at Glaxo have been generally carried out in marmosets using i.v. administration of test compounds. We have reported [62] that the C-3-hydroxymethyl analogue (67) had no effect in lowering serum

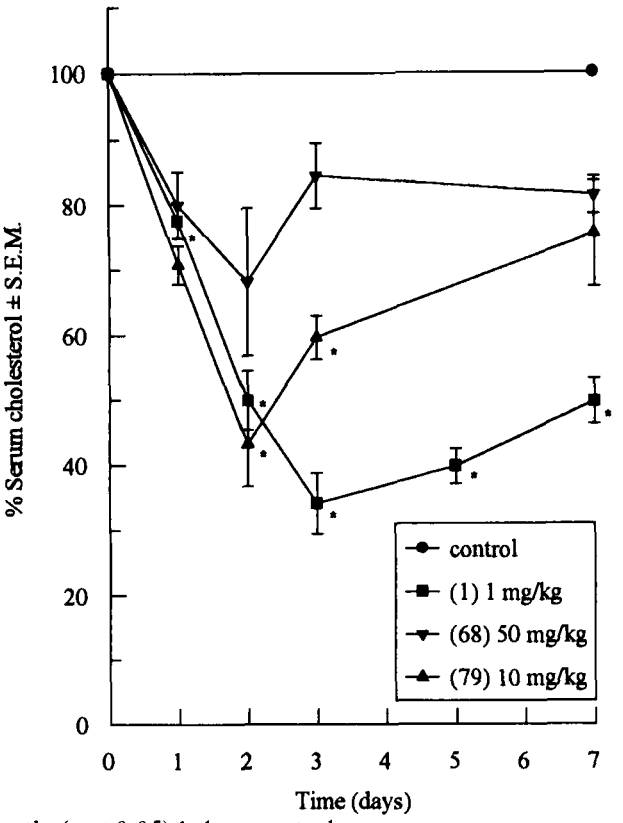


* significantly ($p < 0.05$) below control

Figure 7.6. Duration of effect of S1 and C-4-deoxyS1 (79) on cholesterol biosynthesis in rats after i.v. administration.

cholesterol after a single i.v. dose of 10 mg/kg and only a marginal effect of 31% 2 days after an i.v. dose of 50 mg/kg. By dosing (67) to marmosets 3 times a day at 16.7 mg/kg i.v. for 3 days, a significant cholesterol lowering of 61% was observed which returned towards base-line 7 days post-dose. Confirmation of the different handling *in vivo* of (67) and S1 came from studies in perfused marmoset liver which established that tritiated material was abstracted by the liver more quickly than S1 but was cleared more rapidly into bile.

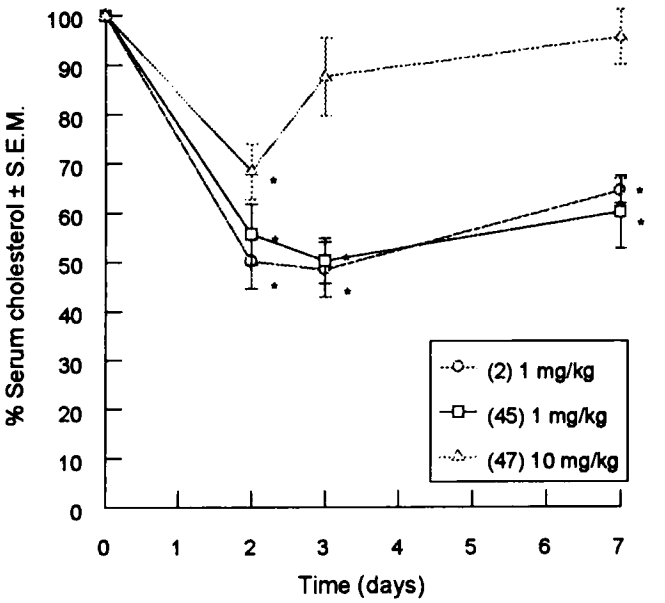
Differences in the *in vivo* durations of action of these compounds were also seen using an *in vivo* model of cholesterol biosynthesis in rat liver. Thus, significant inhibition of cholesterol biosynthesis was seen for at least 8 h following a single i.v. dose of S1 at 1 mg/kg. In contrast, the C-3-hydroxymethyl analogue (67) showed a much shorter *in vivo* duration of effect even after administration of a single dose of 10 mg/kg i.v. Thus whilst an effective block on cholesterol biosynthesis was observed after 1 h, comparable to that for S1, significant inhibition of cholesterol biosynthesis was seen with (67) for only up to 2 h post-dose [62].



* significantly ($p < 0.05$) below control

Figure 7.7. S1 , C-3-decarboxyS1 (68) and C-4-deoxyS1 (79) on serum cholesterol levels of marmosets after a single i.v. dose.

These studies at Glaxo were extended to the *in vivo* evaluation of both the C-3-decarboxy (68) and C-4-deoxy (79) analogues. The duration of action of the C-4-deoxy analogue (79) relative to S1 was examined in the rat at equipotent doses of 1 mg/kg (*Figure 7.6*). Both compounds showed maximal inhibition of hepatic cholesterol biosynthesis *in vivo* at 1 h post-dose. Significant inhibition was observed for at least 7 h with S1 but only up to 4 h with the C-4-deoxy analogue (79). The C-3-decarboxy analogue (68) effectively blocked cholesterol biosynthesis at 1 h post-dose (1 mg/kg) but had no effect on cholesterol biosynthesis in rats at doses up to 10 mg/kg at 6 h post-dose [63].



* significantly ($p < 0.05$) below control

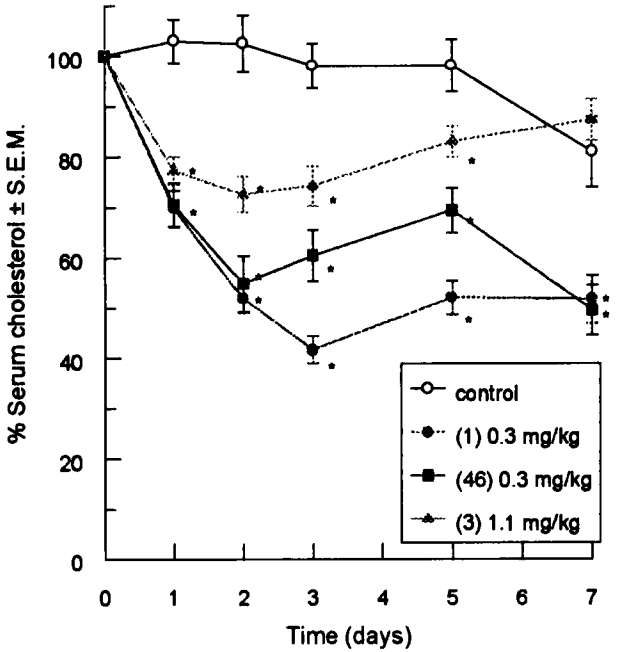
Figure 7.8. Effect of S2, dihydroS1 (45) and tetrahydro desacetoxy S1 (47) on serum cholesterol levels in marmosets after a single i.v. dose.

The C-4-deoxy analogue (79) at 10 mg/kg retained good lipid lowering ability whilst the C-3-decarboxylated analogue (68) had only a marginal affect even when dosed at 50 mg/kg (*Figure 7.7*).

C-1 AND C-6 MODIFIED ANALOGUES

The durations of action of inhibiting hepatic cholesterol biosynthesis in the rat have been reported for tetrahydro S1 (46) and H1 in comparison with S1 [39]. When tested at equipotent doses, all three compounds showed maximal inhibition at 1 h post-dose. Significant inhibition was observed for at least 7 h with S1 and H1 and only up to 4 h with (46).

The natural product S2 possesses good cholesterol lowering properties in marmosets (*Figure 7.8*) [39], however, neither the allylic system in the C-1 side-chain nor the C-6 α,β-unsaturated ester are essential for good lipid-lowering abilities [39]. Thus, in single i.v. dose studies in this species, dihydro S1 (45) and tetrahydro S1 (46) retain good lipid lowering properties (*Figures 7.8 and 7.9*). Removal of the acetoxy group from (46) to generate



* significantly ($p < 0.05$) below control

Figure 7.9. Effect of S1, tetrahydro S1 (46) and H1 on serum cholesterol levels in marmosets after a single i.v. dose.

(47), however, resulted in a major reduction of its serum cholesterol lowering ability (*Figure 7.8*). The C-6 methyl carbonate (30) possesses a closely similar lipid lowering profile in marmosets to that of S1; serum cholesterol reductions of 58% and 85% were obtained when (30) was dosed p.o. (10 mg/kg/d) and i.v. (1 mg/kg/d) for 7 days. Furthermore, in a single i.v. dose (1 mg/kg) study a maximum effect was seen after 3 days and serum cholesterol was still reduced by 47% at 7 days post-dose [unpublished results]. The more hydrophilic natural product H1 did not lower serum cholesterol levels in marmosets when dosed orally at 10 mg/kg/d for 7 days [unpublished results]. A reduction of 56% was obtained following i.v. administration of H1 at 1 mg/kg/d for 7 days whilst it showed reduced lipid-lowering ability in related single i.v. dose lipid lowering studies (*Figure 7.9*) [39].

The C-6-methyl carbonate (30), H1, tetrahydro S1 (46) and C-4-deoxy S1

(79) all showed evidence of hepatotoxicity in primates at equivalent lipid-lowering doses [unpublished results].

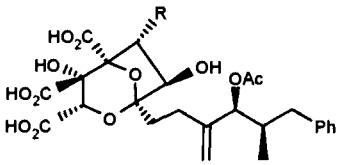
ORAL ABSORPTION STUDIES

Merck have reported that whilst S1 blocks cholesterol biosynthesis when administered s.c. in mice with an ED_{50} of 0.2 mg/kg [31], it is only weakly active in this model when administered p.o. (ED_{50} 100 mg/kg) [31].

As part of a programme to find analogues with improved oral absorption, Merck have reported that whilst series of C-6 long-chain esters, carbonates and ethers with potent SQS inhibitory activity possessed poor activity in inhibiting cholesterol biosynthesis in mice when administered p.o., the corresponding series of C-6 short-chain derivatives appear to have enhanced *in vivo* potency in this model. The most active analogue reported was the n-butyryl ester (95) (ED_{50} 4.5 mg/kg); related series of short-chain C-6-carbamates, carbonates and ethers (95) - (97) (*Table 7.25*) show similar potency (ED_{50}s values of 6 - 9 mg/kg) in this species [50].

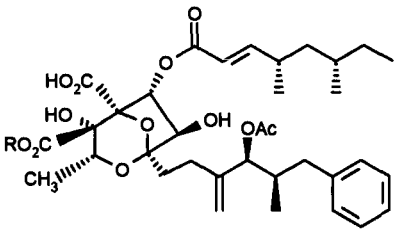
In the same way, Merck have investigated compounds with reduced ionic character. The C-3-methyl analogue (99) showed 22% inhibition of cholesterol biosynthesis at 24 mg/kg in their oral mouse assay whereas its C-4-pivaloyloxymethyl ester (100) exhibited an ED_{50} of 1.6 mg/kg [68]. Comparable findings were reported for a series of C-3 and C-4 alkyl esters

Table 7.25. ACTIVITY OF C-6 MODIFIED SQUALESTATINS IN ORAL MOUSE MODEL OF HEPATIC CHOLESTEROL BIOSYNTHESIS INHIBITION



Compound	*R*	*SQS Assay RA*	*Oral Mouse Assay ED_{50} (mg/kg)*
(95)	$Me(CH_2)_2OCO$	0.07	4.5
(96)	$Me_2CHNHCO_2$	0.05	6
(97)	$MeCH_2OCO_2$	0.02	9
(98)	$Me(CH_2)_2O$	0.05	8

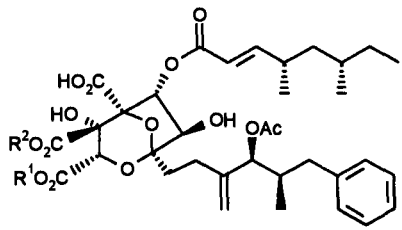
[a] RA = relative activity of the test compound expressed as the quotient of the IC_{50} determined for S1 divided by the IC_{50} of the test compound.



(99) R = H
(100) R = $tBuCO_2CH_2$

(*Table 7.26*) [58, 69]. Thus, within a series of C-3 esters, the isopentyl ester (101) was preferred showing 62% inhibition at 40 mg/kg whilst the C-4-methyl ester (102) showed 43% inhibition at the same dose. The C-3, C-4 bis esterified analogue (103) was the most active compound reported (ED_{50} 18 mg/kg) in this study. Since the 3,4-diester (103) lacks potent enzyme inhibitory activity, an explanation for the further increased activity *in vivo* was its enhanced oral absorption and possible ester hydrolysis to derivatives with greater intrinsic enzyme inhibitory activity. The C-5

Table 7.26. ACTIVITY OF SQUALESTATINS WITH REDUCED IONIC CHARACTER IN AN ORAL MOUSE MODEL OF HEPATIC CHOLESTEROL BIOSYNTHESIS INHIBITION



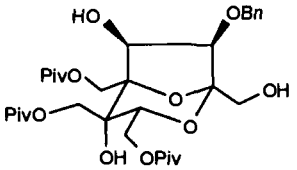
Compound	*Rat* IC_{50} *(nM)*	R^1	R^2	*Oral Mouse Assay* ED_{50} *mg/kg*
(101)	0.66	$iPrCH_2CH_2$	H	a
(102)	4.5	H	Me	b
(103)	c	$iPrCH_2CH_2$	Me	18
(104)	d	$iPrCH_2CH_2$	$CH_2OCOtBu$	9
(105)	d	$iPrCH_2CH_2$	CH_2OAc	6

[a] 62% inhibition at 40 mg/kg [b] 43% inhibition at 40 mg/kg; [c] not active at 13 nM in rat liver SQS assay; [d] not active at 30 ng/ml

carboxylic acid is essential for SQS inhibitory activity (see earlier section) and 3,5-diesters and 4,5-diesters were inactive in the mouse *in vivo* model (data not shown) [58]. In a separate study [69], the C-3-isopentyl-C-4-pivaloyloxymethyl and C-3-isopentyl-C-4-acetoxymethyl esters, (104), (105), gave ED_{50} values of 9 mg/kg and 6 mg/kg, respectively, in the oral mouse assay.

TOTAL SYNTHESES

A number of academic groups fascinated by the complex structure of S1 possessing ten chiral centres but moreover by the unique, highly oxidised and highly functionalized 2,8-dioxabicyclo[3.2.1]octane ring system, are currently involved with the total synthesis of the squalestatins. A number of publications describe the synthesis of the squalestatin and zaragozic acids' side-chains [70–72] or the synthesis of less highly substituted 2,8-dioxabicyclo[3.2.1]octane ring systems as model compounds of the squalestatin nucleus [73–77]. The first asymmetric synthesis of the fully substituted 2, 8-dioxabicyclo[3.2.1]octane ring system (106) and a late stage intermediate for the preparation of S1 was described by the Roberts-Glaxo group starting from (D)-(+)-1, 6-anhydrogalactose (107) [78, 79].



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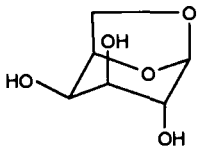
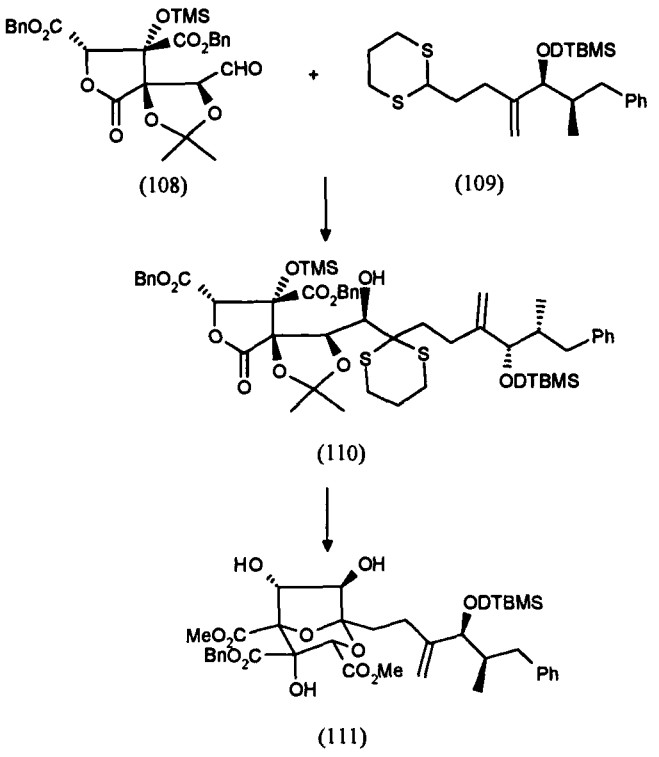


(107)

Bn = CH_2Ph; Piv = tBuCO

The first total synthesis of S1 has recently been described by Nicolaou *et al.* [80, 81] involving the condensation of the aldehyde (108) with the lithio derivative of the dithiane (109) constructing thus the C-1–C-7 bond of the squalestatins. Removal of the dithiane ring from (110), unmasking thus the C-1 ketone, followed by rearrangement in methanolic HCl provided under thermodynamically controlled conditions the squalestatin nucleus (111) possessing all the correct functionality and stereochemistry.

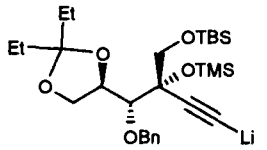
The first total synthesis of zaragozic acid C was published by Carreira and Du Bois [82]. The key step in this synthetic route involved the



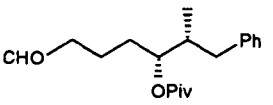
DTBMS = tBu_2MeSi

condensation of the C-1 side-chain aldehyde (112) with the acetylene (113) to provide propargylic alcohol (114). Oxidation of the mixture of propargyl alcohols to the ynone, partial reduction to the enone, Sharpless dihydroxylation, and ketalization in methanolic HCl provided the squalestatin nucleus (115) which on further functional group modifications provided zaragozic acid C.

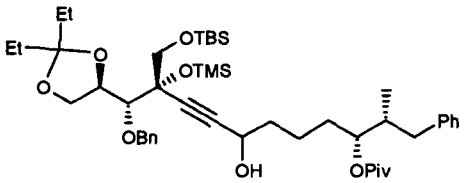
A second asymmetric synthesis of zaragozic acid C has been published by the Evans-Merck group [83]. The key step in this synthetic route involved the condensation of the lithio derivative of the C-1 side-chain (116) with lactone (117) to give the lactol (118) possessing all the required functionality and correct stereochemistry. Replacement of the *p*-methoxybenzyl ether with the acetyl group, followed by acid catalyzed ketalisation, and re-esterification of the carboxylic acids provided the bicyclic core of zaragozic acid C (119).



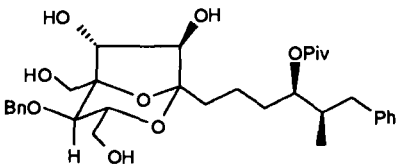
(113)



(112)



(114)



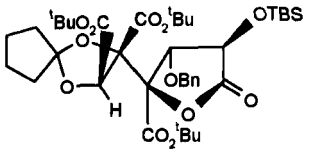
(115)

TBS = $tBuMe_2Si$

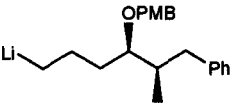
CONCLUSION AND PROSPECTS

Developments in the last few years have shown that one of the most effective approaches to treating hypercholesterolaemia is by inhibiting cholesterol biosynthesis. A number of clinically effective agents are available, such as lovastatin, simvastatin and pravastatin, which work by inhibiting HMGR and these typically reduce serum cholesterol levels by 30% in man. Major toxic effects are not commonly associated with this class of compounds, and an improvement in survival has been demonstrated for patients with coronary heart disease by lowering their lipid levels with simvastatin [10].

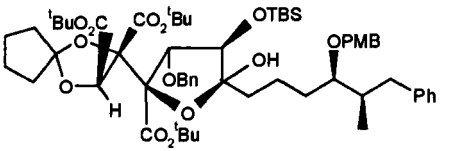
SQS is the first committed step to sterol biosynthesis and inhibitors of this enzyme represent attractive potential new therapies for hypercholesterolaemia. The squalestatins (zaragozic acids) are potent, selective inhibitors of both the rat and fungal enzymes and exhibit potent broad-spectrum antifungal activity *in vitro*. They inhibit hepatic cholesterol biosynthesis *in*



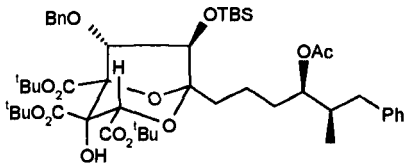
(117)



(116)



(118)



(119)

PMB = p-$MeOC_6H_4CH_2$

vivo and possess excellent cholesterol-lowering abilities in primates. Thus, for example, in marmosets, a species which has been shown to be sensitive to HMGR inhibitors, oral administration of S1 at 10 and 100 mg/kg/d for 7 days results in a 50% and 75% reduction in serum cholesterol levels, respectively. An 86% reduction in serum cholesterol levels is observed when S1 is dosed i.v. (1 mg/kg/d) for 7 days to marmosets and in single i.v. dose studies (1 mg/kg) in this species a profound and extended lipid-lowering effect is observed. These findings have both provided evidence that SQS activity in hepatic cells is similar to the flux through the biosynthetic pathway, consistent with its proposed role as a rate-determining step [5–9] and provided the first evidence that a potent inhibitor of this enzyme can affect serum cholesterol levels *in vivo*.

The isolation of this class of compounds has stimulated considerable interest throughout the scientific community. Additional highlights of the wealth of information generated by the Glaxo and Merck groups include delineation of the biosynthesis of the squalestatins and its application to the

directed biosynthesis of novel analogues; the development of procedures by both groups for the selective chemical manipulation of this unique polyfunctionalized system together with the interest stimulated throughout the academic community in its total synthesis is worthy of a separate review.

The poor oral absorption associated with S1 has been investigated by the Merck group who have identified analogues with improved oral absorption compared with the natural product. However, the key issue of hepatotoxicity at threshold lipid lowering doses with S1 and selected analogues remains to be addressed if this series is to provide compounds of therapeutic value.

With the development of totally synthetic routes to the squalestatins, access to structurally modified analogues not readily available by modification of the natural products should be possible. Biological evaluation of such compounds together with the continuing interest in structurally dissimilar series of SQS inhibitors [51, 52] and the possibility of designing alternative structural classes of inhibitor through the possible cloning and X-ray crystal structure of the derived purified protein will allow the therapeutic potential of SQS inhibitors in the treatment of hypercholesterolaemia to be fully evaluated.

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