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Edited by

F.D. KING and A.W. OXFORD

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Progress in Medicinal Chemistry 36

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Preface

The six chapters presented in this volume review recent advances in important areas of medicinal chemistry. Chapter 1 describes the rational drug design of inhibitors of the influenza virus enzyme, sialidase, from which zanamivir was identified. This compound is representative of a new generation of agents for the treatment of human influenza infections. Non-peptide small molecule fibrinogen antagonists are reviewed in Chapter 2, indicating the extent of progress to identify compounds with good oral pharmacokinetics. The clinical trials of these potent antithrombotic agents are giving encouraging results in angioplasty and unstable angina and as an adjunct to thrombolysis therapy in myocardial infarction.

Combinatorial chemistry, one of the most rapidly evolving techniques for drug discovery, is already the subject of many reviews but most are concerned with synthetic methodology. In contrast, Chapter 3 considers the value of combinatorial chemistry to the medicinal chemist by reviewing the scope of its application to therapeutically important targets and reveals there is much progress to report.

Advances in the molecular biology and pharmacology of the neurotransmitter, GABA, have established there is considerable heterogeneity in $GABA_A$ receptors, the receptors known to be modulated by all the clinically effective benzodiazepine anxiolytics. The consequent surge of activity to find anxiolytic agents which are more selective and possess fewer side-effects than the early benzodiazepines has led to the synthesis of a variety of novel structures. One such series of pyrido[1,2-a]benzimidazoles is described in Chapter 4.

There is good evidence to suggest that cyclooxygenase-2 (COX-2) inhibitors will have important advantages over conventional non-steroidal anti-inflammatory drugs in arthritic disorders, and may be of value in Alzheimer's disease. The numerous compounds that exhibit COX-2 inhibitory activity are reviewed systematically in Chapter 5.

One of the most recent technologies to be developed is that of molecularly imprinted polymers. These materials have considerable potential in a variety of settings, including chromatographic separation of chiral molecules, and in the high throughput screening of complex mixtures such as combinatorial libraries. A survey of this field is given in Chapter 6.

We are most grateful to our contributors for assessing the enormous literature of these topics, and to our publishers for their help and encouragement.

We wish to bring to the attention of our readers that Dr. G. P. Ellis and Professor D. K. Luscombe, who for many years have served as editors to

the series, have decided to stand down following the publication of Volume 35. We pay tribute to their efforts in maintaining the consistently high standard of content and presentation of PMC and in establishing the high standing it enjoys amongst scientists of many disciplines. We owe a particular debt of gratitude to Dr. Ellis who has been an editor to the series from the year it was founded in 1961. We wish him and his wife, Gill, a happy and fulfilling retirement.

July 1998

Dr. F. D. King
Dr. A.W. Oxford

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1 Influenza Virus Sialidase: A Target for Drug Discovery

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INTRODUCTION

Influenza has probably been in existence for much of the history of man. Evidence of the potentially lethal nature of the disease dates from as early as 430 BC, in the fateful plague of Athens. The 1918–1919 influenza pandemic (Spanish flu) swept across the world in three waves and was responsible, directly or indirectly, for over 20 million deaths [1]. Since then at least ten lesser pandemics, and numerous milder more localised epidemics, of influenza have been recorded. As recently as 1989 an outbreak of influenza was reported to have been responsible for about 26,000 deaths in England and

Wales [2]. The term influenza was introduced in Italy in the 15th century to describe an epidemic attributed to the influence of the stars.

For centuries there was wild speculation about the cause of influenza but the 1918–1919 pandemic placed influenza on the world stage and prompted a substantial research effort to find a preventative agent and/or cure for this fatal disease. It was not until 1933 [3], however, that a virus was identified as the causative agent, which marked the start of a better understanding of the disease.

Several excellent articles describing various aspects of influenza virus, both with respect to the virus itself as well as approaches towards the development of anti-influenza agents, have been published in recent years [4–16]. In view of the comprehensive nature of these reports it is the aim of this account, apart from providing the background information necessary to a fundamental understanding of influenza, to focus on advances in the development of influenza virus sialidase inhibitors over the last decade.*

THE INFLUENZA VIRUS

Influenza viruses are members of the orthomyxoviridae family which are further classified, on serological differences, into three distinct types, *viz.* A, B, and C. The human population appears to be most affected by types A and B [17], with the elderly, the very young and those with existing conditions such as chronic pulmonary and heart disease being most susceptible.

The influenza virus undergoes frequent and rapid mutations in its surface antigens. It is this characteristic of the virus which results in the limited efficacy of influenza vaccines and, until recently, has hindered the development of effective specific anti-influenza agents. Of more importance in terms of the fight against influenza is the fact that every so often the virus undergoes a major antigenic transformation, resulting in a pandemic strain of the virus.

Electron microscopic examination of the influenza virion reveals a sphere of $\sim 1 \times 10^3$ Å in diameter [18]. The RNA, which contains the virus's genetic code, is divided into eight separate single-stranded segments. The influenza virus RNA polymerase is prone to a high error rate which, in conjunction with the single strand genomic structure, is manifested as a poor editing function [19,20]. This results in the production of minor mutations of the

*The following abbreviations are used in this article. Neu5Ac, 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid; Neu5Ac2en, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enoic acid; Neu5Acα2Me, methyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid; Boc, *tert*-butoxycarbonyl.

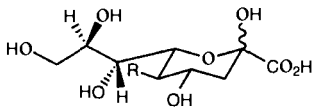
virus, which escape antibody recognition and therefore allow infection of individuals with previous exposure to different influenza strains. However, major antigenic variations, which have to date only been detected in type A influenza, are believed to occur *via* a genetic recombination or reassortment of viral RNA of different influenza A strains.

Such genetic mixing is thought to occur in a susceptible animal (e.g. swine) infected with both human and avian influenza strains, resulting in a strain in which the avian surface proteins have replaced those of the human strain [7, 13, 21, 22].

There are two major surface antigenic proteins, haemagglutinin (HA) and sialidase (neuraminidase, *N*-acetylneuraminate glycohydrolase, EC 3.2.1.18), and their functions in the infective cycle of influenza are well understood. These membrane glycoproteins are seen as 'spikes' covering the surface of the virus particle, with an average of about 500 haemagglutinin and 100 sialidase 'spikes' on each virion [23,24]. In addition to these two major surface proteins, type A influenza virus has a small hydrophobic protein (M2) which functions as an ion channel [25]. Type B influenza virus contains an analogous protein, designated NB, which is encoded by a second reading frame on the sialidase gene.

ROLE OF HAEMAGGLUTININ

The haemagglutinin glycoprotein is a trimer of identical subunits and extends ~135 Å from the lipid membrane of the viral surface [26]. It has a dual function in that it is involved in the binding of the virus to multiple α-ketosidically linked sialyl [*N*-acetylneuraminic acid, Neu5Ac (1)] moieties on the surface of mammalian epithelial cells in the upper respiratory tract, and also aids the penetration of the viral genome through membrane fusion [16, 26–28]. The X-ray crystal structure of influenza haemagglutinin has been resolved to 3 Å [29] (*Figure 1.1*) and co-crystallisation of haemagglutinin with sialyllactose [30, 31] has resulted in a better understanding of the binding site architecture of this protein. The binding site is composed of conserved amino acids surrounded by antibody-binding sites [29–32]. The three sialic acid binding sites of the haemagglutinin trimer have been determined to be ~40 Å apart from analysis of the X-ray crystallographic data [29].



(1) R = NHAc

Since haemagglutinin is involved in the adhesion of influenza virus to epithelial cells, it is an attractive target for therapeutic intervention. The design and synthesis of potential inhibitors of haemagglutinin has been reviewed elsewhere [5, 7, 14, 17, 33, 34] and, apart from a brief discussion of the general approaches employed towards such anti-influenza agents, is outside the scope of this article.

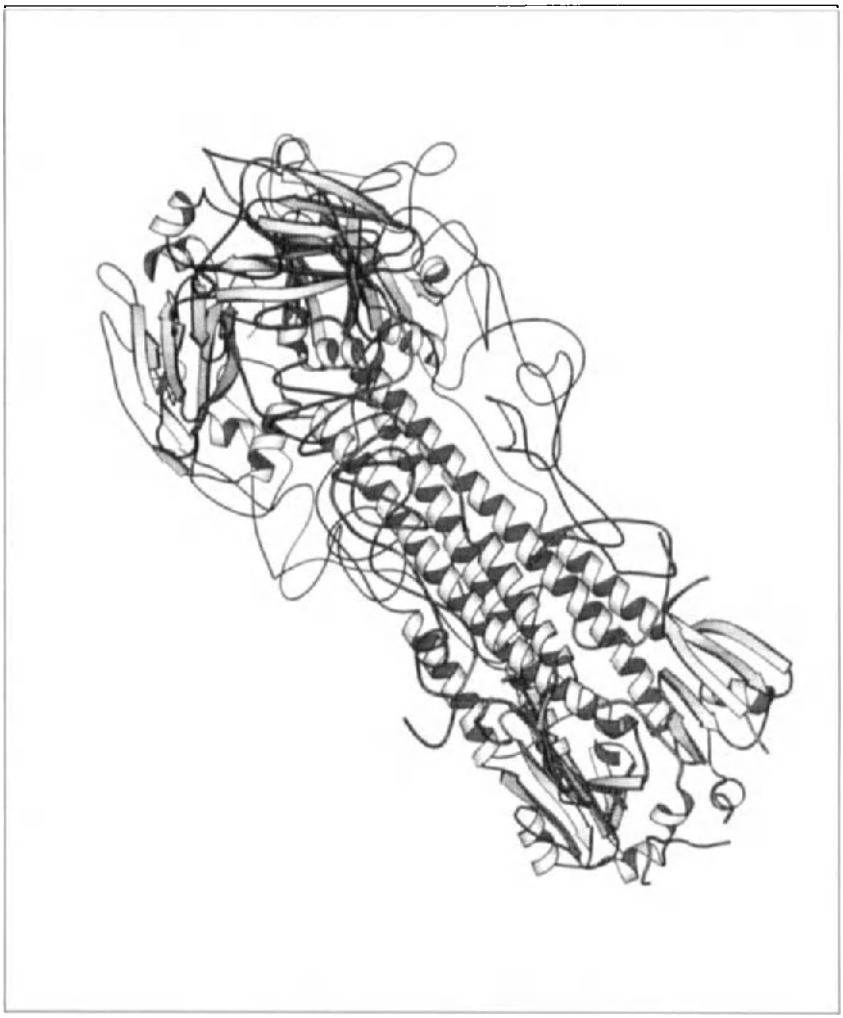
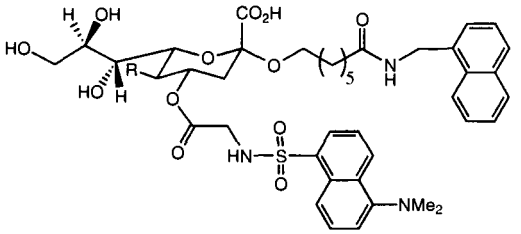
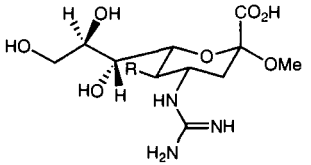


Figure 1.1. A view of a monomeric subunit of influenza virus haemagglutinin.



(2) R = NHAc



(3) R = NHAc

The synthesis of several structurally modified α-glycosides of Neu5Ac as potential inhibitors of haemagglutinin have been reported [14,32–40], however such compounds generally suffer from a poor affinity for a single haemagglutinin binding site. Of the multitude of simple monomeric sialic acid derivatives prepared for this purpose, some of the best inhibitors (e.g. 2) contain large hydrophobic groups at C-2 and C-4 [37]. More recently the synthesis of 4-deoxy-4-guanidino-Neu5Acα2Me (3) has been reported [41], and the compound is currently being evaluated for its affinity towards haemagglutinin.

Real progress in the design and synthesis of potential inhibitors of haemagglutinin rests with the trimeric nature of the protein, and the observation that it binds to multiple sialoglycoconjugates on the cell surface [16, 27, 28, 42]. These multiple simultaneous haemagglutinin-sialic acid interactions form the basis of some elegant work towards the development of polyvalent sialosides [33, 43–49]. A number of polyvalent sialosides have been reported, including polymer bound [33, 43, 44, 46, 48, 49], liposomal bound [33, 36] and dendritic compounds [33, 45]. Often such multivalent sialosides contain either *C*- [43, 47–49] or *S*-sialoside [44–46] linkages, resulting in compounds which are sialidase resistant. These polyvalent sialosides appear to have a significantly increased affinity over monomeric sialosides [33, 47],

showing highly effective inhibition (up to $K_i \sim 10^{-10}$M) of *in vitro* replication of influenza virus [33, 48, 49]. However, the potential therapeutic utility of these haemagglutinin inhibitors remains to be determined.

ROLE OF INFLUENZA VIRUS SIALIDASE

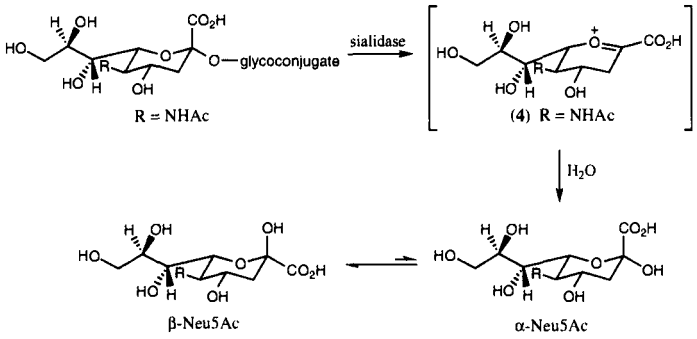
Influenza virus sialidase (EC 3.2.1.18) is a tetramer consisting of four identical disulfide linked subunits (M_r 60 kDa) and extends ~60 Å [18,50] from the viral membrane *via* a long thin stalk. The X-ray crystallographic determination of influenza virus sialidase has been resolved to 2.9 Å [51–54] (*Figure 1.2*), and backbone chain-tracing of the monomer shows a β-sheet propeller topology with an approximate six-fold symmetry passing through the centre of each subunit [51–54]. Examination of a sialidase–sialic acid complex located the catalytic site in a large pocket on the top face of each monomer lying near the pseudo-symmetry axis [51–54]. The walls of the pocket are lined with several charged amino acids pointing towards the sialic acid binding site [51–53]. Importantly, especially from a drug design and development point of view (*vide infra*), the catalytic site residues are conserved across type A and B influenza sialidase subtypes [55].

Sialidase, unlike haemagglutinin, is an enzyme (an exo-glycohydrolase) and cleaves α-ketosidically linked sialic acids from glycoconjugates [15, 55–59]. Sialidases are widespread among animals and some microorganisms [55, 56] and, in the latter case, have been implicated as playing a pivotal role in a number of pathogenic processes [10, 15, 56–60]. The biochemistry of influenza virus sialidase has been thoroughly studied [13, 15, 59, 61–63] and it is believed to play a dual role in the infective cycle. It has been suggested that the enzyme is essential in the release of virion progeny away from infected cells [64–66], as well as assisting the movement of the virus through the mucus in the respiratory tract and also reducing the propensity of the virus particles to aggregate [13, 15, 59, 67]. At the molecular level sialidases cleave terminal α-ketosidically linked sialic acid residues (*Scheme 1.1*) leading to the formation of the proposed endocyclic sialosyl cation transition-state intermediate (4) which is subsequently released as α-Neu5Ac [62, 65, 68]. The proposed intermediacy of the sialosyl cation (4) has found support through kinetic isotope experiments [62] and computational chemistry [69]. The cationic intermediate (4) is believed to be stabilised by a general negatively charged environment within that region of the sialidase catalytic site. That Neu5Ac is released as the α-anomer (which rapidly mutarotates to the thermodynamically more favoured β-anomer) was

Figure 1.2. A monomeric subunit of influenza virus sialidase showing the β-sheet propeller topology. The catalytic site is located near the pseudo-symmetry axis.

confirmed by ^{1}H n.m.r. spectroscopic experiments performed using the 4-methylumbelliferyl α-glycoside of Neu5Ac as substrate [62].

As with haemagglutinin, the presence of sialidase as a surface glycoprotein and its intimate involvement in the infective process of influenza make it an attractive drug design target. Early attempts at the development of inhi-

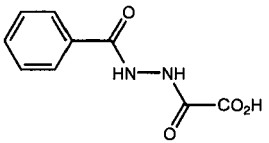


Scheme 1.1

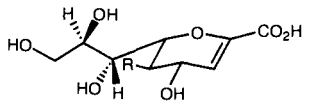
bitors of influenza virus sialidase [6, 8, 54, 57, 59] resulted in oxamic acid derivatives (e.g. 5) being studied [70], wherein their activity was rationalized by comparison with sialic acid using the carboxylate moiety as a common structural feature [8, 70]. Several compounds were subsequently investigated as sialidase inhibitors based on the results of random screening programmes [9, 70]. However, most of these early compounds suffered from either poor potency, lack of selectivity, and/or lack of *in vivo* activity. The successful strategy which has resulted in the development of potent substrate-based influenza virus sialidase inhibitors relies on several factors: the information from the X-ray crystallographic studies of influenza virus sialidase; advances in computational chemistry and therefore rational drug design techniques; and an understanding of the enzyme mechanism.

INHIBITORS OF INFLUENZA VIRUS SIALIDASE

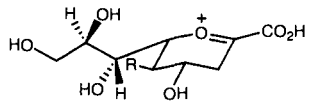
The first substrate-based influenza virus sialidase inhibitor described was Neu5Ac2en (6) [71, 72]. It is proposed that the olefin in (6) mimics, to a certain extent, the conformation of the proposed sialosyl cation transition-state intermediate (4) [62, 68, 69]. Neu5Ac2en shows reasonably potent inhibition of influenza virus sialidase (with a K_i in the range of 10^{-5} to 10^{-6} M) *in vitro*



(5)



(6) R = NHAc

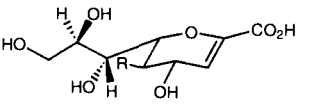


(4) R = NHAc

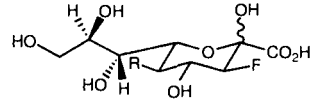
[72,73], but is not selective for influenza sialidase. Interestingly, this unsaturated inhibitor initially failed to demonstrate any beneficial effect in animal models of influenza infection [74].

Despite the apparent limited efficacy of Neu5Ac2en (6) *in vivo*, which has been ascribed to the rapid excretion of such sialic acid derivatives [75], and the possibility that such compounds may not be suitable candidates as *in vivo* influenza inhibitors, a considerable research effort has nonetheless been devoted to the development of sialic acid based sialidase inhibitors. Several excellent overviews of the work in this area have been published in recent years [4, 5, 8,12, 14,17, 34, 54, 76].

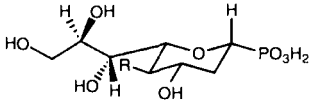
Simple structural modifications to Neu5Ac2en, to give compounds such as the trifluoroacetamido derivative (7), resulted in a slight improvement in *in vitro* inhibition of influenza sialidase [72], but again such derivatives showed no apparent *in vivo* activity. Several articles have described in detail the preparation of a multitude of Neu5Ac derivatives as potential inhibitors of influenza virus sialidase [8, 12, 14, 17, 34]. Of these many examples, some of those which exhibit reasonable *in vitro* inhibition of influenza sialidase include fluorinated derivatives (e.g. 8) ($K_i = 8 \times 10^{-6}$ M) [77,78], phosphonic acid analogues (e.g. 9) [79–81], sialosides (e.g. 10) ($K_i = 10^{-4}$ to 10^{-5} M) [82, 83] and thioglycosidic analogues of gangliosides ($K_i = 10^{-5}$ to 10^{-6} M) [84–86]. However, none of these sialic acid analogues shows significant improvement in inhibition of influenza virus sialidase over Neu5Ac2en (6).



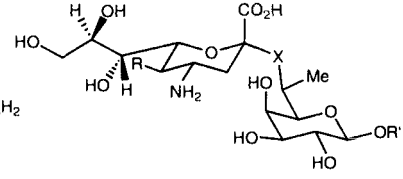
(7) R = $NHC(O)CF_3$



(8) R = NHAc



(9) R = NHAc

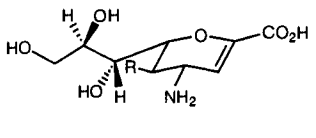


(10) R = NHAc, X = O, S

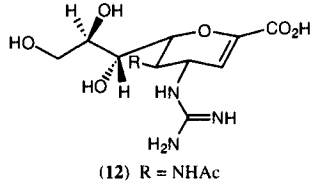
DESIGN AND SYNTHESIS OF ZANAMIVIR

The determination of the crystal structure of influenza virus sialidase [51, 52, 54] provided the basis of the information necessary to prepare the appropriate structural modification of Neu5Ac2en in an attempt to develop more potent inhibitors. With the data from the crystallographic studies, particularly those with inhibitors bound in the catalytic site [51–55], the von Itzstein group set about a rational drug design approach. Using computational chemistry these workers probed the catalytic site of influenza sialidase in order to establish what structural modifications of Neu5Ac2en would potentially be tolerated by the enzyme [69, 87, 88]. In particular the use of the GRID program [89], which allows the determination of energetically favourable interactions between various functional groups and the residues within the binding pocket, revealed that replacement of the C-4 hydroxyl group in Neu5Ac2en by an amino group would be beneficial [87,88]. These studies predicted that the introduction of an amino substituent at C-4, to give 4-amino-4-deoxy-Neu5Ac2en (11), should produce a significant increase in overall binding interaction due to the formation of a salt bridge with Glu-119 [87,88]. In addition, inspection of the region of the catalytic site about C-4 revealed a conserved pocket large enough to accomodate a basic functional group bigger than an amino substituent. Further computational analysis, using the more basic guanidino group at C-4, predicted an even higher affinity of the substituted Neu5Ac2en analogue 4-deoxy-4-guanidino-Neu5Ac2en (12) for the binding site, with the terminal nitrogens of the guanidino group exhibiting lateral binding [90] to both Glu-119 and Glu-227 (*Figure 1.3*) [69, 87, 88].

The preparation of the two target molecules, (11) and (12), is depicted in *Scheme 1.2* [91,92]. Briefly, the oxazoline derivative (13), which is readily prepared by reaction of Neu5Ac2en with $BF_3{\cdot}Et_2O$ [93], was treated with azide to afford the 4-azido-4-deoxy-Neu5Ac2en derivative (14). Reduction of (14), and subsequent deprotection, gave the 4-amino derivative (11), which was treated with aminoiminomethanesulphonic acid in base to give 4-deoxy-4-guanidino-Neu5Ac2en (12) in moderate yield [91]. Subsequently,



(11) R = NHAc



(12) R = NHAc
(zanamivir)

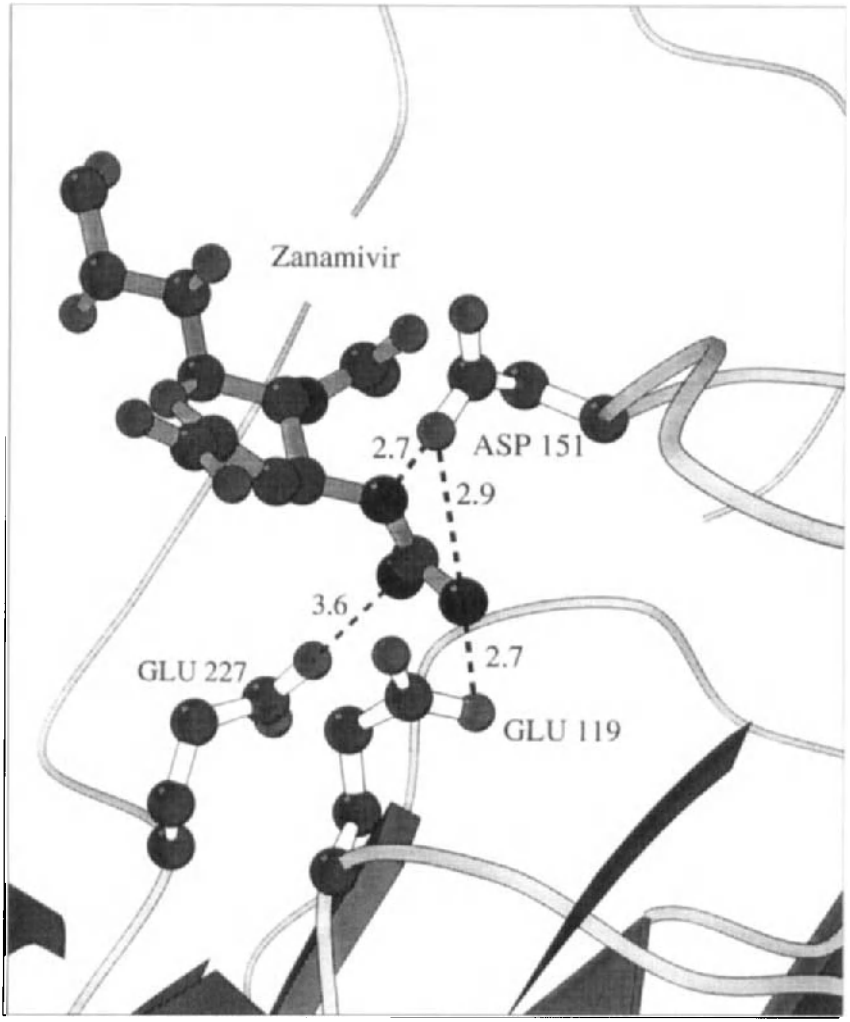


Figure 1.3. Zanamivir (12) bound into the active site of influenza virus sialidase showing interactions to some of the conserved amino acid residues.

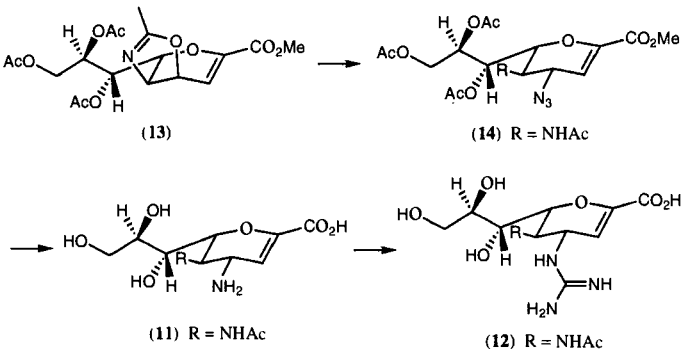
minor modifications and improvements in the synthesis of (12) have been reported [94, 95].

Both the 4-amino and 4-guanidino compounds, (11) and (12) respectively, have been complexed to influenza virus sialidase and examined crystallographically. In both instances the computational and molecular modelling stu-

dies are in general agreement with the crystallographic findings [87, 88, 96]. The predicted lateral binding between the terminal guanidinyl nitrogens and the carboxylate of Glu-227 does occur [87, 88] whilst Glu-119, although slightly further removed than predicted, is found within a distance close enough for electrostatic interaction with the other terminal guanidinyl nitrogen [88].

More importantly, (11) and (12) were found to be both competitive inhibitors of influenza virus sialidase and exceptionally potent *in vitro* and *in vivo* inhibitors of virus replication of both influenza A and B strains [73, 87, 97–100]. Indeed, the 4-guanidino compound (12) inhibits influenza sialidase in the subnanomolar range ($K_i \sim 10^{-11}$ M) [73, 87, 97, 99], and at the time of writing, has completed phase III clinical trials and has been submitted for registration as a pharmaceutical. 4-Deoxy-4-guanidino-Neu5Ac2en (12) has been referred to as 'GG167' in various publications, its generic name is 'Zanamivir' and its tradename is 'Relenza™'. For the purposes of this article compound (12) will be referred to as zanamivir.

Another important feature, especially from the viewpoint of rational drug design, zanamivir (12) shows a remarkable degree of selectivity towards influenza virus sialidase. In experiments aimed at determining the specificity of (12) towards influenza sialidase it was shown that zanamivir [as well as the 4-amino derivative (11)] did not show any increase in inhibition of other viral, bacterial, or mammalian sialidases when compared with Neu5Ac2en (6) [73, 87]. The results of some of these studies are presented in the clinical summary section of this article. This selectivity has been explained on the basis of the active site architecture of the different sialidases, in that it is only influenza virus sialidase which can accomodate the relatively bulky and basic C-4 substituent in its catalytic site [73].



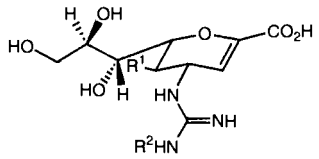
Scheme 1.2

SYNTHESIS OF ANALOGUES OF ZANAMIVIR

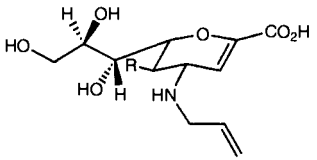
The discovery, in the early 1990s, that zanamivir was a potent and selective inhibitor of influenza virus sialidase prompted several researchers to investigate the synthesis of Neu5Ac2en based analogues of zanamivir. Much of this effort was a consequence of the fact that zanamivir (12) must be administered as a nasal spray, due to its poor oral bioavailability and rapid excretion [101,102], and the desire to identify new sialidase inhibitors with modified physicochemical properties. Several researchers have described structure-activity relationship studies based on zanamivir (*vide infra*), with most modifications reported at C-4, C-5, and the glycerol side-chain.

In terms of modifications at the crucial C-4 position, substitution of the guanidino nitrogens, to give compounds such as (15) [94], or the use of other nitrogen containing substituents such as the mono- or dialkyl- substituted derivatives (16) and (17), respectively [73], resulted in considerably weaker inhibitors of influenza sialidase. These C-4 nitrogen substituted compounds were prepared from the 4-amino-Neu5Ac2en derivative (11) [73, 94]. Changing the orientation of the C-4 substituent, for example to give 4-*epi*-amino-4-deoxy-Neu5Ac2en (18) results in a compound which is an order of magnitude weaker inhibitor ($K_i \sim 10^{-7}$ M) than 4-amino-4-deoxy-Neu5Ac-2en (11) [88].

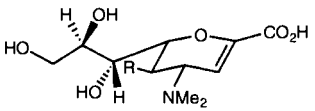
The 5-acetamido group in zanamivir is also important in terms of influenza sialidase inhibition. Removal of this group, to give the 5-desacetamido derivative (19) resulted in a compound with a 25,000-fold reduced affinity for influenza virus sialidase [103]. However both the 5-trifluoroacetamido zanamivir analogue (20) and the 5-sulphonamide derivative (21) retain potent inhibitory activity against both influenza A and B strains, although the reported [104] IC_{50} values [2×10^{-8} M and 9×10^{-8} M, respectively, for (20)



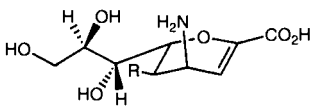
(15) R^1 = NHAc, R^2 = NO_2, CO_2Et, Me, OH



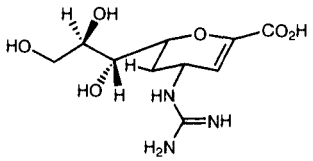
(16) R = NHAc



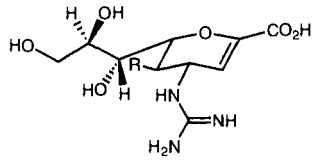
(17) R = NHAc



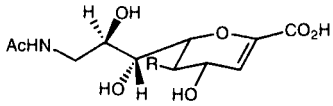
(18) R = NHAc



(19)



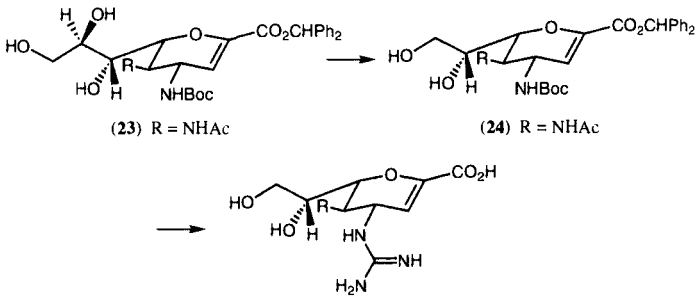
(20) R = $NHC(O)CF_3$
(21) R = $NHSO_2CH_3$



(22) R = $NHC(O)CF_3$

and (21) against influenza A] are an order of magnitude lower than zanamivir. Of several C-9, C-5 disubstituted Neu5Ac2en analogues prepared chemoenzymically using Neu5Ac aldolase [105], compound (22) was found to be the most potent inhibitor ($IC_{50} = 8 \times 10^{-6}$ M) of influenza A sialidase.

Side-chain modifications to zanamivir have resulted in compounds which also provide some insight into the structure-activity relationships necessary for activity against influenza virus sialidase. Truncation of the glycerol side-chain, providing 8, 7, and 6-carbon analogues of zanamivir resulted in a progressive loss, respectively, of activity [106]. Removal of the terminal CH_2OH, by an oxidation ($NaIO_4$)/reduction ($NaBH_4$) sequence carried out on the C-4 Boc protected amine (23) (*Scheme 1.3*) gave (24) which, after amine deprotection and guanidation afforded the 8-carbon analogue (25) of zanamivir [106]. The 7-carbon guanidino analogue (26) of zanamivir was obtained directly from (12) using an oxidation/reduction sequence similar to that shown in *Scheme 1.3* [106].

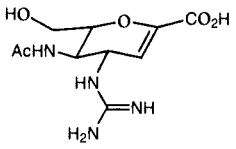


(23) R = NHAc

(24) R = NHAc

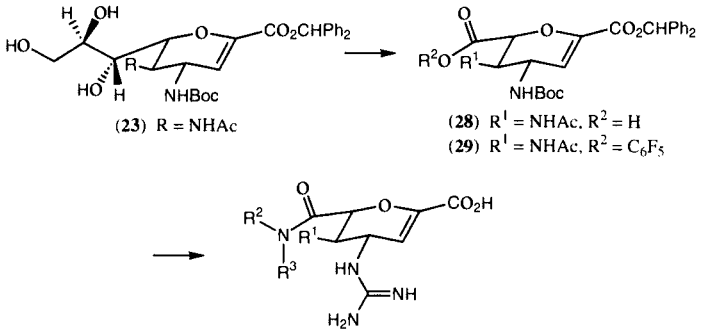
(25) R = NHAc

Scheme 1.3



(26)

The most interesting side-chain modified zanamivir analogues to date are those represented by the general structure (27, R^2 &/or R^3 = alkyl, phenethyl, aryl) (*Scheme 1.4*), in which workers at Glaxo Wellcome [107–109] have placed a carboxamide at C-6. The synthesis of these compounds centres on the ready preparation of the carboxylic acid derivative (28) from the C-4 Boc protected amine (23), followed by conventional amide formation *via* the activated pentafluorophenyl ester (29) (*Scheme 1.4*) [107]. Of the many compounds prepared in this work it appears that tertiary amides containing short alkyl groups, such as (30) and (31), show comparable *in vitro* activity to zanamivir against influenza A sialidase [107–109]. However, the intranasal efficacy of compounds like (30) (ED_{90} = 2.1 mg/kg) is significantly worse than zanamivir (ED_{90} = 0.03 mg/kg) [108]. This difference is attributed to a lower concentration of (30) at the site of virus replication as compared with zanamivir administered at the same dose [108]. The replacement of the glycerol side-chain with heterocyclic groups (e.g. oxadiazoles and triazoles) has also resulted in compounds which exhibit inhibition of influenza sialidase. The most potent inhibitors appear to be those containing a disubstituted triazole ring (e.g. 32) [110].



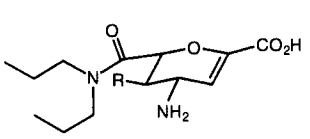
(23) R = NHAc

(28) R^1 = NHAc, R^2 = H

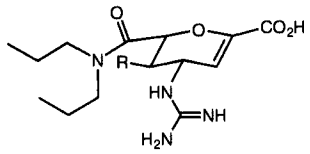
(29) R^1 = NHAc, R^2 = C_6F_5

(27) R^1 = NHAc, R^2/R^3 = alkyl, phenethyl, aryl

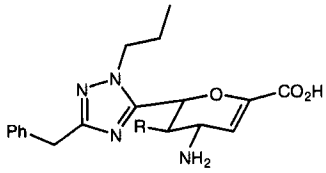
Scheme 1.4



(30) R = NHAc



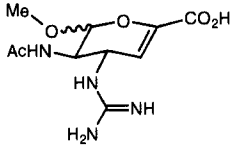
(31) R = NHAc



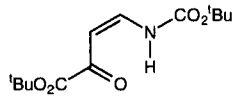
(32) R = NHAc

Interestingly, all of the carboxamides and the heterocyclic containing zanamivir analogues described by Glaxo Wellcome [107–110] exhibit a marked selectivity for influenza A sialidase. This has been attributed, on the basis of protein crystallography and molecular modelling experiments [111], to the binding mode of the tertiary carboxamide group. In influenza B sialidase the binding of (30) results in a significant distortion of the residues in that region of the catalytic site where the glycerol side-chain normally resides [111]. Although the carboxamide moiety in (30) binds in a similar way in influenza A sialidase, there is only a minor effect on the positions of the surrounding residues.

Much of the chemistry devoted to side-chain modified zanamivir analogues has been driven by a desire to prepare structurally simpler derivatives, especially from the viewpoint of easier chemical syntheses. Replacement of the glycerol side-chain of zanamivir with an achiral ether substituent has



(33)



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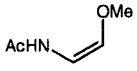

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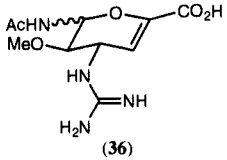
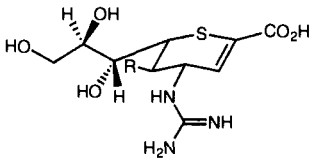
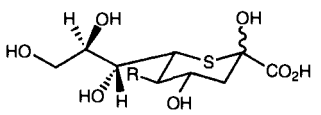


(36)



(37) R = NHAc



(38) R = NHAc

led to the synthesis of compounds of the type (33), which was prepared *via* an inverse demand hetero Diels–Alder reaction between (34) and (35) [112]. Unfortunately, the zanamivir analogue (33) exhibits only modest inhibitory activity against influenza A sialidase, as does the regioisomeric analogue (36), obtained as a by-product in the synthesis of (33) [112].

Other carbohydrate based analogues of zanamivir which have been synthesized as potential influenza virus sialidase inhibitors include the sulfur isostere (37), which was prepared from the known [113] 6-thio-Neu5Ac derivative (38) *via* a sequence analogous to that shown in *Scheme 1.2* for the preparation of zanamivir [114]. The sulphur isostere (37) was found to have comparable activity ($IC_{50} = 5 \times 10^{-9}$ M) to zanamivir [114].

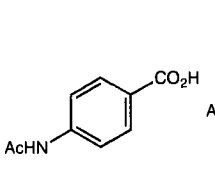
Despite all of these investigations into the structure-activity relationships of zanamivir, none of the carbohydrate-based analogues prepared to date have resulted in an improvement of *in vitro* or *in vivo* activity against influenza virus sialidase. From these studies it appears that only minor structural changes to the zanamivir template are tolerated by influenza virus sialidase. However, the recent findings associated with zanamivir analogues containing carboxamide substituents at C-6, provides valuable information for any future efforts in this regard.

ZANAMIVIR MIMETICS

Apart from the synthesis of Neu5Ac2en based zanamivir analogues, which has met with mixed success in terms of developing new inhibitors of influenza virus sialidase, there has also been considerable effort expended recently in the search for 'non-carbohydrate' based zanamivir analogues. Much of this research is driven by the search for less polar influenza sialidase inhibitors, which should possess modified physicochemical properties as well as being structurally simpler and therefore synthetically more accessible. In this regard, the major area of interest to date has involved moving away from the dihydropyran core of zanamivir, and has resulted in the development of compounds which have exciting activity against influenza sialidase.

Replacement of the dihydropyran ring by a planar benzene ring has resulted in the development of several benzoic acid based mimics of zanamivir [115–118]. It was rationalised, and shown crystallographically, that simple 4-acetamido-benzoic acid derivatives (e.g. 39) would bind in the same orientation as Neu5Ac2en in the catalytic site of influenza sialidase [115]. Further structural modification of the benzoic acid template, with a view to increasing the similarity to zanamivir, led to the influenza sialidase inhibitor (40) [116]. This compound contains a guanidino substituent in the appropriate position to mimic the 4-guanidino group of zanamivir, and exhibits activity ($IC_{50} = 2.5 \times 10^{-6}$M) comparable to Neu5Ac2en [116]. Interestingly, crystallographic analysis of (40) bound to influenza sialidase revealed that the guanidino group in (40) was occupying the site where the glycerol side-chain binds [116]. This difference in binding mode was rationalized on the basis of the inherent symmetry in the parent 4-acetamido-benzoic acid [116]. In an attempt to explore this unexpected binding mode further, the benzoic acid analogue (41) of zanamivir has been prepared [117], as has a benzoic acid derivative containing two guanidino groups [118]. However, the inhibitory activity of these trisubstituted benzoic acids did not improve over (40). Of the multitude of benzoic acid derivatives prepared as potential influenza sialidase inhibitors, including a recent report describing the results of testing 94 such compounds [116], the 3-guanidino benzoic acid derivative (40) remains the most potent inhibitor *in vitro*. Unfortunately (40) exhibits no *in vivo* activity (in mice models), possibly due to its rapid removal from the lung by metabolism and/or absorption [116]. Despite the lack of highly potent benzoic acid based influenza sialidase inhibitors, the huge amount of data generated from these structure-activity relationships has provided several interesting insights which may ultimately lead to more viable compounds.

Of all the influenza virus sialidase inhibitors based on zanamivir reported to date, the most promising compounds are those which contain a carbocyclic ring in place of the dihydropyran ring. Early work in this regard involved the use of Diels–Alder chemistry to ultimately provide access to the side-



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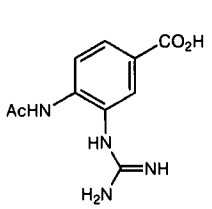

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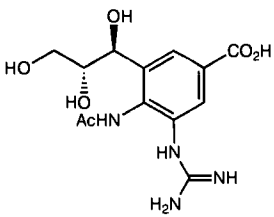

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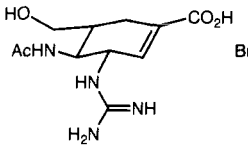

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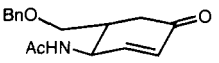

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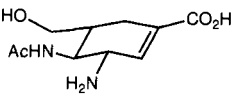
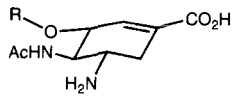


(44)

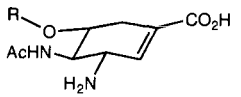
chain truncated carbocyclic zanamivir analogue (42) *via* the key enone intermediate (43) [119]. The carbocyclic zanamivir mimetic (42), together with the corresponding amino derivative (44), display similar levels of activity to those shown by their respective side-chain truncated dihydropyran containing counterparts [119].

Recently, Kim and coworkers have described the design and synthesis of several carbocyclic compounds of the general structure (45) [120–123]. It was reasoned that the cyclohexene ring in (45) would adopt a similar conformation to the putative sialosyl cation transition state intermediate (4) [120]. The location of the double bond in the cyclohexene ring, either as shown in (45) [which is analogous to the position of the oxonium ion double bond in (4)] or in a position analogous to that found in Neu5Ac2en (6) [as in (46)] was found to be crucial. Molecular modelling analysis showed that the isomers (45) and (46) overlay well [120], so compounds (45, R = H) and (46, R = H) were prepared in order to determine the effect, if any, of the double bond position on inhibition. Significantly, it was found that compound (45) showed a markedly higher activity ($IC_{50} = 6.3 \times 10^{-6}$ M) than the isomeric derivative (46) ($IC_{50} > 2 \times 10^{-4}$ M) in influenza sialidase inhibition studies [120].

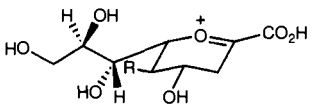
The strategy employed for synthesizing compounds such as (45) started with shikimic acid (47) and employed the aziridine derivative (48) as the key intermediate (*Scheme 1.5*) [120]. Exposure of the aziridine (48) to alcohols in the presence of $BF_3{\cdot}Et_2O$ and subsequent amine acetylation gave



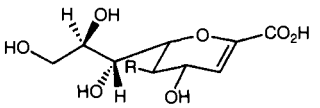
(45)



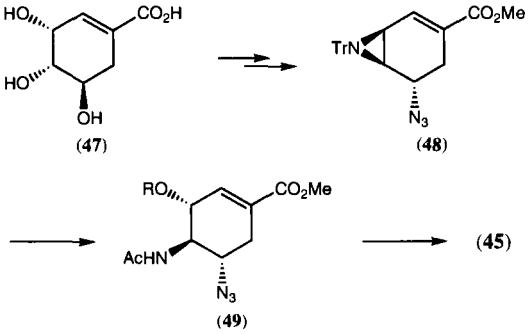
(46)



(4) R = NHAc



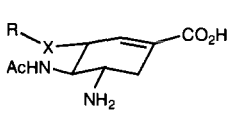
(6) R = NHAc



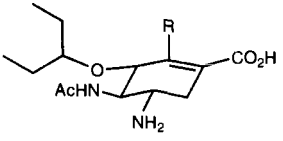
Scheme 1.5

the azido ethers (49). Reduction of the azide in (49) and ester saponification gave the required carbocyclic derivatives (45).

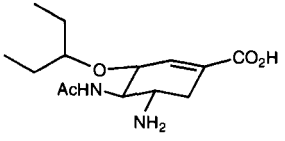
Many carbocyclic derivatives have been prepared by these workers, including C-3* thio- (50) and C-3* carba-isosteres (51) [121], C-2* substituted analogues (52) [122], and C-4* and C-5* functionalised compounds [123], with the most potent inhibitor reported being compound (53) (GS4071) [120] (*positions refer to cyclohexene numbering). As can be seen by comparison with Neu5Ac2en, the glycerol side-chain has been replaced by the more lipophilic 3-pentyl ether residue. The use of an ether linkage was chosen in an attempt to reduce the electron density in the cyclohexene double bond, since the oxonium ion double bond in (4) is electron deficient, as well as facilitating the introduction of alternative ether residues (see the transformation (48) to (49) in *Scheme 1.5*) [120]. It was postulated that a more lipophilic group would optimise any hydrophobic interactions within the glycer-



(**50**) X = S
(**51**) X = CH_2



(**52**) R = Cl, SMe, Me



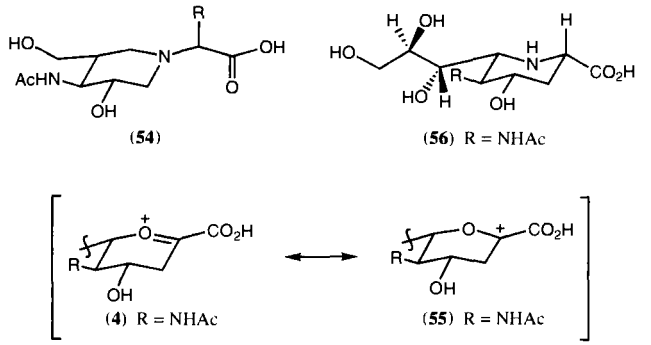
(**53**) GS4071

ol side-chain binding region of the sialidase catalytic site, leading to a new class of inhibitor with increased lipophilicity and different pharmacological properties [120, 123].

The carbocyclic zanamivir mimetic (53) inhibits influenza virus sialidase ($IC_{50} = 1 \times 10^{-9}$ M) with equal potency to zanamivir [120]. X-Ray crystallographic information derived from (53) bound to influenza sialidase shows that the compound binds in a similar orientation to that observed with zanamivir. However, the lipophilic 3-pentyl ether residue in (53), which sits in the same region as the glycerol side-chain of zanamivir, shows hydrophobic interactions with Glu-276, Ala-246, Arg-224 and Ile-222 [120]. The carboxylate of Glu-276, which interacts with the C-8 and C-9 hydroxyls of zanamivir [53], is oriented away from the binding pocket when (53) is bound [120].

The ethyl ester of (53) (designated GS4104), which acts as a prodrug of (53), has shown good oral bioavailability in a number of animal models and has recently entered clinical trials [120]. At the time of writing, results from the phase II clinical trials have not appeared in press although they have been presented at conferences [124]. Replacement of the amino group in (53) with a guanidino group is reported [120, also see ref. 123] to provide a 'significant increase' in activity, although at this stage further details are not available.

An alternative approach to the synthesis of compounds which mimic the sialosyl cation transition state intermediate (4) centres on *N*-functionalised piperidines like (54) [125]. It is proposed that, upon protonation, the structure (54) would be electronically equivalent to that of resonance contributor (55) of the transition state intermediate (4) which bears the positive charge on the anomeric carbon. Whilst no information is given regarding the activity of compounds such as (54) against influenza sialidase [125], modest inhibition of bacterial sialidases is reported. It remains to be seen what effect, if any, the incorporation of some form of glycerol side-chain in this new struc-



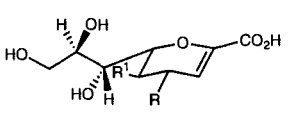
tural class of sialidase inhibitor has on activity. Nitrogen isosteres of Neu5Ac, such as (56), have also been prepared as sialidase inhibitors [126], although these compounds exhibit only moderate inhibition of sialidases.

CLINICAL SUMMARY

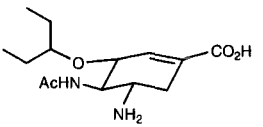
Zanamivir has been shown to act as a slow-binding competitive inhibitor of sialidase from both influenza A and B viral strains [97,99]. It has been proposed that the slow binding nature of the inhibitor is a result of the displacement of an active site-bound water molecule by the bulky guanidinium moiety at C-4 [97]. Importantly zanamivir, as well as GS4071, appear to be highly specific for influenza virus sialidase. As *Table 1.1* shows, zanamivir exhibits potent activity against both influenza A and B strains, but shows significantly less inhibition of sialidases from bacterial origins or some endogenous mammalian sialidases. Similar results have been obtained for GS4071 (*Table 1.1*).

Influenza virus replication in human respiratory epithelial cells is efficiently inhibited by zanamivir. Little, if any, cytotoxicity has been reported

Table 1.1. INHIBITION OF SIALIDASE FROM VARIOUS SOURCES.
Sialidase activity was assayed using a modification [127] of the fluorimetric assay described by Potier [128]. Results of K_i determinations and IC_{50} measurements have been sourced as indicated.



R[1] = NHAc



GS4071

compound	Sialidase K_i (M) *Flu A*	*Flu B*	*C. perf.*	*V. chol.*	*A. urea.*	IC_{50} (M) Human[d,e]
(6) R = OH[a]	4×10^{-6}	2×10^{-5}	8×10^{-6}	3×10^{-6}	1×10^{-6}	1×10^{-5}
(11) R = NH_2[a]	4×10^{-8}	1×10^{-6}	7×10^{-4}	3×10^{-4}	3×10^{-6}	9×10^{-3}
Zanamivir[a,b] (R = NHC(NH)NH_2)	5×10^{-10}	1×10^{-9}	$> 10^{-4}$	6×10^{-5}	$> 10^{-2}$	1×10^{-3}
GS4071[c]	5×10^{-10}	1×10^{-9}	4×10^{-4}	5×10^{-4}	–	5×10^{-4}[e]

[a]Ref [73]
[b]Ref [99]
[c]Ref [129]
[d]Human lysosomal sialidase assays are reported in [87] for compounds (6), (11) and zanamivir.
[e]Human liver sialidase assay for GS4071 is reported in [129] and is expressed as a K_i.

in a range of mammalian cell types [100]. Using both the mice and ferret models for *in vivo* influenza infection studies, zanamivir has demonstrated good efficacy [87, 101, 102]. Notably, as a consequence of the charged nature of zanamivir, the bioavailability of the drug, following either oral or intraperitoneal administration, is poor, resulting in rapid elimination of the compound unchanged *via* the kidneys [101, 102].

A number of human trials have been conducted with zanamivir [98, 130] and the results from these studies strongly suggest that the drug is useful in both the prophylaxis and treatment of human influenza infection. Whilst the poor oral bioavailability of zanamivir may be considered an issue by some, the nature of influenza infection is such that delivery of the drug directly to the site of infection, *via* inhalation as a dry powder, may hold advantages.

The carbocyclic zanamivir mimetic GS4104, the ethyl ester of (53), entered phase II/III clinical studies towards the end of 1997. Although the detailed results from the clinical trials with this compound remain to be published, it appears [124] that it is also useful in the prophylaxis and treatment of experimentally infected patients.

The development of resistance to zanamivir is under thorough investigation [131–136]. There is no doubt that resistant mutants can emerge by exposure of influenza virus to high doses of zanamivir under experimental conditions. Similarly, it appears [124, 136] that influenza viruses with decreased susceptibility to GS4104 have been detected. Whilst the clinical relevance of these mutants remains to be determined, it has been shown under experimental conditions [136] that influenza strains with mutations in previously conserved active site residues have significantly reduced activity with respect to viral replication. This reduction in activity may well have an effect on the virulence of the virus.

CONCLUSION

The benefits of using a rational drug design strategy are exemplified in the development of zanamivir, a highly potent inhibitor of influenza virus sialidase. The recent findings with respect to structurally modified analogues of zanamivir for example, GS4071 and the carboxamide compounds such as (31), especially from the point of view of ease of synthesis, as well as the possibility of altering the physicochemical properties, clears the way for the emergence of next generation compounds with different pharmacological properties.

The success of these structure-based drug design studies with influenza

virus, drawing on the information derived from protein crystallographic studies, molecular modelling and computational chemistry analysis, an understanding of the enzyme mechanism, and synthetic chemistry, may provide encouragement for future efforts targeted at other disease states.

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2 Fibrinogen Receptor Antagonists: Design and Clinical Applications

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INTRODUCTION

MECHANISM OF ARTERIAL THROMBUS FORMATION

The concept of a fibrinogen receptor antagonist as a powerful new antithrombotic mechanism arose from consideration of the central role of the platelet in thrombosis (*Figure 2.1*). This aspect of platelet biology has been reviewed in detail elsewhere [1–5]; therefore, only brief details will be presented here.

In healthy blood vessels, 'resting' platelets do not adhere to the non-thrombogenic surface of intact endothelium. However, platelets can adhere

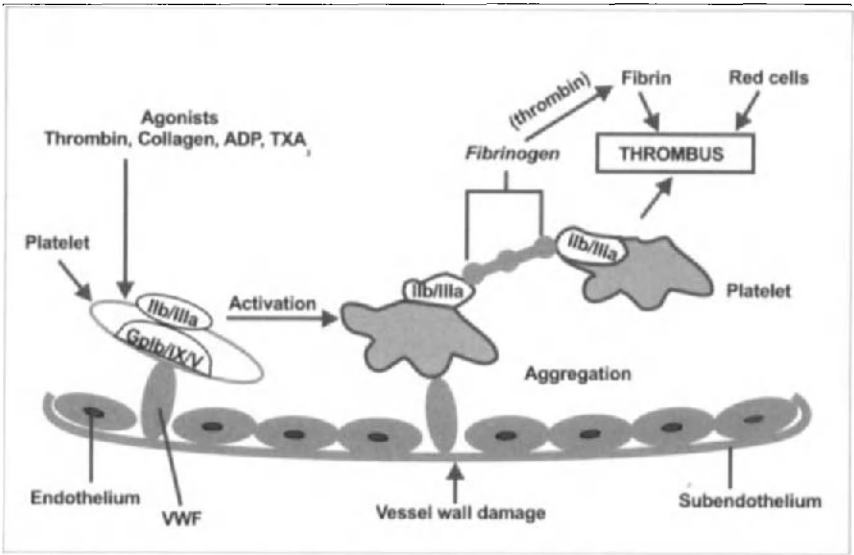


Figure 2.1. Role of the platelet in thrombosis. Platelets adhere to sub-endothelial structures of a damaged vessel wall via the glycoprotein Ib/IX/V complex and sub-endothelial von Willebrand Factor (vWF). Stimulation of platelets by a range of 'agonists' such as thrombin, collagen, adenosine diphosphate (ADP) or thromboxane A_2 (TXA_2) causes platelet activation, manifested in shape change, internal cytoskeletal rearrangement, and secretion of granule contents. Subsequent conformational change of the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa, the 'fibrinogen receptor') allows cross-linking of platelets by the dimeric plasma protein fibrinogen, leading to platelet aggregation. Aggregated platelets combine with polymeric fibrin (formed by the action of thrombin on fibrinogen) and red cells to form a clot or thrombus.

to sub-endothelial structures of a damaged vessel wall, e.g. following rupture of an atherosclerotic plaque [3], resulting in initiation of the process of clot formation. Platelet adhesion is mediated *via* receptors on the platelet surface; in particular the glycoprotein Ib/IX/V complex on unactivated platelets is crucially involved in adhesion to sub-endothelial von Willebrand Factor (vWF) [6–9], or in adhesion to sub-endothelial collagen *via* bridging interactions with plasma vWF [10].

Once adhered to the vessel wall, platelets undergo stimulation by a range of 'agonists', generated locally and acting on specific platelet surface receptors to cause platelet activation [6]; this activation is manifested in shape change, internal cytoskeletal rearrangement, and secretion of granule contents. The most important primary ('strong') agonists include thrombin, generated at the surface of activated platelets *via* the intrinsic coagulation pathway [11], or at the site of injury *via* the extrinsic coagulation pathway

[12]; and collagen, which acts as an anchoring surface in the sub-endothelial matrix as well serving as a potent agonist. Secondary agonists secreted from activated platelets which serve to amplify the initial stimulus include ADP (released from platelet dense granules), and thromboxane A_2 (generated *via* activation of phospholipase A_2 following thrombin receptor stimulation) [13].

One crucial consequence of platelet activation and shape change is a change in conformation of the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa, the 'fibrinogen receptor'). This abundant cell surface receptor, unique to platelets and megakaryocytes, is inactive in the resting state, but on activation it binds to the dimeric plasma protein fibrinogen [14]; cross-linking of platelets by fibrinogen then occurs, leading to platelet aggregation. Under conditions of high shear, vWF can also mediate platelet aggregation *via* binding to $\alpha_{IIb}\beta_3$ [8]. Aggregated platelets combine with polymeric fibrin (formed by the action of thrombin on fibrinogen) and red cells to form a clot or thrombus; arterial thrombi contain a high proportion of platelets [3], whereas venous thrombi are rich in fibrin and red cells and contain relatively few platelets.

RATIONALE FOR A FIBRINOGEN ANTAGONIST, AND ADVANTAGES OVER EARLIER ANTIPLATELET MECHANISMS

Inappropriate clot formation in atherosclerotic arteries manifests itself in the familiar categories of thrombosis such as myocardial infarction (MI), thrombotic stroke, or unstable angina. In spite of improved outcomes in the treatment of acute MI owing to the advent of thrombolytic agents [15], there remains a major need for powerful antithrombotic agents for prevention of thrombosis in high risk populations, such as patients with a history of MI, unstable angina or undergoing coronary angioplasty. In addition, the relative inefficiency of thrombolysis of platelet rich thrombi demonstrates the need for adjunctive agents to enhance the benefits of thrombolysis in the acute treatment of MI.

An agent designed to bind to $\alpha_{IIb}\beta_3$ and hence inhibit the cross-linking of platelets by fibrinogen (or vWF) should prevent platelet aggregation whatever the initial stimulus for activation. Hence such an agent would be expected to provide a very powerful 'broad spectrum' antithrombotic effect for chronic or acute use. A finding of clinical significance is that some thrombolytic agents can activate platelets and hence retard the speed of reperfusion, providing a rationale for the use of a fibrinogen antagonist as an adjunct to thrombolysis [16]. The effectiveness of antiplatelet agents, such as aspirin, which are targeted at only one platelet activating agonist (i.e., for as-

pirin, thromboxane A_2), are limited by the continued operation of alternative pathways to platelet activation and aggregation [3].

DISADVANTAGES OF THE FIBRINOGEN ANTAGONIST MECHANISM; BLEEDING RISK

Since the mechanisms underlying arterial thrombosis and haemostasis (prevention of blood loss due to physical injury) are similar, the powerful anti-aggregatory action provided by a fibrinogen antagonist must necessarily entail a degree of risk of clinical bleeding [1]. Evidence in nature for such a bleeding risk is provided from two sources. Firstly, a number of snake venoms contain peptide inhibitors of $\alpha_{IIb}\beta_3$, used to induce bleeding in the host [3,17,18]. Secondly, the inherited bleeding disorder Glanzmann's thrombasthenia results from either a numerical or a functional deficiency in $\alpha_{IIb}\beta_3$. However, the condition is not normally life-threatening, and varies widely in its severity [19,20]. It has been argued that we may have evolved an over-active haemostatic system [2]; hence there may be scope for a significant degree of inhibition of this system with an acceptable level of safety. A proper assessment of bleeding risk has only recently become possible with the emergence of clinical trial data (see below).

Whilst providing potent inhibition of platelet aggregation, central to the formation of platelet rich arterial thrombi, fibrinogen antagonists leave the enzymes of the coagulation pathways unaffected; hence these agents are expected to be relatively ineffective in venous thrombosis, where only a minor degree of platelet aggregation is involved [21].

STRUCTURE AND FUNCTION OF FIBRINOGEN AND ITS RECEPTOR, INTEGRIN $\alpha_{IIb}\beta_3$

FIBRINOGEN: STRUCTURE AND LIGAND RECOGNITION MOTIFS

Fibrinogen is a dimeric plasma protein, each half containing α, β and γ-chains linked by disulphide bridges [3] (*Figure 2.2*). The α-chains each contain two Arg-Gly-Asp (RGD) sequences (at residues 95–97 and 572–574). This tripeptide moiety serves as a receptor recognition motif in a variety of integrin ligands (including vitronectin, fibronectin and von Willibrand factor) [22]. RGD in fibrinogen is thought to bind to the β-chain of $\alpha_{IIb}\beta_3$, within the region β (109–171) [23]. A further recognition region, unique to fibrinogen, is the dodecapeptide 400–411 of the γ-chain; this sequence is thought to bind to residues 294–314 of the α-chain of $\alpha_{IIb}\beta_3$ [24]. The RGD

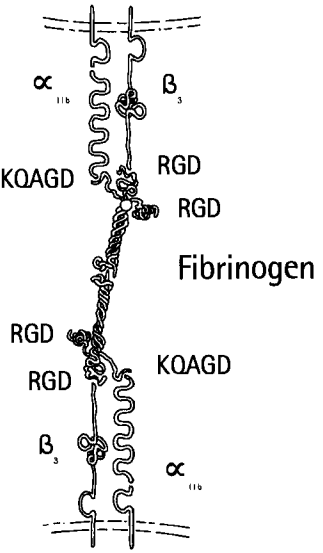


Figure 2.2. Fibrinogen structure and interactions with its receptor $\alpha_{IIb}\beta_3$. The α-chains of fibrinogen each contain two RGD sequences which serve as receptor recognition motifs thought to bind to the β-chain of $\alpha_{IIb}\beta_3$. The dodecapeptide 400–411 of the γ-chain of fibrinogen, containing the sequence KQAGD, is thought to serve as a further recognition region binding to the α-chain of $\alpha_{IIb}\beta_3$. (Reproduced with permission from Harker et. al. in Colman RW. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia: JB Lippincott, 1994:1638–60).

and γ-chain motifs bind to $\alpha_{IIb}\beta_3$ competitively, i.e. binding by one sequence prevents binding by the other [25]. The importance of the RGD sequence as an $\alpha_{IIb}\beta_3$ recognition motif is further borne out by its occurrence in several snake venom peptides ('disintegrins'); mutational studies revealed that the RGD sequence in these peptides is crucial to their potent $\alpha_{IIb}\beta_3$ binding and platelet aggregation inhibitory activity [18].

THE FIBRINOGEN RECEPTOR AS A TYPICAL INTEGRIN

The fibrinogen receptor represents the best characterised member of the integrin family of cell adhesion receptors, which are involved in a wide variety of cell-cell and cell-extracellular matrix interactions (reviewed in detail elsewhere) [14,26–29]. The integrins consist of non-covalently associated transmembrane α and β subunits [27], and are commonly grouped into classes defined by shared β subunits; over twenty receptors in eight classes are now known [26]. Using the α/β nomenclature, the fibrinogen receptor is now

Table 2.1. PLATELET SURFACE INTEGRINS

Integrin	*Ligand(s)*	*Function*
$\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa)	Fibrinogen Fibronectin Vitronectin von Willebrand Factor	Aggregation
$\alpha_V\beta_3$ (glycoprotein Ic'/IIa)	as for $\alpha_{IIb}\beta_3$	Adhesion
$\alpha_2\beta_1$ (glycoprotein Ia/IIa)	Collagen	Adhesion
$\alpha_5\beta_1$ (glycoprotein Ic/IIa)	Fibronectin	Adhesion
$\alpha_6\beta_1$	Laminin	Adhesion

termed $\alpha_{IIb}\beta_3$ (α_{IIb} = glycoprotein IIb; β_3 = glycoprotein IIIa). This nomenclature will be used for the remainder of this review.

$\alpha_{IIb}\beta_3$ is the major platelet integrin (each platelet containing approximately 100,000 copies) [26], and is unique to platelets. By contrast, the related integrin $\alpha_V\beta_3$, the receptor for the extracellular matrix protein vitronectin, is widely distributed [26]. Several other integrin receptors occur on the surface of platelets, which together with non-integrin glycoprotein receptors such as glycoprotein Ib, are involved in adhesion of platelets to sub-endothelial structures prior to platelet aggregation (see *Table 2.1*) [4].

RECEPTOR STRUCTURE AND REGULATION

In common with other integrins [27], $\alpha_{IIb}\beta_3$ consists of a calcium dependant complex of two transmembrane glycoproteins, with long extracellular domains forming a globular head containing calcium and ligand binding regions, and short cytoplasmic tails (see *Figure 2.3*) [20,25]. The structure and function of the receptor domains is described in detail elsewhere [26]. In the resting state, $\alpha_{IIb}\beta_3$ does not bind plasma fibrinogen; it can, however, bind other RGD containing peptides in which the RGD sequence is more accessible [18], or RGD related small molecules. In addition, resting $\alpha_{IIb}\beta_3$ can bind to fibrinogen coated on a surface [14].

Complex intracellular signalling pathways (known as 'inside-out' signalling) ensuing from stimulation by platelet agonists are thought ultimately

to lead to activation of $\alpha_{IIb}\beta_3$. This activation is hypothesised to occur initially *via* a conformational change in the cytoplasmic domains of the receptor, which is then transmitted across the membrane to cause a conformational change in the extracellular domains and exposure of ligand binding sites

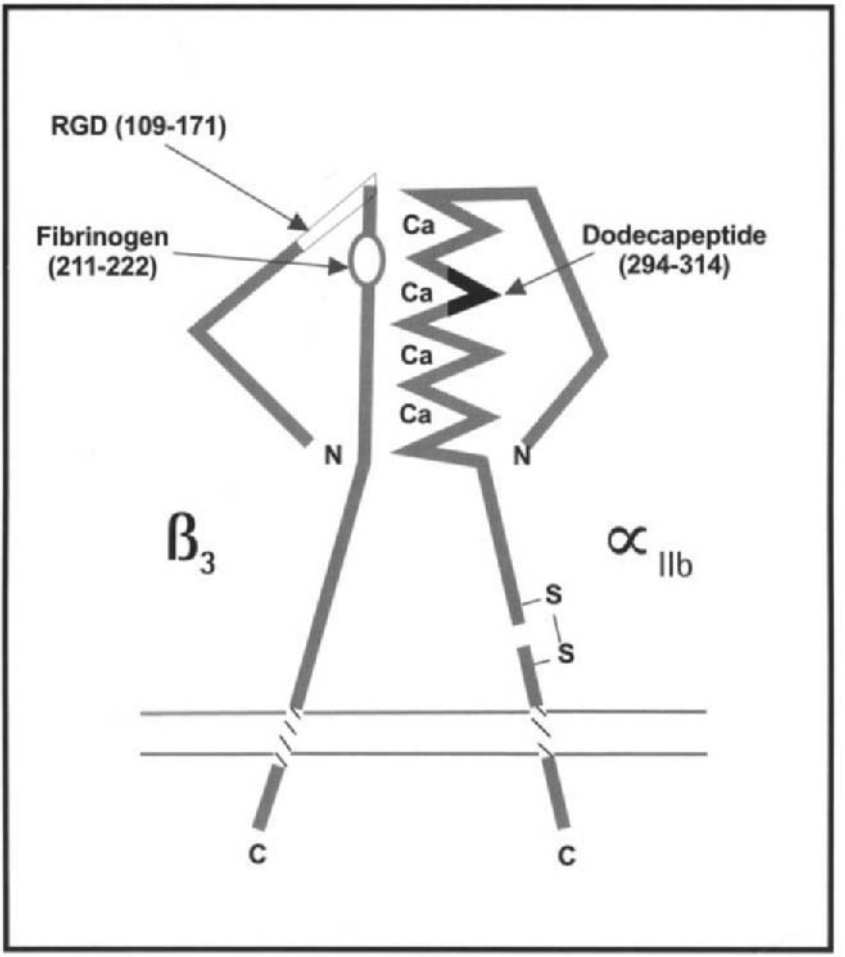


Figure 2.3. Structure of the $\alpha_{IIb}\beta_3$ complex. Integrin $\alpha_{IIb}\beta_3$ consists of a calcium dependant complex of two transmembrane glycoproteins, α_{IIb} (glycoprotein IIb) and β_3 (glycoprotein IIIa), with long extracellular domains forming a globular head containing calcium and ligand binding regions as indicated, and short cytoplasmic tails. (Adapted from Charo et al. in: Colman RW. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia: JB Lippincott, 1994:489–507).

[28]. Although full details of these pathways remain to be established, progress in this area has been the subject of several reviews [14,26,28].

Upon activation, the interface of the extracellular domains of $\alpha_{IIb}\beta_3$ becomes reoriented, exposing a ligand binding pocket which is thought to consist of discontinuous regions of both the α and β sub-units [26]. Several possible ligand binding sequences have been identified [30], including the putative calcium binding loop around Asp-119 of the β_3 chain; it is suggested that the Asp residue of the RGD sequence may interact with a metal ion in this region [24]. Regions of the α_{IIb} chain which may be involved in ligand binding include residues 657–665 and sequence 296–312 [26]. Despite this knowledge of the residues involved in binding of $\alpha_{IIb}\beta_3$ to fibrinogen, no precise 3D structural information on the binding site is available which might facilitate *de novo* drug design. In addition to binding plasma fibrinogen, $\alpha_{IIb}\beta_3$ is also able to bind other RGD containing ligands such as fibronectin, vitronectin, and vWF. However, owing to its high plasma concentration, fibrinogen is the major ligand involved in platelet aggregation.

On binding of ligand to $\alpha_{IIb}\beta_3$, further conformational change in the receptor is thought to occur, leading to the generation of intracellular signals ('outside-in signalling') [26,28]. One consequence of these signals is further platelet activation [31]; hence inhibition of ligand binding to $\alpha_{IIb}\beta_3$ should not only prevent aggregation, but should also reduce the extent of platelet activation. Reducing platelet activation and secretion might confer benefits in restenosis, since secreted platelet-derived growth factor provides an important stimulus for smooth muscle cell proliferation [32]; however, in practice, secretion in response to strong agonists is largely unaffected by fibrinogen antagonists [33].

RECEPTOR SELECTIVITY: $\alpha_{IIb}\beta_3$ vs. OTHER INTEGRINS

The vitronectin receptor, $\alpha_V\beta_3$ shares the β_3 subunit with $\alpha_{IIb}\beta_3$, and the RGD recognition sequence is used by integrin ligands other than fibrinogen to recognise their receptors, including $\alpha_V\beta_3$ and the fibronectin receptor, $\alpha_5\beta_1$ [4]. It is therefore to be expected that potential selectivity issues may exist for inhibitors of $\alpha_{IIb}\beta_3$, especially if designed to block the β_3 subunit, or if based on the RGD moiety. Several naturally occurring inhibitors of $\alpha_{IIb}\beta_3$ are indeed non-selective vs. other integrins; most of the disintegrins and leech proteins (also RGD containing) inhibit $\alpha_V\beta_3$ and $\alpha_5\beta_1$ as well as $\alpha_{IIb}\beta_3$. The relative affinity for the different integrins is influenced by the residues adjacent to the RGD sequence [18]. Small RGD containing peptides such as Gly-Arg-Gly-Asp-Ser (GRGDS) are also non-selective inhibitors of $\alpha_{IIb}\beta_3$ [33]. Whilst selective inhibition of $\alpha_{IIb}\beta_3$ is generally presumed

desirable, non-selectivity vs. $\alpha_V\beta_3$ might have advantages in some circumstances; for example, inhibition of $\alpha_V\beta_3$ is thought to have an inhibitory effect on restenosis following angioplasty [34] (see later).

BIOLOGICAL TEST SYSTEMS FOR FIBRINOGEN RECEPTOR ANTAGONISTS

IN VITRO ASSAYS

Measurement of fibrinogen receptor binding affinity

Receptor binding affinity of fibrinogen antagonists has been estimated by measuring the inhibition of [^{125}I]-fibrinogen binding to ADP stimulated gel filtered platelets [33,35]. A more direct measure of affinity is provided by measuring the inhibition of biotinylated fibrinogen binding to purified $\alpha_{IIb}\beta_3$ coated on enzyme-linked immunosorbent assay (ELISA) plates [35]. In either case, IC_{50} values represent the concentration of compound required to reduce ligand binding to 50% of the control value.

Measurement of inhibition of platelet aggregation

Although a receptor binding assay gives the simplest initial measure of drug-receptor interactions, a functional assay measuring inhibition of platelet aggregation is of more relevance for predicting activity prior to *in vivo* work. The functional consequences of drug binding to $\alpha_{IIb}\beta_3$ have been evaluated using a variety of assays in which the extent of aggregation of agonist stimulated platelets is measured in the presence and absence of drug. In each case, potency is expressed as an IC_{50} value, which represents the concentration of drug required to reduce the extent of platelet aggregation to 50% of the control value.

Large discrepancies are often seen between the IC_{50} for drug affinity as measured in a fibrinogen receptor binding assay, and the IC_{50} from a functional platelet aggregation assay. This can occur for very high affinity compounds (very low IC_{50} values in a binding assay) because the relatively high concentration of $\alpha_{IIb}\beta_3$ receptors in plasma imposes a theoretical minimum on the concentration of drug required to bind half the receptors present, and hence produce 50% inhibition of aggregation; this minimum IC_{50} value has been estimated as *ca.*10nM [36]. In addition, IC_{50} values in functional assays can vary with the conditions employed (in particular the presence or absence of plasma proteins). The most commonly used assays are as follows:

Partially purified platelet preparations

Assays designed to be free of possible interference from red cells or other blood constituents have been employed as primary screens in structure-activity work. One procedure relatively free of plasma proteins involves inhibition of platelet aggregation in a preparation derived from platelet rich plasma by centrifugation, followed by washing and resuspending the platelets in physiological saline [37]. Alternatively, inhibition of ADP induced platelet aggregation has been measured using gel filtered platelets (GFP) [33,38], or platelet rich plasma (PRP) [35]; however, the last two platelet preparations both contain plasma proteins which can affect potency owing to plasma protein binding. In all three assays, aggregation is measured in an optical aggregometer, and is observed as an increase in light transmittance.

Whole blood assay

For prediction of likely *in vivo* activity, inhibition of ADP or U-46619 (a stable thromboxane A_2 mimetic) induced platelet aggregation has been measured in citrated whole blood. The extent of aggregation is determined using a single platelet counter [33, 35, 38]. Compounds may be less potent in the whole blood assay than in the partially purified platelet assays described above if the drug is significantly bound to plasma proteins.

Specificity assays

Numerous assays have been employed to demonstrate the specificity of action of fibrinogen antagonists. These fall into two main categories as follows:

Assays for inhibitory effects on platelet stimulus-effect coupling

The lack of an inhibitory effect on platelet shape change or secretion rules out any direct antagonist action on platelet agonist (e.g. thrombin) receptors or intracellular events such as G protein coupling, as has been demonstrated for the peptidomimetic fibrinogen antagonist GR91669 [33]. The effect of compounds on platelet shape change is measured in U-46619 stimulated platelet rich plasma, prevented from aggregating by addition of the calcium chelating agent EGTA. Shape change is detected by a fall in light transmittance in an optical aggregometer [33]. The effect of compounds on platelet secretion is determined using platelet rich plasma incubated with ^{14}C-5HT. Secretion of ^{14}C-5HT is measured along with platelet aggregation following platelet stimulation with U-46619, collagen, ADP or thrombin [33].

Table 2.2. INTEGRIN SPECIFICITY ASSAYS

Integrin	*Ligand binding assay: purified receptor*	*Functional (cell adhesion) assay*
$\alpha_v\beta_3$	Inhibition of binding of biotinylated vitronectin to $\alpha_v\beta_3$	1. Inhibition of human umbilical vein endothelial cell adhesion to a fibrinogen coated surface. 2. Inhibition of SK cell adhesion to a vitronectin coated surface.
$\alpha_4\beta_1$		Inhibition of Jurkat cell adhesion to a fibronectin coated surface.
$\alpha_5\beta_1$	Inhibition of binding of biotinylated fibronectin to $\alpha_5\beta_1$	
$\alpha_M\beta_2$		Inhibition of granulocyte adhesion to a fibrinogen coated surface.

Assays for actions at other integrin receptors

Owing to the close homology between integrins and the known lack of specificity of some peptide fibrinogen antagonists (see above), a variety of assays has been employed to test for specificity versus other integrins and their physiological ligands [33,35] (see *Table 2.2*).

IN VIVO ASSAYS

Ex vivo inhibition of platelet aggregation

The pharmacodynamic duration of action of fibrinogen antagonists has generally been assessed by measuring inhibition of platelet aggregation *ex vivo* in samples of blood taken at intervals following i.v. or p.o. administration of drug, for example in the marmoset [33]. The duration of action is normally defined as the time taken for the response to platelet agonist (such as ADP) to return to 50% of its pre-dose (control) value. This measure of duration is dose dependent; a high value may simply reflect a dose far in excess

Table 2.3. ANIMAL MODELS OF ARTERIAL THROMBOSIS AND THROMBOLYSIS

Model [reference]: induction of thrombosis	*Nature of thrombus*	*Detection of thrombosis/thrombolysis*	*Type of study*
Folts model: artery stenosis and intimal damage [40], produced by vessel clamping causing exposure of sub-endothelial collagen and tissue factor	Platelet rich	Cyclic variations in arterial blood flow caused by repeated thrombus formation and embolisation	Efficacy of antithrombotic agents
Electrically induced thrombosis [41], [42], [43]: electrode placed directly into a coronary artery causing endothelial disruption	Platelet rich, but significant fibrin/ red cell content	Coronary artery blood flow (e.g. electromagnetic flow probe)	Efficacy of antithrombotic agents; adjunctive agents in thrombolysis
Arteriovenous graft [44], [45], [46]: segment with thrombogenic vessel wall (e.g. dacron graft, coronary stent) grafted into arteriovenous shunt.	Platelet rich, but significant fibrin/ red cell content	Imaging of ^{111}In platelet deposition and ^{125}I-fibrin accumulation; blood flow rate through graft; thrombus weight in shunt	Efficacy of antithrombotic agents
Photochemically induced thrombosis [47]: light activated photosensitiser causing endothelial injury.	Platelet rich, but significant fibrin/ red cell content	Coronary artery blood flow	Efficacy of antithrombotic agents; adjunctive agents in thrombolysis; effects on restenosis
Thrombin induced thrombosis [48]: stenosis, endothelial damage (by arterial clamping) and thrombin/blood injection	Fibrin and red cell rich initial thrombus; platelet rich reocclusion	Coronary artery blood flow	Adjunctive agents in thrombolysis (but not for effects on lysis of platelet rich thrombus)
Everted coronary artery graft with stenosis [48]	Platelet rich (rt-PA resistant)	Coronary blood flow	Adjunctive agents in thrombolysis of rt-PA resistant platelet rich thrombus

of that required to give initial 100% inhibition of platelet aggregation. In addition some compounds having a particularly high affinity for the fibrinogen receptor (irreversible binding or slow off-rate) may show a longer duration of action than might be suggested from the plasma levels detected in pharmacokinetic studies (see below).

Animal models of thrombosis and thrombolysis

A large number of animal models have been used to study the effects of antithrombotic agents in thrombosis and as adjunctive agents in thrombolysis. The models most commonly used in the study of fibrinogen antagonists are summarised in *Table 2.3*. A variety of species have been employed, including baboon, cynomolgus monkey, dog, rabbit and guinea pig; the rat is an unsuitable species owing to the very low sensitivity of rat $\alpha_{IIb}\beta_3$ receptors to fibrinogen antagonists [39]. Coronary, carotid, or femoral arteries, or various types of grafted shunt vessels have been used depending on the purpose of the study.

DESIGN AND STRUCTURE-ACTIVITY RELATIONSHIPS

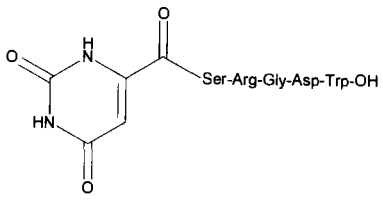
PEPTIDES AND CYCLIC PEPTIDES

The observation that the RGD sequence on the α-chain of fibrinogen binds to the receptor $\alpha_{IIb}\beta_3$ on platelets led many groups to begin their programs by synthesizing simple linear RGD containing peptides. These studies showed that the basic arginine and acidic aspartic acid side-chains were essential for activity, although potencies were invariably low in functional assays [49–56]. Additionally, their peptidic nature led to a short duration of action *in vivo*, e.g. the pentapeptide (1) had an IC_{50} of 4μM in human PRP*, making it one of the most potent linear peptides, but had a short duration of action in a guinea pig model of thrombosis [50].

An additional problem with linear peptides is non-selectivity against other integrin receptors; as mentioned earlier, GRGDS is a weak inhibitor of platelet aggregation ($IC_{50} = 25\mu M$), but has affinity for the vitronectin receptor $\alpha_v\beta_3$ as well as $\alpha_{IIb}\beta_3$ [33].

It is well known that cyclic peptides are able to bind to receptors at re-

*All IC50 values quoted are for inhibition of platelet aggregation in human PRP unless stated otherwise.



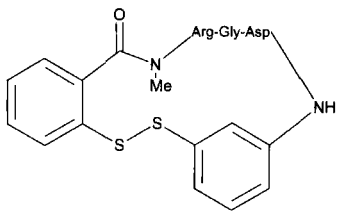
(1)

duced entropic cost compared to their acyclic counterparts. Thus they are more likely to be potent and selective provided the correct conformation for binding of the pharmacophore can be adopted. In the case of peptidic $\alpha_{IIb}\beta_3$ antagonists, the conformation of the RGD sequence of the cyclic peptide will influence potency and selectivity. Cyclic RGD peptides which have selectivity for $\alpha_v\beta_3$ over $\alpha_{IIb}\beta_3$ have been disclosed [57]. The approaches to peptidic $\alpha_{IIb}\beta_3$ antagonists which various groups have adopted are summarised below.

Workers at SmithKline Beecham constrained the RGD sequence into the cyclic peptide disulphide (2) which had modest potency (IC_{50} 16200nM; dog PRP), but additional modifications to further reduce flexibility lead to the discovery of SKF 106760 (3) (IC_{50} 360nM, dog PRP; IC_{50} 175nM, human PRP) and SKF 107260 (4) (IC_{50} 90nM). These two leads were used to design non-peptidic antagonists (see later). Nmr studies suggested that SKF 106760 exists in two principal conformations in solution both of which have the Arg and Asp residues as part of a β-turn with an extended central glycine fragment [58,59]. *Cyclo*-(Pro-Arg-Gly-Asp-Gly-D-Pro) ($IC_{50} = 5.3\mu M$) was also studied [60], and was shown to adopt a different conformation in which the Gly and Asp fragments occupied the i+1 and i+2 positions of a β-turn respectively. The conformation of the RGD sequence of this compound is significantly different from that of (3) above, sug-

(2)

(3)



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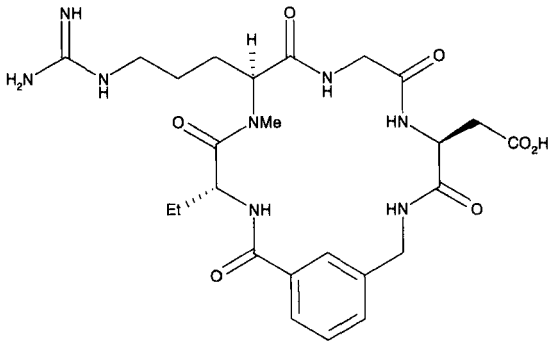
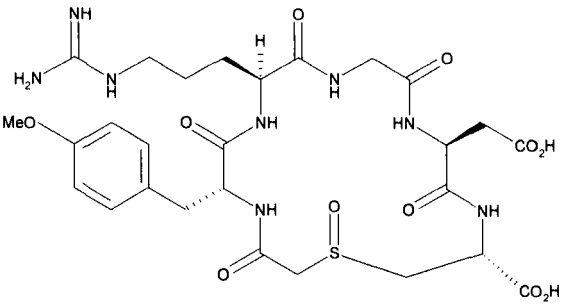


(5)

gesting that there may be two separate cationic binding sites on the receptor [61].

The Dupont Merck group used SKF 106760 as their starting point, and employed 3-aminomethylbenzoic acid as a linker to develop DMP 728 (5) (IC_{50} 20nM) [62]. In solution, this compound was shown by nmr to exist in one conformation in which there is a C_7 turn at Asp and a type II' β-turn at the *N*-Me(Arg) fragment [63]. As for SKF 106760, the central glycine residue is in an extended conformation. Scientists at Genentech developed a sulphoxide tether to hold the RGD unit in a favourable conformation [51], typified by compound G4120 (6) (IC_{50} 150nM). Nmr analysis [64] indicated that the molecule adopts a type II' β-turn, in which the D-tyr and Arg fragments occupy the i+1 and i+2 positions respectively, with the RGD sequence adopting a 'cupped' conformation.

Cor Therapeutics [65] synthesized a number of KGD containing cyclic



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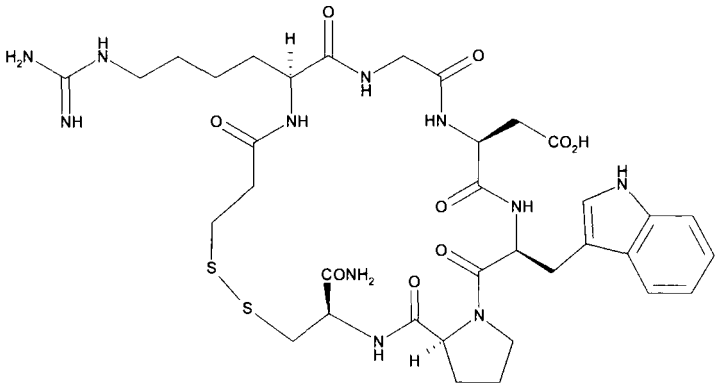
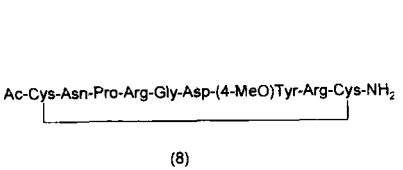


(7)

peptides with IC_{50}'s of between 1–10μM; the hexapeptide integrilin (7, Eptifibatide) [66a], is in phase III clinical trials (see later). Telios have synthesized several cyclic peptides [67], which showed that a lipophilic amino acid after the RGD unit enhanced potency; the cyclic nonapeptide TP 9201 (8) (IC_{50} 220nM) has been studied *in vivo* [68]. The Glaxo group used a thiazoline derived tether [52] in the pentapeptide GR 83895 (9) (IC_{50} 900nM) in which the RGD unit was held in an extended conformation with the Asp-Phe bond as part of a type II' β-turn.



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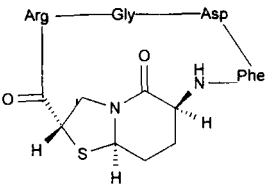
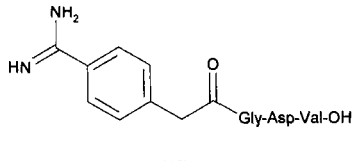


GR83895 (9)

DEVELOPMENT OF NON-PEPTIDE LEADS

In order to improve upon the poor pharmacokinetics of the peptidic $\alpha_{IIb}\beta_3$ antagonists, many groups have developed non-peptidic analogues from their peptide leads. The conformational studies on cyclic peptides described above indicated that the most potent had the RGD sequence in an extended or 'cupped' conformation. Non-peptidic analogues have been designed from these by using an appropriate spacer between the acidic and basic side



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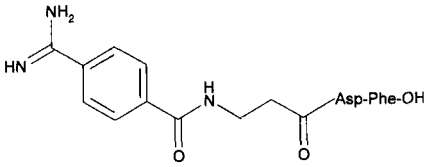

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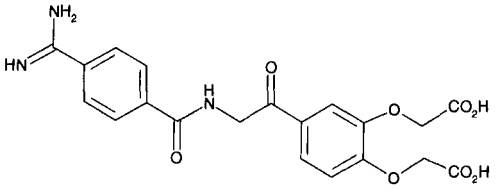
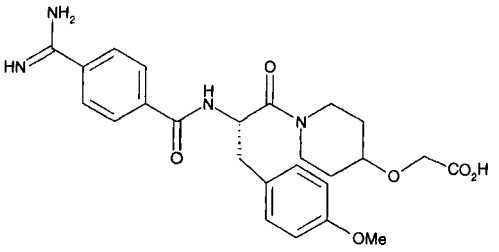


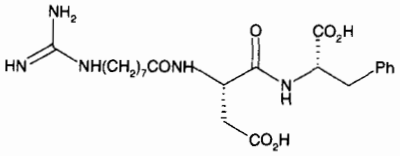
(12)

chains (which constitute the essential pharmacophore) to match those of the RGD sequence in cyclic peptides. A considerable degree of ingenuity is displayed in the various approaches summarised below. Two major categories of compounds are described in which the arginine side-chain of linear peptides is replaced by a benzamidine moiety or by a cyclic amine. However, a number of different starting points were employed.

Workers at Roche [36] modified earlier linear peptides by replacing the arginine side-chain with a benzamidine unit, which had been used previously as an effective arginine mimetic in the serine protease area [69]. The resulting semi-peptidic structures (10) (IC_{50} 300nM) and (11) (IC_{50} 60nM) were potent and selective $\alpha_{IIb}\beta_3$ antagonists. Substitution of the remaining peptidic fragments lead *via* phenoxyacetic acids such as (12) (IC_{50} 70nM) to the piperidine analogue (13, Ro 44–9883, lamifiban) (IC_{50} 30nM) which is being investigated in phase III clinical trials (see later).



(13) (lamifiban)



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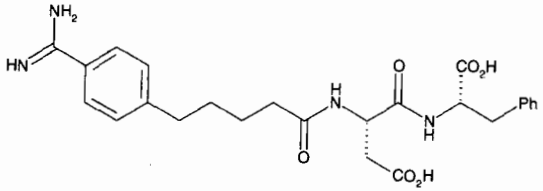
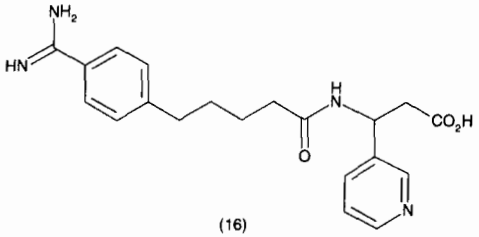


(15)

Chemists at Searle modified the tetrapeptide Arg-Gly-Asp-Phe to give the weakly active guanidine (14) (IC_{50} 1600nM; dog PRP) [70] which was further modified to give the benzamidine (15) (SC 52012) (IC_{50} 42nM; dog PRP) [71]. Further modifications by removal of the terminal phenylalanine and substitution by either a 3-pyridyl group or alkynyl group lead to (16) (IC_{50} 150nM; dog PRP) and (17) (IC_{50} 67nM) [72], both of which were orally active in ethyl ester prodrug form (see later).



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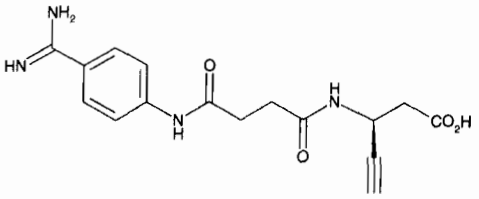

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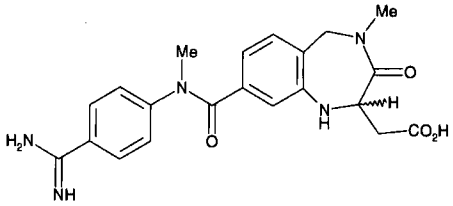

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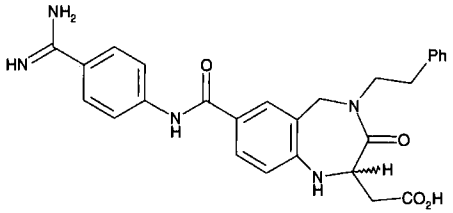
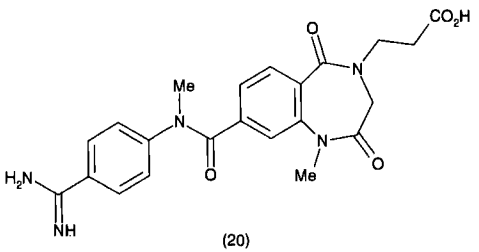


(19)

The previously described extended RGD conformation found in SKF 106760 provided a basis for the design by the SmithKline Beecham group of the benzodiazepine (18) (IC_{50} 65nM) [58,73]. Interestingly, the corresponding 7-substituted benzodiazepines e.g. (19) were also active (IC_{50} 380nM) [61]. Modelling studies indicated that the acid and base functionalities of (19) were not superimposable with those of SKF 106760, but were found to match the favoured conformation of the previously mentioned cyclic peptide *cyclo*-(Pro-Arg-Gly-Asp-Gly-D-Pro). Genentech also employed benzodiazepines as central spacers; modelling studies indicated that they would adopt a similar cupped conformation to that seen in the RGD sequence of the cyclic peptide G4120 (see above). The benzamidine (20) was more potent (IC_{50} 120nM) than G4120 in PRP; however, no *in vivo* data was disclosed [74,75].

Conformational studies of the cyclic peptide DMP 728 were employed by



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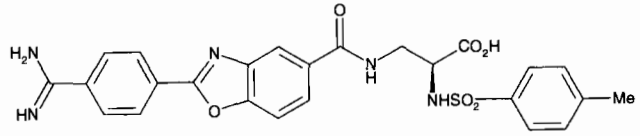

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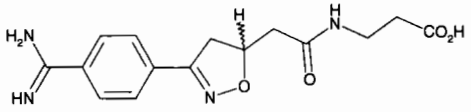
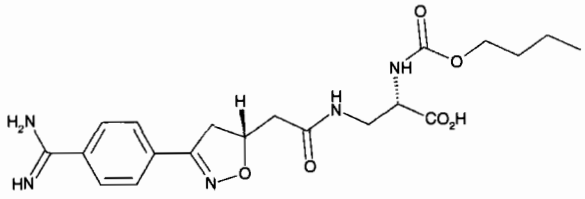


(22)

Dupont Merck to generate benzimidazole/benzisoxazole templates e.g. (21) (IC_{50} 10nM) and isoxazolines such as XR 299 (22) (IC_{50} 240nM) [76,77]. Substitution α and β to the carboxylic acid group in XR 299 was tolerated [78], for example the carbamate (23) was potent (IC_{50} 50nM). Administration of (23) to dogs at 0.025mg/kg i.v. resulted in 90–100% inhibition of platelet aggregation *ex-vivo*, which declined to 40% after 5h. As in the case of L-738,167 (see later), compound (23) also binds to both activated and unactivated platelets [78].

Lilly [79] demonstrated by nmr that a hydrogen bond existed between the Trp and Arg of the cyclic peptide (24) (IC_{50} 300nM), with the RGD fragment adopting a Gly-Asp type II' turn. This conformation suggested the use of a 6,6-central spacer and isoquinolinones such as (25) were found to be potent. Several 5,6-systems were also synthesized [80,81] , of which the benzofuran (26) (IC_{50} 270nM) was the most potent.

Karl Thomae [82] initially designed pyrollidinone (27) based on the observation that tetrapeptides Arg-Gly-Asp-AA (AA = Ser, Val, Phe) were more potent inhibitors of platelet aggregation than Arg-Gly-Asp alone. They deduced that the tetrapeptides adopted a β or γ-turn (illustrated) which pre-



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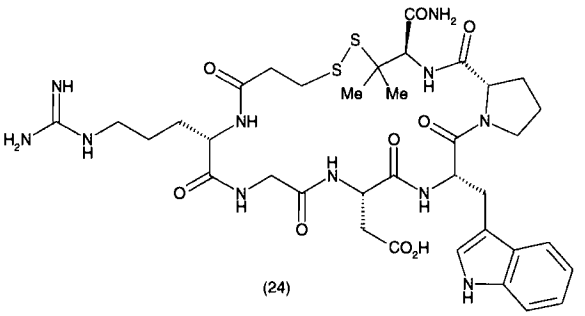

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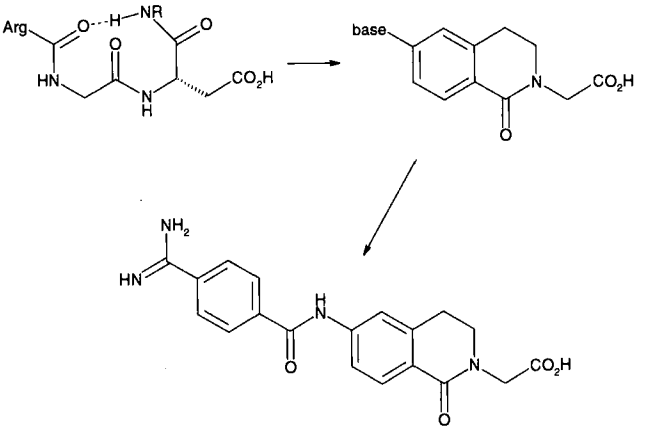

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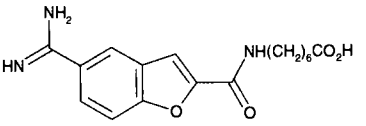
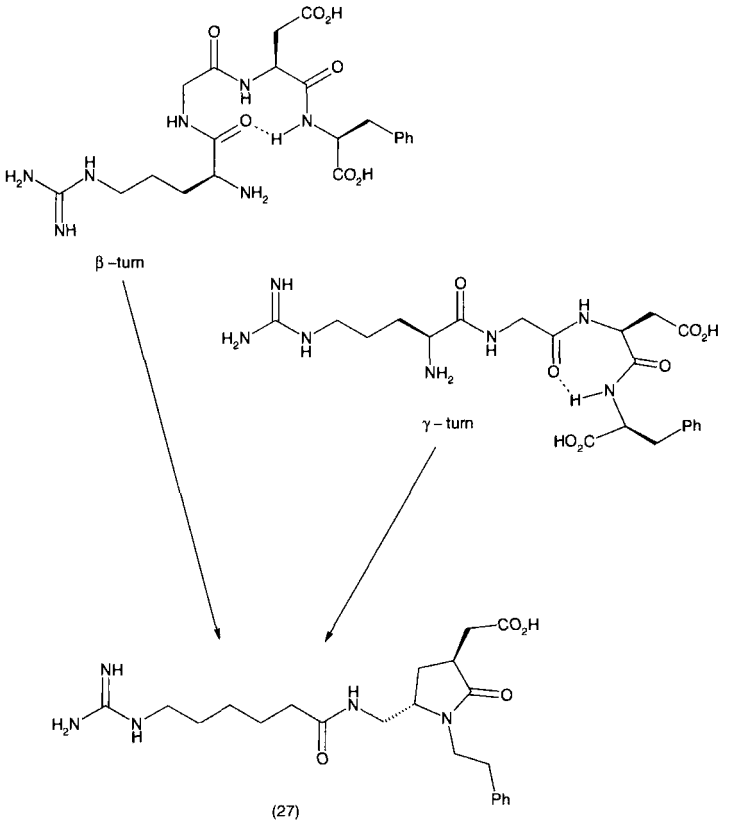


(26)

sented the Arg and Asp side-chains in a more favourable conformation for binding. The pyrollidinone ring was selected to mimic both β and γ-turn conformations, to give the weakly active inhibitor (27) (IC_{50} 94000nM). Further modification gave a number of much more potent analogues, e.g. BIBU 52 (28) (IC_{50} 84nM). BIBU 52 had activity *in vivo* in a pig model of recurrent thrombosis at 1mg/kg i.v., but was inactive orally. However, oral activity was obtained using a pro-drug strategy (see later).

The weakly active bicyclohexyl lead (29) (IC_{50} 5400nM) was discovered at Glaxo by directed screening of their in-house compound collection [83,84]. Structural information from the conformationally constrained cyclic peptide GR 83895 (9) (see earlier) was used to enhance the potency of (29). Elaboration *via* iso-thioureas such as (30) (IC_{50} 27000nM; GFP) and (31) (IC_{50} 430nM; GFP) gave the potent benzamidines (32) (IC_{50} 55nM; GFP) and GR144053 (33) (IC_{50} 37nM; GFP). When administered to the marmoset at 1mg/kg i.v., GR 144053 gave >50% inhibition of ADP induced platelet aggregation *ex-vivo* for up to 6.5h; when given orally at 3mg/kg, the duration was 5.7h.

Monosubstitution (but not disubstitution) of the glycine CH_2 adjacent to the carboxylic acid of GR 144053 was tolerated [85]; however only the α-



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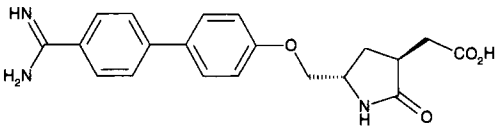
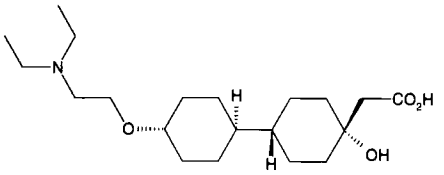


(28)

phenyl substituents retained the full potency of GR 144053 (*Table 2.4*). Substitution of bulky groups in the 4-position of the aryl group was also tolerated, allowing the synthesis of the potent, fluorescent antagonist (38) (IC_{50} 44nM; GFP).



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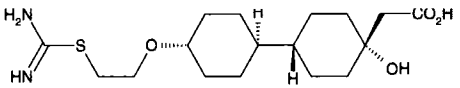

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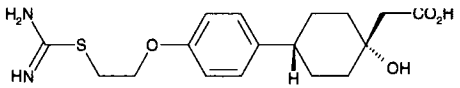

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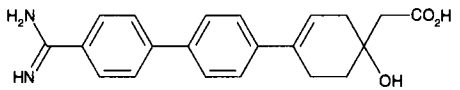

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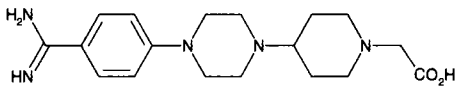
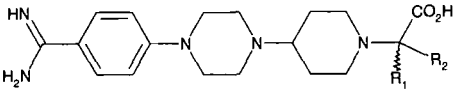
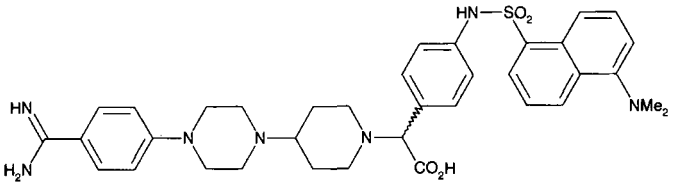


(33)

Table 2.4. α-SUBSTITUTED ANALOGUES OF GR144053 (33)





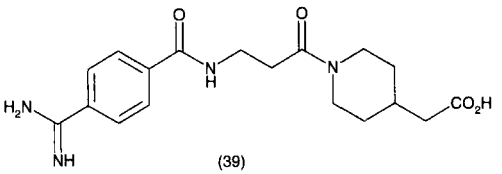
(38)

Compound	*R^1*	*R^2*	*IC_{50} (nM) (GFP)*
(34)	Me	H	340
(35)	Me	Me	20000
(36)	Ph	H	33
(37)	4-$MeSO_2C_6H_4$-	H	35

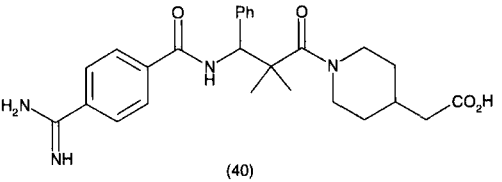
Using a solid phase combinatorial strategy, workers at Nippon Steel [86] combined various acidic, basic and aminoacid derived spacer groups to give the initial lead (39) (IC_{50} 2500nM), which was further optimised combinatorially to NSL-95301 (40) (IC_{50} 92nM) (absolute stereochemistry unknown). Related compounds such as (41) (IC_{50} 210nM) have been described recently [87] though no *in vivo* data has been disclosed.

Sandoz [88] used a γ-lactam group as an isostere for the Gly-Asp fragment in peptides to design analogues such as (42) (IC_{50} 40nM; resuspended platelets). As an alternative approach, screening of their in-house compound collection gave a weak lead (43) (IC_{50} 20000nM; resuspended platelets) which on further elaboration yielded the clinical candidate SDZ-GPI-562 (44) (IC_{50} 15nM; resuspended platelets) [89]. This compound administered at 1mg/kg i.d. to the guinea pig gave 65% inhibition of ADP induced platelet aggregation *ex-vivo* after 5h.

Sanofi have described [90] thiazole benzamidines e.g. (45) (IC_{50} 48nM)



(39)



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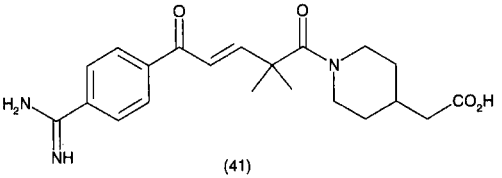

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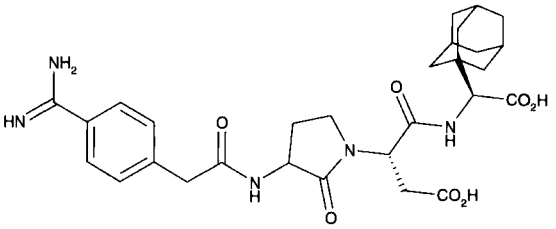

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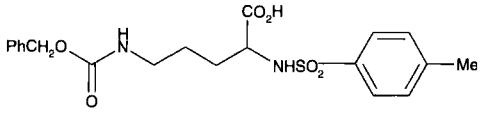

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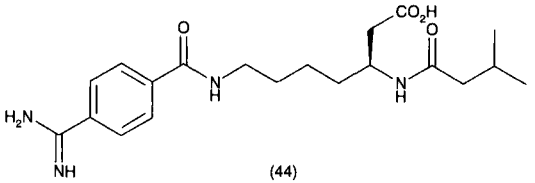

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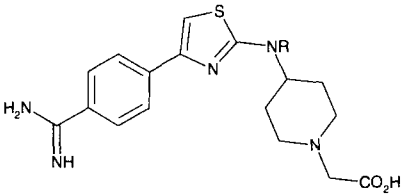
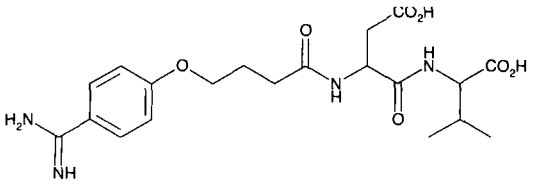


(45) R=H
(46) R = $(CH_2)_2CO_2H$

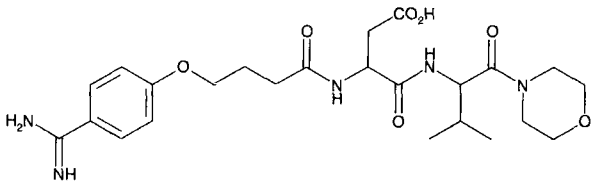
and (46) (IC_{50} 46nM). The -NH compound (45) administered to the baboon at 0.1mg/kg i.v. gave a duration of action of <4h assessed by inhibition of ADP induced platelet aggregation *ex-vivo*. In contrast the corresponding diacid (46) gave maximal inhibition for up to 8h, with 50% inhibition after 24h.

Semi-peptidic antagonists have been disclosed by workers at Fujisawa based on previously disclosed publications of others [36,71], and on computer simulation of the RGD sequence in a type II β-turn with Gly at the (i+1) position, which had been previously observed in other RGD peptides. This analysis lead to FK 633 (47) (IC_{50} 100nM) [91,92], which had low bioavailability in the dog and rat, believed to be due in part to hydrolysis by peptidases. The analogue FR 158999 (48) (IC_{50} 79nM) was much more stable, giving >50% inhibition of ADP induced platelet aggregation in the dog *ex-vivo* for 3h at 3.2mg/kg p.o.

Takeda have recently described [93] the piperazinone TAK-029 (49), which was derived from the weak lead (50) (IC_{50} 1100nM), which was itself



(47)



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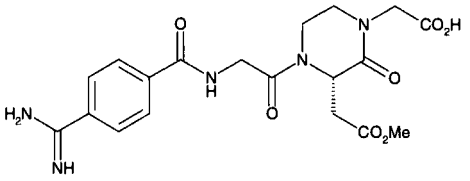
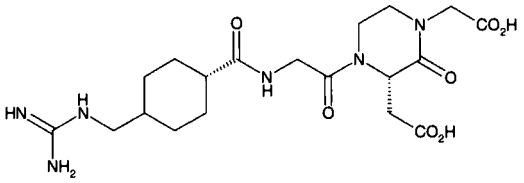
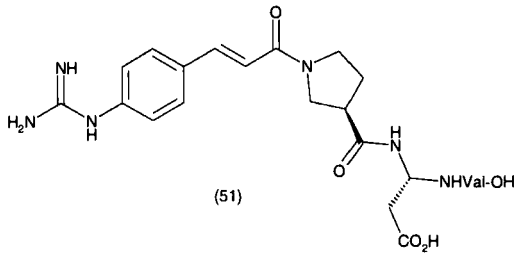


(49)



(50)

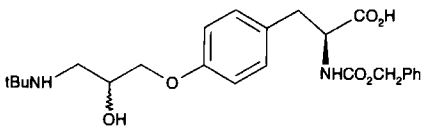


(51)

derived from the tetrapeptide RGDF. TAK-029 (49) (IC_{50} 30nM) inhibited ADP induced platelet aggregation for 4h in the guinea-pig *ex-vivo* at 0.1mg/kg i.v., and for 8h at 3mg/kg p.o.. Klein *et. al.* have described the synthesis and evaluation of guanidine cinnamates such as (51) (IC_{50} 65nM; fixed activated platelets), though no *in-vivo* data is disclosed [94].

The group at Merck have been very active in the area, publishing details of several series [95–107]. An initial lead (52) (IC_{50} 27000nM; GFP), detected by directed screening, was developed using a classical molecular modification strategy [95] into tirofiban (Aggrastat) (53) (IC_{50} 9nM; GFP), the first example of a fibrinogen antagonist using a simple amine as the basic moiety. Tirofiban has been launched in the US for the treatment of unstable angina.

Concurrently, the Merck group also modified a cyclic pentapeptide lead c[(Ac)CRGDC] (IC_{50} 680nM) to give L-734,217 (54) (IC_{50} 32nM GFP) [98], which exhibited oral activity; in the dog at 1.0mg/kg p.o., *ex-vivo* inhibition of ADP induced platelet aggregation was >90% for up to 5h after administration, and in the chimpanzee at 2.0mg p.o. was >50% for up to 16h



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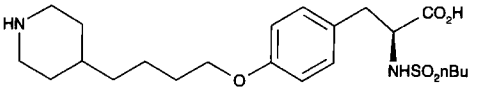
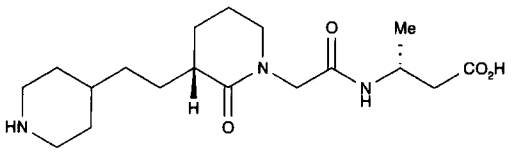


(53) Tirofiban

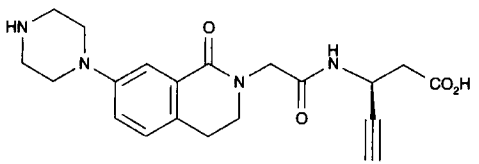
after administration. Further modification of L-734,217 by fusing a phenyl ring onto the central piperidine group and introduction of the β-alkynyl substituent previously described for (17) afforded L-767,679 (55) (IC_{50} 12nM; GFP) [103]. When (55) was administered to the dog at 0.3mg/kg p.o., inhibition of ADP induced platelet aggregation *ex-vivo* was maintained at $>80\%$ for up to 3h.

In addition, Merck have modified the central tyrosine moiety of tirofiban to develop several other leads (56)-(60) *(Table 2.5)*. The indole (60) was particularly effective orally, giving sustained inhibition of ADP induced platelet aggregation over a 24h period when dosed once daily at 0.25mg/kg to rhesus monkeys.

More recently, L-738,167 (61) (IC_{50} 8nM; GFP) was developed which interestingly binds to $\alpha_{11b}\beta_3$ of both activated and unactivated platelets, and

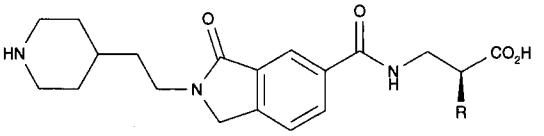


(54)

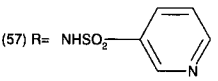


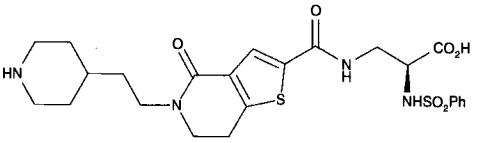
(55)

Table 2.5. MERCK FIBRINOGEN ANTAGONISTS



(56) R=H





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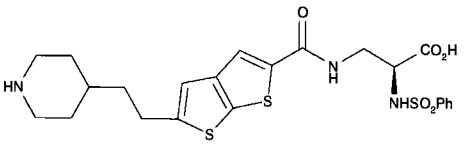
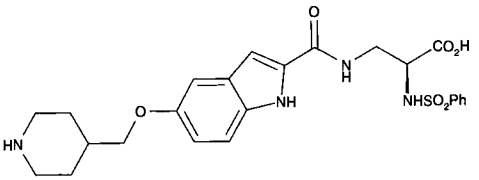
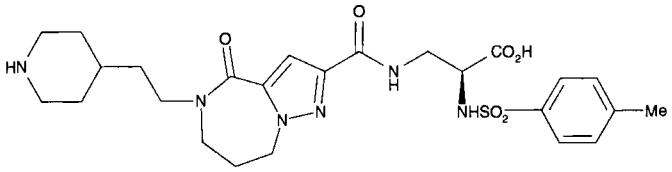


(59)



(60)

Compound	*IC_{50} (nM) (GFP)*	*Reference*
(56)	25	[97]
(57)	15	[101]
(58)	7	[102]
(59)	8	[104]
(60)	13	[105]



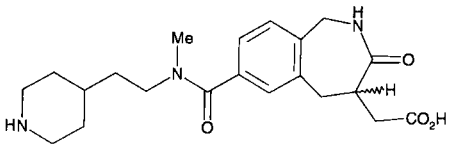
(61)

has a long duration of action *in vivo* (see later) despite having modest plasma pharmacokinetics ($t_{1/2}$ *ca.* 0.5h) [106].

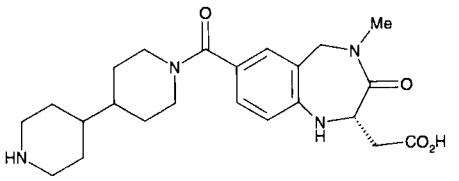
The group at SKB have also employed the piperidinylethyl fragment as a replacement of the benzamidine group [108]. The benzazepine (62) (IC_{50} 71nM) had an improved duration of action in the dog (4h at 3mg/kg i.d., *ex-vivo*) compared with the benzodiazepine (18) [108]; bioavailability was estimated to be approximately 10%. Modification of the benzamidine (19) lead to SB 214857 (63) (IC_{50} 28nM), which is under pre-clinical evaluation [109], giving an extended duration of action at 1mg/kg p.o. in the dog (>8h, $>80\%$ inhibition of collagen induced platelet aggregation *ex-vivo*).

The piperidinylethyl fragment has also been employed to advantage by workers at Karl Thomae, leading to BIBU 251 (64) (IC_{50} 100nM). Klein *et. al.* [110] and incorporated trimethylenedipiperidine as a template into (65) (IC_{50} 110nM; fixed activated platelets).

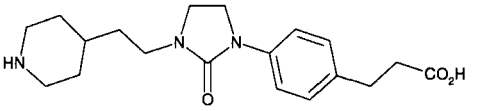
More recently, Glaxo Wellcome have disclosed in the patent literature



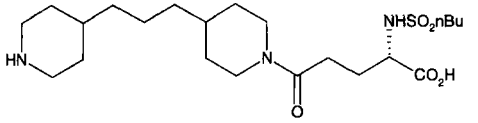
(62)



(63)



(64)

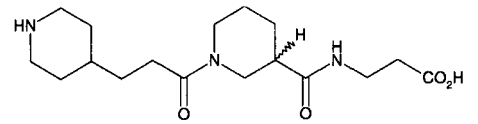


(65)

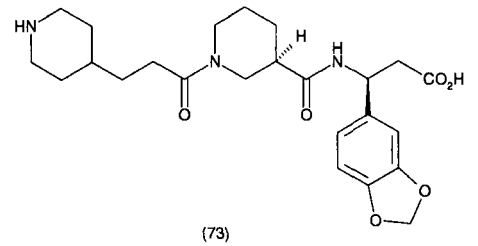
[37,111–113] a series of indazole containing antagonists, for example (66)-(71) (*Table 2.6*).

Based on nmr analysis of the structures of RGD containing antagonists [114], workers at RW Johnson designed 3-substituted piperidines as β-turn mimetics, the most potent being (72) (IC_{50} 700nM; GFP vs. thrombin). Further optimisation [115] using solid phase chemistry gave (73) (IC_{50} 20nM; GFP vs. thrombin) which possessed some oral activity in the dog (ED_{50} = 3mg/kg, duration > 3h for *ex-vivo* inhibition of ADP induced platelet aggregation).

Katano *et. al.* at Meija Seika Kaisha [116] described modification of the weak lead (74) (IC_{50} 4300nM) into the more potent analogue (75) (IC_{50} 160nM), though no *in vivo* data is disclosed. Zeneca have published patents [117,118] disclosing 4-aminopyridine based antagonists, detected by ran-

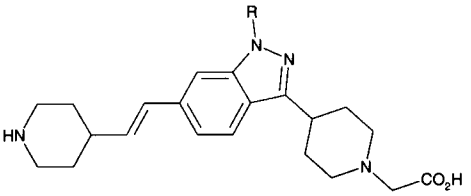


(72)

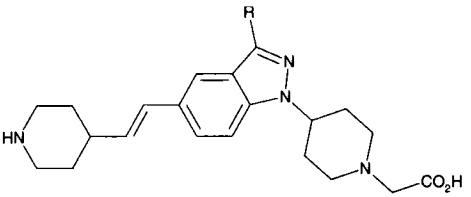


(73)

Table 2.6. INDAZOLE CONTAINING FIBRINOGEN ANTAGONISTS



Compound	*R*	*IC_{50} (nM) (RP)*[a]	*Reference*
(66)	H	67	[112]
(67)	$PhCH_2$-	107	[112]
(68)	4-$FC_6H_4CH_2$-	126	[112]
(69)	4-$ClC_6H_4CH_2$-	72	[112]



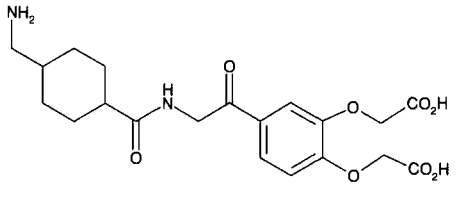
Compound	*R*	*IC_{50} (nM) (RP)*[a]	*Reference*
(70)	H	46	[113]
(71)	-SO_2Me	100	[111], [37]

[a]Resuspended platelets assay (see above)

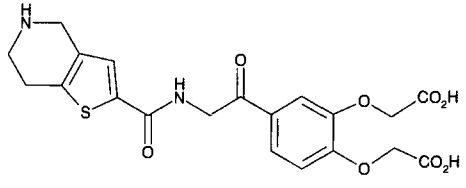
dom screening, e.g. (76) (ZD2486) (IC_{50} 7.65nM; ELISA). This compound is now in phase II trials and is the only known fibrinogen antagonist which has high bioavailability as a parent entity, being 65% bioavailable in the dog at 5mg/kg p.o..

Patent literature

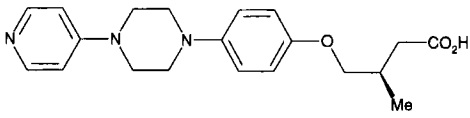
Numerous patents have been published disclosing fibrinogen antagonists; these have formed the subject of several reviews [66,119].



(74)



(75)



(76)

PHARMACOKINETICS OF FIBRINOGEN ANTAGONISTS

The narrow therapeutic window of fibrinogen antagonists imposes relatively stringent criteria for the most desirable pharmacokinetic profile for this class of agents. These criteria normally include low plasma clearance, long pharmacokinetic half-life, and high oral bioavailability if the drug is intended for oral dosing; such parameters should minimise undesirable fluctuations and unpredictable inter-patient variations in plasma levels. However, for some indications where i.v. infusion is appropriate, a short half-life may be desirable.

The above attributes, especially high oral bioavailability, have been difficult to achieve owing to the zwitterionic fibrinogen antagonist pharmacophore. This acid-base feature results in poor ability to cross biological membranes, usually leading to poor oral absorption (very few of the compounds described above achieves $> 10\%$ oral bioavailability) and low volume of distribution. Because the drugs are largely confined to the plasma compartment, they can be eliminated quite rapidly, resulting in short plasma half-lives, even with relatively low plasma clearance values. Pharmacokinetic

Table 2.7. PHARMACOKINETIC DATA FOR REPRESENTATIVE FIBRINOGEN ANTAGONISTS

Compound	*Species/ dose*	*Cl_p ml/min/kg*	*V_d L/kg*	*$t_{1/2}$ h*	*F/ dose*	*Reference*
m7E3 Fab	Man 0.25mg/kg i.v.	NA	NA	0.6	NA	[120]
Tirofiban	Man 0.15μg/kg/min i.v.	4±1.6[a]	0.89±0.3[a]	1.6±0.2	NA	[121]
SKF 106760	Dog 1mg/kg i.v.	3.4±0.8	0.26±0.03	1.1±0.2	3–6% 3mg/kg i.d.	[122]
L-703,014	Dog 0.2mg/kg i.v.	8	0.61	2.0	4.9% 2.0mg/kg p.o.	[123]
L-738,167	Dog 3μg/kg i.v.	0.018[b]	0.15	98	NA	[124]
RO 44–3888	Rhesus Monkey 0.2mg/kg i.v.	4.4±1.8	0.8±0.4	2.5±0.8	NA	[125]
Sibrafiban	Rhesus Monkey 1.0mg/kg p.o.	NA	NA	5.1±1.6[c]	33±6%[c]	[125]
SC-54701A	Dog 2.5mg/kg i.v.	5.0	2.8	6.5	8.5%[d]	[126]
Xemilofiban	Dog 2.5mg/kg p.o.	NA	NA	3.0[e]	61.5%[e]	[126]

Notes Cl_p, plasma clearance; V_d, steady state volume of distribution; $t_{1/2}$, plasma half-life; F, oral bioavailability (except where stated); a, figures adjusted assuming 70kg body weight; NA, data not available; b, total blood clearance; c, pharmacokinetic parameters for parent acid RO 44–3888; d, oral administration at 2.5mg/kg; e, pharmacokinetic parameters for parent acid SC-54701A.

data for some representative fibrinogen antagonists are shown in *Table 2.7*. Several approaches have been adopted to overcome problems of short duration of action and low oral bioavailability.

Enhancement of duration of action

The pharmacodynamic duration of action may be prolonged if the drug has very high affinity for the receptor, as for Abciximab [127] or L-738,167

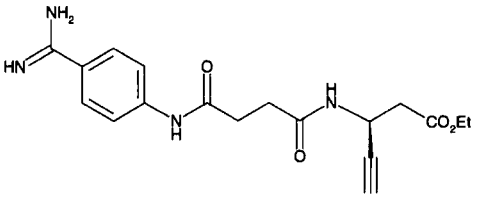
[124]. Alternatively, plasma half-life can be increased by reducing clearance as a result of high plasma protein binding [128]. A sustained release approach has been adopted using injectable copoly(dl-lactic/glycolic) acid microspheres; a single injection of TAK-029 (20mg/kg s.c.) contained in microspheres achieved plasma levels in rats within the therapeutic range (20–100ng/ml) over 3 weeks [129].

Enhancement of oral bioavailability

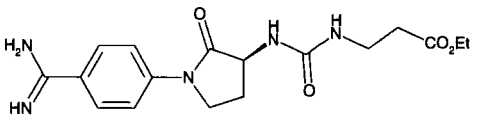
Prodrugs

The most common approach used to increase bioavailability of fibrinogen antagonists is to mask one or both of the charged entities thereby increasing log D and increasing the likelihood of absorption through lipophilic membranes. Thus, Searle's xemilofiban (77), the ethyl ester of (17), is bioavailable in the dog (F = 62%), despite the high basicity of the benzamidine group (pKa ca. 11.5) [72,130]. Once absorbed, the ester is rapidly hydrolysed to the corresponding carboxylic acid, the active drug. By contrast, the oral bioavailability of the acid (17) itself in the dog is only 8.5% [126]. Searle have disclosed another prodrug, orbofiban (78), which, like xemilofiban, is in phase III trials.

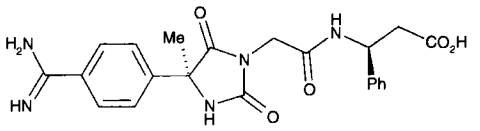
Dupont Merck have also employed a 'single prodrug' strategy to obtain oral activity [76,78]. The acid XR 299 (22) was orally inactive, but the corresponding ethyl ester XR 300 gave >90% inhibition of platelet aggregation *ex-vivo* at 1mg/kg p.o. in the dog, falling to 70% over 6h. Workers at Hoechst



(77) xemilofiban



(78) orbofiban



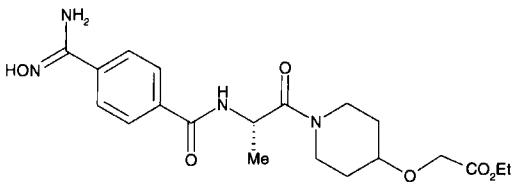
(79)

[131,132] have described hydantoin containing antagonists e.g. (79) (IC_{50} 20nM), whose modest bioavailability (F = 10%) in the dog was much improved as the ethyl ester prodrug S 5740 (F = 42%).

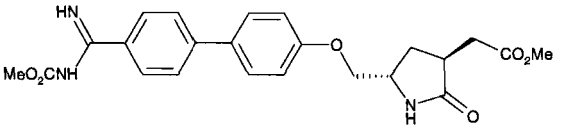
The Merck group synthesized the ethyl ester of the potent antagonist L-767,679 (55) and found it gave total inhibition of platelet aggregation *ex-vivo* at 0.5mg/kg p.o. in the dog for up to 8h [103]. A more detailed *in vivo* study using liver microsomes of this and other esters showed that only the low molecular weight esters (such as methyl, ethyl, and isopropyl) were cleaved to the parent acid. The higher molecular weight esters (e.g. benzyl) only gave small amounts of the parent acid and the presence of other metabolites was observed [133].

Other groups have masked both charged entities of their antagonists to increase bioavailability. For example, whereas Roche's lamifiban (13) had very low bioavailability (F = 1% p.o. in the mouse), the prodrug sibrafiban (80) of a related analogue (Ro-3888) was much more bioavailable (F = 25% in the dog, 33% in the rhesus monkey) [125] and is undergoing phase III trials. The amidine in sibrafiban is masked as an amidoxime (pKa *ca.* 5) giving an uncharged molecule at physiological pH. Amidoximes are known to undergo reductive metabolism to amidines *in vivo* [134].

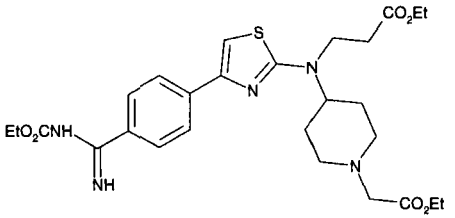
Karl-Thomae [82] have also used a 'double prodrug' strategy on BIBU 52 (28) by masking the amidine as a methylcarbamate. Carbamates of amidines are more labile to hydrolysis than normal amine derived carbamates owing to the presence of the electron-withdrawing imino group. The parent BIBU 52 (28) showed no oral activity in the rhesus monkey, whereas the cor-



(80) sibrafiban



(81) lefradafiban



(82)

responding prodrug form BIBU 104 (lefradafiban) (81) dosed at 1mg/kg p.o. gave substantial inhibition of collagen induced platelet aggregation *ex-vivo* for >8h, and is now in phase II trials (see later).

Sanofi's dicarboxylic acid (46) on oral administration to the baboon at 0.5mg/kg gave 35% inhibition of platelet aggregation *ex-vivo* after 4h [90]. However, the 'triple prodrug' SR121787 (82) gave nearly complete inhibition of platelet aggregation for 8h at the same dose, indicating an improved oral bioavailability over the parent.

Formulation approaches

A formulation approach applied to the cyclic peptide SKF 106760 (3) employed a water-in-oil microemulsion (composition Captex 355/Capmul MCM/Tween 80/Aqueous 65/22/10/3, %w/w) to enhance intestinal absorption, improving bioavailability after i.d. administration in rats from 0.5% to 27% [135]. A modest improvement in oral bioavailability (from 5–19%) of another cyclic peptide, DMP 728 (5) has been achieved in dogs using the fatty acid excipient sodium caprate [136].

Alternatives to the oral route

The difficulty in obtaining fibrinogen antagonists with high oral bioavailability has led to an interest in alternative routes of administration. For example, enhanced bioavailability of the ester prodrug DMP 755 compared

with the oral route was achieved by intranasal administration [137]. Administration of fibrinogen antagonists by iontophoretic patch has also been described [138,139]. This route, suitable for molecules which are polar, charged and water soluble, has the potential to overcome the problem of fluctuating drug plasma levels associated with the oral route, since controllable delivery equivalent to extended i.v. infusion may be achievable.

IN VIVO STUDIES

Numerous *in vivo* studies have been described demonstrating the efficacy of fibrinogen antagonists as antithrombotic agents, and as adjunctive agents in combination with thrombolysis. Only studies with the clinically more advanced agents are described here, along with several studies suggesting further possible indications.

EFFICACY OF FIBRINOGEN ANTAGONISTS AS ANTITHROMBOTIC AGENTS

Intravenous agents

Abciximab (monoclonal antibody c7E3 Fab)

The value of abciximab as an antithrombotic agent has been evaluated in a wide variety of animal models in several species. The object of these studies was to determine antithrombotic efficacy, bleeding risk, and duration of action, and to provide a comparison with conventional antithrombotic agents and combination therapy.

Abciximab (or the earlier, non-chimeric version 7E3 F(ab')$_2$) showed antithrombotic efficacy, with $>80\%$ of $\alpha_{IIb}\beta_3$ receptors blocked, in the Folts model in the dog (0.8mg/kg i.v.) [140] or monkey (0.8mg/kg i.v.) [141]; or in the electrically induced thrombosis model in the dog (0.8mg/kg i.v.) [142] or cynomolgus monkey (0.20 mg/kg i.v) [143]. In none of these studies was spontaneous bleeding or excessive bleeding from surgical sites observed. In addition, 7E3 F(ab')$_2$ (0.8mg/kg i.v.) reduced stent thrombus weight by 95% in an arteriovenous graft model of stent thrombosis in the dog [45].

In the cynomolgus monkey, the minimum effective antithrombotic dose (0.20 mg/kg i.v) maintained its effect 3.5h after drug administration; after 5h receptor blockade had only declined from 80% to 64% [143].

In contrast to the efficacy of abciximab in the electrically induced thrombosis model and in stent thrombosis (see above), aspirin and heparin, or combinations of the two, were ineffective in these models. However, in the electrically induced thrombosis model in the dog, combination of 7E3

$F(ab')_2$ (0.4mg/kg) with the thrombin inhibitor efegatran (2h infusion at 0.25mg/kg/h) gave an antithrombotic effect equivalent to that obtained using 7E3 $F(ab')_2$ alone (0.8mg/kg), but with a smaller increase in bleeding time [144].

Tirofiban (MK-0383)

In the Folts model, tirofiban (0.3mg/kg i.v.) gave 85% inhibition of ADP or collagen induced platelet aggregation, and abolished cyclic flow variations in the canine left circumflex coronary artery, with a short duration of action (18min). In the electrically induced thrombosis model, an infusion of tirofiban (10μg/kg/min), which produced 90% inhibition of platelet aggregation *ex vivo*, delayed or fully prevented occlusive thrombosis in the canine left circumflex coronary artery [145].

Lamifiban (Ro 44–9883)

Lamifiban (1mg/kg infused over 20 min) [146] abolished cyclic flow variations in a Folts type model using the guinea pig carotid artery. This model is thought to be of relevance to transient ischaemic attacks and cerebral infarction; aspirin, heparin or hirudin were only partially effective [146].

BIBU-52

BIBU-52 inhibited thrombus formation in Folts type models in the guinea pig, pig and marmoset [147]. In the pig, a dose of 1mg/kg i.v. reduced the frequency of occlusions by 100% for 40min, and by 91% over the next 20min; collagen induced platelet aggregation *ex vivo* was reduced by 89%, 70%, and 52% after 15, 30 and 60min respectively. In the marmoset, BIBU-52 was effective at inhibiting thrombosis at a lower dose (10μg/kg), reflecting the greater potency of the compound in marmoset PRP (similar to that in human PRP).

Orally active agents

Xemilofiban (SC-54684A)

When the prodrug xemilofiban was administered orally twice daily in the conscious dog, peak plasma levels of the active entity (SC-54701A) were about four times trough levels [148]. At 2.4mg/kg p.o. or 1.4 mg/kg p.o. twice daily, the minimum level of inhibition of *ex vivo* platelet aggregation (at trough plasma levels) was 75% or 27% respectively. In the canine electrically induced thrombosis model, SC54701A (infused at 0.87μg/kg/min for 15min, followed by 0.39μg/kg/min maintenance infusion) fully inhibited thrombosis in the left circumflex coronary artery [149]. This dose inhibited platelet aggregation by 92%; however, an equal antithrombotic effect was

achievable using half the dose of SC-54701A combined with heparin, even though inhibition of platelet aggregation with this regimen was reduced to 67%. Addition of aspirin to the above combination increased inhibition of aggregation to 96%, and provided some further benefit in terms of reduced cyclic flow variations.

L-738,167

L-738,167 (5μg/kg i.v.) was fully effective in preventing occlusion and reducing thrombus mass in a canine model of electrically induced thrombosis [150,151]. This dose produced initial *ex vivo* inhibition of platelet aggregation of 67+/-20%, and maintained inhibition between 65% and 55% over 8h. Once daily oral dosing to conscious dogs at either 10μg/kg or 30μg/kg maintained trough levels of *ex vivo* inhibition of platelet aggregation to within *ca.* 60–80%. Three doses of 100μg/kg p.o. (administered immediately prior to vessel injury, and 12h pre- and post injury) fully prevented thrombus formation, and achieved >90% inhibition of ADP induced platelet aggregation in the same model [150].

S5740

The ester prodrug S5740 (0.1mg/kg i.d.) prevents thrombus formation (94% reduction in cyclic flow variations) in a Folts type model of coronary thrombosis in the dog [132,152].

FIBRINOGEN ANTAGONISTS AS ADJUNCTIVE AGENTS IN THROMBOLYSIS

In addition to studying the ability of fibrinogen antagonists to prevent thrombus formation, the ability of these agents to enhance the outcome of thrombolytic therapy has been studied in several animal models, in which thrombolysis alone is only partially effective and/or is followed by rapid reocclusion. These studies are summarised below.

Abciximab (monoclonal antibody c7E3 Fab)

Effectiveness as adjunctive agent and comparison with other antithrombotic agents

In a model of thrombin induced thrombosis in the dog coronary artery, 7E3F(ab')$_2$ (0.8mg/kg i.v.) markedly accelerated thrombolysis with recombinant tissue plasminogen activator (rtPA), and fully prevented reocclusion [153]. Similar models were used to show the inferiority of aspirin and dipyramidole in the dog coronary artery [154], or of aspirin in the baboon femoral artery [155], compared with 7E3F(ab')$_2$ as adjunctive agents to thrombolysis with rtPA. However, in another dog study using this model, the thrombin in-

hibitor argatroban (200μg/kg/min, 60min infusion) in combination with aspirin was as effective as 7E3F(ab')$_2$ at enhancing thrombolysis with rtPA and preventing reocclusion, but with a smaller increase in bleeding time [156]. The equal effectiveness of a thrombin inhibitor in this study presumably reflects the fibrin rich nature of the initially formed thrombus in this model (see above).

Duration of action

7E3F(ab')$_2$ (0.8mg/kg i.v.) was also effective at preventing coronary artery reocclusion after thrombolysis with rtPA in an electrically induced thrombosis model. Thrombus mass and infarct size were reduced, although coronary blood flow was not fully restored to prethrombosis levels [157]; following the single bolus dose of 7E3F(ab')$_2$, inhibition of platelet aggregation remained above 50% for at least 48h, and vessel patency was maintained over five days [158].

Effect on arterial versus venous thrombolysis

In order to compare the effects of 7E3F(ab')$_2$ on arterial versus venous thrombolysis, a canine model was employed in which thrombosis was electrically induced simultaneously in the carotid artery and jugular vein [159]. Whilst 7E3F(ab')$_2$ (0.8mg/kg i.v.) prevented reocclusion in the carotid artery after thrombolysis, no effect was seen on rethrombosis in the jugular vein, reflecting the different thrombotic mechanisms in the arterial and venous circulation.

Integrilin and Tirofiban

The effectiveness of these two agents in enhancing thrombolysis with rtPA has been studied in a canine model of electrically induced coronary artery thrombosis. Integrilin combined with the thrombin inhibitor r-hirudin (i.v. infusion for 90min at 2.5μg/kg/min and 10μg/kg/min respectively) reduced the number of reocclusions, and prolonged the time to reocclusion [160]. This combination of $\alpha_{IIb}\beta_3$ and thrombin inhibition provided enhanced benefit over integrilin (5μg/kg/min for 90min) or r-hirudin (10μg/kg/min for 90min) alone, both of which prolonged the time to reocclusion without significantly altering the number of animals reoccluding.

Tirofiban (i.v. infusion for 165min at 10μg/kg/min) in the presence of background heparin enhanced the frequency and speed of reperfusion, and delayed or prevented reocclusion during the infusion period [145]. However, tirofiban failed to prevent reocclusion 1h after the infusion was terminated, presumably owing to the short duration of the initially high level (>95%) of inhibition of platelet aggregation.

Xemilofiban

The effect of xemilofiban on restenosis during chronic therapy following thrombolysis with rtPA has been studied in a photochemically induced thrombosis model in the guinea-pig femoral artery [161]. Xemilofiban (30mg/kg p.o. prior to occlusion and thrombolysis, then 30mg/kg twice daily for 3 weeks) reduced the degree of intimal thickening after 3 weeks, whereas aspirin was ineffective. The beneficial effect on restenosis was postulated to be due to reduced platelet deposition on the damaged vessel wall, resulting in reduced secretion of growth factors such as platelet derived growth factor which stimulate smooth muscle cell migration and proliferation.

OTHER *IN VIVO* STUDIES

Tirofiban and integrilin: preservation of platelet function during cardiopulmonary bypass

Cardiac operations involving cardiopulmonary bypass suffer from post-operative bleeding problems owing in part to loss of platelet numbers during the bypass procedures. In a study of cardiopulmonary bypass in baboons, tirofiban (0.3μg/kg/min infusion) preserved platelet numbers and accelerated the restoration of normal bleeding times after bypass [162]. By inhibiting platelet aggregation, adhesion and secretion in the bypass circuit, tirofiban provided 'platelet anaesthesia', allowing return of normal haemostatic function once the drug plasma levels had declined.

In a similar study in dogs, integrilin (90μg/kg i.v. bolus followed by 2μg/kg/min infusion) given during cardiopulmonary bypass preserved platelet number and function (assessed as the aggregation response to ADP) 6h after the bypass procedure, and reduced post-operative blood loss [163].

SDZ GPI 562: effect on cardiac xenograft survival

It has been postulated that platelet aggregation and microvascular thrombosis may play a role in xenograft rejection. In a study involving guinea pig to rat cardiac transplantation, SDZ GPI 562 (0.5mg/kg i.v., then 0.5mg/kg s.c. twice daily), used as an adjunctive agent with cobra venom factor, prolonged xenograft survival time, and reduced intragraft microthrombus formation and leukocyte infiltration [164].

^{99m}Tc-P280; [^{99m}TcO(L1-V)]$^-$: thrombus imaging agents

Since platelets are the only blood constituent to carry the $\alpha_{IIb}\beta_3$ receptor, a suitably radiolabelled agent which binds to $\alpha_{IIb}\beta_3$ should be of potential use as an imaging agent, e.g. for diagnosis of deep vein thrombosis or pulmonary embolism. The technetium-99m labelled peptide ^{99m}Tc-P280 has high affinity and specificity for $\alpha_{IIb}\beta_3$ and provided images of femoral vein thrombi in a canine model of venous thrombosis [165]. Similarly, the labelled cyclic peptide [^{99m}TcO(L1-V)]$^-$ was incorporated into growing thrombus under both arterial and venous conditions in a canine arteriovenous shunt model, and gave thrombus images in a canine model of deep vein thrombosis [166].

GR144053: effect on restenosis in combination with losartan

In a model of restenosis in the injured hamster carotid artery, GR144053 (i.v. infusion at 1.0mg/kg/h for 7 days) combined with post-injury treatment with the angiotensin II antagonist losartan (10mg/kg/day p.o. for 2 weeks), but not GR144053 alone, significantly reduced neointima formation after 2 weeks [167].

CLINICAL STUDIES

INTRAVENOUS AGENTS

Results from many of these studies have been described in detail in a recent review [168]; key findings from these trials are grouped below by indication. In addition, several more preliminary studies examining novel indications are mentioned. The major named clinical trials with the more advanced fibrinogen antagonists are summarised in *Table 2.8*.

Coronary angioplasty

The revascularisation procedure in percutaneous transluminal coronary angioplasty (PTCA) inevitably results in vessel wall damage, leaving a thrombogenic surface prone to complications such as rethrombosis, restenosis and possibly the need for repeat angioplasty [169]. The early trials with fibrinogen antagonists aimed to investigate whether blockade of the $\alpha_{IIb}\beta_3$ receptor could reduce such complications.

The EPIC (Evaluation of c7E3 in Preventing Ischaemic Complications) trial was the first large scale trial of a fibrinogen antagonist, and proved to

be a landmark in demonstrating the efficacy of this new class of antithrombotic agent [168,170]. Abciximab (0.25mg/kg i.v. bolus followed by 10μg/min infusion for 12h) was administered in addition to standard aspirin/heparin treatment to high risk patients undergoing coronary angioplasty. This regime maintained 85–90% $\alpha_{IIb}\beta_3$ receptor blockade, and the main findings validated the fibrinogen antagonist approach, demonstrating both short and long term clinical benefit. At 30 days, a composite endpoint of adverse events (including death, myocardial infarction, and urgent repeat angioplasty) was reduced by 35% compared with placebo. This benefit was maintained, albeit at a reduced level, at six months (23% event rate reduction) and even at the three year follow-up (13% reduction) [171].

The EPILOG (Evaluation of PTCA to Improve Long-term Outcome by c7E3 GPIIB/IIIA Receptor Blockade) trial was designed to evaluate whether the safety profile of abciximab could be improved by using low dose heparin, and to investigate its use in all risk categories of angioplasty patients [168,172]. The results at 30 days confirmed the marked reductions in adverse events seen in the EPIC trial, and outcomes were as good with low-dose, weight-adjusted heparin as with standard dose heparin (*ca.* 55% relative reduction in the composite event rate in each case). In addition, improved safety results were achieved compared with the EPIC trial (see below).

The use of abciximab in a 'rescue' manner (as opposed to the usual prophylactic use) when newly formed intracoronary thrombus occurs as a complication of coronary angioplasty has been examined in a small trial [173]. Dissolution of thrombus and restoration of coronary blood flow were reported to be readily achieved using abciximab (0.25mg/kg i.v. bolus followed by 10μg/min infusion for 12h).

The efficacy of integrilin has been examined in all types of coronary intervention and all risk category groups in the IMPACT II (Integrilin to Minimise Platelet Aggregation and Prevent Coronary Thrombosis II) trial [168,170,174]. Treatment with integrilin (135μg/kg i.v. bolus and 20–24h infusion at 0.75μg/kg/min) in conjunction with aspirin and heparin resulted in a 30–35% relative reduction in the composite endpoint (as for the EPIC trial) after 24h. However, although the results at thirty days and six months showed favourable trends, the benefits were not statistically significant. This trial confirmed the utility of a shorter acting and receptor specific fibrinogen antagonist; however, it has been suggested [175,176] that the determination of $>80\%$ inhibition of ADP induced platelet aggregation in the phase II trial [177] may have been an over-estimate, leading to a less than optimal dosing regimen being employed for the IMPACT II trial. *Ex vivo* inhibition of platelet aggregation was determined in plasma containing citrate; this

anti-coagulant reduces plasma calcium concentration, and enhances the inhibitory activity of integrilin [176]. These considerations led to higher doses being selected for the PURSUIT trial for unstable angina (see below).

Tirofiban has been investigated in high risk patients undergoing coronary angioplasty or directional atherectomy in the RESTORE (Randomised Efficacy Study of Tirofiban for Outcomes and Restenosis) trial [168,170,178]. Treatment with tirofiban (10μg/kg i.v. bolus followed by infusions for 12h at 0.15μg/kg/min and 24h at 0.1μg/kg/min), in conjunction with aspirin and heparin, resulted in a 38% relative reduction in the composite endpoint (similar to EPIC but including non-urgent repeat angioplasty) at 2 days. At 30 days the reduction in event rate was 16% (or 24% if non-urgent repeat angioplasty was excluded as in the EPIC trial).

In summary, of the agents which have undergone large-scale trials in coronary angioplasty, abciximab has thus far shown the greatest efficacy; however, it is as yet unclear whether this was due to the longer duration of action of abciximab, its non-selectivity versus the vitronectin receptor, or to suboptimal dosing in some of the other trials.

Unstable angina

Results are now available from phase III studies in unstable angina with abciximab, integrilin, lamifiban and tirofiban. In the CAPTURE (c7E3Fab Antiplatelet Therapy in Unstable Angina) trial, patients with unstable angina undergoing angioplasty were treated with abciximab using a dosing regimen similar to that employed in the EPIC trial. The significant 30% relative reduction in adverse events seen at 30 days was not maintained at six months [168]. A trial with abciximab in unstable angina patients not undergoing angioplasty (GUSTO-4) is planned.

Integrilin was shown in a phase II trial to reduce the number and duration of ischaemic events in unstable angina patients compared with aspirin [179]. The PURSUIT (Platelet IIb-IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy) trial employed integrilin at higher doses (180μg/kg i.v. bolus, 1.3–2.0μg/kg/min infusion for 72h) than the IMPACT II trial, and achieved a modest but significant 10% relative reduction in adverse events (death, myocardial infarction) at 30 days [168,175,180].

The PARAGON A (Platelet IIb-IIIa Antagonist for the Reduction of Acute Coronary Syndrome Events in a Global Organisation Network) trial with lamifiban [168,181] failed to reproduce the promising reductions in death and myocardial infarction seen at 30 days in the dose-ranging Canadian Lamifiban study [182]. However, the lower of the two doses employed (300μg/kg i.v. bolus followed by 1μg/kg/min infusion) did show a significant

benefit at six months when combined with heparin; the higher dose (750μg/kg i.v. bolus followed by 5μg/kg/min infusion) was not beneficial, possibly because of increased bleeding complications.

The PRISM (Platelet Receptor Inhibitor for Ischaemic Syndrome Management) and PRISM PLUS (Platelet Receptor Inhibitor for Ischaemic Syndrome Management in Patients Limited to very Unstable Signs and Symptoms) trials allowed the comparison of the efficacy of tirofiban with (PRISM PLUS) and without (PRISM) the addition of background heparin [168,181]; in each case comparison was made with control groups given heparin alone. In the PRISM study, whilst tirofiban alone (0.6μg/kg/min and 0.15μg/kg/min infusions for 30min and 48h respectively) achieved a significant 36% relative reduction in combined death, myocardial infarction or refractory ischaemia at 48h, little benefit was seen at 30 days. In contrast, in the PRISM PLUS study, the inclusion of heparin together with a reduced dose (0.4μg/kg/min and 0.10μg/kg/min infusions for 30min and 48–96h respectively) of tirofiban led to significant (34% and 33%) reductions in these events after 7 and 30 days respectively. However, owing to differences in the patient populations in these two trials, the merits of adding heparin to therapy with tirofiban remain controversial [181].

Adjunctive agent in thrombolysis

Studies with fibrinogen antagonists in combination with thrombolytic agents in acute myocardial infarction are less advanced than the trials described above; however, phase II results have been published with abciximab, integrilin and lamifiban. In the TAMI-8 dose-ranging study, c7E3Fab was given in escalating bolus/infusion doses following initiation of thrombolysis with tPA, aspirin and heparin [15,168]. Favourable trends were observed in reocclusion and coronary artery patency rates at 24h. Interim results are now available for abciximab in the TIMI-14 trial, the first major trial of a fibrinogen antagonist combined with thrombolysis [183]. The main conclusion thus far is that a combination of the standard dose of abciximab and lower than normal doses of tPA is as effective in thrombolysis as full dose tPA alone, and may provide a safer therapy in terms of the haemorrhagic stroke rate. Higher doses of both abciximab and tPA are being investigated as the trial continues.

The IMPACT-AMI phase I/II trial investigated the combination of various doses of integrilin (36–180μg/kg i.v. bolus and 0.2–0.75μg/kg/min 24h infusion) with thrombolysis using tPA, aspirin and heparin [15,168]. Although an improvement in coronary artery patency at 90min was observed with the highest dose, no significant benefit in clinical outcomes was

demonstrated; however, residual levels of platelet aggregation (35% after 2h infusion) suggested that higher doses may be necessary for maximal therapeutic effect.

In the PARADIGM II trial, lamifiban is being studied using three dosage regimens in combination with tPA, aspirin and heparin; or with streptokinase and aspirin [127,168]. Full results are yet to appear.

Restenosis

Clinical trials investigating the influence of fibrinogen antagonists on restenosis following coronary angioplasty are of great interest, since restenosis represents a major clinical limitation to the angioplasty procedure, and none of the many classes of drug previously studied in this indication has shown conclusive benefit [186]. Theoretical grounds exist for anticipating a potential effect of fibrinogen antagonists on restenosis, especially if combined with vitronectin receptor blockade (as with abciximab) (see earlier). The effects of abciximab, integrilin and tirofiban on restenosis have been examined as part of the trials of these agents in coronary angioplasty (see above).

The EPIC trial provided a ground-breaking result, when a reduced rate of clinical restenosis at six months was manifested in a 36% reduction in the need for repeat angioplasty, following inhibition of platelet aggregation with abciximab for only 36–48h after the original angioplasty procedure [186,187]. This led to the concept of 'vessel wall passivation' after short-term fibrinogen receptor blockade; however, there was no angiographic examination to confirm the mechanism of this effect, and it is unclear whether the effect is attributable to fibrinogen and/or vitronectin receptor inhibition.

Results from the EPILOG trial, designed to examine long term outcomes following use of abciximab in angioplasty, included angiographic examination, but disappointingly failed to show improvements in restenosis at six months. Similarly, neither integrilin nor tirofiban has shown significant improvements in the rate of restenosis at six months in the IMPACT-II and RESTORE trials respectively. Hence a conclusive benefit in this indication has still not been confirmed [186,187].

Other indications

The use of fibrinogen antagonists in other indications is being examined in a number of trials, summarised in Table 2.9.

Fibrinogen antagonists can be used as adjunctive agents in coronary stenting, either prophylactically or during the procedure; prospects in this

Table 2.8. CLINICAL TRIALS: IV ONLY AGENTS

Agent/ synonyms	*Trial*	*Study type*	*Status*	*Reference*
Percutaneous coronary angioplasty				
Abciximab *ReoPro, c7E3 Fab*	EPIC	Phase III	Completed	[170], [168]
Abciximab	EPILOG	Phase III	Completed	[170], [168], [172]
Integrilin *Integrelin, Intrifiban, Eptifibatide*	IMPACT II	Phase III	Completed	[170], [168], [174]
Tirofiban *Aggrastat MK-383*	RESTORE	Phase III	Completed	[170], [168], [184], [178]
Unstable angina				
Abciximab	CAPTURE	Phase III	Completed	[168]
Abciximab	GUSTO-4	Phase III	Planned	[185]
Integrilin	PURSUIT	Phase III	Completed	[168], [175], [180]
Lamifiban *Ro-44–9883*	PARAGON	Phase III	Completed	[181], [168]
Tirofiban	PRISM PRISM PLUS	Phase III Phase III	Completed Completed	[168], [184], [181]
Adjunctive agent in thrombolysis				
Abciximab	TAMI-8	Dose-ranging	Completed	[15], [168]
Abciximab	TIMI-14	Phase III	Ongoing	[183]
Abciximab	GUSTO-4	Phase III	Planned	[183]
Integrilin	IMPACT-AMI	Dose ranging/ Phase II	Completed	[15], [168]
Lamifiban	PARADIGM PARADIGM II	Phase II Phase III	Completed Planned	[127], [168] [168]

Table 2.9. CLINICAL TRIALS: NOVEL INDICATIONS

Agent	*Trial/study type*	*Status*	*Reference*
Coronary stenting			
Abciximab	EPIC, EPILOG, CAPTURE	Completed	[185]
Abciximab	EPILOG stent	Ongoing	[188], [185]
Abciximab	ERASER	Ongoing	[188], [185]
Abciximab	Pilot studies	Completed	[189]
Integrilin	IMPACT-II	Completed	[188]
Venous thrombosis imaging			
Tc-99m-P280	Phase III	Ongoing	[190]
Stroke			
Abciximab		Planned	[185]

area have recently been reviewed [188]. In the EPIC, EPILOG and CAPTURE trials, stent patients experienced a reduction in myocardial infarction with abciximab [185]. The ongoing ERASER and EPILOG/Stent trials will specifically address the use of abciximab in stenting; the former will examine the effects of abciximab therapy on restenosis following stenting, whilst the latter will compare outcomes after abciximab therapy following angioplasty versus stenting [188].

The use of a high affinity technetium-labelled $\alpha_{IIb}\beta_3$ ligand as a thrombus imaging agent is an additional novel indication. Tc-99m-P280 has completed a phase III trial in deep vein thrombosis and a phase II trial for carotid thrombus imaging; results suggest this agent may be able to detect thrombi in all areas of the body [190].

The application of abciximab in the treatment of thrombotic stroke is to be studied in a trial starting in 1998. Possible advantages cited over tPA include benefit longer after the onset of symptoms, and a lower risk of haemorrhagic stroke compared with tPA [185].

ORALLY ACTIVE AGENTS

The extension of the range of clinical indications for the use of fibrinogen an-

tagonists to include chronic therapy, e.g. for secondary prevention of myocardial infarction, requires a form of administration suitable for long-term use. Despite the difficulty in obtaining compounds of this class with good oral pharmacokinetics (see earlier), a number of orally active agents are now in development, and the most advanced agents undergoing clinical trials are summarised in *Table 2.10.*

The EXCITE trial with Xemilofiban will examine the safety and efficacy of long term therapy (10mg or 20mg p.o. three times daily for 6 months post angioplasty). Results from the Phase II ORBIT study (15mg or 20mg p.o. three times daily) showed promising reductions in the incidence of cardiac events, at the cost of an increased rate of minor bleeding [191]. Sustained inhibition of platelet aggregation was demonstrated over two weeks following stent deployment [193], and over 30 days following angioplasty [192]. In patients receiving abciximab during stenting, an enhanced inhibition of platelet aggregation was observed when Xemilofiban was administered 8–18h after termination of abciximab infusion [195].

Table 2.10. CLINICAL TRIALS: ORALLY ACTIVE AGENTS

Agent/ synonym	*Trial/ indication*	*Study type*	*Status*	*References*
Xemilofiban *SC-54684A* *SC-54701A*	EXCITE/ angioplasty	Phase III	In progress	[191]
Xemilofiban	ORBIT/ angioplasty	Phase II	Completed	[191]
Xemilofiban	Angioplasty/ unstable angina	Phase II	Completed	[192]
Xemilofiban	Stent deployment	Phase II	Completed	[193]
Orbofiban *SC-57099B*	OPUS-TIMI Unstable angina/ myocardial infarction	Phase III	In progress	[191]
Sibrafiban *RO 48–3657*	SYMPHONY Unstable angina/ myocardial infarction	Phase III	In progress	[191]
Lefradafiban *BIBU-104*	Angioplasty	Phase II	Completed	[194]

The OPUS-TIMI and SYMPHONY trials will investigate long term therapy with orbofiban and sibrafiban, respectively, commencing shortly after myocardial infarction or unstable angina. Up to 50% inhibition of platelet aggregation by sibrafiban over 28 days was well tolerated in terms of bleeding risk in the TIMI 12 phase II trial [196,197].

A phase II dose-ranging study of lefradafiban in angioplasty indicated that doses up to 45mg p.o. three times daily were well tolerated, but 60mg p.o. doses gave unacceptable bleeding rates [194]; an earlier phase I study showed that during chronic dosing over 7 days, 50mg and 75mg p.o. doses three times daily gave minimum levels of inhibition of platelet aggregation of 53% and 88% respectively [198].

BLEEDING COMPLICATIONS AND SAFETY

Because fibrinogen antagonists inhibit a key step in the clot forming process which is fundamental to haemostasis, their clinical use carries an inherent risk of bleeding complications. Hence assessment of bleeding risk has been a safety issue of major importance for all the clinical trials with these agents; available data are summarised briefly in *Table 2.11.*

The data on bleeding risk and efficacy available so far suggest that the irreversible, long duration inhibition of $\alpha_{IIb}\beta_3$ by abciximab provides the most dramatic clinical benefit, but at the cost of a higher risk of bleeding complications compared with the short half-life, reversible inhibitors such as integrilin and tirofiban. Whilst the bleeding risk with abciximab can be reduced by careful management of the heparin regimen, the disadvantages of antibody formation and thrombocytopenia cannot be avoided. This topic has been the subject of several reviews [199–203].

CONCLUSIONS

The uniquely powerful antithrombotic mechanism of fibrinogen receptor antagonists has provided a new class of agents for the treatment of resistant arterial thrombosis. The evolution of these agents has broken new ground in several respects. Fibrinogen antagonists constituted the first class of inhibitors of the integrin family of cell adhesion molecules to be investigated, and the first to progress to the clinic. Progress in the medicinal chemistry of these compounds has provided a rare example of the successful design of small molecule non-peptide drugs from an endogenous peptide starting point, assisted by knowledge of the tripeptide RGD recognition motif. The acid-base pharmacophore posed severe difficulties in achieving long-acting,

Table 2.11. CLINICAL TRIALS: BLEEDING RISK AND SAFETY

Trial	*Bleeding events/ other safety data*	*Reference*
Abciximab		
EPIC	• doubling of rates of major bleeding (14% *cf.* 7% placebo) and transfusions (standard heparin dose) • increased rate of thrombocytopenia • no increase in haemorrhagic stroke • 6.5% incidence of antibody formation	[199], [200]
EPILOG	• no increase in major bleeding (low dose weight-adjusted heparin)	[199]
CAPTURE	• doubling of rates of major bleeding (3.8% *cf.* 1.9% placebo)	[199]
TAMI-8	• no increase in major bleeding or transfusion rates • major bleeding events not correlated with bleeding time	[168], [15]
Integrilin		
IMPACT II	• no increase in rates of major bleeding, transfusion, thrombocytopenia or haemorrhagic stroke • no antibody formation	[199]
IMPACT-AMI	• no increase in rates of major bleeding, transfusion, or thrombocytopenia	[199]
PURSUIT	• 6 month results awaited	[168]
Tirofiban		
RESTORE	• no significant increase in rates of major bleeding or thrombocytopenia	[168]
Xemilofiban		
ORBIT	• no increase in major bleeding • significant increase in minor bleeding	[191]

orally active molecules; however, these were eventually overcome using prodrug and other approaches.

The use of animal models of arterial thrombosis has been important for demonstrating the superiority of fibrinogen antagonists over other classes of antithrombotic agents, in assessing the level of inhibition of platelet aggregation required, and in providing an initial assessment of bleeding risk. Clinical studies have shown a high degree of success in reducing cardiac ischaemic events following angioplasty; promising results have also been obtained in the settings of unstable angina and acute myocardial infarction (providing adjunctive therapy in thrombolysis), and one trial has suggested a favourable impact on restenosis. Bleeding risk has proven to be controllable for acute use; long term safety assessment awaits further results from ongoing trials.

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3 Combinatorial Chemistry as a Tool for Drug Discovery

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INTRODUCTION

Over the last three decades the practising medicinal chemist has been bombarded with a plethora of new methods and technologies to make the job of drug discovery more efficient. These include QSAR analysis, rational drug design, molecular modelling and structure-based design. Each has in its time been associated with a degree of over optimism and it is only with the benefit of hindsight that a particular technique's true merit and worth can be assessed. The picture is often confused by medicinal chemistry papers in which one or more of the above techniques has been used to rationalise observed structure-activity relationships (SAR) rather than having been employed in a true predictive sense to design improved drug molecules. Nevertheless, for each of the above techniques there are notable examples of pharmaceuticals that have been discovered with their aid. Combinatorial chemistry is a very new addition to the medicinal chemists' tool box [1]. It encompasses an ever increasing range of chemistries and techniques all of which are associated with making more and screening more compounds. It is therefore different to the other drug discovery methods mentioned above which involve data analysis rather than the generation of the primary data.

Combinatorial chemistry has created a lot of excitement within the pharmaceutical industry with specialist companies being created and the established pharmaceutical companies setting up often very large combinatorial chemistry teams. Many advances have been made in a very short period of time since the first reported combinatorial chemistry experiments in the early 1990s, so it is perhaps an appropriate time to ask what is the worth of combinatorial chemistry to the practising medicinal chemist. It is with this question in mind that we have undertaken this review of combinatorial chemistry. As such a rapidly growing field it has already been the subject of many reviews [1–96] starting from the seminal overview by the Affymax group in 1994 [2, 3], which was followed by two other important reviews from Terrett and co-workers [4] and Balkenhohl and co-workers [5]. However, for the most part these have focused on synthetic methodology and tactics. In this review we have focused particularly on those reported combinatorial libraries which have been screened against therapeutically interesting targets.

COMBINATORIAL TECHNOLOGIES

There is considerable variety in the methods and approaches that have been taken to both prepare combinatorial libraries and for the subsequent screen-

ing. In the context of combinatorial chemistry directed to the discovery and/or optimisation of biological activity, the approach taken for screening can influence the choice of methods for library production and vice-versa. For screening there are three options: (a) to screen single compounds in solution; (b) to screen mixtures of compounds in solution; (c) to screen compounds attached to a solid phase. These options are discussed in turn below.

SCREENING SINGLE COMPOUNDS IN SOLUTION

Methods are well established for screening single compounds in solution [97]. In fact it is the advances that have been made in high-throughput screening (HTS) technologies that have created the demand for large libraries of compounds [98]. The main advantage of screening single compounds in solution is that activity can be directly correlated with chemical structure. However, this assumes that the 'single compounds' are relatively pure and are not contaminated by impurities that may affect biological assays. Thus a key decision is the extent of purification to perform on each compound produced. In the context of smaller focused libraries targeted for lead optimisation, compound purification can be readily achieved by preparative HPLC [99–102] or parallel flash chromatography. For the preparation of larger libraries an optimised solid phase synthetic route can lead to compounds in good purity [35, 46–54, 103–108]. Alternatively, solution phase chemistry [42–43] incorporating solid phase purification protocols (Solid Phase Extraction SPE [109], Solid supported Liquid Extraction SLE [110], ion exchange resins [111–114], polymer supported chemical scavengers [62, 115–122], or resin capture and release [57, 58]) can be equally effective. Product identity can be confirmed by high-throughput mass spectrometry techniques [123–125] but the accurate quantification of product yields for large libraries is an issue that still remains to be resolved. It is a matter of debate whether compounds need to be analytically pure for high-throughput screening but there can be time penalties to determining the structure of an active component when the library compounds have not been purified. Nevertheless, in the context of lead discovery there are certainly proponents for the screening of crude reaction products. A logical extension of this philosophy is the screening of compound mixtures and this is discussed below.

The screening of single compounds in solution necessitates that the synthesis of the compound libraries are conducted in an array format. This is often termed rapid parallel synthesis. As mentioned above, either solution phase or solid phase synthetic methods may be used. Certain of the pioneering companies developed their own proprietary apparatus for automated

parallel synthesis [63–65, 126–134] but there is now a wide variety of commercially available systems [99, 135–138]. This is a rapidly changing field and many of the commercial systems suffer from a lack of rigorous evaluation before marketing and consequently can be difficult to use and are unreliable. For the preparation of larger libraries by parallel synthesis there is an attraction to performing the chemistry in the same format that will be used for screening. Solution phase chemistry can be conducted in 96-well plates [139–141] with reagents being added using an X-Y pipetting robot and solid phase chemistry can be performed either with resin beads in 96-well filter plates [142, 143], (a wide variety of which are now available commercially) or on functionalised polyacrylic-acid-grafted pins arrayed in the same format as a 96-well plate [144–150]. However, whilst the 96-well format is convenient it is perhaps not so versatile if heating, cooling or air sensitive reagents are required. An alternative solid phase procedure that involves alphanumeric or radio frequency tagging [58, 79, 80] for a 'split-mix' synthesis is described below.

SCREENING MIXTURES OF COMPOUNDS IN SOLUTION

Screening mixtures of compounds has the advantage that fewer assays need to be performed and that by use of combinatorial synthetic methods many compounds are prepared in fewer reaction steps than in array or parallel synthesis. However, there is the disadvantage that once an active mixture is identified the structure of the active compound must be deconvoluted. A problem with the screening of mixtures is that additive and / or cooperative effects of weakly active compounds can lead to false positives which can only be identified following lengthy deconvolution. Thus, there is considerable debate about the ideal number of components for screening mixtures [4]. One might expect that the identification of the active component from a mixture will be easier if the component compounds are structurally diverse with respect to each other. Nevertheless, there are examples of active compounds that have been identified from the screening and subsequent deconvolution of large compound mixtures of structurally related compounds. Obviously, the use of mixture screening negates classical SAR and data analysis as applied traditionally by medicinal chemists.

Mixtures of compounds have been prepared directly in solution by mixing multiple reagents together to achieve a one-pot simultaneous synthesis (*Figure 3.1c*) [44, 151–155]. Following screening the active compound can be identified by a subtractive deconvolution process in which subset mixtures are prepared, each of which are missing one building block [44, 151–155]. The subtractive subsets are screened, and those that lose the greatest activity

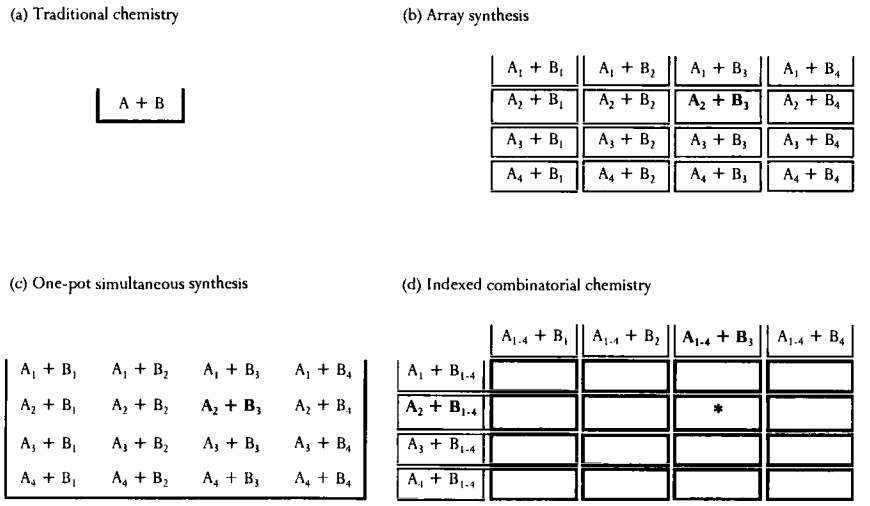


Figure 3.1. A comparison of (a) traditional chemistry with three approaches to combinatorial chemistry; (b) array synthesis directly identifies an active compound; (c) one-pot simultaneous synthesis neccessitates deconvolution; and (d) indexed library indirectly indicates active compounds.

relative to the parent mixture identify the building blocks that are responsible for activity. If the chemistry gives rise to regioisomeric compounds, as in the case where the library is prepared by simultaneous addition of functional groups to multiple sites on a core scaffold, the synthesis of a further subset of compounds containing the identified building blocks is required [44, 151–155]. The most active compound in this subset is selected either by individual synthesis or by iterative deconvolution (see below).

Active compounds may be identified directly from combinatorial libraries prepared as a series of mixtures in an indexed manner (*Figure 3.1d*) [29, 76, 156–160]. In this process the building blocks are usually assigned to two orthogonal groups as in a matrix format. Two series of subsets are synthesized that define the matrix, using either solution phase or solid phase methods, and screened. The orthogonal deconvolution process involves synthesis and testing of the single unique compound that is present in the most active subset of each series. By preparing compound mixtures considerably fewer reactions are performed than compounds are produced but in the case of indexed libraries each compound is synthesised at least twice. The advantage of the orthogonal deconvolution process is that the minimum of resynthesis is required to confirm activity, though it does rely on the assumption that the two halves (building blocks) within a molecule do not influence the bind-

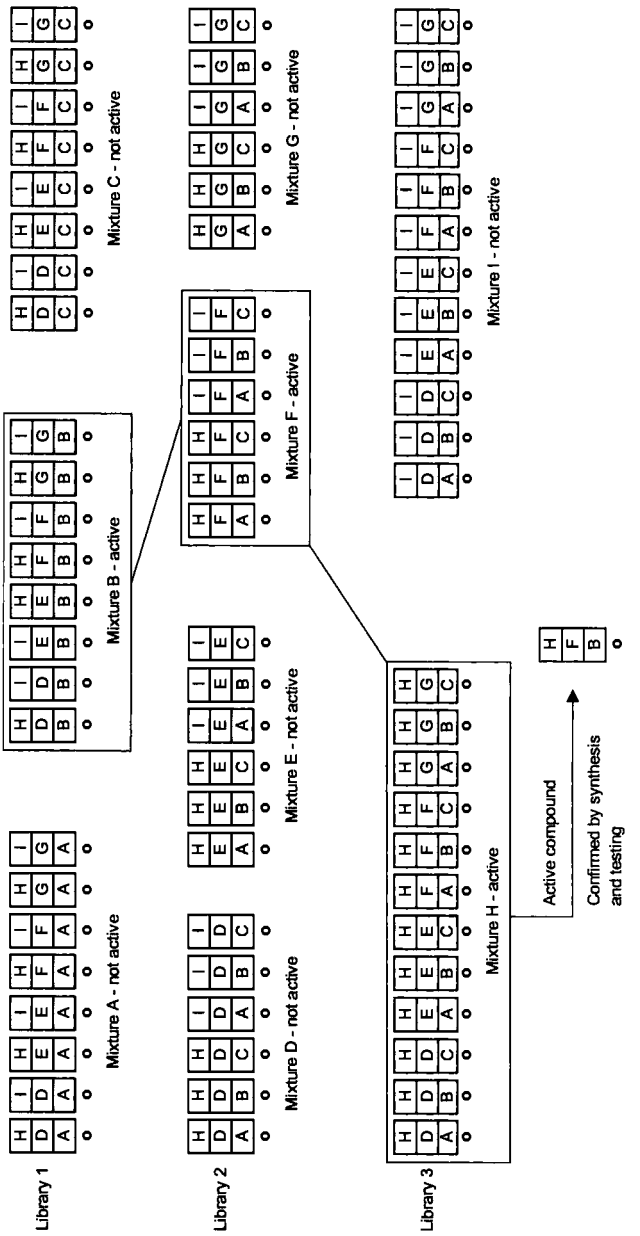


Figure 3.2. Schematic representation of the positional scanning process

ing of each other to the macromolecular target of interest. However, this approach provides qualitative structure-activity data for each building block used in the synthesis.

A related procedure that enables active compounds to be identified directly from screening is the positional scanning approach (*Figure 3.2*) [12–15, 160–165]. It has been applied to both peptide and non-peptide libraries and involves the synthesis of a series of subset mixtures which contain a single building block at one position and all building blocks at the other positions. The structure of the most active compound is assigned by selecting the building block from the most active subset at each position. Synthesis and testing of this compound is required to confirm activity. Positional scanning libraries have been synthesized by solid phase methods in which a predefined ratio of building blocks in excess is used to achieve approximately equimolar incorporation of each building block at each position. Alternatively, a mixture of equal amounts of all building blocks that is equimolar relative to the reaction site is used and subsequently excess reagents are used in order to ensure complete reaction. A potential disadvantage of this approach and related methods that rely on mixtures of reagents to achieve large compound numbers in library synthesis is that differing reactivities may lead to unequal quantities or even omissions of certain library members.

This can be overcome in principle by employing an excess quantity of a single reagent and mixtures of solid phase beads in each step. This process has been termed the 'split-mix', 'divide, couple and recombine' and 'one bead – one compound' method and has proved to be a cornerstone for large combinatorial library synthesis [166–168]. In this process (*Figure 3.3*) multiple copies of just one library member are attached to each bead since each bead 'sees' only one building block at a time. The final groups of beads can be subjected to cleavage conditions to provide the corresponding mixtures of compounds in solution for screening. An approach to identifying an active compound from a 'split-mix' library is the iterative deconvolution process (*Figure 3.4*) [164, 165, 169]. This involves the screening of the final mixtures for which the identity of the building block that was introduced in the last step is known. The most active mixture identifies the 'preferred' building block for the last position. The subset is then resynthesized with the 'preferred' final building block by a similar 'split-mix' protocol such that mixtures are obtained in which the second to last building block is defined. Each mixture is evaluated for biological activity in order to select the 'optimal' building block at the penultimate defined position. This deconvolution process or iterative resynthesis and screening is repeated in order to define all of the positions. This process has been applied with success to both pep-

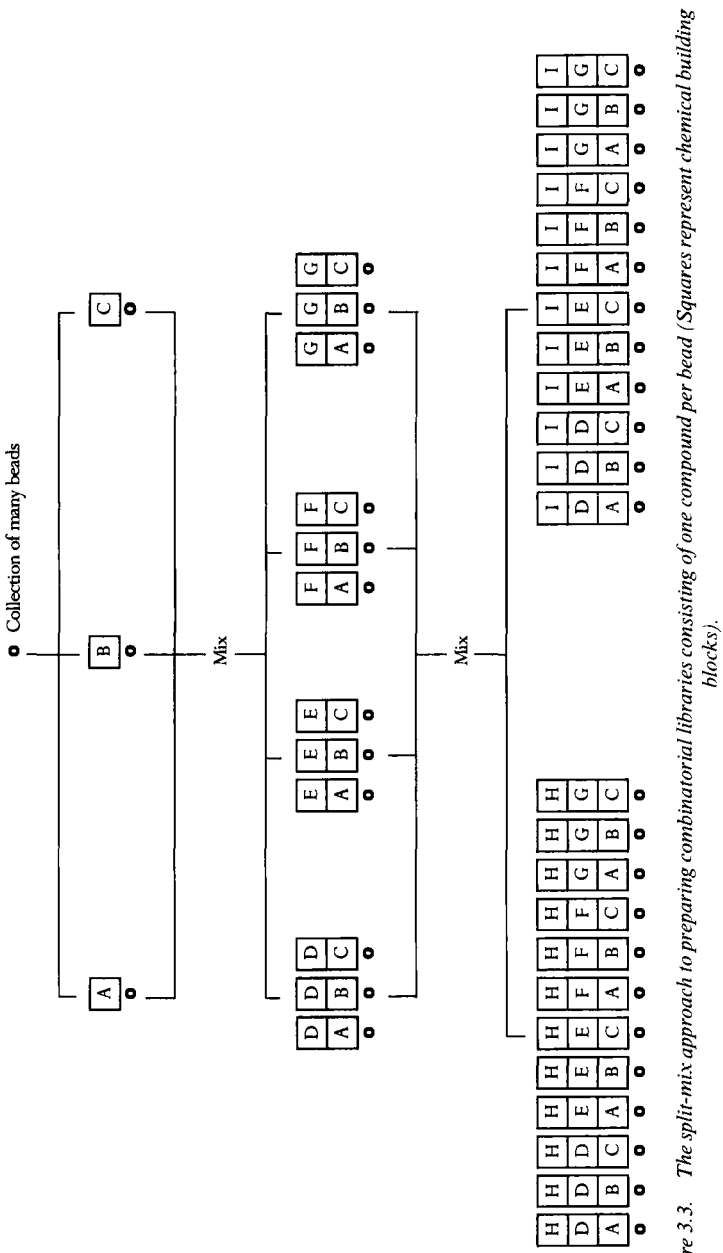


Figure 3.3. The split-mix approach to preparing combinatorial libraries consisting of one compound per bead (Squares represent chemical building blocks).

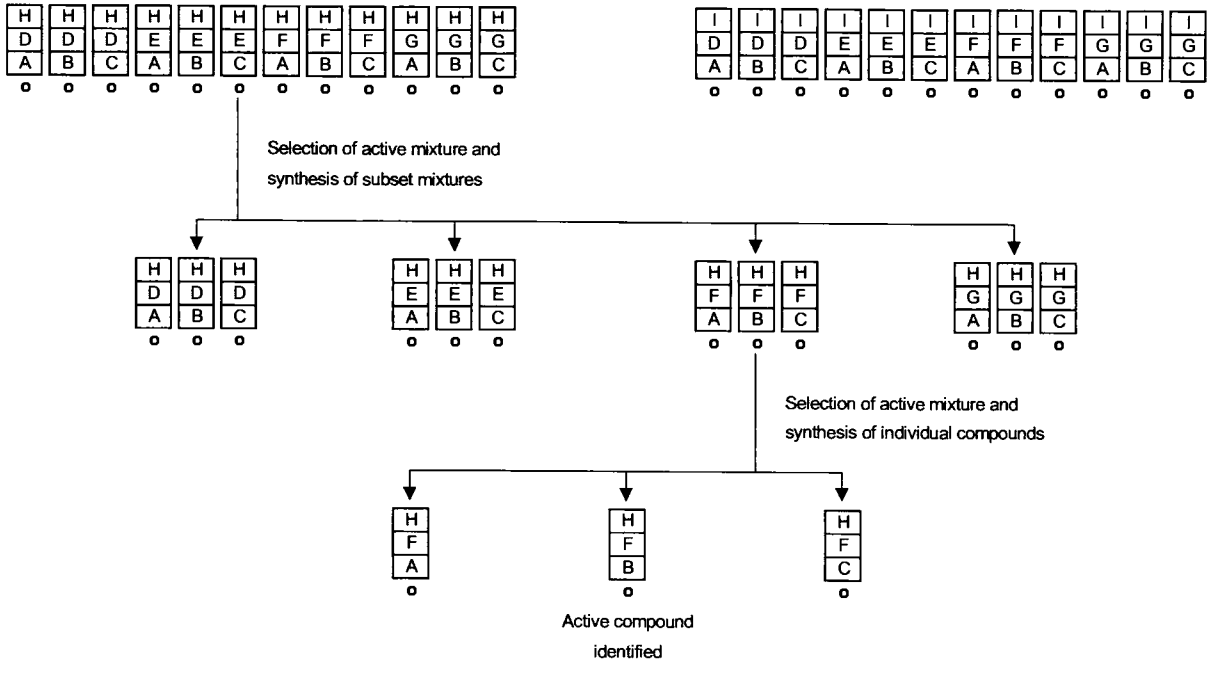


Figure 3.4. Schematic representation of the iterative deconvolution process.

tide and non-peptide libraries. However, a potential problem of this method and indeed all procedures that involve the screening of mixtures is that the observed activity of a mixture depends on both the number of active compounds in the mixture and their individual activity. Thus the most active mixture does not necessarily contain the most potent compounds. The use of large numbers of compounds in each mixture may result in an additional disadvantage in that lower concentrations of each compound may be required in order to maintain solubility in the initial screen. Finally, the iterative deconvolution process necessitates laborious resynthesis for each screen in which the library is evaluated, though the amount of chemistry required can be reduced by 'recursive deconvolution' which involves the archiving of intermediate solid phase beads for later use during the 'split-mix' resynthesis process [170].

There has been considerable interest in developing methods that circumvent the necessity of performing iterative deconvolution but which still make use of the 'split-mix' process to prepare many compounds in few synthetic steps. These involve performing the assays in a bead associated manner using an orthogonal release system; i.e. the compound is screened in the presence of its parent bead such that an active compound can be identified by reading information that is still attached to the bead. This approach has been successfully applied to peptide libraries [10, 11, 171–172]. Following completion of a 'split-mix' synthesis the beads are partitioned into groups of 30–500 per well of an assay plate (*Figure 3.5*). Portions of the compound are cleaved off into solution and assayed. The groups of beads corresponding to the most active pools are then split into single beads, before releasing and assaying another portion of the compound. The beads associated with the most active compounds are then submitted for structure determination which can be readily achieved by Edman microsequencing in the case of a peptide library. This process was originally achieved by the use of a three-arm linker that selectively releases compounds at different pH optima [171–173]. Alternatively, varying ratios of three different linkers can be introduced onto each bead to allow orthogonal cleavage of the compound [174], or staged compound release can be achieved by gaseous TFA cleavage using an acid sensitive linker [175, 176], or by timed photolysis using a single photolabile linker [177, 178]. Staged release enables an alternative screening format to be used in which beads are dispersed in a gel (e.g. agarose) [98]. In such bead lawn assays after partial release from the solid support compounds diffuse into the gel creating inhibition zones around active beads [175, 176]. These are then removed for structural identification. For non-peptides direct structure identification necessitates a very sensitive analytical technique. Whilst mass spectrometry has the required sensitivity, the pro-

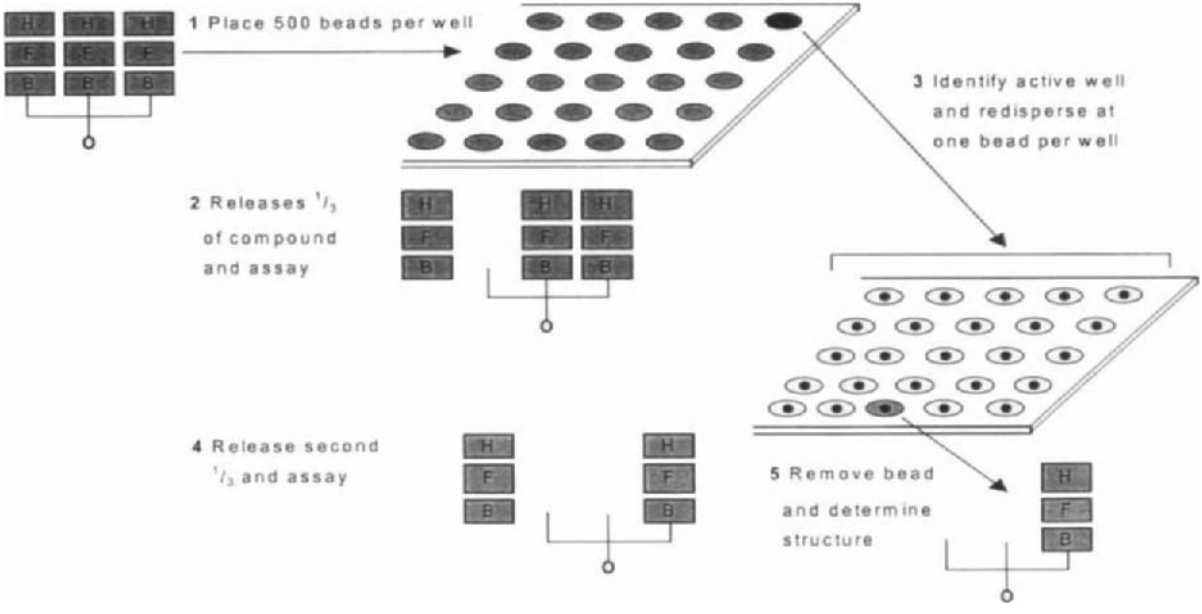


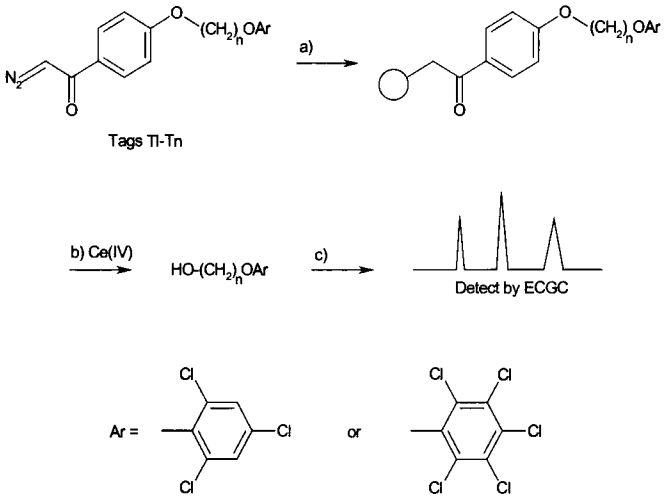
Figure 3.5. The use of a selectively cleavable linker to help assay and identify an active compound in a combinatorial library.

blems of molecular weight degeneracy limit the size of library which can be deconvoluted [179–186]. For peptide libraries the problem of mass degeneracy can be partially solved by introducing a small amount of a capping group at each stage in the synthesis [20, 187–190]. At cleavage the desired peptide is obtained together with a low concentration of fragment peptides from which the sequence can be read by mass spectrometry.

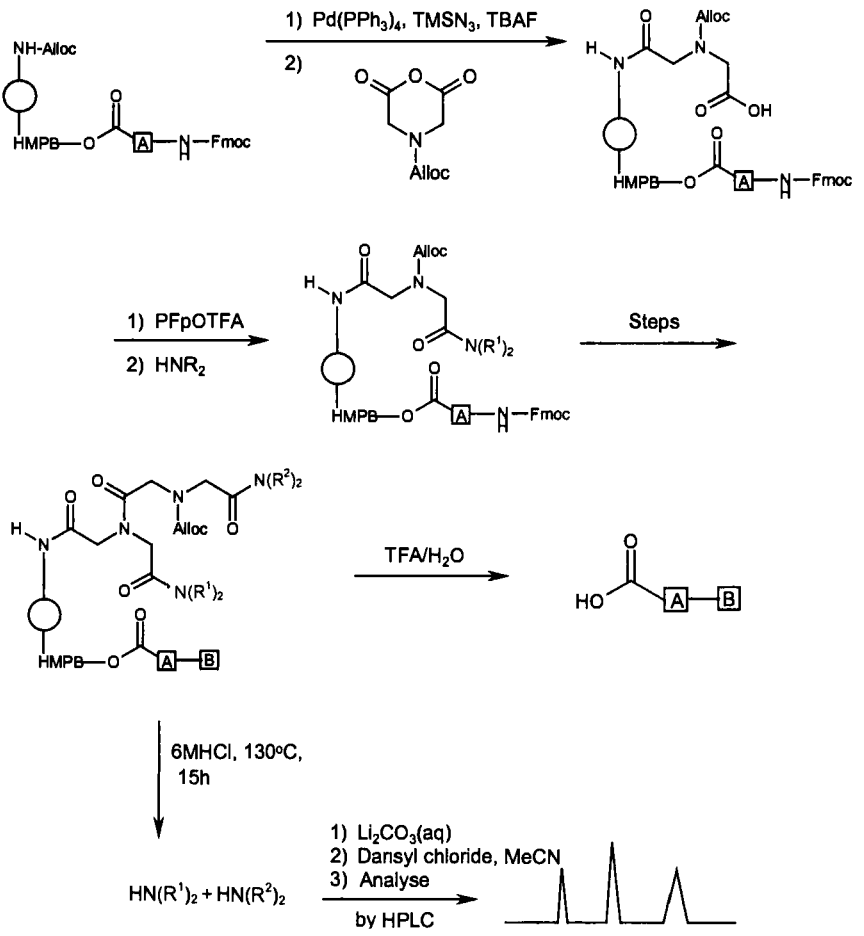
The introduction of a second molecule in addition to the one of interest on each bead which can be analysed by more sensitive techniques enables the indirect determination of structure. This process has been termed tag-encoding and requires that orthogonal chemistry is used for the introduction of the tags which is conducted at each stage of a 'split-mix' synthesis prior to the pooling process. The initially reported coding strategies that involved oligonucleotides (read by PCR amplification and DNA sequencing) [191–194] and peptides (read by Edman microsequencing) [195, 196] have given way to binary coding with chemical tags [197–200]. Two procedures have been developed, both of which rely on chromatographic separation of the tags to read the binary code for each building block. In the procedure originally developed by Still, ω-halophenoxy-l-alkanol ethers are employed as molecular tags which can be detected at levels of less than 0.1 pmol using electron-capture gas chromatography (ECGC) [71–73, 197–198]. The tags are linked to an oxidatively labile linker which may be cleaved with ceric ammonium nitrate (*Scheme 3.1*). Rhodium-catalysed acylcarbene insertion chemistry is used to directly attach the tags to the resin beads. This occurs predominantly into the support because it is present in greater proportion than the compound of interest [198]. Minimal compound modification should result since the tag loading level is only ~ 1% of the compound. An advantage of this approach over other encoding strategies is that a protection scheme is not required. In the binary code format multiple tags are used for each building block with the presence or absence of a tag corresponding to 1 or 0, respectively, in a binary sequence similar to a barcode. An alternative binary coding method involves the use of secondary amine tags [199, 200]. These are incorporated using iminodiacetic anhydride at each stage of the 'split-mix' synthesis. Decoding involves hydrolysis of the tagging strand and dansylation of the secondary amines which are detected at sub-picomolar levels using reverse phase high performance liquid chromatography (RP-HPLC) (*Scheme 3.2*) [199, 200]. From the tag decoding of a number of active compounds SAR information may be obtained [177, 200]. This is in contrast to the iterative deconvolution method which results in the identification of a single active compound. Tagging methods clearly involve more initial synthetic steps but the rapid identification of hits from very large libraries is a distinct advantage. Alternative methods of using mo-

lecular tags have been suggested in which rather than cleaving the encoding tags from an active bead the tags are analysed whilst still attached. This requires a sensitive analytical method such as the use of fluorescent dyes [201, 202], analysed by fluorescent confocal microscopy. A consideration with tag encoded methods and indeed all 'split-mix' synthesis is whether there are sufficiently more beads than library members such that all the possible compounds within the library are actually formed [192, 203–205]. This is because there is no way of controlling which beads are placed into which reaction vessels at each stage of the library synthesis.

Rather than chemically tagging single beads, procedures have been developed for tagging encapsulated collections of beads [58, 78–80, 206–211], laminar solid phase [77, 212] or macroscopic grafted polymer supports [213]. These methods which rely on alphanumeric labelling (i.e. writing on a tea bag of beads) [77, 206, 207, 212], radio frequency (RF) tags [208–211] or laser optical encoding [213] allow the selection of which portions of solid phase are placed in which vessels. This enables the formation of all possible library members. However, because the tagged solid phase is so much larger than a single bead there is a limitation on the size of library (up to 1,000 members) that can realistically be made by such procedures, whereas single bead tagging methods could be utilised for encoding much larger libraries (ca. 100,000). Compound libraries prepared by alphanumeric labelling, RF tagging or laser optical encoding methods are more usually screened as dis-



Scheme 3.1. Haloaromatic tags a) attached via carbene insertion, b) oxidatively released, and c) detected by ECGC.



Scheme 3.2. Secondary amine tag binary encoding method.

crete compounds rather than mixtures. This is because the tags can be read prospectively and each package of encapsulated beads (laminar solid phase or grafted polymer) spacially arrayed for the cleavage and screening processes. The smaller library sizes make this process feasible. Rather than use a single approach for the deconvolution of libraries that are screened as mixtures in solution, the possibility exists of combining approaches. For example, the combination of 'split-mix' synthesis with an indexed library approach (termed Spatially Arrayed Mixture (SpAM) technology) enables the direct determination of the last two building blocks that were introduced,

with the preferred earlier building blocks being identified by a subsequent iterative deconvolution [214–216]. Thus for combinatorial compound libraries prepared by solid phase 'split-mix' methods the design of the deconvolution strategy is as important as the selection of the compounds that make up the library.

An ideal method for the screening of compound mixtures in solution would involve selection of the active by the assay system coupled with direct structure analysis. Procedures based on mass spectrometry coupled to some form of affinity selection are being applied to this problem [123, 217–231]. In these methods a mixture of candidate ligands is mixed with the protein target and the unbound library members are removed. Usually the ligand-receptor complex is dissociated before identifying the released ligands by MS. The possibility also exists of analysing the ligand-receptor complex directly in the mass spectrometer [217, 218]. Methods that have been used to achieve the separation of bound from unbound ligands include affinity capillary electrophoresis with on line ESMS [219–221], size exclusion chromatography followed by reverse phase LC-MS [222, 223], ultrafiltration coupled to ESMS [224–226] and immobilisation of the protein of interest on an affinity chromatography matrix and coupling the affinity chromatography system to ESMS [227–231]. It is questionable as to whether such techniques can be applied to very large libraries because of issues of mass degeneracy. Careful library design and / or the use of ESMSMS may partially circumvent this issue. At present there are four disadvantages with affinity selection MS methods. Firstly, larger quantities of the target protein are required than for biochemical assay systems, though for certain methods the protein is recycled. Secondly, the protein needs to be of a high purity in order for the results to be meaningful. Thirdly, the method only determines whether a library member binds to a target of interest but the binding may not be productive in terms of achieving the desired biochemical effect (e.g. for enzymes the binding could take place other than at the active site). Finally, the protein needs to be either soluble (in the case of ultra filtration and size exclusion methods) or suitable for immobilisation (in the case of affinity chromatography) and so presumably cannot be applied to membrane bound protein targets such as G-protein coupled receptors.

SCREENING OF COMPOUNDS ATTACHED TO THE SOLID PHASE

For compound libraries prepared by solid phase methods following either array or 'split-mix' approaches, the possibility exists of screening the compound whilst still attached to the solid phase [168, 192, 196, 197]. This has also been applied to the screening of spacially arrayed peptide libraries pre-

pared by spot synthesis on cellulose supports [77] and spacially arrayed peptide or oligonucleotide libraries prepared on small silica wafers using photolithography techniques [2, 232]. In these approaches a suitable detection system is required such as measuring the percentage binding of a soluble fluorescently labelled receptor. In the case of compound libraries attached to beads, apparatus for fluorescent cell sorting has been adapted for the separation of beads to which a fluorescent protein is bound from non-binding beads [192]. Alternatively, protein depletion assays can be conducted [233, 234]. These involve the incubation of the protein (e.g. an enzyme) with polymer supported library member(s), separation of the supported compound(s) and subsequent bioassay of the remaining protein. A library member which undergoes a strong interaction with protein will result in protein depletion and a lower signal upon bioassay. There may, however, be problems with such methods: the ligand attached to a solid phase may not behave the same as when in solution, the high effective molarity of ligands on the solid phase may lead to polyvalent interactions, the solid phase and linker may provide non-specific interactions and not be compatible with the detection systems (e.g. fluorescent protein binding may be difficult to detect if the solid phase fluoresces). Nevertheless, the screening of spacially arrayed immobilised oligonucleotide libraries is being successfully applied within the genomics arena [232]. For screening bead attached libraries a deconvolution [233] or tag decoding protocol [197] is required in order to identify actives (see above) unless the library has been prepared in a parallel array format (e.g. using functionalised pins) [234].

LIBRARY DESIGN: DIVERSITY AND INFORMATICS

The medicinal chemist can use combinatorial methods to both identify novel lead molecules and to optimise the activity and properties of an initial lead compound. For lead identification the approach taken to library design is in part dependent on the amount of information available for the target of interest [56]. When the class of target is known it may be possible to design a small focused library based on a known pharmacophore. In contrast to this, when little information is available or novel structures are sought the screening of a larger diverse prospecting library is required to maximise the chances of success. It is in this context that the screening of libraries which are prepared as mixtures using 'indexed', 'split-mix' or other methods is advantageous. For optimising an initial lead, parallel array synthesis of focused analogue libraries is more applicable. In particular in the later stages of an optimisation program it may be necessary to prepare larger quantities of each library member to enable *in vivo* evaluation by cassette-dosing meth-

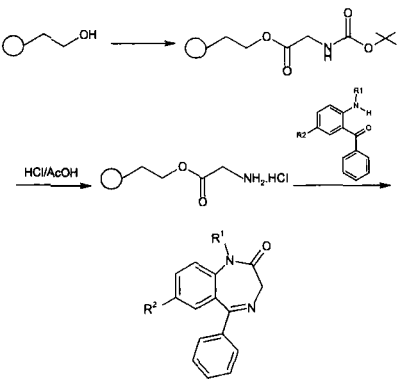
ods [235–237]. For optimisation programs assessment of compound purity profiles is important for generating meaningful SAR comparisons. Thus, for such focused libraries considerable attention tends to be paid to synthesis design and product purification methods.

Compound diversity is a central issue [38, 87, 90–92, 238–259], for the design of both large prospecting and smaller optimisation libraries. For prospecting libraries it is desirable that the compounds are diverse with respect to each other, whereas for optimisation libraries diverse variation of one or more substituents about a preferred template or core may be required. A somewhat bewildering variety of approaches and methods for selecting diverse substituents and even measuring diversity have been developed to date. At the simplest level the medicinal chemist can choose substituents which are selected by eye from groupings such as acidic, basic, aromatic, aliphatic, H-bond donor, H-bond acceptor, and polar, with consideration as to whether the substituent is small, medium or large [87]. Computer based methods (available within commercial software packages [251]) can calculate diversity based on 2D descriptors (finger prints) [238, 239, 246, 247], 3D descriptors [239, 240, 246], 3D lattice analysis [245] and pharmacophore analysis [258]. The medicinal chemist needs to understand the basis for diversity analysis that he or she is using in order to be able to interpret the results. For example, certain methods of diversity analysis treat enantiomers of the same compound as being identical: whereas one enantiomer could be biologically active and the other inactive. Since so many different descriptors can be considered in a diversity analysis there is a need for tools for visualisation and comparison of diversity. Methods such as 'flower plots' have been developed to address this issue [238]. Many of the methods for conducting diversity analysis which consider complete structures rather than substituents rely on the derivation of a virtual library from which a smaller subset is selected for combinatorial synthesis. This requires powerful informatics tools which ideally aid the registration of the compounds made and link to biological data that is obtained from screening [88, 89, 260–262]. The synthesis of libraries based on 'privileged structures' [263] has been adopted by a number of groups [52]. These are often rigid templates about which a variety of functionality may be displayed and which appear in a number of drug types [264]. An example is the biphenyl moiety (which appears in angiotensin II antagonists and gelatinase A selective matrix metalloproteinase inhibitors) and Pavia and co-workers have constructed what they term a 'Universal Library' based on this template [265]. It is clearly questionable whether such a library based on a single template will be truly universal and have sufficient diversity to provide hits against any biological target. In an alternative approach to selecting compounds from a virtual library based

on a diversity analysis, a small random subset of individual compounds is prepared and assayed. Using a genetic algorithm, subsequent compounds are selected for preparation [266–268]. This approach may require a number of rounds of synthesis and screening but has been successfully used for activity optimisation. Genetic algorithms have also been applied to improving combinatorial library design [269–271].

LIBRARY PRODUCTION: SYNTHETIC CHEMISTRY

Early combinatorial chemistry reports tended to focus on solid phase synthetic chemistry methods. Initially this involved the preparation of peptide libraries and subsequently of non-peptidic compounds. In the 1970s solid phase methods were developed for organic synthesis by Frechet [106], Leznoff [103, 104] and others [105, 107]. In the 1990s this work has been re-discovered, re-invented and now extended. For example, the solid phase synthesis of 1,4-benzodiazepin-2-ones was first described by Camps and co-workers in 1974 (*Scheme 3.3*) [272]. Eighteen years later Ellman and co-workers [26, 273–279] and DeWitt and co-workers [25, 280, 281] described sophisticated adaptations of this chemistry and applications to parallel array synthesis of libraries of benzodiazepines. Since the seminal studies of the Ellman and DeWitt groups there has been an explosion of publications describing solid phase synthetic organic chemistry [46–54, 108]. The adaptation of solution phase chemistry to the solid phase is a far from trivial undertaking. It is often necessary to invest considerable time in reaction optimisation in order to achieve a robust library synthesis. While the transfer of existing chemistry to the solid phase is difficult it can hardly be described as



Scheme 3.3. Synthesis of 1,4-benzodiazepin-2-ones after Camps and co-workers.

novel. However, significant innovations are being made in linker design [51–54] and analytical methods [83–86]. The linker provides the attachment between the solid support and the organic molecule that is being synthesized.

Two types of linker have been developed; the first introduces the desired functionality upon cleavage (e.g. carboxylic acid, amide, hydroxamic acid, thiol, etc.) and the second leaves no trace of the site to which the molecule was attached to the resin [51–54]. For these so called 'traceless linkers' the cleavage conditions usually involve replacement of a heteroatom by hydrogen [51, 52, 277, 279, 282–290]. Early linkers often required strong reaction conditions to effect cleavage (e.g. HF or neat TFA). Although linkers have been developed which can be cleaved under mild conditions, there is still a need for linkers for which the conditions are compatible with *in vitro* biological assays [171, 172, 178, 291, 292]. Analytical methods have been developed for the examination of small molecules attached to polymer beads [83–86]. These advances are of great importance for the optimisation of solid phase synthetic organic chemistry routes. In particular, early techniques for gel phase NMR analysis [293, 294] have been improved particularly with the design of magic-angle spinning probes [83–86, 295–311]. Methods have also been developed for single bead infra-red spectroscopy [312–318] and microscale procedures have been developed for cleavage and analysis by mass spectroscopy [83–85, 180–183, 319–322]. Advances are also being made in the chemistry of the solid support. Of particular note are grafted materials which, in comparison to the parent cross-linked polystyrene resin, swell in a wider range of solvents including water [53, 54]. Such polyethylene glycol grafted resins (Tentagel™) can be used with a wide range of chemistries [53, 323] and are suitable for biological assays in which compounds are not cleaved from the solid phase beads [233, 324].

The main advantage of solid phase synthesis is that excess reagents may be used to drive each reaction to completion and these may be removed at the end of each synthetic step by a simple filter and wash protocol. However, there can be problems associated with solid phase chemistry. The extent of resin loading can limit the amount of product obtained though loading can be increased by use of dendrimer modified resins [325]. It is necessary to ensure that the resin is adequately swelled and appropriately mixed with reagents such that all available reaction sites are accessible to the reagents [326]. Excessive agitation and/or heating can lead to resin degradation. Furthermore, resin degradation can occur under the cleavage conditions leading to lower than expected product purities [327, 328]. Nevertheless, at present solid phase methods dominate reports on combinatorial and parallel array libraries. Solution phase methods [42, 43] have the advantage that often less reaction optimisation is required prior to library production but of-

ten suffer from the disadvantage of low product purity. This issue is being addressed in a number of innovative ways by the use of soluble polymer supports [55, 329–339], fluorous phase protocols [340–349], and resin mediated purification techniques [57, 58, 62, 109–122]. Polyethylene glycol monomethyl ether polymers [MeO-PEG] are soluble in a range of solvents but can be precipitated from diethyl ether [55, 329–337]. Thus, chemistry has been developed where the molecule of interest is constructed whilst attached to MeO-PEG. Each step uses excess reagents which are removed by a precipitation / re-crystallisation protocol. Alternatively, dendrimers [338] or non-cross-linked polystyrene [339] may be used as soluble supports. The former has the advantage that purification can be achieved by size-exclusion chromatography techniques [338] while the latter support is compatible with low temperature (-78°C) chemistries [339]. In the fluorous phase strategy a polyfluoroalkyl substituent is attached either to a reagent or to the molecule of interest with isolation / separation being readily achieved by a three phase extraction protocol (fluorous, organic, aqueous) [340–349].

A variety of resin mediated purification techniques are being developed. These include use of solid supported reagents which can be removed by filtration [62, 107, 350–358], removal of by-products or product isolation using ion-exchange resins [111–114], solid phase extraction media [109, 110] or functional polystyrene scavenger resins which form covalent bonds with by-products [62, 115–122]. Resin capture protocols have been developed which represents a true hybrid of solid phase and solution phase methods [57, 58, 359–362]. In this approach the desired product from a solution phase synthesis is covalently trapped onto a functionalised resin (or soluble polymer [362]) and is subsequently released following filtration and washing by an appropriate cleavage method. Whilst attached to the solid phase the compound may be elaborated further prior to cleavage. The advantage of these various methods for product purification is that they enable excess reagents to be used in a solution phase synthesis. However, thought needs to be given to the complementary reactivity that is required for successful product isolation or by-product removal. Whilst perhaps not so elegant, effective methods are available for product purification using rapid chromatography techniques [99–102]. In particular, generic gradient HPLC methods have been developed [99, 100] and equipment is available for parallel processing [101] and molecular mass-selective fraction collection [102].

INTELLECTUAL PROPERTY

Various aspects of combinatorial methods and technology have been the subject of patent applications. These include screening and deconvolution

methods including tag-encoding procedures, solid phase resins, linkers and synthetic methods, apparatus for automating combinatorial chemistry, solution phase chemistries and purification techniques, and combinatorial libraries themselves [93–95]. It will be interesting to see whether such patent applications are granted and, if they are, to what extent they can be effectively defended. However, the extent to which pharmaceutical companies are attempting to patent combinatorial technology reflects the important position that combinatorial chemistry has now assumed in pharmaceutical research.

ENZYME INHIBITORS

The inhibition of enzymes has been a target of interest from the outset of the development of combinatorial methods. Initial work was more concerned with technology developments, illustrating their potential by the discovery of inhibitors for readily available, but often not therapeutically relevant, enzymes. More recently there has been a number of reports on the application of combinatorial methods to the inhibition of therapeutically relevant enzymes. For the most part the approach taken has been to prepare targeted libraries following a 'rational drug design' strategy based on knowledge of the catalytic mechanism and / or structural information. In many cases a pharmacophoric group or 'war-head' can be selected which is known to interact with key functionality within the enzyme active site (e.g. statine group for aspartyl protease inhibitors, aldehyde group for serine and cysteine protease inhibitors and thiol or hydroxamic acid groups for metalloproteinase inhibitors). Such libraries built around the selected 'war-head' have then addressed the effect of the attached substituents on potency and selectivity. For certain therapeutic applications it is important to obtain selectivity for inhibition of one particular enzyme within a class (e.g. the selective inhibition of thrombin over other serine proteases is desirable for a therapeutic agent to be used chronically in stroke patients). However, if the enzyme-inhibitor binding is dominated by a potent 'war-head', obtaining selectivity can be a difficult task. For many therapeutic applications it is advantageous to have a compound that not only is a selective inhibitor but is orally bioavailable with a suitable half-life. In the binding of a peptide substrate to a protease enzyme, hydrogen bond interactions between the amide groups of the substrate backbone and the enzyme play a significant role. The traditional approach to inhibitor design is to attach a 'war-head' to a fragment of a preferred peptide substrate. This results in peptidic based inhibitors which are highly amenable to preparation by combinatorial methods. However, a dis-

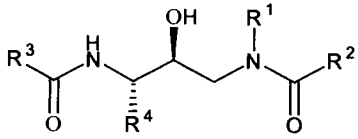
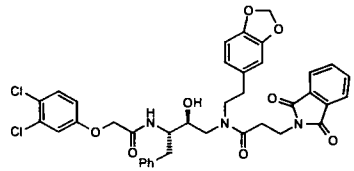
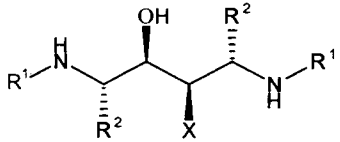
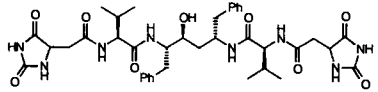
advantage of such an approach is that it is very difficult to obtain oral activity since the N-H group of amides is a known factor in limiting oral absorption [363]. Furthermore, compounds based on natural amino acids may be prone to rapid proteolytic metabolism *in vivo* and hence have an unacceptably short half-life [364]. Thus, there is increasing interest in the discovery of non-peptidic protease inhibitors. This has been achieved in a number of recent examples by structure-based design approaches [365] but it is also clear that targeted library methods are now being applied to this problem, often in conjunction with structure-based design [91, 92, 366].

This section focuses on the construction of compound libraries by solid or solution-phase techniques targeted to discovering or optimising enzyme inhibitors. As in subsequent sections, the libraries will be presented in tabular form (*Tables 3.1–3.4*) and for each library the name, the generic structure, the size, the synthesis, the enzyme screen and the structure and potency of the most active member will be included. We have chosen to include in the tables only libraries for which biological data (K_i or IC_{50}) has been given and in the text only selected libraries are discussed.

ASPARTYL PROTEASE INHIBITOR LIBRARIES

The aspartyl proteases renin and HIV protease have been targets of intense interest to the pharmaceutical industry [365, 367]. A common strategy for the inhibition of this class of protease is to replace the scissile bond of the substrate with an isostere that mimics the geometry of the tetrahedral intermediate. Combinatorial methods have been developed following this approach for the preparation of aspartyl protease inhibitor libraries (*Table 3.1*) [368, 369]. Pioneering work at Lilly included statine, a known transition-state analogue for aspartic acid proteinases, in their library design [368]. They reported the identification of a potent peptide-based inhibitor for HIV protease (library 1; IC_{50} 200 nM) from mixtures consisting of nearly a quarter of a million compounds [368]. Recently, Ellman and co-workers have reported the synthesis of two libraries each of 1,000 compounds, that were prepared by solid phase parallel synthesis [366]. The libraries were of non-peptides based on a hydroxyethylamine core [370] and were targeted for the inhibition of cathepsin D, an aspartyl protease that induces localised increases in vascular permeability, fluid accumulation and inflammation. Reaction optimisation enabled the compounds to be obtained in sufficient purity following cleavage such that purification was not required prior to assay [366, 370]. In the first library substituents were selected to maximise diversity about the hydroxyethylamine core using computational methods. However, more active hits were obtained from a 'directed library' in which

Table 3.1. ASPARTYL PROTEINASE INHIBITOR LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
1 [368]	Statine tetrapeptide $Ac\text{-}Aa_1\text{-}Aa_2\text{-}Statine\text{-}Aa_3\text{-}NH_2$	SPMS - ID (> 240,000)	Ac-Trp-Val-Statine-D-Leu-NH_2	HIV Protease IC_{50} = 200 nM
2 [366]	Diamino hydroxypropane 	SPPS (2,039)		Cathepsin D K_i = 9 nM IC_{50} = 14±2 nM
3 [369]	C_2-Symmetric Diamino alcohols 	SPPS (300)		HIV Protease IC_{50} < 1 nM

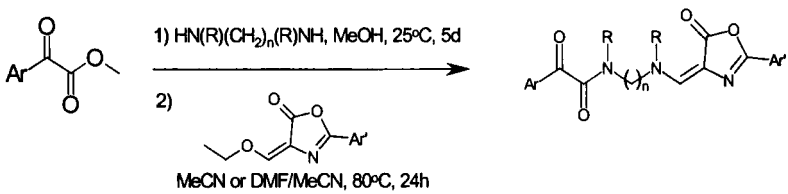
⁺SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution

the substituents were chosen following a structure-based design approach using the crystal structure of cathepsin D complexed with the natural peptide inhibitor pepstatin. This second library gave a 'hit rate' of 6–7% at 1 μM as opposed to 2–3% for the 'diverse library'. A second generation library of 39 compounds was then prepared to optimise the most active hits from the 'directed library'. This provided several potent inhibitors of cathepsin D (library 2; K_i 9nM) [366].

SERINE AND CYSTEINE PROTEASE INHIBITOR LIBRARIES

Many of the known inhibitors of serine and cysteine proteases feature the same types of 'war-head' (e.g. α-ketoamide, ß-lactam, aldehyde) which are able to undergo a covalent interaction with the nucleophilic active site alcohol or thiol group [371–373]. Depending on the reactivity of the 'war-head' this approach has led to irreversible inhibitors or reversible but tight-binding inhibitors. The problem with this approach is that it is difficult to achieve selectivity and so there is considerable interest in the discovery of inhibitors that bind in a non-covalent fashion to the active site.

1,600 Non-peptidic α-keto amide derivatives were prepared by a solution phase parallel synthesis procedure conducted in 96-well plates (*Scheme 3.4*) [374]. Inhibitors with low micromolar K_i values (not reported) were identified from screening this library against the serine proteases thrombin, factor Xa, trypsin and plasmin. The first of these enzymes, thrombin, plays a key role in blood clot formation by converting fibrinogen to fibrin and there has been considerable interest in the discovery of thrombin inhibitors as potential anti-thrombotic agents. A large library of peptides was generated with the aim of increasing potency of the known inhibitor of thrombin based on the active site binding tripeptide (D-Phe-Pro-Arg) [375, 376]. From on-bead screening and subsequent micro-sequencing of the single optimisation library a novel structure 1,000-fold more active than the original lead was identified (library 4; K_i 25 nM) (*Table 3.2*) [375, 376]. Following a similar approach involving screening of compounds whilst still attached to Tentagel™



Scheme 3.4. Parallel solution phase synthesis of α-ketomide serine protease inhibitor.

beads, a pentapeptide incorporating unnatural amino acids was identified as a selective inhibitor of factor Xa over thrombin (library 5) [377]. Recently there have been reports of thrombin inhibitors, discovered by library methods, that are non-peptides and importantly lack a reactive 'war-head' group. A benzylamino derived sulphonamide library of 200 members provided a 10 nM inhibitor of thrombin (library 6) [378]. An interesting approach adopted by workers at Merck was to design a focused library based on a potent and selective *trans*-(4-aminocyclohexyl)methyl amide derivative thrombin inhibitor which lacked oral availability. A rapid solid-phase synthesis of 200 analogues in which the P3 residue was replaced with a range of carboxylic acid derivatives afforded a novel inhibitor (library 7; K_i 1.5 nM) which exhibits good efficacy, enzyme selectivity and oral bioavailability in several animal species. A common characteristic of many thrombin inhibitors is the presence of a basic P1 substituent. The Merck group generated a focused benzylamide library in order to explore a lipophilic binding region in the S1 pocket (suggested by structural studies) in a search for a neutral thrombin inhibitor [380]. A potent inhibitor was identified (library 8; IC_{50} 10 nM) and SAR from the other library members suggested that lipophilic substitutents placed in the 2 and 5-positions of the P1 benzyl group were optimal. This was confirmed by the preparation of a 2,5-dichlorobenzyl analogue (K_i 3 nM) which was synthesized by a conventional method [380]. In an earlier study Weber and co-workers prepared a small focused library of thrombin inhibitors which was also designed on the basis of structural information (library 9) [381]. A three component 1,3-dipolar cycloaddition conducted in the solution-phase was the key step in the construction of the library [381]. Weber has also reported the optimisation of thrombin inhibitory activity from a virtual library of 160,000 compounds by use of a genetic algorithm (library 10; K_i 220 nM) [266]. In this study 400 different solution-phase Ugi reactions were conducted over 20 generations with the crude reaction products being screened directly without purification. The most active reaction product was found in generation 18 and when repeated on a larger scale both the expected Ugi product and a more active three-component adduct were isolated [266]. Genetic algorithm optimisation has also been applied to peptides that inhibit trypsin (library 11) [267]. It is unclear at present whether the genetic algorithm evolutionary optimisation paradigm will find widespread application in medicinal chemistry or remain a curiosity [382].

A weak inhibitor of trypsin was obtained from a large library of dodecapeptides synthesized on cotton-carriers following a combined positional scanning and iterative deconvolution strategy (library 12) [383]. Rebek's approach to the discovery of trypsin inhibitors was to generate a large library

Table 3.2. SERINE AND CYSTEINE PROTEINASE INHIBITOR LIBRARIES

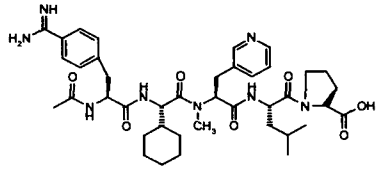
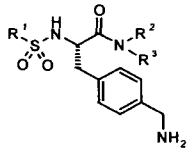
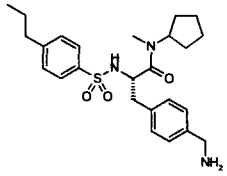
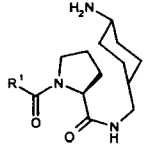
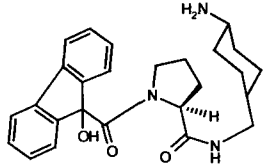
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
4 [375, 376]	Nonapeptide D-Phe-Pro-Arg-Pro-Aa_1-Aa_2-Aa_3-Aa_4-Aa_5-OH	SPMS - Tag (280,054)	D-Phe-Pro-Arg-Pro-Phe-Gly-Tyr-Arg-Val-β-Ala-OH	Thrombin K_i = 25 nM
5 [377]	Peptide	SPMS - Tag (not given)		Factor Xa K_i = 0.003 μM Thrombin K_i = 40 μM
6 [378]	Benzylamino-derived sulphonamides 	SPPS (200)		Thrombin K_i = 0.01 μM
7 [379]	(Aminocyclohexyl) methyl amide 	SPPS (200)		Thrombin IC_{50} = 4 nM K_i = 1.5 nM Trypsin K_i = 860 nM

Table 3.2 continued. SERINE AND CYSTEINE PROTEINASE INHIBITOR LIBRARIES

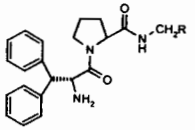
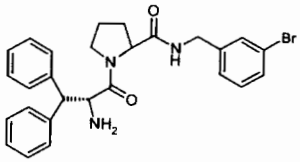
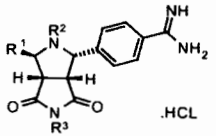
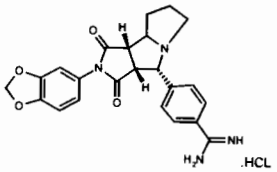
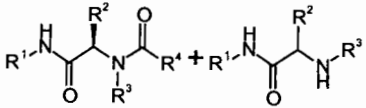
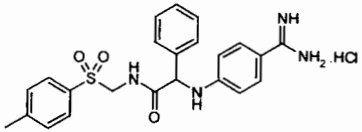
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
8 [380]	Benzylamide 	SPPS (ca 20)		Thrombin IC_{50} = 10 nM
9 [381]	Benzamidinium 	SolPS (14)		Thrombin K_i = 90 nM
10 [266]	Ugi Reaction Products 	SolPS (400 Reaction products)		Thrombin K_i = 220 nM
11 [267]	Hexapeptide $Ac\text{-}Aa_1\text{-}Aa_2\text{-}Aa_3\text{-}Aa_4\text{-}Aa_5\text{-}Aa_6\text{-}NH_2$	SPPS (140)	Ac-Thr-Thr-Lys-Ileu-Phe-Thr-NH_2	Trypsin 89% inhibition @ 190 μM

Table 3.2 continued. SERINE AND CYSTEINE PROTEINASE INHIBITOR LIBRARIES

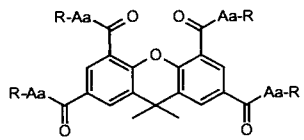
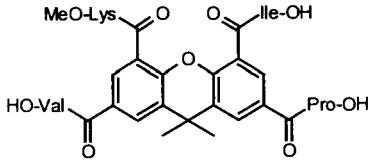
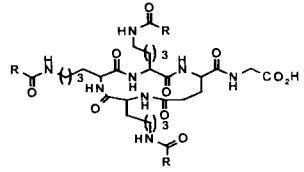
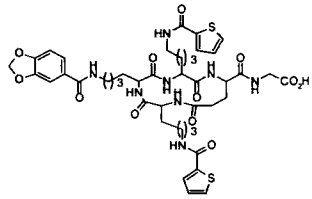
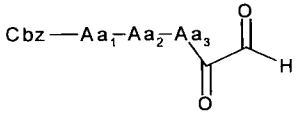
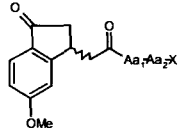
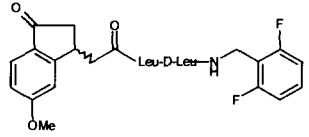
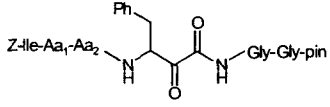
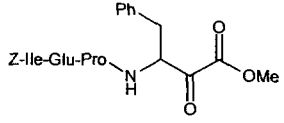
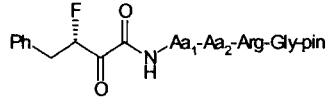
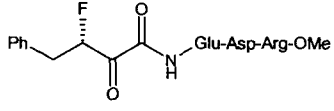
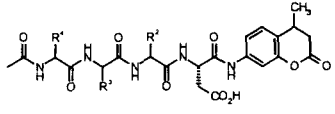
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
12 [383]	Dodecapeptide Ac-Aa_1-Aa_2-Aa_3-Ala-Lys-Ile-Tyr-Arg-Pro-Aa_4-Aa_5-Aa_6-NH_2	SPPS-cotton carrier (50 million)	Ac-Tyr-Tyr-Gly-Ala-Lys-Ile-Tyr-Arg-D-Pro-Asp-Lys-Met-NH_2	Trypsin IC_{50} = 12 μM
13 [151–153]	Xanthene tetra-amide 	SolPS (ca 65,000)		Trypsin IC_{50} = 9.4 ± 0.8 μM
14 [384]	Cyclic Lysine 	SPPS - ID (25,230)		Chymotrypsin IC_{50} = 51 μM
15 [385]	Peptidyl α-ketoaldehydes 	SPPS (9)	Cbz-Leu-Leu-Tyr-COCHO	'Chymotrypsin-like activity of proteasome' K_i = 3.1 ± 0.4 nM

Table 3.2 continued. SERINE AND CYSTEINE PROTEINASE INHIBITOR LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
16 [386]	Indanone peptides 	SPPS (400)		'Chymotrypsin-like activity of proteasome' IC_{50} = 0.14 μM
17 [234]	Peptidyl α-ketoamide 	SPPS - Pins (324)		Human heart chymase K_i = 1.0 nM Chymotypsin K_i = 10 nM
18 [234]	Peptidyl fluorobenzyl-pyruvamide 	SPPS - Pins (400)		Human heart chymase K_i = 1.0 μM Chymotrypsin K_i = 100 μM
19 [391]	Tetrapeptide 	SPMS - ID (3 × 8,000)	Ac-WEHD-CHO	caspase-1 K_i = 56 pM

of small organic molecules in solution by the derivatisation of a xanthene tetracarboxylic acid core with mixtures of amino acid esters [151–153]. A subtractive deconvolution procedure was developed which enabled the identification of a trypsin inhibitor with micromolar activity (library 13) [152, 153]. The ε-amino group of lysine present in the Lys-xanthene adduct is thought to bind into the S1 subsite of the enzyme.

Houghten and co-workers generated a 25,000 member cyclic peptide library to find inhibitors of the serine protease chymotrypsin [384]. The cyclic peptide template was composed of three orthogonal protected lysine residues and one glutamic acid residue. Diversity was introduced by derivatisation of the ε-amino groups with non-amino acid capping functionality following a positional scanning approach. A weak chymotrypsin inhibitor (library 14; IC_{50} 51 μM) was identified following deconvolution and resynthesis [384].

A series of peptidyl α-ketoaldehydes have been synthesized as putative inhibitors of the chymotrypsin-like activity of proteasome [385]. The most potent peptide Z-Leu-Leu-Tyr-COCHO exhibits a K_i value of 3.0 nM (library 15), the lowest so far reported for tripeptidyl aldehyde-based proteasome inhibitors. A novel indanone peptide derivative (library 16; IC_{50} 0.14 μM) was identified as potent competitive inhibitor of the chymotrypsin-like activity of the 20S proteasome from a 400 member library [386]. The SAR indicates a strong preference for lipophilic side-chains L-Leu and D-Leu at the Aa_1 and Aa_2 positions.

Two libraries of α-ketoamide derivatives were generated on pins to map the S and S' subsites of human heart chymase [234]. The libraries were screened directly on the pins by a method that measured enzyme absorption onto the pins and by an enzyme depletion assay. From the first library which was based on the sequence Z-Ile-Aa_2-Aa_1-Phe-CO-Gly-Gly it was found that Pro-Gly provided the P3-P2 combination that gave rise to potent inhibition and maximum selectivity over chymotrypsin (library 17; K_i 1.0 nM). From the second library of peptidyl-3-fluorobenzylpyruvamides, (F)-Phe-CO-Glu-Asp-Arg-OMe was identified as an inhibitor of the chymase (library 18; K_i 1 μM) but it was less active than the corresponding ketoamide. However it exhibited 100-fold selectivity over chymotrypsin [234].

Protease substrate libraries can provide useful information about the enzyme sub-site preferences which can be applied to inhibitor design [268, 387–392]. Researchers at Merck have applied positonal-scanning combinatorial substrate libraries to identify an optimal substrate (Ac-WEHD-ACM) for caspase-1 which is a cysteine protease implicated in inflammation and apoptosis [390, 391]. This information was then used to generate the po-

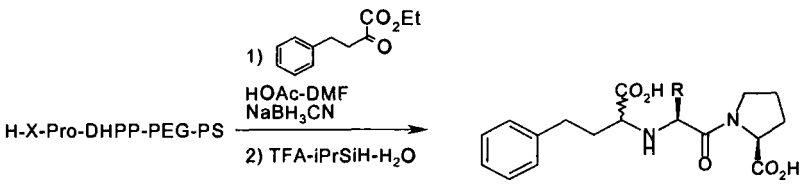
tent peptide aldehyde inhibitor Ac-WEHD-CHO (library 19; K_i 56pM) [391].

METALLOPROTEINASE INHIBITOR LIBRARIES

The inhibition of metalloproteinase enzymes has provided effective therapeutics for the treatment of hypertension (inhibition of ACE) and holds the promise of providing new ways of treating cancer and arthritis (inhibition of MMPs [393, 394]). Proteolysis by this class of enzyme involves activation of the substrate amide carbonyl to hydrolysis by the active site metal ion which is usually a zinc(II) ion. The principle approach taken to discovering MP inhibitors has been to use a zinc binding group (ZBG), such as a thiol, *N*-carboxyalkyl, carboxylic acid or hydroxamic acid as the 'war-head'.

The identification of a potent ACE inhibitor (library 20; K_i~160 pM) from a targeted library of 480 proline derivatives that feature a thiol ZBG demonstrated the power of iterative deconvolution to discover active compounds from a library of modest diversity (*Table 3.3*) [395]. A recent report from the same group at Affymax describes the use of secondary amine encoding tags [199] for the direct identification of the identical compound that had been discovered by iterative deconvolution from the same library [200]. Library preparation involved a key 1,3-dipolar cycloaddition step on solid phase with tags introduced during the split/pool process. It was concluded that the encoding strategy was a more efficient means of extracting information from the ACE inhibitor library than iterative deconvolution since it provided SAR data on a large number of active structures.

The known ACE inhibitor enalaprilat was identified as the most active compound in a mixture of 19 compounds using an affinity selection screening technique [223]. This small targeted library was generated on the solid phase and involved a reductive alkylation step to generate the *N*-carboxyalkyl ZBG functionality (*Scheme 3.5*). The screening process involved the incubation of the library following cleavage in solution with ACE and subsequent size exclusion chromatography to separate unbound compounds



Scheme 3.5. Solid-phase synthesis of ACE inhibitor library.

Table 3.3. METALLOPROTEINASE INHIBITOR LIBRARIES

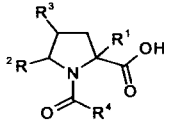
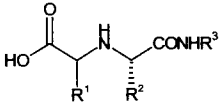
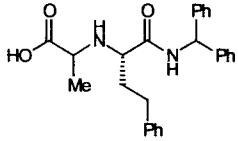
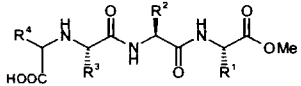
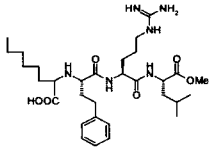
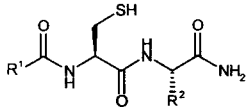
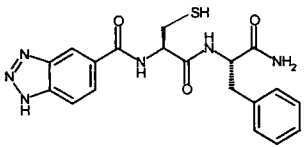
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
20 [200, 395]	Mercaptoacyl proline 	SPMS - Tag (480)		ACE inhibitor K_i = 160 pM
21 [396]	N-carboxyalkyl amino acid 	SPPS (100)		Stromelysin-1 33% inhibition at 100 μM 72% inhibition at 200 μM
22 [215, 216]	N-Carboxyalkyl tripeptide 	SPMS (SpAM) (100 × 200)		Stromelysin-1 IC_{50} = 0.4 μM
23 [397]	Acyl cysteine dipeptide 	SPPS ('Several hundred')		Collagenase IC_{50} = 8 nM Gelatinase IC_{50} = 24 nM

Table 3.3 continued. METALLOPROTEINASE INHIBITOR LIBRARIES

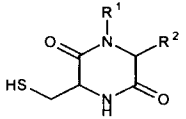
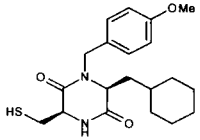
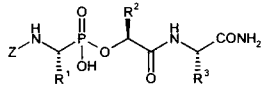
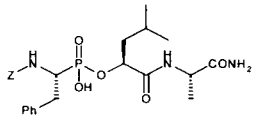
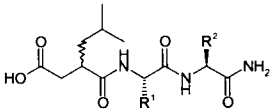
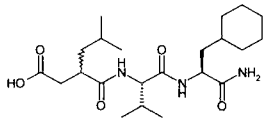
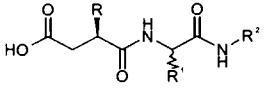
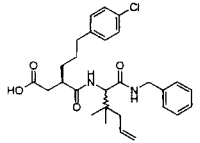
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
24 [398]	2,5-pyrazinedione derivatives 	SPMS - ID (2 × 684)		Collagenase IC_{50} = 30 nM
25 [233]	Peptidyl phosphinic acid ester 	SPMS - ID (540)		Thermolysin K_i = 49 nM
26 [177]	Dipeptide succinates 	SPMS - Tag (324)		Matrilysin IC_{50} = 165 nM
27 [400, 401]	Succinates 	SolPS (> 90)		Gelatinase A IC_{50} = 60 nM

Table 3.3 continued. METALLOPROTEINASE INHIBITOR LIBRARIES

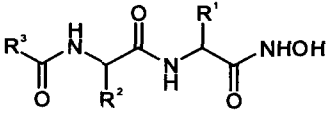
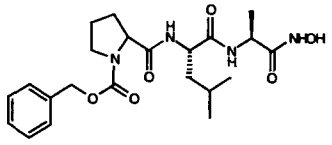
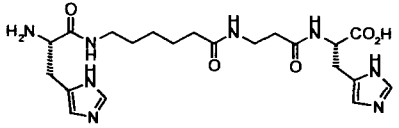
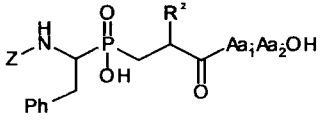
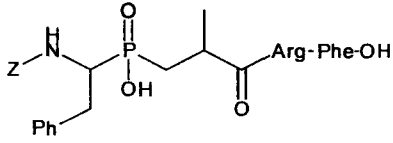
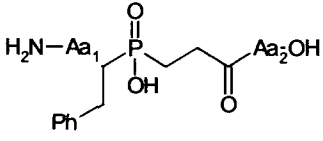
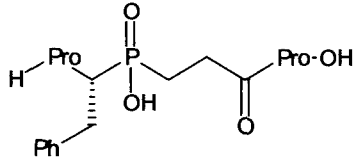
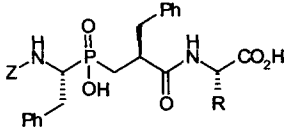
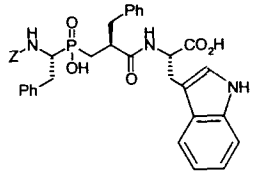
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
28 [404, 405]	Tripeptide hydroxamic acid 	SPMS - ID (500)		Collagenase-1 IC_{50} = 8 μM Stromelysin-1 IC_{50} = 3.5 μM Gelatinase A IC_{50} = 8.0 μM
29 [414]	Tetrapeptide	SPMS - ID (24 × 13,824)		Gelatinase A IC_{50} = 400 μM Gelatinase B IC_{50} = 300 μM
30 [415]	Peptidyl phosphinic acid 	SPMS - ID (ca 800)		Thimet oligopeptidase K_i = 0.16 nM Neurolysin 24–16 K_i = 530 nM
31 [416]	Peptidyl phosphinic acid 	SPMS - ID (ca 800)		Neurolysin 24–16 K_i = 4 nM Thimet oligopeptidase 24–15 K_i = 8000 nM

Table 3.3 continued. METALLOPROTEINASE INHIBITOR LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis[+] (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
32 [417]	Peptidyl phosphinic acid 	SPPS (23)		Endothelin converting enzyme IC_{50} = 1100nM Neutral endopeptidase IC_{50} = 1400 nM

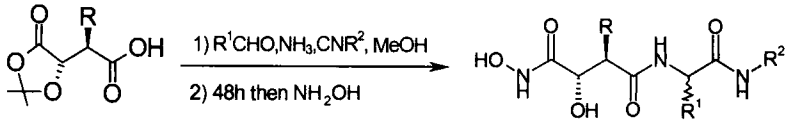
[+]SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis

from protein which were then analysed by electrospray ionisation mass spectrometry. This process was then used to identify active inhibitors from the corresponding dipeptide library of 722 compounds in which the C-terminal proline of enaloprilat was varied (e.g. $Ph(CH_2)_2CH(CO_2H)$-Aa_1-Aa_2-OH) [223].

The *N*-carboxyalkyl ZBG has also been incorporated into combinatorial libraries targeted against the MMPs [215, 216, 396]. The DuPont Merck group prepared a library of over 100 members using parallel solid phase synthesis [396]. This library was targeted to identify variations of the C-terminal amide substituent of the *N*-carboxyalkyl library. Weak inhibition of stromelysin-1 (MMP-3) (library 21; 72% @ 200 μM) was observed for a benzhydryl P2' derivative. In subsequent non-combinatorial studies, introduction of this modification into succinyl hydroxamic acid derivatives gave more potent compounds [396]. A 20,000 member library of *N*-carboxylalkyl tripeptides has been prepared by solid phase synthesis following a combination of split / pool and indexed techniques (SpAM) [215, 216]. The library was screened against fibroblast collagenase (MMP-1), gelatinase A (MMP-2) and MMP-3 as 100 mixtures each of 200 compounds [215]. Due to the indexed library approach for R^4 and R^3 introduction, SAR information was obtained directly for the P1 – P1' modifications and this was consistent with literature data. Deconvolution resulted in the identification of active inhibitors (library 22) [215, 216].

Cysteine containing dipeptides were identified by Glaxo as possessing MMPI activity and parallel solid phase synthesis of compounds RCO-L-Cys-Aa-NH_2 led to the identification of inhibitors selective for MMP-1 over gelatinase B (MMP-9) (e.g. CF_3CO-L-Cys-L-Phe-NH_2; MMP-1 IC_{50} 40 nM; MMP-9 IC_{50}>1,000 nM) and for MMP-9 over MMP-1 (e.g. $PhCH_2CH_2CO$-L-Cys-L-Phe-NH_2; MMP-1 IC_{50} 3,498 nM; MMP-9 IC_{50} 38 nM) as well as broad spectrum inhibitors (library 23) [397]. A recent report from the Affymax group describes the corresponding cysteine based diketopiperazines which were prepared by solid-phase synthesis on Tentagel™ resin as libraries targeted for MMP inhibition (library 24) [398].

Affymax have explored libraries of MMPIs focused around ZBGs other than thiols, including phosphonates [233, 399] and more recently carboxylates [177]. In the former study compounds were screened against the bacterial enzyme thermolysin whilst attached to beads following an enzyme depletion protocol. Iterative deconvolution was then employed for identification of actives (library 25; K_i 49 nM) [233]. In the latter study, a library of 324 dipeptide succinates was prepared on Tentagel™ beads following a 'split-mix' protocol using secondary amine encoding tags [199]. Following photolytic cleavage the library was screened against matrilysin (MMP-7) in a novel

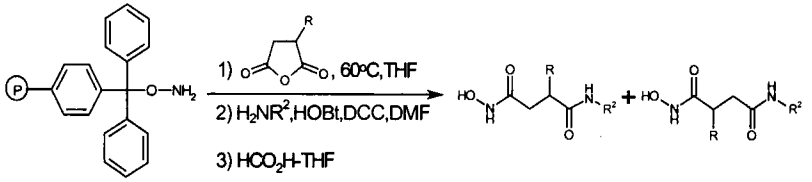


Scheme 3.6. One-pot five-component solution phase synthesis of MMP inhibitors.

high-density nanowell array format. This study provided direct SAR for the P2' and P3' amino acids and the re-synthesis of two inhibitors led to the identification of one diastereoisomer of (*R,S*)-3-isobutyl-L-Val-L-homo-Phe-NH_2 (R^1=iPr, R^2=CH_2CH_2Ph) as a potent MMP-7 inhibitor (library 26; IC_{50} 165 nM) [177].

Related succinate inhibitors have been prepared by parallel solution phase synthesis using the Ugi multi-component reaction [400, 401]. The carboxylic acid products, whilst active as MMP inhibitors (library 27; MMP-2 IC_{50} 60 nM), were converted into the more potent hydroxamic acids [402]. The transformation to the hydroxamic acids is not a very efficient process and requires product purification by preparative HPLC which, however, does separate the diastereoisomers. In the special case where a hydroxy group is present alpha to the hydroxamic acid the desired hydroxamates were obtained directly in a one-pot five-component condensation (*Scheme 3.6*) [400, 402]. The introduction of the hydroxamate ZBG into succinyl based MMPIs has been achieved albeit as a mixture of regioisomers by the reaction of succinic anhydrides with an O-hydroxylaminetrityl resin (*Scheme 3.7*) [403]. The use of hydroxylamine presenting resins for the synthesis of MMPIs had been reported earlier [404, 405] and subsequently has been the subject of intense interest [406–413]. In particular such resins have been utilised in the preparation of a 500 member library of tripeptide hydroxamates Z-Aa_3-Aa_2-Aa_1-NHOH (library 28) [404, 405].

From the screening of a large library of tetrapeptides the compound H-His-ε-Ahx-β-Ala-His-OH was identified as a weak inhibitor of MMP-2 (IC_{50} 400 μM) and MMP-9 (IC_{50} 300 μM) (library 29) [414]. Phosphinic acid derivatives have been prepared by solid-phase methods and screened



Scheme 3.7. Use of O-hydroxylamine resin for preparation of MMP inhibitors.

against thimet oligopeptidase, neurolysin and endothelin converting enzyme (libraries 30–32) [415–417].

NON-PROTEOLYTIC ENZYME INHIBITORS

The screening of a synthetic peptide library led to the identification of the hexapeptide His-Cys-Lys-Phe-Tyr-Tyr as an inhibitor of HIV integrase (library 33; IC_{50} 2.0 μM) (*Table 3.4*) [418]. This enzyme is required for integration of the viral DNA into the host chromosome and is essential for viral replication. The peptide inhibits integrin-mediated processing and integration of other retroviral integrases and is believed to bind to a region conserved among the integrase proteins [418].

Two libraries (289 and 256 members) were constructed by preparing tripeptide derivatives of 4-carboxybenzenesulphonamide as carbonic anhydrase II (CAII) inhibitors [218]. Inhibitors of this enzyme are known to be useful in ameliorating the symptoms of glaucoma. The screening by a ESI-FTICR mass spectrometry method identified (L)-Leu-(L)-Leu-β-Ala as a tight binding inhibitor (library 34; binding constant K_b=14 nM).

Binary encoding technology [71–73] has been used to identify selective CAII inhibitors from two libraries (6,727 members and 1,143 members) [419, 420]. The first library was composed of acyclic and cyclic amino acids and the second library was composed of dihydrobenzopyrans. The arylsulphonamide moiety, a known pharmacophore for CAII inhibition, was included in order to bias the library. The active compounds identified from the first library exhibited a preference for lipophilic groups at R^2 (library 35; K_d 4 nM) [419]. Again active members from the 1143 dihydrobenzopyran compound library were 4-carboxybenzene sulphonamide derivatives (library 36; K_d 15 nM) [420].

Three 4-thiazolidinone libraries were prepared and assayed for inhibition of the enzyme cyclooxygenase-1 (COX-1), a key enzyme in the conversion of arachidonic acid to prostaglandins [421, 422]. From the carboxylic acid, ester and carboxamides libraries only the methyl ester library showed significant activity. A series of three rounds of testing and deconvolution led to a compound (library 37; IC_{50} 3.7 μM) with equivalent *in vitro* activity to the commercially available COX-1 inhibitors ibuprofen and phenylbutazone.

The screening of 810 compounds for inhibitors of the enzyme phospholipase A_2 (PLA_2) was conducted in order to compare several different pooling strategies for combinatorial libraries. PLA_2 catalyses the hydrolysis of phospholipids to arachidonic acid. It was found that position scanning and all iterative strategies tested, with the exception of 'hard pooling', identified the same inhibitor of PLA_2. The selected compound has an IC_{50} of 5 μM

Table 3.4. NON PROTEOLYTIC ENZYME INHIBITOR LIBRARIES

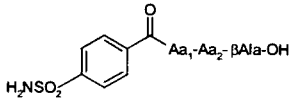
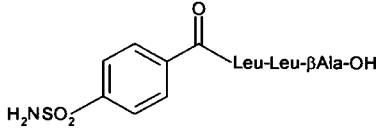
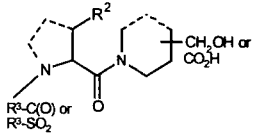
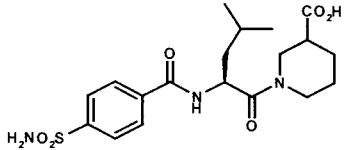
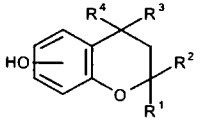
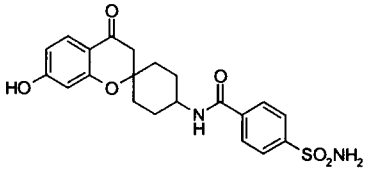
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
33 [418]	Hexapeptide $H\text{-}Aa_1\text{-}Aa_2\text{-}Aa_3\text{-}Aa_4\text{-}Aa_5\text{-}Aa_6\text{-}NH_2$	SPMS - ID (> 2 million)	H-His-Cys-Lys-Phe-Tyr-Tyr-NH_2	HIV integrase IC_{50} = 2.0 μM
34 [218]	Peptidyl carboxysulphonamide 	SPMS - ID (545)		Carbonic anhydrase II K_b = 14 nM
35 [419, 420]	Acylpiperidine 	SPMS - Tag (6,727)		Carbonic anhydrase II K_d = 4 nM
36 [420]	Dihydrobenzopyran 	SPMS - Tag (1,143)		Carbonic anydrase II K_d = 15 nM

Table 3.4 continued. NON PROTEOLYTIC ENZYME INHIBITOR LIBRARIES

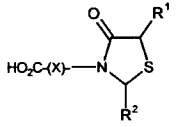
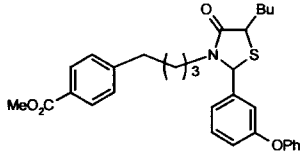
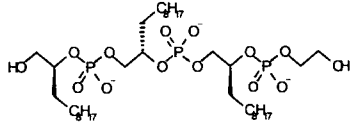
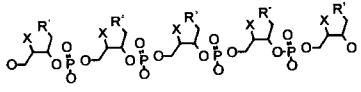
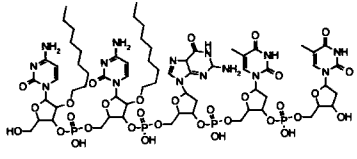
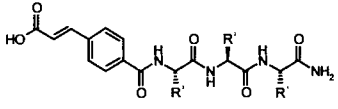
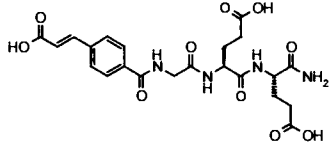
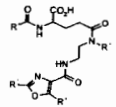
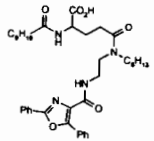
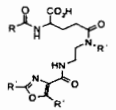
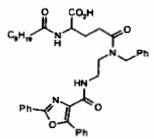
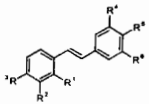
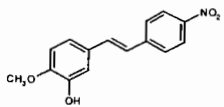
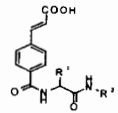
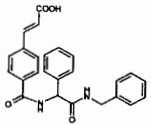
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
37 [422]	Thiazolidinone 	SPMS - ID (1,620)		Cyclooxygenase I IC_{50} = 3.7 ± 1.4 μM
38 [423]	Phosphate diester R-OP(O)(O^-)O-X_1-OP(O)(O^-)O-$(CH_2)_2$-OH	SPMS - ID (810)		Phospholipase A_2 (PLA_2) IC_{50} = 5 μM
39 [424]	Pentahydroxyethylphosphate 	SPMS (SURF) (12 × 1,728)		PLA_2 IC_{50} = 2 μM
40 [208]	Peptidyl cinnamic acid 	SPMS - Tag (RF) (125)		Protein Tyrosine phophatase (PTPIB) IC_{50} = 44 nM K_i = 79 nM

Table 3.4 continued. NON PROTEOLYTIC ENZYME INHIBITOR LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
41 [425]	Oxazole amide 	SPPS (18)		Cdc25A, -B, -C (DSPases) $K_i \sim 10\ \mu M$ PTPIB (PTPases) $K_i = 0.85\ \mu M$
42 [426]	Oxazole amide 	SPPS (18)		PP2A (PSTPases) $IC_{50} < 100\ \mu M$
43 [427, 428]	Hydroxystilbene 	SPPS (22)		Protein tyrosine kinase $IC_{50} = 250\ \mu M$
44 [429]	Cinnamic acid derivatives 	SPPS (18)		Hematopoietic protein tyrosine phosphatase (HePTP) $IC_{50} = 3.9\ \mu M$

⁺SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis

and is a trimeric repeat of monomers with hydrophobic alkyl chains linked via phosphate diester groups (library 38) [423]. In an earlier study the ISIS group used an iterative method of synthesis and screening, SURF, to select a PLA_2 inhibitor from a phosphodiester-based library [424]. Twelve subsets (each containing 1,728 compounds) of pentamers were synthesized and screened. Deconvolution of the library led to the identification of the inhibitor nonylC-nonylC-dG-T-T (library 39; IC_{50} 2 μM) [424].

Protein tyrosine phosphatases play a key role in regulating phosphorylation levels during signal transduction events. The protein tyrosine phosphatase PTP1B was the target for a 125-member tripeptide substituted cinnamic acid library (library 40) [208]. The library was prepared by the split-pool protocol using radio frequency transponders to encode each member of the library. Glutamate residues were always present in the first and second position of the most active compounds. The observation is consistent with PTP1B structural data in which numerous positively charged residues surround the active site. It is suggested that electrostatic interactions occur between the glutamic acid carboxylate and the surface residues of the protein [208].

Recently, Lazo and co-workers reported a combinatorial library of PTP1B inhibitors based on a pharmacophore derived from the structure-activity relationships for several natural product inhibitors of PSTPases such as okadaic acid, microcystins and calyculin A [425, 426]. The pharmacophore model involved a carboxylate, a non polar aromatic group and hydrogen-bond acceptors and donors and was used as a platform for functional group variation. Among the 18 library compounds generated by parallel solid-phase chemistry, a non-competitive inhibitor for PTP1B was identified (library 41, K_i=0.85 μM) [425] and as well a serine/threonine phosphatase inhibitor (library 42, $IC_{50} < 100$ μM) [426].

A small library based on the hydroxy-*trans*-stilbene core structure (library 43) was synthesized and screened for selective disruption of specific tyrosine phosphorylation events during B cell activation [427, 428]. One member of the library, 3-hydroxy-4-methoxy-4'-nitro-*trans*-stilbene was found to selectively inhibit the phosphorylation of several proteins in the B cell receptor mediator cascade while not affecting other cellular phosphorylation events [428].

A library of cinnamic acid derivatives was prepared on solid phase using a four-component Ugi condensation [429]. The results from screening compounds against the hematopoietic protein tyrosine phosphatase (HePTP) indicate that the variations in R^2 has a more pronounced effect on potency than do changes in R^1. The most potent compound in the series was found for R^1=phenyl and R^2=benzyl (library 44; IC_{50} 3.9 μM) [429].

It is clear that targeted library methods are an effective means for discovering enzyme inhibitors especially when used in conjunction with structure-based design methods. To date the use of solid phase synthesis has predominated over solution based methods for the preparation of targeted (focused) libraries. Key strategic decisions in the synthetic planning are the attachment point to the resin and whether to use parallel or 'split-mix' methods. Attachment via the 'war-head' group as used in the synthesis of certain libraries (e.g. libraries 2 and 21) enables greater diversity of substitution. Conversely, attachment elsewhere allows the generation of libraries to discover novel 'war-heads' but this approach has not been explored to any great extent. An advantage of parallel synthesis is that complete SAR data are obtained. However, it is now clear that similar data can be obtained from the first round of screening of 'split-mix' libraries either by tag encoding [177, 200] or indexing methods [215]. Whilst, in some reports on protease inhibitor libraries the compounds were screened whilst still attached to the solid phase [233, 234, 375–377], the favoured methods of *in vitro* library testing involve screening of compounds in solution. However, recently it has been demonstrated that the serine protease trypsin can cyclise certain polymer supported peptidic amino esters and it is suggested that this will provide a new paradigm for enzyme inhibitor discovery from combinatorial libraries [430].

RECEPTOR ANTAGONISTS AND OTHER PHARMACOLOGICAL AGENTS

In this section compounds other than enzyme inhibitors are considered, the majority of which are receptor antagonists (*Tables 3.5–3.8*). Cell receptors have long been a target for therapeutic agents. For those receptors which accept peptides as the natural agonist the use of libraries to search for new agonists or antagonists is a standard method of investigation to which combinatorial methodology is ideally directed. Using any of the approaches described above a biological target can be investigated with up to millions of peptide combinations and active compounds can be identified (libraries 45–52) (*Table 3.5*) [170, 431–437].

PEPTIDIC COMPOUNDS

The question of whether to prepare focused or diverse prospecting libraries was highlighted in the previous sections. In situations other than when a randomly selected library is being screened in order to identify an initial hit, there is always likely to be some data to guide the design of target molecules,

Table 3.5. PEPTIDE-BASED LIBRARIES

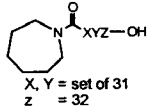
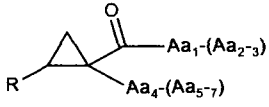
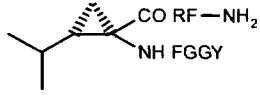
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
45 [170]	Bead bound Pentapeptide Aa_1-Aa_2-Aa_3-Aa_4-Aa_5-Tg	SPMS - ID (1,024)	YGGFL-Tg Testing on bead	Binding to β endorphin $\alpha\beta$
46 [431]	cyclic hexapeptide c-(Pro-Aa_1-Trp-Aa_2-Thr-Phe)	SPPS (~ 20)	c-(Pro-1-Nal-Trp-*p*-F-Phe-Thr-Phe)	NK_1 receptor IC_{50} = 2 nM
47 [432]	Acylated hexapeptide Ac-^{D}BHG-Leu-Aa_1-Aa_2-Ile-Trp-OH	SPMS - ID (19 × 361)	Ac-^{D}BHG-Gln-Asp-Val-Ile-Trp	Endothelin ET_A 1.7 nM
48 [433]	Urethane capped tripeptide 	SPMS - ID (30,752)	C_6H_{12}N-CO- Leu-(D)-Trp-(D)-Phe	Endothelin ET_A IC_{50} = 2.5 nM
49 [434]	Cyclic pentapeptide c-(Aa_1-Aa_2-Aa_3-Aa_4-Aa_5)	SPMS - ID (82,944)	BQ-123 c(P-^{D}V-L-^{D}W-^{D}D)	Endothelin ET_A

Table 3.5 continued. PEPTIDE-BASED LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis*[+] *(Lib. Size)*	*Active Compound Identified*	*Target and Activity*
50 [435]	Pentapeptide Aa_1-Aa_2-Aa_3-Aa_4-Aa_5	SPMS - Ind (2.5×10^6)	Asn-Trp-Met-Met-Phe	Glycosonal phosphoglycerate kinase of *Trypanosoma Brucei* IC_{50} = 80 μM
51 [436]	Pentapeptide His-Aa_1-Aa_2-Ser-Aa_3	SPMS - ID (6,859)	His-His-Arg-Ser-Tyr	Ligands specific for Sugar Binding Site of Concanavaldin A
52 [437]	Constrained heptapeptide 	SPPS (96)		μ & δ opiod receptor K_i = 6.3 nM

[+]SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis

whether it be from SAR data already accumulated or from some measure of diversity known (or believed) to be appropriate for the system under investigation. Two studies using combinatorial libraries to exploit lead endothelin ETa antagonists illustrate this point [433, 434]. The antagonists BQ-123 and FR-139,312 had been developed by scientists at Banyu and Fujisawa respectively using 'classical' medicinal chemistry methods which followed the isolation of the original cyclic pentapeptide lead from microbial sources. The Pfizer group investigated the Fujisawa compound, a *N*-capped tripeptide, by using solid phase chemistry to prepare their compounds as mixtures followed by subsequent iterative deconvolution methods. They chose to prepare the libraries as pools of ~900 compounds which gave them 3 active pools from an original 30. Deconvolution led to the desired Fujisawa compound along with some novel more potent analogues (library 48) [433]. Interestingly, as highlighted by the investigators, one of the initial active pools led to the preparation of subsequent compounds with little activity in the second round iterative process. This is not an uncommon finding with deconvolution methods, the presence of 'false positives', and is one reason why the current trend is to work either with smaller mixtures (~30 compounds) or to use parallel synthetic methods to prepare single compounds. The Spatola group identified the cyclic pentapeptide BQ-123 using a positional scanning approach which provided the active compound very directly (library 49) [434].

These studies show the use of mixed peptide libraries in particularly good light. Possibly the inherent extra diversity with cyclic peptides or the incorporation of non-natural peptides or some non-peptidic component makes the various pools of mixtures distinctly different so as to allow rapid deconvolution. This is not always the case with some peptide libraries for reasons discussed above so the optimal size and composition of any particular library is still a matter of debate, although there are still persuasive advocates for the use of very large peptide libraries. Due to the ease of amide bond formation on the solid-phase many libraries feature one or more natural or non-natural amino acid residues (libraries 53–61) (*Table 3.6*) [438–445]. However, the inherent disadvantages of many peptidic compounds as potential drugs [363, 364] has led to the exploration of other oligomeric units which mimic peptides in the ability to project side-chain groups into receptor binding pockets. Vinylogous sulphonamides [446–450], carbamates [451] and ureas [452] have been investigated, though oligomers based on *N*-substituted glycines ('peptoids') have been the most widely explored [214, 364, 453–461].

Table 3.6. AMINO ACID-BASED LIBRARIES

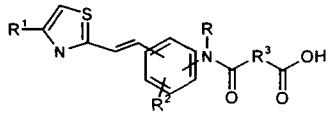
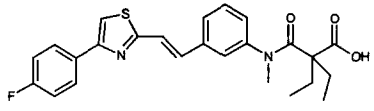
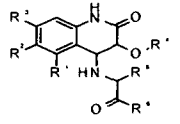
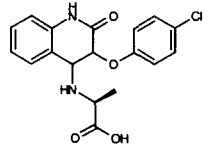
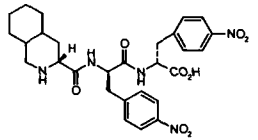
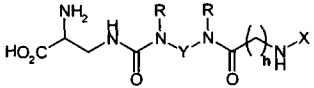
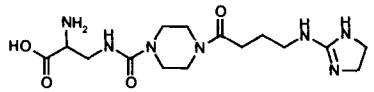
Library No. [Reference]	*General Structure*	*Synthesis+ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
53 [438]	Thiazolostyrene 	SolMS (700)		Leukotriene D_4 antagonist
54 [439]	Tetrahydroquinolone 	SPMS - ID (4,260)		Antagonist at K- and μ-opioid receptors
55 [157]	Tripeptide Aa_1-Aa_2-Aa_3	SPMS - Ind (15,625)		V_2-Vasopressin IC_{50} = 63 nM
56 [440]	Substituted amino acid 	SPPS (~ 300)		$\alpha v\beta_3$ integrin IC_{50} = 1.1 nM

Table 3.6 continued. AMINO ACID-BASED LIBRARIES

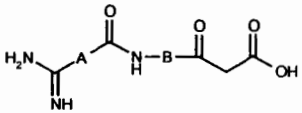
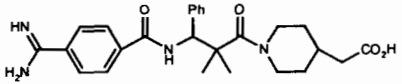
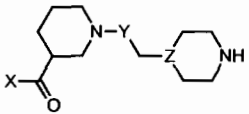
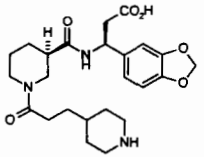
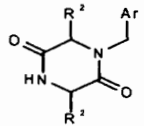
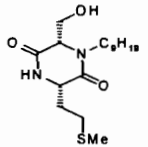
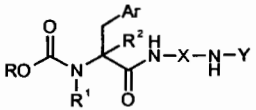
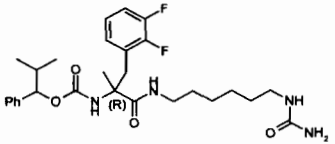
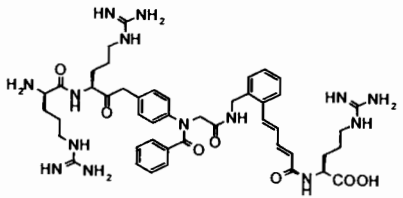
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
57 [441]	Acylated amidine 	SPPS (27)		GP11b/111a receptor IC_{50} = 190 nM (collagen induced platelet agg)
58 [442]	Nipecotic acid 	SPPS (250)		GPIIb / IIIa Fibrinogen receptor IC_{50} = 20 nM Plat Agg IC_{50} = 0.5 nM binding
59 [443]	Diketopiperazine 	SPMS - ID (~ 1,000)		NK_2 receptor IC_{50} = 313 nM
60 [444]	Substituted phenylalanine 	SolPS (256 / 64)		NK_3 receptor

Table 3.6 continued. AMINO ACID-BASED LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis*[+] *(Lib. Size)*	*Active Compound Identified*	*Target and Activity*
61 [445]	Pentapeptide (D)-Arg-Arg-Aa_1-Aa_2-Arg	SPMS - ID (> 300)		Bradykinin Receptor K_i = 36 nM

[+]SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis

PEPTOIDS

Peptoids have now been well described [214, 364, 453–461]. Their application has been advanced by two of the themes outlined earlier: an easily applied solid phase method of synthesis which is readily adapted to automation along with the ability to introduce a greater degree of diversity than is available with common amino acids. For instance, different substituents on a tripeptoid base scaffold has led to the identification of selective antagonists against the G-protein effector linked adrenergic α-1 and μ-opiate receptors from the same 5,000 member library (library 62) (*Table 3.7*) [455]. Iterative deconvolution was used to identify antagonists of these two receptors [455]. The same adrenergic α-1 antagonist identified by the Chiron group was discovered by workers at Merck using the 'Spatially Arrayed Mixture' (SpAM) approach (library 63) [214]. The advantage of the SpAM technique is that less synthesis was required and that greater SAR information can be obtained from the initial screening. From the screening of peptoid libraries a novel urokinase receptor antagonist (library 64) [456] and a ZAP-70 SH2 domain antagonist (library 65) [457] have been identified. The peptoid concept is not seemingly restricted to small oligomeric units. The Chiron group have recently described the use of a library of much larger oligomers (30–40 mers) in the identification of gene delivery agents that condense plasmid DNA and hence may mediate gene transfection [461]. Workers at ISIS have identified leukotriene (LTB_4) antagonists from a large phosphodiester oligomer library (library 66) [424] and more recently have reported the discovery of anti-microbials from a polyamine library (library 67) [462] and from a hydroxamate library (library 68) [412]. This illustrates how one company appears to be re-directing their combinatorial library production away from oligomeric compounds to small molecules. This would appear to be the trend throughout the pharmaceutical industry.

NON-PEPTIDIC COMPOUNDS

Although compounds derived directly from natural substrates or oligomeric compounds can provide interesting lead candidates, the development of receptor binding antagonists based on structures with no similarity has always been pursued by medicinal chemists. Once again combinatorial approaches have contributed to these investigations. The 'scaffold and side-chain' concept, based on some preferred core molecule mentioned earlier is the basis for many approaches. By the judicious choice of a suitable core molecule with a series of handles the attachment of side-chains displaying a range of functionalities can give rise to a whole family of structures which can be

Table 3.7. PEPTOID AND OLIGOMER-BASED LIBRARIES

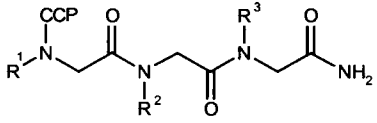
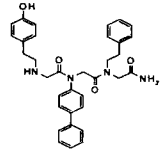
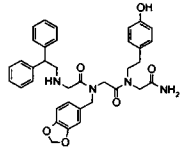
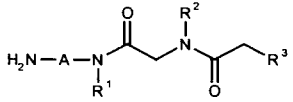
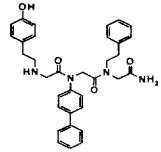
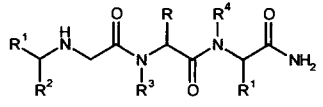
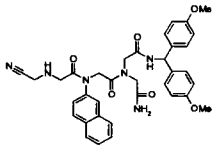
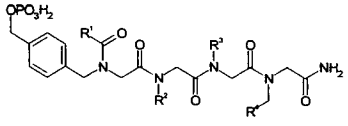
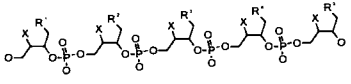
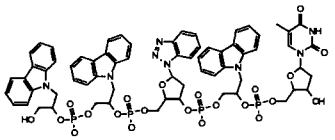
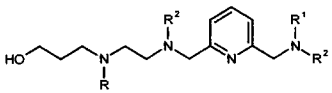
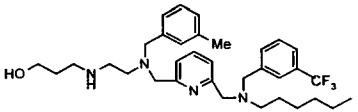
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
62 [455]	Peptoid 	SPPS (~ 5,000)		α_1 -adrenergic receptor K_i = 5 nM
				μ-opioid receptor K_i = 6 nM
63 [214]	Peptoid 	SPMS (SpAM) (9,216)		α_1- adrenergic receptor K_i = 5 nM

Table 3.7 continued. PEPTOID AND OLIGOMER-BASED LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis+ (Lib. Size)*	*Active Compound Identified*		*Target and Activity*
64 [456]	Peptoid 	SPPS (~ 60)			Urokinase receptor IC_{50} = 33 nM
65 [457]	Peptoid 	SolPS (27)	$R_1 = C_5H_{11}$ R_3 = Isobutyl	$R_2 = C_2H_5$ R_4 = Isobutyl	ZAP-70 SH2 domain IC_{50} = 25 μM
66 [424]	Pentahydroxyethylphosphate 	SPMS (SURF) (1,728 × 12 ~ 20,000)			LTB_4 IC_{50} = 0.68 μM
67 [462]	Polyamine 	SolMS (1,638)			Antimicrobial MIC = 1–5 μM *S.Aureus* *S.Pyrogenes*

+SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis

used to explore receptor binding. In essence this is how medicinal chemistry has always been applied: the combinatorial approach is to develop chemistry and techniques to prepare these targets in families of compounds and then integrate the synthesis with the assay and informatics.

A justification of working with 'privileged structures' is that nature itself seems to have applied this concept in the design of receptor binding entities [263]. The most striking example is that compounds based on a benzodiazapine core have been used as antagonists for a whole series of G-protein coupled receptors (GPCRs) and are used clinically for an array of different disorders. Although this may be a consequence of a common evolutionary ancestor for all these receptors, the high preponderance of clinically significant GPCR linked pharmacological effects can provide some justification for the core molecule approach. As indicated above, the benzodiazapine skeleton has become a favoured target for combinatorial methods with the development of some very elegant syntheses which are amenable to the generation of families of variants (libraries 69 and 70) (*Table 3.8*) [25, 26, 273–281, 458, 463].

Other popular scaffolds have been derivatives of biphenyls which are now readily prepared using solid phase methods [265, 464–469]. A solution phase approach has been to react a polyfunctionalised core containing reactive groupings – acid chlorides or isocyanates are easily prepared examples – with a mixture of reagents. One of the earliest synthetic examples showed the use of cubane tetracarboxylic acid chloride [151]. Two recent methods have used functionalised xanthene (library 71) [470] or diphenylmethane cores (library 72) [471] to identify DNA and urokinase receptor antagonists respectively.

Historically the majority of successful pharmaceuticals have been based on core heterocycles. Few heterocycles can be prepared using the linear methods developed for oligomeric compounds so classical synthetic methodology has been adapted to allow preparation of such compounds in library formats. Various methods have now been described in which libraries of heterocyclic compounds, including those with pharmaceutically interesting structures, can be prepared, especially on solid phase. A typical example is the preparation of a family of quinazolinediones from a urethane linked anthranilic acid derivative [472]. Anthranilic acids are familiar starting materials in many heterocyclic series and a diverse range of them are available. In this example the anthranilic acid can be attached to the resin via the amino group, coupled to a range of amines and then released from the support with concomitant cyclisation simply by heating (*Scheme 3.8*) [472]. With simple equipment the cleavage step can be performed directly in vessels suitable for distributing in the assay format.

Table 3.8. NON-PEPTIDIC-BASED LIBRARIES

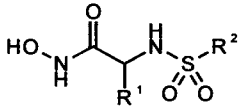
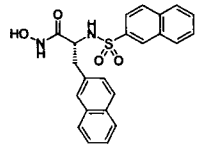
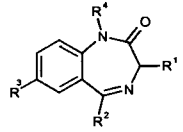
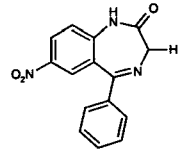
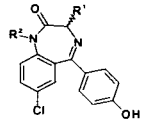
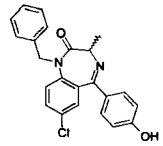
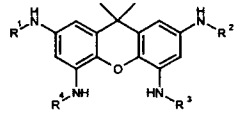
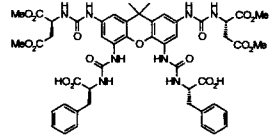
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
68 [412]	Sulphonamidohydroxamic acid 	SPPS (ca. 1,200)		Anti-microbial MIC = 0.7 -1.5 μM *Escherichia coli*
69 [280]	Benzodiazepine 	SPPS (40)		Inhibition of Fluoronitrazepam binding @ bovine cortex :IC_{50} = 15nM
70 [463]	Benzodiazepine 	SPPS (1,680)		Blocking of DNA - Protein interactions IC_{50} < 20 μM
71 [470]	Xanthene tetra-amide 	SolMS - ID (2,080)		DNA binding

Table 3.8 continued. NON-PEPTIDIC-BASED LIBRARIES

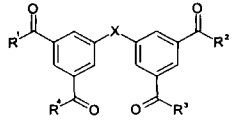
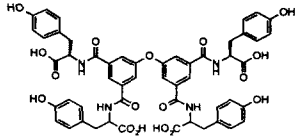
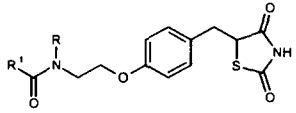
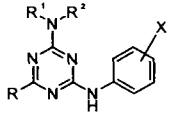
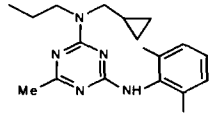
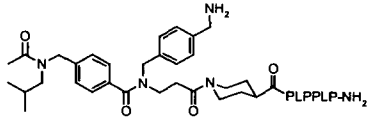
Library No. [Reference]	*General Structure*	*Synthesis+ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
72 [471]	Tetra-amide 	SolMS - ID (~ 10,000)		Urokinase receptor binding 3 μM
73 [473]	Thiazolidinediones 	SPPS (24)	$R_1 = C_8H_{17}$ R = Me	Peroxisome Activated Receptor antagonist K_i = 18 nM
74 [474]	Triazines 	SolPS (350)		Corticotropin-Releasing Factor (CRF) IC_{50} = 5 nM
75 [475]	Triamidohexapeptide amine Ac-X_1-X_2-X_3-PLPPLP-NH_2	SPMS - ID (1.1×10^6)		Src SH_3 domain K_d3.4 μM

Table 3.8 continued. NON-PEPTIDIC-BASED LIBRARIES

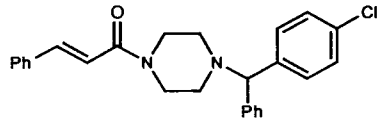
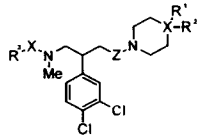
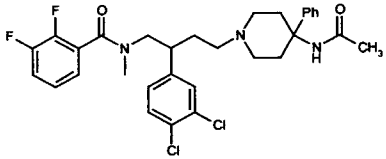
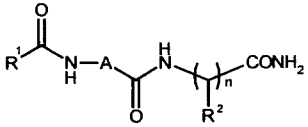
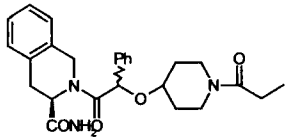
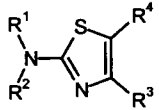
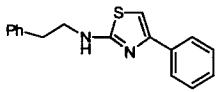
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
76 [156]	Amides RNR^2COR^3	SolMS - Ind (1,600)		NK_3 receptor IC_{50} = 60 μM
77 [476]	Dichlorophenyl piperidine 	SolPS (4 × 295)		NK_2 receptor IC_{50} = 2.5 nM in CHO cell
78 [212]	Acylamide 	SPMS (100 × 100)		NK_2 receptor IC_{50} = 59 nM
79 [477]	Thiazoles 	SolPS (20)		Anti-inflammatory

Table 3.8 continued. NON-PEPTIDIC-BASED LIBRARIES

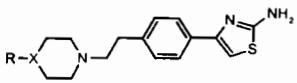
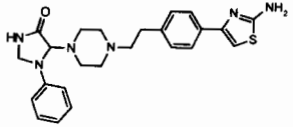
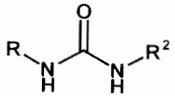
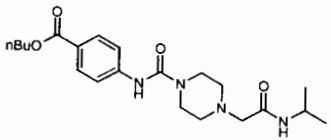
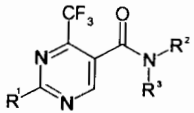
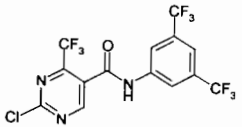
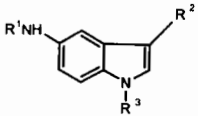
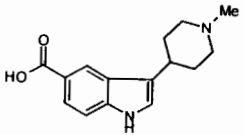
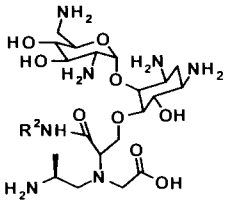
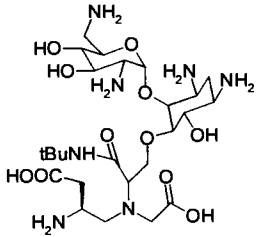
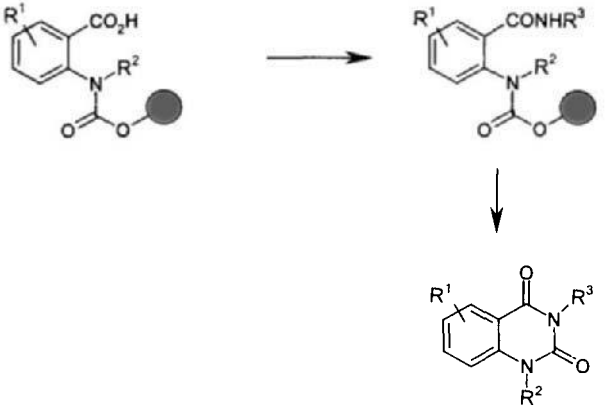
Library No. [Reference]	*General Structure*	*Synthesis⁺ (Lib. Size)*	*Active Compound Identified*	*Target and Activity*
80 [476]	Aminothiazole derivatives 	SolPS (60)		HSV-1 plaque reduction *in vitro* IC_{50} = 1.6 μM
81 [478]	Substituted ureas 	SolMS - ID (18)		HRV-14 IC_{50} = 1.8 μM TC_{50} > 40 μM
82 [479]	Pyrimidine amides 	SolPS (> 80)		Inhibition of NF-kB Gene transcription factor K_i = 0.05 μM
83 [480]	Indoles 	SolPS (~ 60)		Serotonin receptors IC_{50} = 2.8 nM $5HT_{1F}$ = 6.1 nM $5HT_{1A}$

Table 3.8 continued. NON-PEPTIDIC-BASED LIBRARIES

Library No. [Reference]	*General Structure*	*Synthesis*[+] *(Lib. Size)*	*Active Compound Identified*	*Target and Activity*
84 [332]	Neomycin B mimetics 	SPPS (PEG linked) (52)		Rev responsive unit of HIV mRNA IC_{50} = ~200 μM

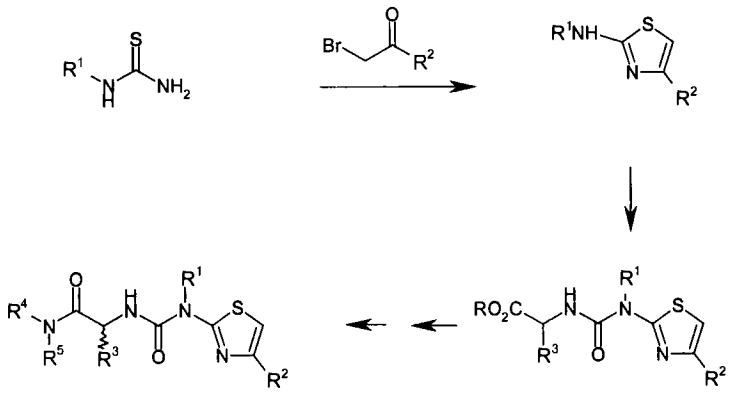
[+]SPPS - Solid phase parallel synthesis; SPMS - Solid phase split-mix synthesis; Ind - Indexed; ID - Iterative deconvolution; Tag - Tag encoded; SolPS - Solution phase parallel synthesis; SolMS - Solution phase mixture synthesis



Scheme 3.8. Solid-phase route to quinazolinediones.

A whole range of other heterocycles have now been prepared using solid phase or solution phase technology (libraries 73–83) [156, 212, 473–480]. These include diketopiperazines [398, 443, 481], quinazolines [472, 482–484], pyrazoles [485, 486], quinolines [487], benzimidazoles [488], quinolones [328, 489, 490], indoles [491–494], pyridines [495], benzofurans [496–499] and hydantoins [280, 281, 500–504] among other heterocycles: many of those that have historically produced biologically valuable lead compounds. A representative example is the synthesis of antibacterial quinolones related to Ciprofloxacin using the 'diversomer' apparatus [328, 492]. Much of the diversity in these particular compounds is introduced via readily available amines. The application of multicomponent reaction procedures to solid phase substrates can also be used to prepare heterocycles such as imidazoles [505] and dihydropyrimidines [506]. Although the biological assay of many of these novel libraries has not yet been described in detail, there is no doubt that the methodology is now available to the medicinal chemist for the preparation of a whole range of heterocyclic libraries.

Potentially useful heterocyclic libraries can also be prepared by the application of solution-phase combinatorial chemistry. Although multiple reactions in solution have often been complicated by the difficulties with liquid-liquid extractions the introduction of solid scavengers and equipment to automate these extraction processes allows hundreds of reactions to be managed simultaneously. A typical example is a series of aminothiazole derivatives which has been prepared starting from acyclic precursors (*Scheme 3.9*). Naturally one-pot multicomponent condensations such as the Ugi (library 84) [332], Passerini or Biginelli reactions present one of the simplest



Scheme 3.9. Solution-phase combinatorial sysnthesis of substituted thiazoles.

methods of preparing a variety of new entities from a range of uncomplicated starting materials. These reactions can be readily adapted to prepare a diverse set of compounds, either as individual compounds or as a series of mixtures [57–61].

Tables 3.5–3.8 illustrate some of the libraries prepared by combinatorial methods which have been assayed against a range of receptor binding targets. Although with smaller libraries there is often a fine distinction between what is or is not a combinatorial approach, when in doubt we have included those syntheses which could in theory be expanded to prepare much larger compound collections.

SUMMARY

The question 'will combinatorial chemistry deliver real medicines' has been posed [96]. First it is important to realise that the chemical part of the drug discovery process cannot stand alone; the integration of synthesis and biological assays is fundamental to the combinatorial approach. The results presented in *Tables 3.1* to *3.8* suggest that so far smaller directed combinatorial libraries have obtained equivalent results to those obtained previously from traditional medicinal chemistry analogue programs. Unfortunately, because of the long time it takes to develop pharmaceutical drugs there are no examples yet of marketed drugs discovered by combinatorial methods. There are interesting examples where active leads have been discovered from the screening of the same library against multiple targets (e.g. libraries 13, 39, 43, 66, 71 and 76). It is now possible to handle much larger libraries

of non-oligomeric structures and the chemistry required for such applications is becoming available. Whether combinatorial approaches can also be adapted to deal with all the other requirements of a successful pharmaceutical (lack of toxicity, bioavailability etc.) is open to question but there are already examples such as cassette dosing [235–237]. However we can still be optimistic about the possibility of larger libraries producing avenues of investigation for the medicinal chemist to develop into real drugs. Combinatorial chemistry is an important tool for the medicinal chemist.

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ABBREVIATIONS

ACE - Angiotensin converting enzyme
CAII - Carbonic anhydrase-II
COX-1 - Cyclooxygenase-I
GPCR - G-protein coupled receptor
MMPs - Matrix metalloproteinases
MMP-1 - Fibroblast collagenase
MMP-2 - Gelatinase A
MMP-3 - Stromelysin-1
MMP-7 - Matrilysin
MMP-9 - Gelatinase B
MMPI - Matrix Metalloproteinase Inhibitor
MP - Metalloproteinase
SAR - Structure activity relationship
RF - Radio Frequency
ZBG - Zinc binding group

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4 New Directions in Anxiolytic Drug Research

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INTRODUCTION

As part of the spectrum of human emotions, anxiety occurs with varying intensity but serves a useful purpose by increasing awareness in dangerous or stressful situations. While most people deal with anxiety as a normal and periodic component of daily life, others find that their emotional responses are out of control with respect to the circumstances from which they are generated. These individuals, which include more than 23 million Americans (9% with a lifetime incidence of 16%) [1], suffer from various types of anxiety which include generalized anxiety disorder (GAD), panic disorder, post-traumatic stress disorder, phobias, and obsessive compulsive disorder (OCD). The most notable signs which anxiety sufferers display are irritability, abnormal or inappropriate fear, impaired concentration, sleep disturbances, heightened motor tension, palpitations, chest pain, nausea, and dizziness, all of which can be physically and mentally incapacitating if left untreated.

Generalized anxiety disorder is the most frequently occurring anxiety disorder (50% of anxiety diagnoses) [2]. The essential feature of GAD is unrealistic or excessive anxiety where the symptoms are present for at least six months and the patient experiences at least three or more of a series of distressing physical symptoms.

Panic disorders, with or without agoraphobia, affect 1.6% of the adult population (>3,000,000 people) in the United States at some time in their lives. In panic disorder, brief episodes of fear are accompanied by multiple physical symptoms, such as terror, fear of dying, heart palpitations, difficulty in breathing, and dizziness. Panic attacks recur and the victim develops an intense fear of having another attack, which is termed anticipatory anxiety. In addition, the victim may develop irrational fears, called phobias, that relate to situations in which a panic attack has occurred. This condition may coexist with other phobias (agoraphobia, simple phobia, social phobia), depression, obsessive-compulsive disorder, alcohol and drug abuse, suicidal tendencies and irritable bowel syndrome.

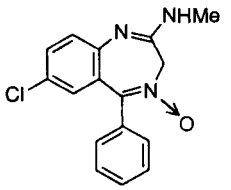
Obsessive compulsive disorder is characterized by recurrent obsessions or compulsions severe enough to cause marked distress and to interfere with daily life. Attempts to resist the compulsion lead to mounting anxiety that can be relieved by yielding to the compulsion. This disorder affects as much as 2–3% of the United States population [3].

TREATMENT OF ANXIETY DISORDERS

The treatment of anxiety throughout human history has involved a variety of natural agents which were administered to relieve tension and induce a state of altered consciousness, with ethanol in its various forms the most widely used [4]. Within the last century, general CNS depressants such as barbiturates, bromide salts, and ethanol surrogates such as chloral hydrate and paraldehyde have been employed to treat anxiety. Because of side-effects of the other drugs, barbiturates were used predominantly in the first half of this century as anxiolytics, but their clinical utility was limited by tolerance and dependence liability. Propanediolcarbamates such as meprobamate were also used to treat anxiety but displayed many of the barbiturate side-effects.

The discovery of the benzodiazepines (BZDs) at Hoffmann-La Roche in the late 1950s provided a more formidable arsenal for the treatment of anxiety [5]. Following the introduction of chlordiazepoxide (1, Librium®) in 1960, a large number of analogues were synthesized and developed, including diazepam (2, Valium®), lorazepam (3, Ativan®), prazepam (4, Centrax®), oxazepam (5, Serax®), chlorazepate (5a, Tranxene®), and alprazolam (6, Xanax®). Although other structural classes of compounds for treatment of anxiety have been discovered such as hydroxyzine (7, Atarax®), buspirone (8, Buspar®), and chlormezanone (9, Trancopal®), the prescriptions written for (1)-(7) over the years since their introduction equal or exceed that for any disorder [4, 6], presumably because of their effectiveness and rapid onset of action [7].

GAD is responsive to pharmacotherapy with BZDs and early intervention is important because this may shorten the episode and prevent the development of complications. Patients rarely exhibit tolerance to the anxiolytic actions of BZDs [8]. The NIH Consensus Panel has recommended that doses be tapered down before discontinuing therapy to avoid physical withdrawal symptoms or a recurrence of anxiety. The most serious risk with BZDs is physical dependence. Other risks include sedation, fatigue, drowsi-



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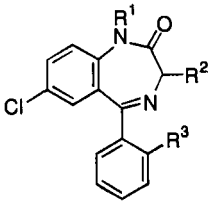
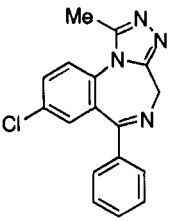
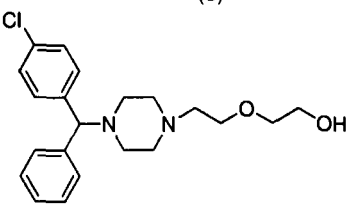




(6)

	R^1	R^2	R^3
(2)	Me	H	H
(3)	Me	OH	Cl
(4)	CH_2-cyclopropyl	H	H
(5)	H	OH	H
(5a)	H	CO_2H	H



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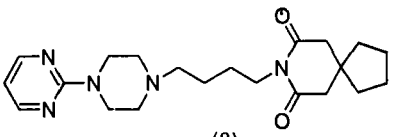

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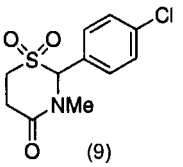


(9)

ness, psychomotor impairment, and adverse interactions with ethanol. Tolerance usually develops to sedation, fatigue, and drowsiness after 4 to 6 weeks.

MECHANISM OF THE ANXIOLYTIC ACTION OF BENZODIAZEPINES

The neurologist James Papez described a neuroanatomical pathway (the limbic system) serving the transmission of emotions, including anxiety [9]. The amygdaloid nucleus is the component of this pathway having the prime role in anxiety. The hypothalamic mammillary body is thought to play a minor role. The amygdala projects to associational and cingulate regions of the cerebral cortex, the hippocampus and to deep brain regions that influence the function of the autonomic nervous system. The input to these brain regions from the amygdala, which is responsible for symptoms of anxiety,

such as unpleasant or irrational thoughts (cerebral cortex), anxiety-induced forgetfulness (hippocampus), increased stress response and visceral disturbances (hypothalamus), is inhibited by the interaction of γ-aminobutyric acid (GABA) with the $GABA_A$ receptor. GABA is a major inhibitory neurotransmitter involved in the vertebrate central nervous system (CNS) and released by about one-third of all neurons in the CNS. Neurons which use GABA as a neurotransmitter are referred to as GABAergic neurons and most are small interneurons that refine the impulse activity of output neurons that project from one area of the CNS to another.

Because GABAergic neurons are nearly all inhibitory, GABA reduces neuronal excitability and exerts a depressant effect on CNS function. Therefore, conditions that impair the functional activity of GABAergic neurons tend to shift the CNS to an excitable state. For example, compounds that act as antagonists at GABA receptors or block the synthesis of GABA induce anxiety and are convulsants or proconvulsants. Alternatively, conditions that result in a generalized increase in the activity of GABAergic neurons can depress CNS function. For example, several anaesthetics and sedatives nonspecifically potentiate the ability of GABA to activate the $GABA_A$ receptor. GABA receptors are currently divided into two general types, $GABA_A$ and $GABA_B$. Since this review is concerned with compounds which interact with the $GABA_A$ receptor and are anxiolytic, the $GABA_B$ receptor will not be discussed further.

The anxiolytic efficacy of BZDs arises from their ability to modulate the physiological activity of GABA at $GABA_A$ receptors [10–14]. Most, if not all, highly effective anxiolytic drugs are known to potentiate the action of the inhibitory transmitter, GABA, by potentiating its ability to activate the $GABA_A$ class of receptors, thereby increasing the intraneuronal conductance of chloride ion and reducing neuronal excitability. Many subtypes of $GABA_A$ receptors exist with different pharmacological properties, but the specific type(s) of $GABA_A$ receptors associated with anxiolytic activity or other CNS effects of $GABA_A$ receptor modulators have not been identified [15–17]. Therefore, it has not been possible to identify compounds with an ideal profile based solely on their affinity for different subtypes of $GABA_A$ receptors and their intrinsic modulatory activity.

Neuroanatomically, $GABA_A$ receptors that are modulated by BZDs have been investigated using receptor autoradiography. These receptors are extensively distributed throughout the CNS with different densities in different structures including the amygdala [18, 19]. This suggests that BZDs are capable of modulating activity in many regions and circuits and this may underlie the multiplicity of behavioural effects evoked by these drugs. There is, however, substantial evidence that the amygdala plays an important role in

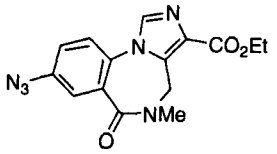
the anxiolytic activity of this class of drugs. Local injection of BZDs into various nuclear regions of the amygdala evoked an anxiolytic effect in several rodent models of fear and anxiety [20, 21] and in one study these effects were blocked by the BZD antagonist flumazenil [22]. Enhancement of neuronal inhibition brought about by the interaction of BZDs with the $GABA_A$ receptor ties in with results from studies showing that lesions of the amygdala and the mammillary body of the hypothalamus evoke anxiolytic effects in rodent models of anxiety [23, 24].

CHARACTERISTICS OF THE $GABA_A$ RECEPTOR

The $GABA_A$ receptor is a heteropentameric complex of several proteins which, when activated, undergoes one or more conformational changes to form a functional chloride ion channel [25]. Based on molecular biology studies, the $GABA_A$ receptor is known to be comprised of a family of subtypes, each a pentameric aggregate of various combinations of three kinds of subunits, usually two α, two β, and one γ. Each of the subunits has several subtypes [26–30]. Theoretically, several thousand different types of $GABA_A$ receptors are possible, because of the many subtypes of $GABA_A$ receptor subunits that exist, and more than fifteen of these receptors exhibiting different pharmacological profiles have already been cloned and expressed [31, 32]. On the basis of these findings, there is a possibility that anxiolytic agents may be discovered which selectively bind to those subtypes that promote anxiolytic activity and not to those which cause side-effects [31].

Currently, there is considerable research into the exact types or subtypes which mediate anxiolytic activity and unwanted side-effects. The $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ subtypes may be involved in anxiolysis [31], whereas cognitive function may be influenced by $\alpha_3\beta_2\gamma_2$ and ethanol potentiation may be due to the effects of the $\alpha_6\beta_2\gamma_2$ subtype [33]. Site-directed mutagenesis studies have localized the BZD binding site to the interface between the α and γ subunits [29, 34] where His_{102} [35] and Gly_{200} [36] of the α_1 subunit and Thr_{142} [37] of the γ_2 subunit are involved in ligand binding. The pharmacological characteristics of the BZD site are influenced by the subtype of each subunit. For example, the BZD sites formed by the interface of an α_1 or α_2 subunit with a γ_2 subunit exhibit high affinity for most BZDs that possess agonist activity, whereas those formed by the interface of an α_4 or α_6 with a γ_1 or γ_3 subunit exhibit low affinity for most agonist BZDs [30]. Recently, it has been reported that Ro 15–4513 (10) binds selectively to a subtype of $GABA_A$ receptor that is exceptionally high in the hippocampus and probably contains an α_5 subunit [15].

The activation of $GABA_A$ receptors is probably a multi-step process in



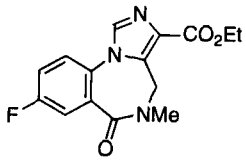
(10) Ro 15-4513

which GABA binds first to sites causing a conformational change that primes the receptor, possibly by unmasking additional binding sites, which GABA binds to and then induces a conformational change that shifts the receptor into an active state. In addition to the binding sites for GABA, $GABA_A$ receptors contain at least three other types of ligand binding sites, which can alter the sensitivity of the receptors to GABA. These modulatory sites are named according to the class of compound which binds to them: (i) BZD, (ii) barbiturate and (iii) steroid. For the first two sites there may be no natural ligand.

COMPOUNDS WHICH BIND TO THE BENZODIAZEPINE SITE

The BZD site is the most thoroughly studied of the three modulatory sites and numerous drugs have been identified that bind to it. About a dozen of these are currently marketed for treating disorders such as generalized anxiety, panic disorders, depression, some forms of epilepsy (e.g. absence seizures), febrile seizures, some sleep disorders (e.g. insomnia), and muscle spasms and cramps and for use as anaesthetics. Flumazenil (11), a BZD antagonist, is currently marketed for treating BZD agonist overdose.

In terms of structure, compounds which bind to the BZD site are generally flat, and contain a two or three-ring heterocyclic nucleus at their core. They include the BZDs mentioned previously, β-carbolines (e.g. abecarnil, 12), imidazopyridines (e.g. alpidem, 13), pyrazoloquinolines (e.g. CGS 8216, 14), and imidazoquinoxalines (e.g. panadiplon, 15). New series of compounds with BZD site affinity are regularly reported in the medicinal chemistry literature [38–41] with similar overall structural elements. There are



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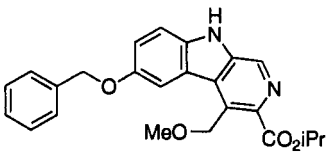

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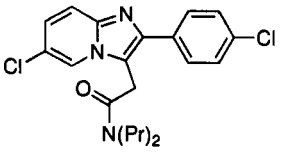

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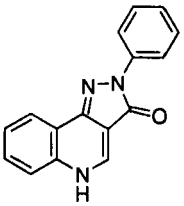

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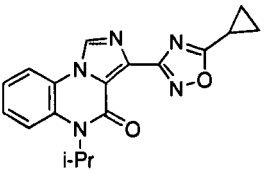
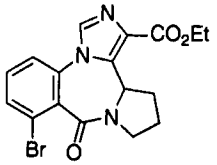


(15)

no known endogenous ligands for the BZD binding site, as supported by the fact that antagonists do not cause any apparent behavioural effects, indicating that endogenous ligands do not play a major pharmacological role. Within a given series, small structural modifications have been found to greatly affect the character of the binding interaction ranging from agonist to inverse agonist, with the ordered behavioural correlates of sedative, anxiolytic, procognitive, anxiogenic to convulsant activities [42, 43]. Solubility is often limited because of the nature of the structures involved, which can cause difficulty in achieving consistent oral activity.

Compounds that interact with the BZD site can exert either of two modulatory effects: an agonist (positive modulatory) effect characterized by a reduction in the concentration of GABA required to activate the receptor, or an inverse agonist (negative modulatory) effect characterized by an increase in the concentration of GABA required to activate the receptor [44–46]. A third type of interaction can also occur, in which a compound binds to the BZD site but does not affect the activity of GABA. Such compounds prevent either agonists or inverse agonists from modulating the activity of GABA, and are therefore called antagonists.

BZD agonists and inverse agonists can be further sub-divided based on their intrinsic activity; i.e. whether they have 'full' or 'partial' modulatory capacity. Because BZD compounds exert only modulatory effects, there may be no absolute definition of a full agonist or inverse agonist. However, the maximum modulatory effect can differ widely; for example, the maximum ability of diazepam (2), lorazepam (3), and alprazolam (6) to potentiate the activity of GABA is much greater than that of bretazenil (16) or abecarnil



(16)

(12) [47]. The former are referred to as full agonists and the latter as partial agonists.

A semi-quantitative classification of the type of binding activity of a particular ligand (agonist, partial agonist, etc.), called the GABA shift (GS), is obtained by dividing the binding affinity of that ligand for the BZD site in the presence of GABA into that found in the absence of GABA [48]. Thus, full agonists have GS values of 2.0 or greater, partial agonists have GS values of 1.0–1.5, antagonists have GS values of approximately 1.0, and inverse agonists have GS values of 0.7 or less. The *in vivo* pharmacology of BZD ligands is generally consistent with the *in vitro* spectrum of full agonist to full inverse agonist activity as indicated in *Table 4.1.*

BZD agonists and inverse agonists can be further differentiated on the basis of differences in affinity and intrinsic activity for different subtypes of GABA receptors [30, 49–54]. This has considerable implications for the discovery of compounds that possess anxiolytic activity without the side-effects of sedation or muscle relaxant activity. Because GABA receptor subtypes can be differentiated by their anatomical localization, physiological relevance, and pharmacological specificity, it seems reasonable to suggest that compounds may be discovered that selectively modulate $GABA_A$ receptors associated with emotional aspects (e.g. fear) of brain function (the limbic system) as opposed to the sleep-wake cycle or motor function.

Table 4.1. COMPARISON OF PHARMACOLOGY OF BZD LIGANDS WITH GABA SHIFT.

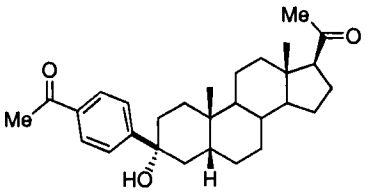
Full Agonist (GS = 2.0 or more)	*Partial Agonist (GS = 1.0–1.5)*	*Antagonist (GS = ~1.0)*	*Inverse Agonist (GS = ~1.0–0.7)*	*Partial Inverse Agonist (GS = 0.7 or less)*
Sedative	Weak Sedative	Inactive	Mild Alerting	Alerting
Anxiolytic	Anxiolytic	Inactive	Weak Anxiogenic	Anxiogenic
Anticonvulsant	Anticonvulsant (?)	Inactive	Proconvulsant	Convulsant
Muscle Relaxant	Muscle Relaxant (?)	Inactive	Mild Muscle Rigidity	Muscle Rigidty

COMPOUNDS THAT BIND TO THE STEROID SITE

Several steroids which have been found to possess CNS activity, have potential use as anticonvulsants, anxiolytics, and sedative-hypnotic agents. The existence of a steroid binding site on $GABA_A$ receptors is now well established, but the location has not been clearly delineated. These steroids have been found to modulate $GABA_A$ receptors in a barbiturate-like manner [55]. Although some steroids exhibit effects *in vivo* similar to the barbiturates, they do not act at the same site. The modulatory activity of steroids differs from that of the benzodiazepines, but they are similar in some respects. For example, steroids can exert either a positive (agonist) or a negative (inverse agonist) modulatory effect. Steroids can also exhibit partial or 'full' agonist activity, as well as receptor subtype selectivity [56, 57].

Steroids that modulate $GABA_A$ receptors are generally in the progestin class, although some glucocorticosteroids have also been reported to be active. Recently, several pregnanes substituted with alkyl groups at the 3β-position, for example (17) and (18), resulted in ligands with high affinity for the neuroactive steroid site on the $GABA_A$ receptors. These compounds displayed anticonvulsant activity in the pentylenetetrazole (PTZ) and maximal electroshock tests following i.p. administration in mice [58].

Progesterone (19) and its metabolites, (20) and deoxycorticosterone (21), are potent modulators of the GABA receptor complex and when adminis-



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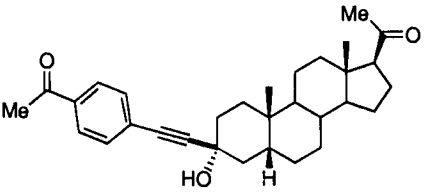

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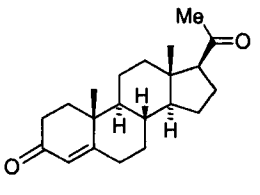
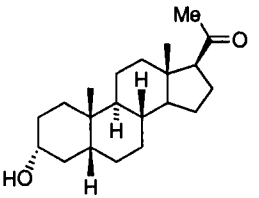


(19) (progesterone)



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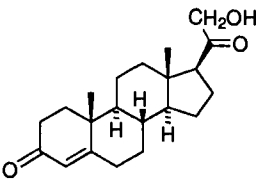


(21) (deoxycorticosterone)

tered to mice, have been found to produce anxiolytic, locomotor stimulant, ataxic, hypnotic and anticonvulsant effects. These behavioural effects are similar to those produced by ethanol, benzodiazepines and barbiturates [59].

RECENTLY DISCOVERED ANXIOLYTIC COMPOUNDS

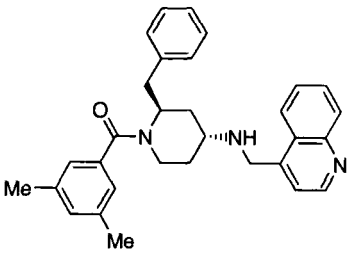
In the last few years new anxiolytic agents have emerged which act by a variety of mechanisms, including $GABA_A$ receptor modulation [60]. More recently, anxiolytics with non-benzodiazepine structures have been reported which are described below.

A neurokinin-1 receptor antagonist, CGP-49823 (22), was shown to have anxiolytic effects in rats [61]. After 3–6 weeks of treatment, some signs of tolerance to the drug appeared although the anti-anxiety activity remained significant. No withdrawal effects were observed after six weeks dosing.

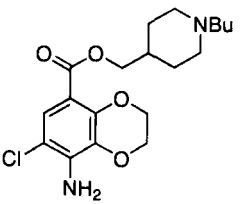
Two selective 5-HT_4 receptor antagonists, SB 204070 (23) and SB 207266 (24), were determined to have modest anxiolytic activity in rats when evaluated in the social interaction test [62]. Rats given either compound and subjected to the elevated-plus maze spent increased amounts of time in the open arms compared with controls. In another model of anxiety, the Geller-Seifter conflict paradigm, neither compound had an effect on either punished or unpunished responding. These compounds had similar efficacy to chlordiazepoxide in the social interaction test, but were less efficacious in the elevated-plus maze.

A high affinity ligand for the 5-HT_3 receptor, the pyrroloquinoxaline (25), was found to display anxiolytic activity in mice which were subjected to the light/dark test [63]. This compound was exceedingly potent with significant activity in the 0.1–100 μg/kg range, p.o. In the [^{14}C]-guanidinium uptake assay, (25) was found to be a 5-HT_3 receptor full agonist whereas *in vivo* (von Bezold Jarisch reflex assay) it displayed partial agonist properties.

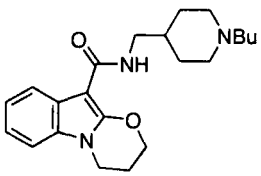
Although PNU-101017 (26) is weakly active in anticonflict anti-anxiety



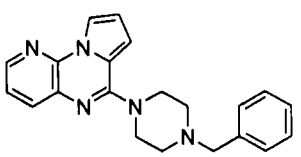
(22) CGP 49823



(23) SB 204070



(24) SB 207266



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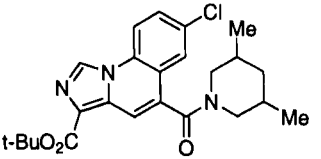


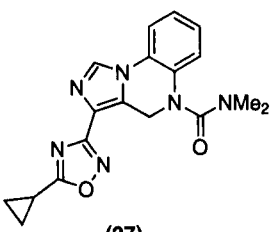
(26) PNU-101017

tests, it decreased cGMP levels and attenuated those cGMP levels which were stressed-induced in mice [64, 65]. This compound, which was determined to be a partial agonist for the BZD site on the $GABA_A$ receptor, antagonized PTZ (pentylenetetrazole, Metrazole®) seizures in mice. Potentiation of the CNS-depressant effects of ethanol was not observed nor did the compound cause physical dependence.

Another high affinity partial agonist for the BZD site of the $GABA_A$ receptor, compound (27), was as effective as diazepam in increasing the number of shocks taken in the Vogel assay and was similarly active in the Geller-Cook conflict anxiety test. This compound also prevented PTZ-induced seizures in mice and was found to be a partial BZD agonist *in vitro*. No typical benzodiazepine-like side effects were observed for (27) [66].

Compounds (28), (29), and (30) are BZD receptor partial agonists which are anxiolytic in mice subjected to an operant punishment test and protect against PTZ-induced seizures in mice and rats [67]. They cause minimum motor impairment in rodents and do not cause withdrawal in mice and squirrel monkeys.

Derivatives of flavonoid natural products display anxiolytic activity. The flavone (31) for example, is a high affinity ligand for the BZD site of the $GABA_A$ receptor in several regions of rat brain and is selective since it does not interact with the $5\text{-}HT_{1A}$, muscarinic and adrenergic receptors [68]. This compound produces anxiolytic activity in mice subjected to the ele-



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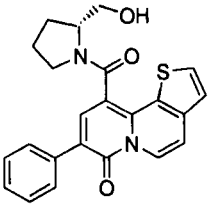
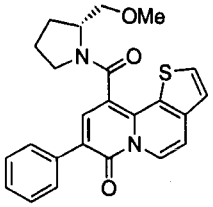
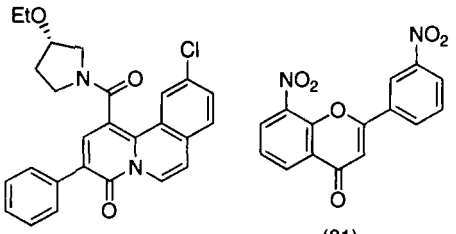


(28) Ro 19-5663



(29) Ro 19-5686



(30) Ro 41-3696 (31)

vated plus maze and is 30–100 times more potent than diazepam. In terms of side-effect liability, (31) did not cause sedation or myorelaxation.

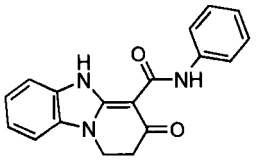
Combined extracts of zingiber and ginkgo biloba (Zingicomb®) elevated the time that rats spent in the open arms of the elevated-plus maze while having no effect on behaviour [69]. At higher doses, however, Zingicomb® appeared to display anxiogenic properties. This combination of natural products may be acting through a serotonergic pathway.

NOVEL ANXIOLYTIC PYRIDO[1,2-*a*]BENZIMIDAZOLES WHICH INTERACT AT THE BZD SITE OF THE $GABA_A$ RECEPTOR

Interest in CNS diseases was the basis for an extensive programme directed to the discovery of novel anxiolytic agents at The R. W. Johnson Pharmaceutical Research Institute. Binding affinity determinations at the BZD site of the $GABA_A$ receptor and two *in vivo* assays, the Vogel test (anticonflict) and the PTZ seizure test (anticonvulsant), were employed to evaluate the potential anxiolytic activity of test compounds.

The Vogel test is an experimental animal model of environmental situations that engender an anxious state within an animal [70]. The anxiolytic activity of test compounds was assessed by determining their ability to release (disinhibit) behaviour that had been suppressed by punishment. Anxiolytic drugs reduce behavioural inhibition as indicated by an increase in the number of shocks received by drug-treated rats compared to a vehicle-treated group. In this test, water-deprived rats are fearful of receiving a mild shock delivered through the sipper tube of a water bottle. Rats treated with an active GABA-based anxiolytic drug tolerate significantly more shocks than control rats. The measurement of activity in this assay is the minimum effective dose (MED) which affords a significant increase in tolerated shocks.

The seizure assay uses pentylenetetrazole (PTZ), a chemical convulsant that negatively modulates $GABA_A$ receptors at a non-BZD site. Com-



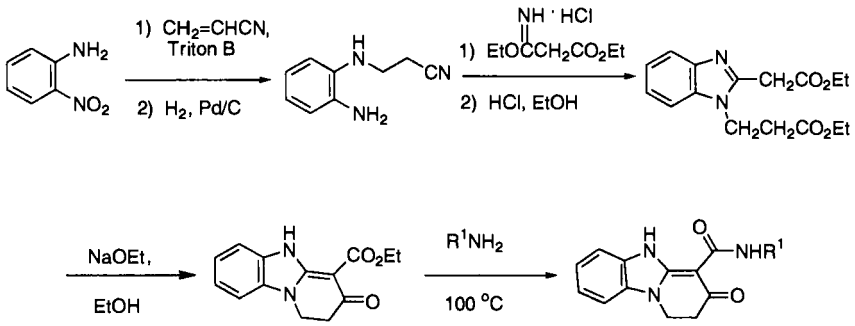
(32)

pounds that positively modulate the activity of GABA at $GABA_A$ receptors can block these convulsions [71]. Historically, the ability of a compound to block PTZ convulsions has been found to be highly correlated with and predictive of anxiolytic activity.

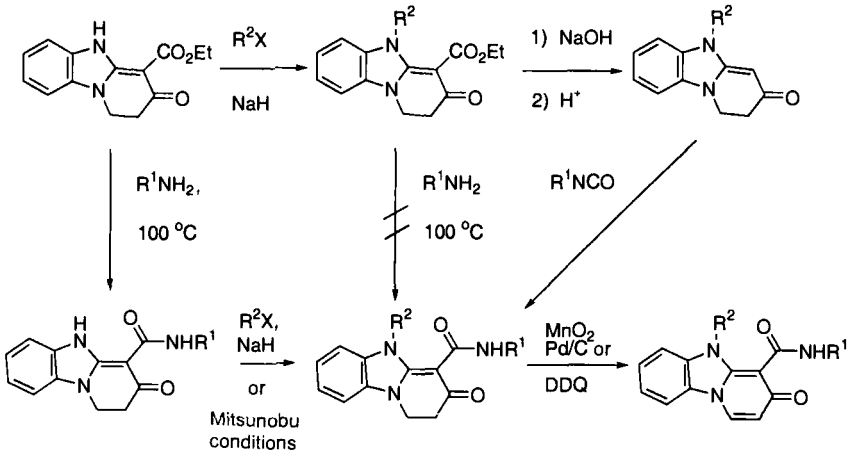
In the late 1970s, using the procedures described above, Drs. Russell Taylor and Joseph Gardocki discovered the pyrido[1,2-*a*]benzimidazole (PBI) RWJ-16979 (32), a potential anti-anxiety agent designed and synthesized by Dr. Winston Ho, which exhibited good binding affinity for the BZD site (IC_{50} = 9.1 nM) and showed moderate *in vivo* activity in the PTZ seizure and conflict assays [72]. The challenge in the PBI drug discovery programme has been to obtain potent oral anxiolytic activity with minimal side-effects, while retaining the high level of $GABA_A$ receptor binding seen with compound (32).

The synthesis of (32) and other analogues, described below, was carried out as shown in *Scheme 4.1* by treating 2-nitroaniline with acrylonitrile followed by catalytic hydrogenation to a phenylenediamine. From this, the PBI nucleus was formed with carbethoxyacetimidate hydrochloride, followed by Dieckmann condensation of the resulting diester. Condensation of the ester with an appropriate amine afforded the target PBIs.

N-Alkylated PBIs were obtained from the PBI ester, as shown in *Scheme*



Scheme 4.1



Scheme 4.2

4.2, by first alkylating to give the *N*-substituted PBI ester. However, the target amides could not be formed from this ester by treatment with amines, but were obtained after decarboethoxylation to the enone by treatment with an appropriate isocyanate [72]. Alternatively, the method of Mitsunobu [73] or recently reported modified procedures [74, 75] were employed. Treatment of a PBI with an appropriate alcohol and 1–5 equivalents of a suitable activating agent such as diethyl azodicarboxylate (DEAD), azodicarbonyldipiperidine (ADDP), or 1,1-azobis(*N*,*N*-dimethylformamide) (TMAD) and triphenylphosphine or tributylphosphine in benzene, THF, or DMF provided the desired *N*-5-(heteroaryl)alkyl pyridobenzimidazole. Oxidation of the appropriate PBIs with MnO_2, Pd/C or DDQ afforded the corresponding unsaturated C-ring derivatives [72, 76].

STRUCTURAL ASPECTS OF THE PYRIDO-BENZIMIDAZOLES

As with many of the previously mentioned compounds which bind to the BZD site, PBI (32) is essentially a planar molecule. Two tautomers involving the B ring hydrogen are possible as shown in figure 4.1, the keto form (32) and the enol (32a), of which the predominant form in solution is the keto tautomer, as shown by ^{13}C-NMR measurements [72]. This is supported by X-ray data which also shows the C-ring slightly puckered out of the plane defined by the tricyclic system, the amide 30° out of plane and a hydrogen bond between the N-H and C-ring carbonyl [72].

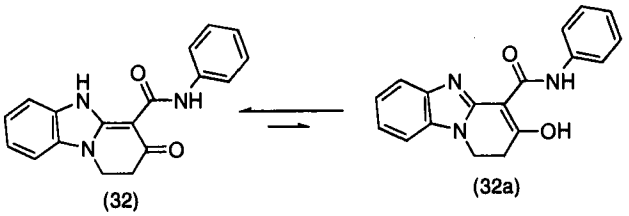
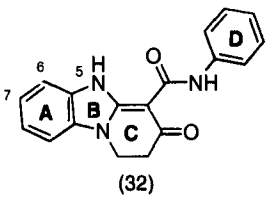


Figure 4.1

STRUCTURE-ACTIVITY RELATIONSHIPS OF ANXIOLYTIC PYRIDO-BENZIMIDAZOLES

As mentioned previously, the structure of the PBIs consists of a tricyclic nucleus (rings A, B, and C) to which is appended an aryl amide (ring D) as illustrated in *Figure 4.2* [72, 76]. The chemistry described above allowed the preparation of a large number of analogues based on variations of A, B, C, and D [77].

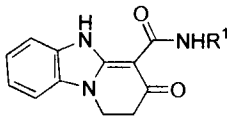
Elaboration of the D ring of (32) (*Table 4.2*) employing a variety of substituents provided some initial insight into the effect of structure on potential anxiolytic activity [72, 76, 77] of PBIs. The 4-chloro substituted compound (33) was more potent than (32) in the anticonflict test although its affinity for the BZD site is attenuated whereas the 2-chloro compound (35) had essentially the same potency as (32) in this assay. The 3-chloro compound (34) may be an antagonist because of its moderate binding affinity, GABA shift, and little or no *in vivo* activity. The 2-fluoro substituted PBI (36), is ten times more potent than (32) in the anticonflict assay, has four times the binding affinity of (32), and based on its GABA shift, is a partial agonist. An even more potent partial agonist is (41), the 2,6-difluoro derivative, which has an MED = 0.1 mg/kg in the anticonflict assay. The corresponding 2,6-dichloro analogue (40) binds weakly to the BZD site and is inactive *in vivo* in both anticonflict and anticonvulsant tests.



(32)

Figure 4.2

Table 4.2. *IN VIVO* AND *IN VITRO* DATA FOR PBIs



Compound	R^1	*Conflict (rat) mg/kg, i.p.*	*PTZ (mouse) mg/kg, i.p.*	*^{3}H-Flunitrazepam binding IC_{50} (nM)*		
				No GABA	*GABA*	*GS*
32	Ph	10	5.2	9.1	5.9	1.5
33	4-ClPh	1	8.9	620	265	2.3
34	3-ClPh	>10	>30	120	95	1.3
35	2-ClPh	10	0.95	12	5.3	2.3
36	2-FPh	1	0.16	1.7	1.4	1.2
37	4-MeOPh	10	2.0	41	16	2.6
38	3-MeOPh	5	>10	26	16	1.6
39	2-MeOPh	>10	>10	990	940	1.1
40	2,6-Cl_2Ph	>10	>30	320	170	1.9
41	2,6-F_2Ph	0.1	0.85	2.8	1.7	1.6
42	4-(CO_2H)Ph	10	>30	>10,000	>10,000	–
43	4-HOPh	10	>30	46	31	1.5
44	4-H_2NPh	>10	2.6	13,000	3400	3.8
45	2-H_2NPh	10	2.1	50	33	1.5
46	4-Me_2NPh	3	3.1	270	210	1.2
47	3-Me_2NPh	>10	2.8	37	25	1.5
48	2-Me_2NPh	10	4.9	10,000	–	–
49	2-F-4-Me_2NPh	30	1	120	87	1.4
50	2-Me-4-Me_2NPh	>10	3.2	4,000	1,500	2.7
51	c-C_6H_{11}	10	3.0	140	72	2.0
52	c-C_4H_7	10	>30	30	25	1.2
53	c-C_3H_5	>10	>10	16	18	0.87
60	4-Py	3	3.1	160	78	2.1
61	3-Py	10	2.6	51	48	1.1
62	2-Py	10	11.2	59	36	1.7
63	4-(1-MePy)$^+$	>10	>10	6,300	3,100	2.0
64	4-(3-ClPy)	>10	2.6	220	100	2.2
65	4-(3-MePy)	>10	>10	7,100	12,000	0.6
66	4-(3-FPy)	10	1.3	53	20	2.6
67	3-(2-ClPy)	10	3.7	68	65	1.1
68	CH_2(4-Py)	>10	>10	65	69	0.9
69	CH_2(3-Py)	>10	>10	150	140	1.1
70	2-Pyrazinyl	>10	>10	360	400	0.9
71	2-Pyrimidinyl	10	2.7	54	50	1.1
72	4-Pyrimidinyl	10	>10	480	370	1.3

The methoxy substituted analogues display a different profile of activity. The 4-methoxy PBI (37) displays much less affinity for the BZD site than (32) but has comparable *in vivo* potency; the 3-methoxy analogue (38) has greater receptor binding affinity than (37) and about the same *in vivo* potency; while the 2-methoxy analogue (39) is inactive *in vivo* and has very low binding affinity.

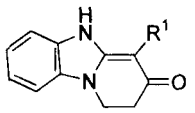
The 4-carboxy analogue (42) exhibits strongly reduced binding affinity whereas the 4-hydroxy compound (43) has reasonably high binding affinity, but only modest *in vivo* activity. Amino substitution in the 4-position (44) drastically reduces binding affinity whereas the 2-amino analogue (45) has moderately reduced binding affinity. Both compounds are more active *in vivo* than (32). A different profile is observed with *N,N*-dimethyl analogues (46)-(48) where the 3-position isomer (47) is better than the 4-isomer (46) which is superior to the 2-isomer (48). Activity in the PTZ seizure assay is better than (32) for all three compounds, but (46) stands out because of its relatively potent anti-conflict activity. Within the 4-*N,N*-dimethyl series, addition of 2-fluoro (49) or 2-methyl (50) substitution results in a slight increase in PTZ seizure inhibition but a decrease in anticonflict activity.

Substituting cyclohexyl for the phenyl ring of (32) to give compound (51) results in a slight increase in potency in the PTZ assay but no change in conflict potency along with a 15-fold reduction in binding affinity. Binding affinity is restored for the cyclobutyl (52) and cyclopropyl (53) analogues, but these compounds are antagonists with GABA shifts of 1.2 and 0.87, respectively. The antagonist properties of compound (53) were further demonstrated when it was found to antagonize chlordiazepoxide-induced impairment of horizontal screen performance in mice [72].

Replacement of the aryl amide moiety by carboxamide or carboethoxy gives compounds (54) and (55), which are weak ligands and have low *in vivo* activity (*Table 4.3*). The thioamide (56) is similar to (55) in terms of binding and *in vivo* activity but the anilide (57) has no binding affinity. Phenyl ester (58) exhibits modest activity in the PTZ seizure assay but phenylthio ester (59) binds strongly to the BZD site (IC_{50} = 29 nM). The lack of *in vivo* activity of (59) may be due to its antagonist activity as reflected in its GABA shift of 1.1.

Pyridyl analogues (60)-(62) (*Table 4.2*) displayed good binding affinity for the BZD site and, with the exception of 2-isomer (62), were more potent than (32) in the PTZ seizure assay [74]. However, the 4-isomer (60) was the only pyridyl analogue which exhibited increased potency in the conflict assay although its binding affinity was less. The methyl quaternary salt (63) was essentially inactive both *in vivo* and *in vitro*. Substitution of the 3-position on the pyridine ring showed that chloro (64) and fluoro (66) were better

Table 4.3. *IN VIVO* AND *IN VITRO* DATA FOR PBIs



Compound	R^1	*Conflict (rat) mg/kg, i.p.*	*PTZ (mouse) mg/kg, i.p.*	*^{3}H-Flunitrazepam binding IC_{50} (nM)*		
				No GABA	*GABA*	*GS*
54	$CONH_2$	10	30	2,400	6,000	0.40
55	CO_2Et		>10	675	840	0.68
56	C(=S)NHPh	>10	>10	360	320	1.1
57	C(=O)NMePh	>10	>10	>10,000	>10,000	–
58	C(=O)OPh	>10	9.9	200	150	1.4
59	C(=O)SPh	>10	>30	29	26	1.1

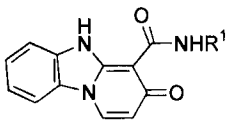
than methyl (65). As illustrated by (66), fluoro substitution improves binding affinity relative to parent PBI (60). The 2-chloro-3-pyridyl analogue (67) shows reasonable binding affinity coupled with good potency in the PTZ seizure assay but no improvement over (32) in the conflict test. Separation of the pyridine ring from the amide nitrogen by one methylene group afforded compounds (68) and (69) with receptor binding affinity similar to the previously mentioned pyridines but without *in vivo* activity. These compounds may be antagonists by virtue of their GABA shift values.

Two pyrimidinyl compounds (71) and (72) and pyrazine (70) were evaluated. Of these, the 2-primidinyl compound (71) displayed slightly less binding affinity than (32) with better potency in the PTZ seizure assay and equivalent potency in the conflict test.

The introduction of unsaturation in the C-ring afforded a wide range of binding affinities and *in vivo* potencies (*Table 4.4*). Compound (73) has good binding activity and, while equipotent with (32) in the conflict assay, is much more potent in the PTZ seizure assay. The 2-fluoro analogue (74), however, exhibits powerful binding affinity (IC_{50} = 0.23 nM) and high potency in both *in vivo* screens. Replacement of the phenyl ring with a 4-pyridyl ring (75) causes a significant reduction of binding affinity and loss of *in vivo* activity.

Excellent *in vivo* activity in both the anticonflict and PTZ seizure assays (*Table 4.5*) was observed with *N*-alkylated PBIs (76) (1 mg/kg MED, i.p. in

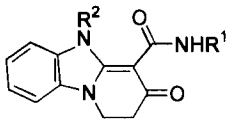
Table 4.4 *IN VIVO* AND *IN VITRO* DATA FOR PBIs



Compound	R^1	*Conflict (rat) mg/kg, i.p.*	*PTZ (mouse) mg/kg, i.p.*	*^{3}H-Flunitrazepam binding IC_{50} (nM)*		
				No GABA	*GABA*	*GS*
73	Ph	10	0.39	24	14	1.7
74	2-FPh	0.1	0.088	0.23	0.11	2.1
75	4-Py	>10	>10	260	150	1.7

conflict), (77) (0.03 mg/kg MED, i.p. in conflict) and (78) (0.3 mg/kg MED, i.p. in conflict). A comparison of (76) with (77) reveals that 2-fluoro substitution imparts powerful binding affinity for (77) (IC_{50} 0.42 nM) compared to (76) (IC_{50} 5.8 nM) along with a 30-fold increase in anticonflict potency. The *N*-benzyl analogue (79) displays less affinity for the BZD site than (76)-(78), but similar to (32) and is more potent in the PTZ seizure assay than (32). The GABA shifts for (73)-(79) suggest that they may be full agonists. Substi-

Table 4.5 *IN VIVO* AND *IN VITRO* DATA FOR PBIs



Compound	R^1	R^2	*Conflict (rat) mg/kg, i.p.*	*PTZ (mouse) mg/kg, i.p.*	*^{3}H-Flunitrazepam binding IC_{50} (nM)*		
					No GABA	*GABA*	*GS*
76	Ph	Me	1.0	0.13	5.8	2.1	2.8
77	2-FPh	Me	0.03	0.019	0.42	0.26	1.6
78	Ph	Et	0.3	0.083	2.1	1.4	1.6
79	Ph	$PhCH_2$	10	1.4	12.0	2.7	4.6
80	4-Me_2NPh	Me	10	1.3	29	16	1.8
81	4-Py	Et	3.0	1.2	80	27	3.0

Table 4.6 EFFICACY AND POTENTIAL SIDE-EFFECT DATA FOR SELECTED PBIs

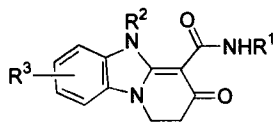
Cmpd	*Anticonflict MED, mg/kg* p.o.	i.p.	*PTZ ED_{50}, mg/kg* p.o.	i.p.	*Ethanol Potentiation MED, mg/kg* p.o.	i.p.	*Horizontal Screen ED_{50}, mg/kg* p.o.	i.p.
32	>10	10	10–30	5.2	30	3	>30	~10
46	10	3	14	3	>60	10	>30	~10
60	10	3	7.8	2.2	>60	>60	>30	>30
81	3	3	10	3	10	–	30	3
Diazepam	5	5	0.3	0.11	1	0.1	1	0.1

tution with a 4-*N*,*N*-dimethylamino group (80) causes a moderate reduction of binding affinity compared with (32), but imparts good potency in the PTZ seizure assay with potency in the conflict test remaining the same as (32). Replacement of the phenyl ring of (78) with a 4-pyridyl ring (81) results in less binding affinity than (32) but with increased *in vivo* potency.

Many of these early PBIs, while displaying good intraperitoneal potency in both *in vivo* assays, were poorly bioavailable and were weakly active orally, most likely due to their poor solubility. However, some compounds (46), (60), and (81) exhibited good oral activity, comparable with diazepam, and showed good separation from side-effects as defined by ethanol sleep potentiation and the horizontal screen test (*Table 4.6*). Since our studies indicated that improvement of *in vivo* anxiolytic activity and binding affinity characteristics related to partial agonism resulted from combinations of fluorine substitution, benzimidazole nitrogen alkylation, and changes in D-ring character, our refinements of the PBIs centered on further changes in the D-ring, expanded substitution of the 5-nitrogen, and substitution and alteration of the A-ring as described below and shown in *Table 4.7*.

The 2-thienyl and 2-thiazolyl rings were used as D-ring replacements (*Table 4.7*) in addition to the pyridine and pyrimidines mentioned previously. Both 2-thiazolyl analogues, (82) which contained an *N*-5 methyl substituent, and (83), where *N*-5 was unsubstituted, were more potent than (32) in the anticonflict test, but receptor binding measurements suggested that (82) was a full agonist. All of the thienyl analogues contained *N*-5 substituents along with various fluorine and methoxy groups on the A and D-rings. Some representative compounds are (84)-(88). One interesting comparison is of the ethoxymethyl derivative (84), which has an oral MED of 3 mg/kg in the conflict assay, with the ethoxyethyl compound (85) which is 10–30 times more potent. In terms of binding affinity, (84) and (85) are comparable and about 10 times better than (32). *N*-Ethyl analogue (86) and propargyl analogue (87)

Table 4.7 *IN VIVO* AND *IN VITRO* DATA FOR PBIs



Cmpd	R^1	R^2	R^3	Conflict (rat) mg/kg, i.p.	PTZ (mouse) mg/kg, i.p.	^{3}H-Flunitrazepam binding IC_{50} (nM) No GABA	GS
82	2-Thiazolyl	Me	H	3.0		8.57	2.12
83	2-Thiazolyl	H	6-MeO	3.0		151	1.37
84	2-Thienyl	$EtOCH_2$	7-F	3.0	0.2	1.05	2.14
85	2-Thienyl	$EtO(CH_2)_2$	7-F	0.1	0.6	0.73	1.97
86	2-Thienyl	Et	7-F	0.3	8.0	1.01	1.91
87	2-Thienyl	Propargyl	7-F	0.3		0.45	1.88
88	3-Thienyl	Et	7-F	1.0	<3	1.07	1.51
89	2-FPh	$AcO(CH_2)_2$	7-F	3.0	3.0	0.025	1.25
90	2-FPh	$EtO(CH_2)_3$	7-F	1.0	0.3	0.28	1.56
91	2-FPh	Propargyl	7-F	1.0	1.0	0.072	2.48
92	2-FPh	$EtOCH_2$	7-F	0.3	0.1	0.14	1.17
93	2-FPh	Me	6-EtO	0.03	0.3	0.16	1.57
94	2-F-4-MeOPh	$EtOCH_2$	7-F	0.03	0.3	0.41	2.16
95	2-FPh	$4\text{-}Br(CH_2)_4$	6-OH	0.01		0.127	1.96

have similar binding affinities (0.46–1.4 nM) to (84) but are 10 times more potent than this compound *in vivo* and all are full agonists. Compound (88), which is a 3-thienyl derivative, is threefold less potent than (86) *in vivo*, but has similar receptor binding affinity.

An especially effective combination of substituents was a fluorine atom at the 2-position on the D-ring coupled with a 7-fluoro substituent on the A-ring, exemplified by compounds (89)-(92). The *N*-acetoxyethyl derivative (89) has better anticonflict activity than (32) and binds strongly to the BZD site whereas ethoxypropyl derivative (90) is more potent in the conflict assay but has one tenth the binding affinity of (89). By virtue of its GABA shift (90) approaches full agonist activity. Propargyl analogue (91) is a full agonist which is as potent *in vivo* as (90) but which displays four times greater BZD site binding. Finally, ethoxymethyl compound (92) has excellent potency in the conflict assay, displays subnanomolar affinity for the BZD site, and is a partial agonist.

The PBIs (93)-(95) which were most potent in the conflict assay contained

a 2-fluorophenyl or 2-fluoro-4-methoxyphenyl D-ring in combination with an *N*-5 substituent and substitution at the 6 or 7-position on the A-ring. Combination of a 4-bromobutyl *N*-5 substituent and an A-ring 6-hydroxy group affords a compound (95) of high potency in the conflict test. When a 4-methoxy group is added in combination with a 2-fluoro substituent on the D-ring, the *in vivo* potency in the conflict assay is increased ten-fold as shown by a comparison of (92) and (94). Compounds (93)-(95) appear to be full agonists based on their GABA shifts.

SAR data were accumulated on approximately 900 PBIs, involving variations of all three rings (A, B, C) of the fused PBI nucleus, the pendant D-ring, and connecting amide bond. Substituents in the 6 and 7-position of the A ring markedly enhance both anticonflict activity and duration. Alkylation of the *N*-5 of the B-ring results in increases of oral anticonflict activity with alkoxyalkyl substituents being superior. The amide carbonyl is necessary for activity and probably acts as a hydrogen bond acceptor while methylation of the amide nitrogen results in decreased activity. Anticonflict potency associated with 2,6-difluoro substitution on the D-ring is much greater when *N*-5 is unsubstituted whereas 2-fluoro and 2,4-difluoro compounds are more active when *N*-5 is alkylated. Separation of side-effects is enhanced when the D-ring is a heterocycle. The carbonyl group of the C-ring may act as a hydrogen bond acceptor and is required for activity. Finally, a 6-membered C-ring is far superior to a 5 or 7-membered ring for anticonflict activity.

PBI-BASED PHARMACOPHORE FOR THE $GABA_A$ BENZODIAZEPINE SITE

From the SAR of the PBI compounds, a $GABA_A$ benzodiazepine site pharmacophore model was constructed in Sybyl 6.1 software [78] using the active analogue approach [79a,79b]. IC_{50} values for competitive binding were available for over 800 compounds. These included the PBI series, benzodiazepines and other structurally diverse reference compounds. The most potent compounds (single digit nanomolar to subnanomolar) where examined for their common features. Since many of the compounds were fairly rigid, all of the conformational changes were done by hand. The Tripos force field [80] and Gasteiger-Huckel charges [81a-81e] were used for full geometry optimization and RMS fits were performed to overlay the individual structures.

A summary of the features of the pharmacophore model is shown in *Figure 4.3*. The *N*-5-benzylated PBI parent compound is shown in white with the benzodiazepine triazolam superimposed on it in orange. The minimal requirements for tight binding are denoted as *H-bond acceptors 1 and 2* as well as *lipophilic site 1*. These requirements are fulfilled by the two carbonyl oxygens on the PBI and the two nitrogens on the benzodiazepines, and there

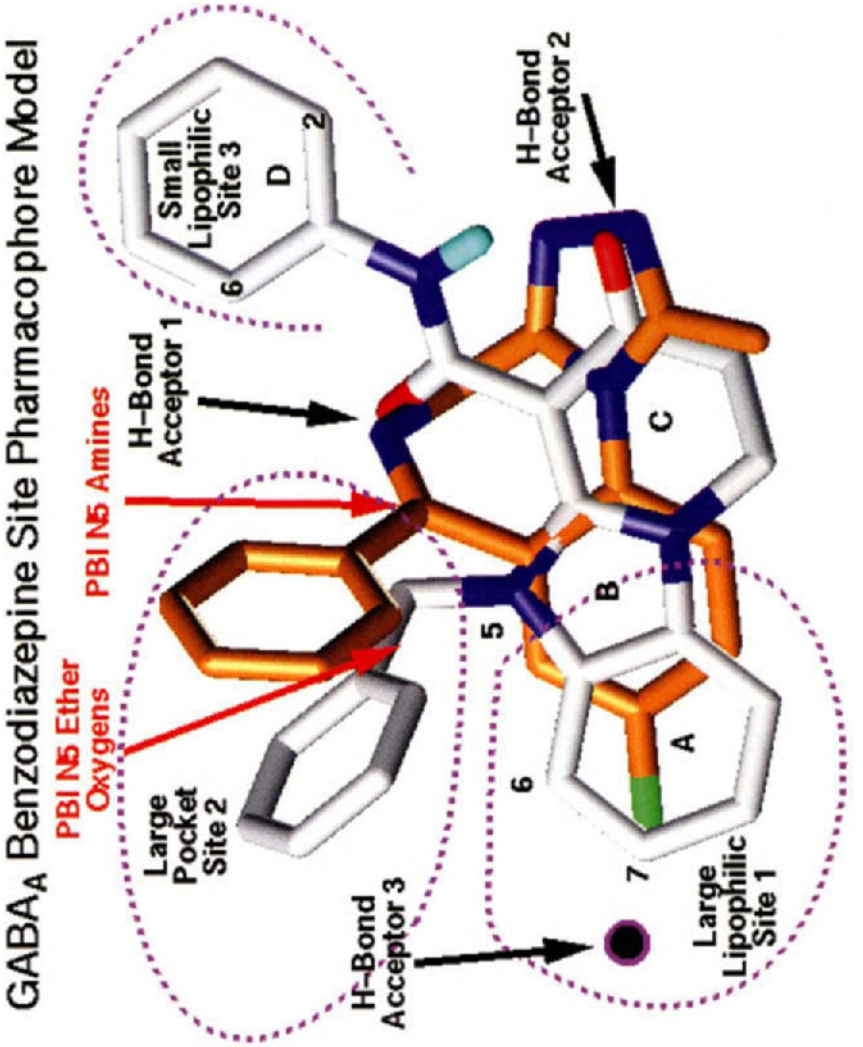


Figure 4.3

is only a small variability in the distance between them. The third requirement is the *large lipophilic site 1* which is filled by the A-ring of the PBI and the chloro-substituent on triazolam. There is greater variability in this region since the overlap of the lipophilic centres is dictated by tighter require-

ments of the two H-bond acceptors. For compounds with high binding affinity, there is a third H-bond acceptor site in this pocket at position-7 in the PBI series which is usually a fluorine atom or an oxygen in a reference compound such as abecarnil.

A second steric pocket exists which is even larger than site 1. This is denoted as *large pocket site 2*. It can accommodate large lipophilic, H-bond acceptors, and protonated amine side-chains substituted at the 5 and 6-positions on the PBI's. Since the IC_{50} data represents binding averaged over all of the different $GABA_A$ receptor subtypes, we believe these chemically diverse substituents bind to different distributions of $GABA_A$ receptor subtypes. Filling this pocket is not a requirement for tight binding in PBIs, but in general it does add potency and modulates the separation between anxiolytic efficacy and side-effects. For benzodiazepines, this pocket is always filled by their pendent phenyl group.

Finally, a third steric pocket exists which is denoted as *small lipophilic site 3*. It is filled by the D-ring of the PBI series. In general, only small substituents are tolerated for tight binding with 2-fluoro and 2,6-difluoro being the best. Large substituents can be accommodated at the 4-position, but this results in a large loss of binding (ca. 100 nM). The fluorine at the 2-position may be interacting with the same receptor site which binds to *H-bond acceptor site 2* while the fluorine at the 6-position may be interacting with *H-bond acceptor site 1*. This pocket is not filled by the benzodiazepines. A different orientation was proposed by Zhang *et al.*[82a, 82b], but their overlay does not exhibit the high degree of volume overlap as does our model between the PBIs and benzodiazepines.

RWJ-51204 AS A POTENTIAL ANTIANXIETY AGENT

Many of the compounds described above were evaluated further for separation of side-effects by their ability to potentiate the effects of ethanol in rats and their effect on the performance of rats on a horizontal screen. From this battery of tests, RWJ-51204 (92) (*Table 4.7*) was chosen for further development. This compound has a high affinity for BZD sites on $GABA_A$ receptors and, based on IC_{50} values, it has a twofold to tenfold higher affinity than lorazepam or clonazepam and a 10 to 100-fold higher affinity than diazepam. A comparison between (92) and eight anxiolytics which bind to the BZD site of the $GABA_A$ receptor indicates that (92), bretazenil, abecarnil and imidazenil all possess similar affinities for the BZD sites on $GABA_A$ receptors in all major areas of the rat CNS (*Table 4.8*). Based on GS values, (92) appears to be a partial agonist or mixed agonist-antagonist.

Our results indicate that (92) binds with particularly high affinity to recep-

Table 4.8 AFFINITY OF RWJ-51204 AND EIGHT GABA-BASED REFERENCE ANXIOLYTICS FOR BENZODIAZEPINE SITES IN FOUR AREAS OF THE RAT CNS USING [^{3}H]-FLUMAZENIL OR [^{3}H]-RO15-4513 AS LIGANDS

	IC_{50} (nM) for CNS Area and Ligand Specified			
Compound	*Cerebral Cortex [^{3}H]flumazenil*	*Hippocampus [^{3}H]Ro15–4513*	*Cerebellum [^{3}H]flumazenil*	*Medulla-Sp. Cd. [^{3}H]flumazenil*
(92)	0.46 ± 0.12	2.9 ± 1.4	0.22 ± 0.10	0.72
Lorazepam	2.6 ± 0.1	12.3 ± 6.7	2.4 ± 0.1	4.3 ± 1.3
Clonazepam	2.6	5.1	2.1	3.4
Alprazolam	13	7.1	14	9.4
Diazepam	20 ± 5	18 ± 2	26 ± 1	23 ± 1
Bretazenil	0.31	0.51	0.19	0.31
Abecarnil	0.38	0.79	0.27	0.44
Panadiplon	2.3 ± 0.9	5.4 ± 1.1	1.6 ± 0.6	2.6 ± 0.2
Imidazenil	0.77	1.32	0.58	0.71

The values represent the results of one or two experiments performed in quadruplicate. Where the results are from two experiments the average ± the range around the average are shown. For each experiment, 11 concentrations of the compound tested were used to generate concentration-inhibition (ligand displacement) curves. IC_{50} values were calculated by fitting a substrate saturation equation to the % inhibition of specific ligand bound as described in detail elsewhere.

tors that contain α_1 or α_2 subunits. For example, the IC_{50} for receptors in the cerebellum was 0.11 or 0.22 nM when [^{3}H]-flunitrazepam or [^{3}H]-flumazenil was used as the ligand, and 0.18 nM for a receptor population in the cerebellum expected to contain primarily the α_1 subunit when [^{3}H]-Ro-15–4513 was used as the ligand. In experiments in which conditions favoured binding to a receptor population in which the α_2 subunit was prevalent, the calculated IC_{50} was 0.33 nM (cerebral cortex, [^{3}H]-flunitrazepam), 0.46 nM (cerebral cortex, [^{3}H]-flumazenil), or 0.76 nM (hippocampus, [^{3}H]-Ro-15–4513, high-affinity site). In experiments in which conditions favoured binding to a receptor population containing primarily the α_6 subunit, the calculated IC_{50} was >12 nM (cerebellum, [^{3}H]-Ro-15–4513, low-affinity site), and when conditions favoured binding to a receptor population containing primarily the α_5 subunit, the calculated IC_{50} was 6.6 nM (hippocampus, [^{3}H]-Ro-15–4513, low-affinity site) and 4.5 nM (medulla-spinal cord, [^{3}H]-Ro-15–4513, low-affinity site). These results show that the affinity of (92) for receptors containing α_5 or α_6 subunits is 10 to 100-fold lower than for receptors containing the α_1 or α_2 subunits.

In acute studies in mice, the oral ED_{50} for (92) in the PTZ test was 0.04 mg/

kg whereas the ED_{50} for motor impairment was 27 mg/kg, indicating a 700-fold separation between efficacy and motor impairment. When compared with lorazepam, (92) was more potent (lower ED_{50}) in the PTZ seizure test, and exhibited a wider separation between the anti-PTZ dose and the dose causing motor impairment. These results are consistent with the concept that (92) in mice is a 'partial agonist'; i.e. it has a lower intrinsic positive modulatory effect than a 'full agonist'. This concept is supported by the observation that (92) antagonized motor impairment caused by chlordiazepoxide.

A study in which (92) and lorazepam were each administered to mice at doses up to 30 mg/kg for 10 days demonstrated that (92) did not cause any withdrawal signs in an experimental condition in which lorazepam produced marked withdrawal signs. This study provides further evidence that (92) exhibits a partial agonist profile, and is less prone to induce a down-regulation of $GABA_A$ receptors when administered over a prolonged period of time.

Evaluation of (92) in rats for acute anxiolytic efficacy in two tests (Vogel conflict test and elevated plus-maze) afforded a minimum effective dose 1 h after oral administration of 60;0.1 mg/kg, with ED_{50} values approximately of 0.4 and 2.0 mg/kg, respectively. The corresponding ED_{50} values for lorazepam were approximately 0.6 and 1.2 mg/kg, respectively. The anxiolytic activity of (92) in the Vogel test was antagonized by flumazenil, verifying that this activity of (92) is mediated by a modulatory effect on one or more types of $GABA_A$ receptors. A comparison of the minimum effective doses and ED_{50} values in tests for efficacy and side-effects (rotarod test for motor impairment, decrease in total arm entries in the elevated plus-maze, decrease in horizontal motor activity in the automated horizontal motor activity test of sedation, and ethanol sleep test for potentiation of alcohol effects) revealed that anxiolytic efficacy did not occur at doses below those causing side-effects in the rat.

In the conflict test in monkeys, (92) was effective at doses in which no physical signs of ataxia, soft body tone, sedation (decreased spontaneous activity or slow ambulation) or a decrease in motor coordination (agility) were observed. By comparison, adverse effects such as ataxia, soft body tone or decreased agility were slight or mild. The difference in the occurrence of side-effects with (92) in rats versus monkeys may be due to better oral absorption of the drug by rats.

When compared with four marketed anxiolytics in monkeys, (92) was approximately tenfold less potent (higher ED_{50}) than lorazepam, alprazolam, clonazepam, and equipotent with diazepam (*Table 4.9*). However, (92) was superior to these reference anxiolytics, when comparing the effective anxiolytic dose (ED_{50}) with that which causes appreciable unwanted CNS effects; i.e. therapeutic index. In addition to a generally wider separation between

Table 4.9 CALCULATED THERAPEUTIC INDEX OF (92) AND OTHER ANXIOLYTICS BASED ON ANXIOLYTIC EFFICACY AND SIDE-EFFECTS IN MONKEYS

Compound	*Anxiolytic Efficacy* ED_{25} *(mg/kg) p.o.*[a]	*Slope*[b]	*Side Effect* ED_{25} *(mg/kg) p.o.*[c]	*Slope*[b]	*Therapeutic Index*[d]
(92)	0.41	0.173	23	0.400	56
Abecarnil	>10	0.003	>10	–	–
Alprazolam	0.04	0.252	0.10	0.834	2.5
Bretazenil	0.01	0.156	5.42	0.167	542
Clonazepam	0.03	0.239	0.18	0.514	6
Diazepam	0.46	0.241	1.0	0.680	2.2
Imidazenil	0.003	0.074	0.44	0.261	147
Lorazepam	0.05	0.194	0.78	0.418	16
Panadiplon	0.03	0.080	>3	–	100

[a]The percent increase in responding was calculated using a random coefficient regression model to fit the data.
[b]The slope of the regression lines was calculated from a double-logarithmic transformation of the data.
[c]The percent incidence in side effects was calculated from a double logarithmic transformation of the data using a robust linear regression model.
[d]Side Effect ED_{25}/Anxiolytic Efficacy ED_{25}

the effective anxiolytic dose, and the dose causing unwanted effects, these effects were much less severe at high doses.

One of the problems associated with benzodiazepine anxiolytics is rebound anxiety and/or insomnia, which may occur between dose intervals or after withdrawal of the drug [83, 84]. In three experiments performed with squirrel monkeys, (92) (up to 20 mg/kg p.o.) did not cause any apparent physical or behavioural signs of withdrawal after abrupt discontinuation of treatment or even after attempted precipitation of withdrawal by administration of flumazenil.

Compound (92) is a high affinity partial agonist for the BZD site of the $GABA_A$ receptor and displays potent and long-lasting efficacy in both rat (Vogel) and monkey anxiety models, which are predictive of anxiolytic activity in humans. A good separation of anxiolytic efficacy from side-effects, superior to that of lorazepam and other marketed anxiolytic agents, is observed with (92) in monkeys and rats. In comparison with lorazepam in general behavioural effects in squirrel monkeys, (92) proved to be superior in most respects at doses ranging from 0.3–20.0 mg/kg, p.o. Based on these results, (92) was subjected to drug safety evaluation in animals and found to be safe. Phase 1 studies of (92) in healthy human volunteers revealed no adverse effects.

SUMMARY

Agents to treat anxiety have gained in acceptance and importance in the fast pace of life in the second half of this century. The discovery and refinement of the benzodiazepines represented a quantum leap in therapy from early compounds which were essentially sedatives. With the advent of molecular biology, an understanding of the basic mechanism by which the benzodiazepines exert their effects was revealed through the discovery and isolation of the $GABA_A$ receptor and its benzodiazepine binding site. This, in turn, has enabled benzodiazepines to be classified into a broad spectrum of pharmacological types ranging from agonist to inverse agonist, thus allowing fine tuning with respect to side-effects. Consequently, newer, more promising agents have emerged which bind at the $GABA_A$ BZD site and have reduced side-effects. An example of this is RWJ-51204 (92), a member of a novel structural type which is superior to several marketed benzodiazepines in animals in terms of efficacy and side-effects. The cost-conscious environment of managed health care presents continuing challenges to the discovery and development of safe, highly efficacious, and cost-effective anxiolytic agents.

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5 Selective Inhibitors of Cyclooxygenase-2 (COX-2)

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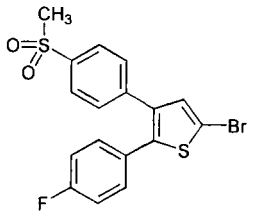
INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin are well known and have been widely used to treat various ailments for over 100 years. As a class, these agents possess anti-inflammatory, analgesic and anti-pyretic activity and are chiefly used to treat chronic inflammatory states

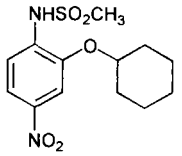
such as rheumatoid arthritis and osteoarthritis and muscoskeletal disorders [1,2]. Four NSAIDs, aspirin, ibuprofen, ketoprofen and naproxen are readily available over the counter but, in addition, more than twenty other drugs of this type are available on prescription with annual worldwide sales exceeding $5 billion. NSAIDs are roughly equivalent in terms of anti-inflammatory efficacy, but differ in their potency. All of them, however, also cause untoward side-effects that limit therapy in a significant fraction of patients. The most common side-effects are gastrointestinal (GI), with haemorrhage and frank ulceration seen in some patients; these lesions can lead to increased morbidity in long term users [3–5]. Renal [6–8] and CNS [9] effects are also observed in some patients. Because of these problems, a major goal of the pharmaceutical industry has been to develop drugs that possess anti-inflammatory and analgesic activity but lacking the side-effects associated with current NSAIDs. To date, no such agent has yet been commercially developed though a drug of this type would undoubtedly command an enormous commercial market.

In 1971 Vane and his associates demonstrated that low concentrations of aspirin and indomethacin inhibited the production of prostaglandins (PGs) suggesting for the first time a biochemical mechanism of action for these drugs [10]. We now know this to be the result of inhibiting the enzyme cyclooxygenase (COX). For many years it was thought that COX was a single enzyme present constitutively in most cells. It was believed that inhibiting the enzyme would lead to decreased production of unwanted PGs (e.g. in inflamed tissue) as well as beneficial PGs produced in the stomach, kidney and elsewhere. However, two distinct COX enzymes were recently identified [11–13]: (i) a constitutive form (COX-1) present in tissues such as gut and kidney that produces PGs that are necessary for normal physiological function [14], (ii) an inducible form (COX–2) whose expression is associated only with inflammation [15–19]. COX-2 is encoded by a second cyclooxygenase gene that is 60% similar to COX-1 in humans [20]. Much of the divergence between the two enzymes occurs at the amino and carboxyl termini but in the central portion of the enzyme there are long stretches of total or near identity, indicative of similar regions for substrate and cofactor binding. A comparison of COX-1 or COX-2 across species (human vs. mouse) indicates a similarity $>85\%$ at the amino acid level [21].

Prior to the identification of the COX-2 enzyme, researchers at DuPont identified a potent anti-inflammatory compound, DuP-697 (1) which was a relatively weak inhibitor of bovine seminal vesicle prostaglandin synthesis, but potent in a variety of anti-inflammatory assays [22]. It was later found that this compound possessed selective inhibitory activity against COX-2. A structurally dissimilar compound, NS-398 (2), was also established to be



(1) DuP-697



(2) NS-398

a selective inhibitor of COX-2 [17]. The results obtained with these two compounds in animal models of inflammation showed that inhibition of prostaglandin production by COX-2 was therapeutically beneficial. DuP-697 and NS-398 [23] are characteristic of most NSAIDs in showing inhibitory activity in standard models of inflammation, analgesia and pyresis. Both compounds inhibit PG production in inflammatory cells. In animal models of NSAID induced gastric damage, high doses of DuP-697 or NS-398 were found to be largely free of typical NSAID gastrointestinal effects.

In addition, NS-398 inhibits PG production at sites of inflammation but not in the stomach or kidney. The role of COX-2 in causing inflammation and pain is now widely accepted [24, 25]. Currently marketed NSAIDs are inhibitors of both COX-1 and COX-2 [26, 27] so there has been a major research effort in the industry aimed at identifying a selective COX-2 inhibitor with an attractive pharmacological profile. A drug that is safe, potent, with anti-inflammatory efficacy comparable to current NSAIDs and a convenient oral dosing regimen. The subject of COX-2 biology, pharmacology, and the medicinal chemistry of selective COX-2 inhibitors has been the subject of several recent reviews [28–40].

In addition to their traditional uses new applications are now emerging for NSAIDs. Epidemiological studies have revealed a significant reduction in the incidence of colon cancer among individuals regularly taking NSAIDs [41]. The epidemiological data suggest a reduction in risk of between 40 and 50%. Prostaglandins have been implicated as contributors to the development and progression of colon cancer because cyclooxygenase expression is substantially elevated in human colorectal carcinomas and adenomas [42]. Knockout mice with an APC gene deletion spontaneously develop colon cancer. Humans with the same genetic defect are also at very high risk of developing colon cancer [43]. Humans and mice with this genetic abnormality show elevated levels of cyclooxygenase activity in their respective adenometous polyps as compared to control colonic tissues. Laboratory research with several selective COX-2 inhibitors, as well as with NSAIDs, in mice with the APC genetic defect showed a marked reduction in the inci-

dence and severity of murine colon carcinogenesis [44]. Azoxymethane treated laboratory animals spontaneously develop colonic aberrant crypt foci, a precursor of colon cancer. Administration of selective COX-2 blockers suppressed the development of aberrant crypt foci in these animals [45]. The results from animal studies and human epidemiological data suggest that COX-2 inhibitors may be beneficial for the prevention and management of colon cancer [46–50].

Finally, prostaglandins from COX-2 may play an important role in certain neurological disorders. For example, the prevalence of Alzheimer's disease seems to be lower in those patients that take NSAIDs [51]. COX-2 immunoreactivity increases in certain brain tissues following cerebral ischemia and the upregulation of COX-2 has been implicated in delayed neuronal death. In rodent studies, selective COX-2 inhibitors attenuated the infarct volume in the hemisphere where the injury was induced. The mechanism by which selective inhibitors showed activity in this model was attributed to inhibition of prostaglandin E_2 (PGE_2) formation by COX-2 [52].

METHODS OF EVALUATING COX-2 INHIBITORS

A comparison of the various inhibitors from different laboratories presents something of a challenge in this field because of the numerous and widely differing methods that have been reported for evaluating COX-1 and COX-2 activity *in vitro*. They have involved the use recombinant enzyme [21, 53–54], human whole blood [55–56], insect cells [57], various mammalian cells and platelets [58–63]. Confusing the issue further is the fact that many of the compounds are irreversible time dependent inhibitors of COX-2 and competitive inhibitors of COX-1 [64, 65]. Thus, the length of time a given inhibitor is pre-incubated with COX-2 prior to adding the substrate, arachidonic acid, can alter the IC_{50} value. Additionally, the concentration of arachidonic acid can effect the IC_{50} value for a given inhibitor [66]. Therefore, it is important to compare compounds against known inhibitors to make a meaningful interpretation of the data from different laboratories.

The *in vivo* assays that have been employed to evaluate COX-2 inhibitors are well known and have been in use for some time. The carrageenan-induced paw oedema assay [67] in the rat has been used as a model for assessing the effect of NSAIDs and COX-2 inhibitors in an acute inflammatory response. Injection of carrageenan into the hind foot pad results in a significant increase in paw volume three hours after injection. Paw volume is measured with a displacement plethysmometer and the volume increase is used

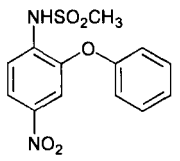
as a direct measure of inflammation. The role of COX-2 in this model has been thoroughly studied [18]. The carrageenan-induced hyperalgesia model, also known as the Hargreaves model, on the other hand is used to assess the analgesic efficacy of anti-inflammatory compounds [68]. The adjuvant-induced arthritis assay is used to measure the activity of anti-inflammatory drugs in chronic inflammation [19, 69]. Finally, in order to measure the ulcerogenicity of drug candidates, fasted rats are administered test compounds and after 72h are sacrificed and the presence or absence of lesions are counted [18]. Alternatively, rats are dosed with the test compound for a given period of time followed by intravenous administration of chromium-51 labelled red blood cells. Evaluation of the collected faeces for the presence of chromium-51 provides a quantitative assessment of gastrointestinal integrity. This model has also been adapted for use with squirrel monkeys [66]. The anti-pyretic activity of test compounds is typically evaluated against an endotoxin-induced pyretic response in rats [66].

MEDICINAL CHEMISTRY

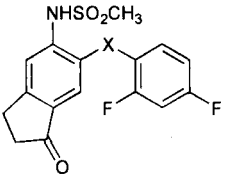
METHANESULPHONANILIDE INHIBITORS

With the recognition that NS-398 (2) was a selective inhibitor of cyclooxygenase-2 came a renewed interest in this general class of anti-inflammatory agents [17, 23, 70]. It was established some time ago that nimesulide (3) [71] and flosulide (4) [72–74] possessed anti-inflammatory activity but re-examination of both compounds showed them to possess some degree of selectivity for the COX-2 isoform [75, 76]. All members of the methanesulphonanilide class of COX-2 inhibitors have the common structural features shown in *Figure 5.1*. Structurally, these compounds are characterized as derivatives of alkylsulphonanilides. The alkyl portion of the sulphonyl group is typically methyl, but halogenated methyl groups such as trifluoromethyl have also been reported [76]. The 2-position is most frequently substituted with an aryl ether or aryl thioether, but ethers and thioethers of cycloalkyl groups are also possible, as in (2). Inhibitors incorporating a heterocyclic ether or thioether at the 2-position have also been described [97]. The 4-position invariably bears an electron-withdrawing substituent, and this may be incorporated as part of a ring.

It appears that nimesulide was the first member of this class to be discovered [77]. The mechanism of action [78], pharmacology [79], and clinical evaluation in rheumatic diseases [80], osteoarthritis [81], and acute inflammatory states [82–84] all demonstrated that nimesulide was a novel anti-



(3)
Nimesulide



(4) Flosulide X=O
(5) L-745,337 X=S

inflammatory agent. Subsequent to the discovery and clinical evaluation of nimesulide, several groups became interested in this unique anti-inflammatory agent. A variety of methanesulphonanilides with different electron-withdrawing groups at the 4-position have been prepared and evaluated for their anti-inflammatory activity. Substituents included acetyl, carboxy, carboethoxy, amide, cyano, trifluoromethyl, sulphonamido, sulphonyl and many others [91, 92]. From the published biological data on these compounds, it appears that selected 4-acetyl, 4-cyano and 4-sulphonamido (6) substituted analogues have the best *in vivo* activity.

Incorporation of the electron-withdrawing group as part of a ring is another structural variant that has been employed. Flosulide (4) possesses many of the structural features of nimesulide [85]. The principal difference between them is the incorporation of the electron-withdrawing substituent into a five membered carbocyclic ring. This structural feature was recently exploited by the Merck Frosst group in L-745,337 (5) [76, 86–88]. The thioether analogue (5) is reported to have greater specificity for COX-2, greater oral bioavailability, improved *in vivo* potency, and a greater gastrointestinal safety profile than flosulide. The *in vitro* activity of L-745,337 and flo-

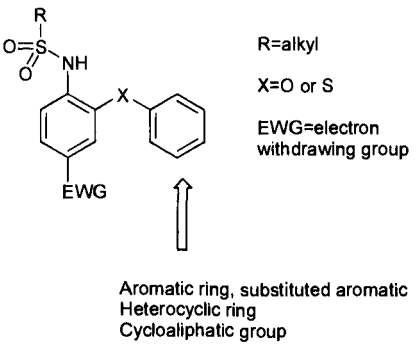
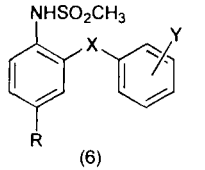
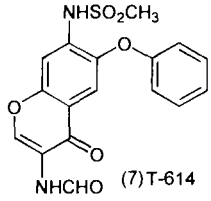


Figure 5.1 General structure of a sulphonanilide COX inhibitor

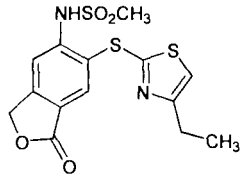


(6)

R=$COCH_3$, CN, SO_2NH_2
X=O or S
Y=Cl, F others



(7)T-614

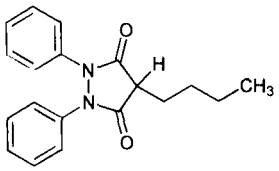


(8)

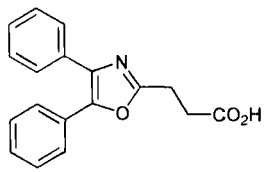
sulide in whole cell COX-2 assays were identical [89], and neither compound showed activity in whole cell COX-1 assays [90]. The electron-withdrawing group may also be incorporated as part of a heterocyclic ring. Toyama's T-614 (7) is an example of this structural class [93–95]. A recent patent application describes a series of highly COX-2 selective isobenzofuranone derivatives (8) [96]. The activity of (8) in cell culture (COX-1 $IC_{50} \geqslant 100$ μM and COX-2 $IC_{50} = 0.005$ μM) showed it to possess exceptional *in vitro* potency and selectivity for COX-2. In the carrageenan paw oedema assay [97] (8) had an ED_{50} of 0.9 mg/kg p.o. It would appear that (8) is the most potent anti-inflammatory methanesulphonanilide prepared to date.

DIARYL HETEROCYCLES AS COX-2 INHIBITORS

The origins of diaryl heterocycles as the pharmacophore for cyclooxygenase inhibition are far from clear but it could be argued that phenylbutazone (9) was the first member of this class of compounds [98]. Phenylbutazone has certainly attracted a great deal of attention from medicinal chemists over the years [99] and its exceptional anti-inflammatory activity undoubtedly provided much of the impetus that led medicinal chemists to explore this type of structure. In the late 1960s the diphenyl oxazole derivative oxaprozin (10) was identified as an anti-inflammatory agent [100–102]. Not long after the discovery of oxaprozin, a large number of 1,2-diaryl heterocycles were prepared and evaluated for their anti-inflammatory activity. Throughout the 1970s and 1980s a large number of analogues were disclosed, primarily in the patent literature. Some of them were based on a central ring containing a thiazole [103–105], oxazole [106–107], furan [108], pyrrole [109–114], imidazole [115–129], pyrazole [130–135], isoxazole [136,137], pyrimidine [138], and thiophene [139–146]. From the evaluation of numerous analogues it was found that 4-halo or 4-methoxy substituted diaryl heterocycles generally possessed enhanced anti-inflammatory potency relative to their unsubstituted congeners [129].



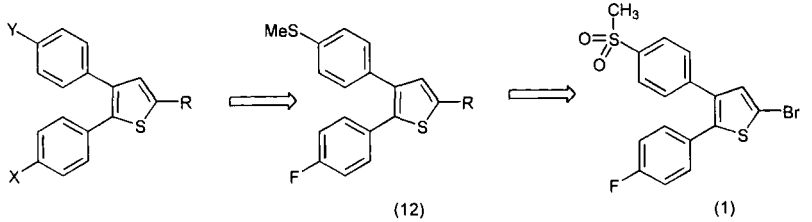
(9)Phenylbutazone



(10)Oxaprozin

In the 1970s a group of researchers at DuPont initiated an extensive evaluation of the diaryl heterocyclic class of anti-inflammatory agents. From their careful evaluation of a large number of compounds they found that certain unsymmetrically substituted analogues had considerable anti-inflammatory activity, see *Figure 5.2*. Starting from an unsubstituted 2,3-diphenylthiophene (11, R = H) a steady increase in potency was observed by first making the 2,3-bis-4-fluorophenyl thiophene (11, X = Y = F, R = Br) or 2,3-bis-4-methoxyphenyl thiophene congeners, and then by replacing one of the fluorine atoms with a methylthio group (12). Oxidation of the methylthio group to the corresponding sulfone afforded very potent anti-inflammatory agents, including DuP-697 [145], see *Table 5.1*.

The biological profile of DuP-697 is illustrated in *Table 5.2*. These data indicate that DuP-697 is a very potent and selective inhibitor of COX-2 under several different assay conditions. In addition, (1) is very potent in the adjuvant arthritis chronic dosing assay. Clinical evaluation of DuP-697 showed it to have an extremely long plasma half-life in humans of 292 h [147] which was attributed to enterohepatic recirculation of the parent compound. DuP-697 is not metabolized to any extent in man. The metabolism of (1) in laboratory animals, however, showed that the 5-bromo substituent of the thiophene was subject to replacement by sulphur containing groups, the



(11)X,Y=H, F, OMe,SMe (12) (1)

Figure 5.2.

Table 5.1. ADJUVANT ARTHRITIS ACTIVITY OF SELECTED 2,3-DIARYLTHIOPHENE DERIVATIVES [143, 145]

Compound	*X*	*Y*	*R*	*Adjuvant arthritis ED_{50} (mg/kg) p.o.*
(11)	F	F	Br	> 60
(11)	OMe	OMe	Br	~4.5
(12)	F	SMe	Br	0.25
(11)	SMe	F	Br	1.4
(11)	SO_2Me	F	Br	0.4
DuP-697 (1)	F	SO_2Me	Br	0.21
Indomethacin				0.25

principal metabolite being the methylsulphonyl derivative (13) [148–151]. In order to overcome these pharmacokinetic shortcomings several groups initiated synthetic programmes around the DuP-697 template. The Fujisawa and DuPont Merck groups prepared a variety of analogues in which the bromine atom was replaced with different substituents [147,152]. Substituents such as carbomethoxy, alkyl, sulphonamide and others were found to be acceptable bromine replacements. The pharmacokinetic parameters of the analogues were not reported, however. From an extensive study of potential replacement groups for the methylsulphone group it was found that only the sulphonamide maintained specificity for COX-2 [153, 154]. The use of the sulphonamide group (SO_2NH_2) generally results in increased potency against COX-2 and improved oral absorption. Replacing the methylsulphone with the sulphonamide, however, generally increased activity against COX-1. In the case of compounds (14) and (15) for example, the sulphone (14) showed a COX-1 IC_{50} of greater than 100 μM and a COX-2 IC_{50} = 0.25 μM whereas the sulphonamide (15) exhited a COX-1 IC_{50} = 1.3 μM and a COX-2 IC_{50} = 0.03 μM.

Regiosiomeric 3,4-diaryl thiophenes were found to be selective inhibitors of COX-2 [147,154,155]. For example, it was found that the *in vitro* activity of (14) and (16) were similar (16, COX-1 $IC_{50} \geqslant 100$ μM ; COX-2 IC_{50} = 0.08 μM). The two regioisomeric mono-bromo derivatives (17) and (18), on the other hand, possessed very different abilities to inhibit COX-2. The analogue (17), where the bromine atom was located in the position adjacent to

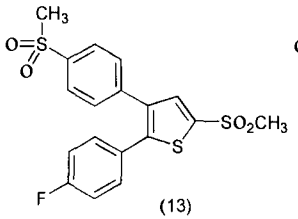
Table 5.2 PHARMACOLOGY OF DUP-697 AND INDOMETHACIN

Compound	*COX-1* $IC_{50}\mu M$	*COX-2* $IC_{50}\mu M$	*Adjuvant Arthritis* ED_{50} *(mg/kg/day) p.o.*	*Gastric Lesions*
DuP-697 (1)	1[a]	0.01[a]	0.18[e]	>400 (mg/kg/day)[e]
	38[b]	7[b]		
	0.6[c]	0.007[c]		
	11[d]	0.02[d]		
Indomethacin	0.01[a]	0.05[a]	0.27[e]	<8 (mg/kg/day)[e]
				5 (mg/kg/day)[d]

[a]Data from Ref.[87] [c]Data from Ref.[155] [e]Data from Ref. [22]
[b]Data from Ref.[147] [d]Data from Ref.[152]

the aromatic ring bearing the methylsulphone, was only weakly active at the screening concentration, i.e. COX-1 $IC_{50} \geqslant 100\ \mu M$ and a COX-2 $IC_{50} = > 100\ \mu M$, in contrast to the isomer (18) with the bromine atom distal to the methylsulphonylphenyl ring which had a COX-1 $IC_{50} \geqslant 100\ \mu M$ and a COX-2 $IC_{50} = 0.08\ \mu M$. The 2,4-diaryl (19) or 2,5-diarylthiophene (20) positional isomers had significantly lower activity at all test concentrations. Thus, so long as the aromatic groups were on adjacent positions in the thiophene ring the compounds possessed considerable inhibitory activity.

In the SAR study of 3,4-diarythiophenes it was found that the 4-methylsulphonyl (CH_3SO_2) or 4-sulphonamido (SO_2NH_2) substituent was the key to selective activity against COX-2. Other substituents afforded compounds that were either inactive as cyclooxygenase inhibitors or were selective for COX-1. For example, the methylthio derivative (21) had a COX-1 $IC_{50} = 0.05\ \mu M$ and a COX-2 $IC_{50} \geqslant 100\ \mu M$. In addition, the observation was made that the substituents on the other aromatic ring could effect the potency and/or specificity for COX-2. The presence of a 4-methoxy substituent



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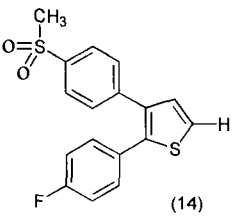

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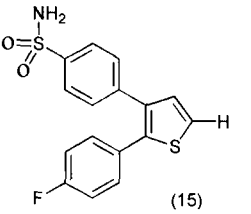

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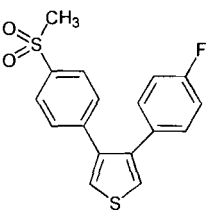

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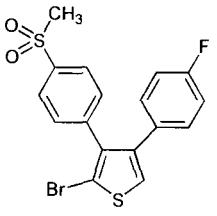

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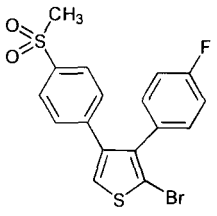

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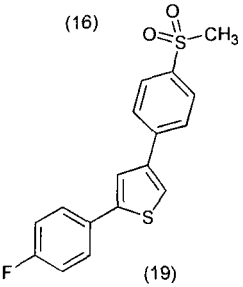

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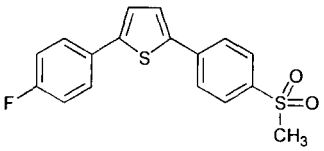
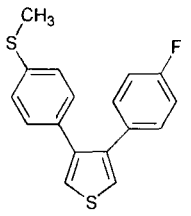


(20)

invariably led to an increase in COX-1 blockade. For example, 4-methoxy substituted 3,4-diarylthiophene (22) showed a COX-1 IC_{50} = 0.9 μM and a COX-2 IC_{50} = 0.05 μM. The specificity could be raised by the introduction of a substituent adjacent to the 4-methoxy group. In particular, it was found that introduction of a 3-fluoro substituent adjacent to the methoxy group substantially improved the selectivity for COX-2, thus (23) had a COX-1 $IC_{50} \geqslant 100$ μM and a COX-2 IC_{50} = 0.03 μM. In general, a wide range of substituents were tolerated on the aromatic ring that didn't bear the sulphone or sulphonamide moieties.

With the knowledge gleaned from the study of diarylthiophene deriva-



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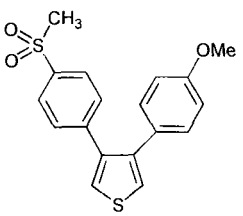

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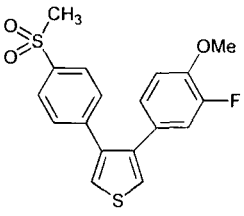
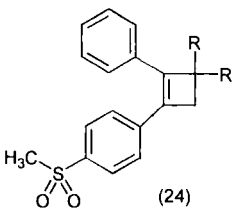


(23)

tives, it was determined that an important function of the thiophene ring was to properly orientate in space the two appropriately functionalized aromatic rings. In this class of compound, the central carbocycle or heterocycle can effect the *in vitro* activity of a given inhibitor candidate very considerably. The nature of the central ring plays a very important role in the pharmacological profile of a given molecule. Subtle structural changes can alter the physicochemical properties such as aqueous solubility and polarity. Alteration of the physicochemical characteristics may in turn alter pharmacokinetic parameters like oral absorption, tissue distribution, and plasma half-life. Some of the structural variations on diaryl heterocycles that have been made to date will be presented in the next section.

Derivatives with a central 4-membered ring

Cyclobutene derivatives (24), (25), and (26) are representative members of the 4-membered ring class of inhibitors [156]. In this series the potency and selectivity was found to be very sensitive to minor steric and electronic changes. While the unsubstituted cyclobutene derivative (24, R = H) was a very weak inhibitor (COX-1 IC_{50} not determined; COX-2 $IC_{50} > 5$ μM) incorporation of a ketone functionality improved the inhibitory activity, (24, R = O) (COX-1 $IC_{50} = 2$ μM; COX-2 $IC_{50} = 0.11$ μM). Introduction of a geminal dimethyl substituent in the cyclobutene ring also had a very favourable effect on potency in this series (25, R = H) (COX-1 $IC_{50} = 0.12$ μM; COX-2 $IC_{50} = 0.002$ μM). The selectivity for COX-2 could be improved without adversely affecting the exceptional potency by incorporating a ketone moiety, (25, R = O) (COX-1 $IC_{50} = 2$ μM and COX-2 $IC_{50} = 0.003$ μM). Regioisomeric geminal dimethyl ketone (26) was about thirty times less potent against COX-2 in cell culture than (25, R = O) (COX-1 $IC_{50} = 2$ μM and COX-2 $IC_{50} = 0.096$ μM). In spite of its lower potency in cell culture, (26) was essentially equivalent to (25, R = O) in the carrageenan-induced paw oedema assay, $ED_{50} = 2.4$ and 2.7 mg/kg p.o. respectively. To date no other 4-membered ring based tricyclic inhibitors have been reported.



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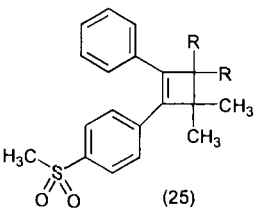

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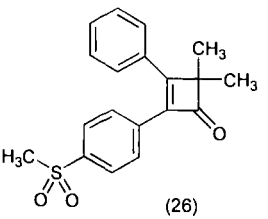

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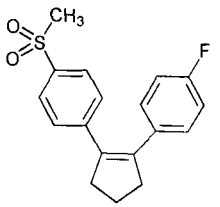
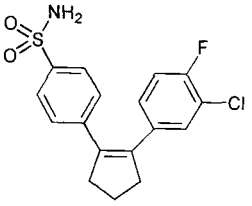


(27) SC-57666

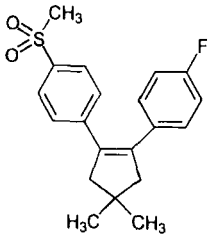


(28)

Derivatives with a central 5-membered carbocyclic ring

Among the first series of diaryl heterocycles to be disclosed in the literature, including considerable experimental detail, were derivatives of cyclopentene [157–160]. SC-57666 (27) for example, possesses a very high degree of specificity for COX-2 (COX-1 $IC_{50} \geqslant 1000$ μM and a COX-2 $IC_{50} = 0.026$ μM). In the adjuvant induced arthritis assay, (27) showed an ED_{50} of 1.7 mg/kg p.o. No gastrointestinal lesions were observed for this compound in adjuvant arthritis, after chronic administration of the effective dose, because of the high degree of specificity for COX-2. In contrast to this most of the currently available NSAIDs cannot be dosed for extended periods in laboratory animals because they are lethal near the effective dose. The generally modest oral bioavailability and relatively short plasma half-life of SC-57666 could be improved by replacing the methylsulphone by the sulphonamide moiety. Thus, (28) with a COX-1 $IC_{50} = 5.1$ μM and a COX-2 $IC_{50} = 0.01$ μM, had an oral bioavailability of 56% and a plasma half-life of 2.1 h in Sprague-Dawley rats. In spite of the greater activity against COX-1, (28) was found to be free of gastrointestinal toxicity even at doses as high as 200 mg/kg. The cyclopentene ring has also been dehydrogenated to afford diaryl-cyclopentadiene inhibitors, but they appear to be less active *in vivo* [158].

Some of the structural modifications to the cyclopentene that have been reported focussed on substitution in the 4-position. While 4,4-dimethylcyclopentene derivative (29) had potency against COX-2 comparable to (27) it was less selective (COX-1 $IC_{50} = 18.3$ μM; COX-2 $IC_{50} = 0.015$ μM). Incorporating the 4,4-substituents into a spirocyclic ring (30) increased the potency (COX-1 $IC_{50} = 5.4$ μM; COX-2 $IC_{50} = 0.008$ μM), but increased further the activity against COX-1 [161,162]. Increasing the size of the spirocyclic ring to a cyclobutane ring (31) restored the specificity for COX-2 (COX-1 $IC_{50} \geqslant 100$ μM; COX-2 $IC_{50} = 0.004$ μM), but the *in vivo* activity suffered. Increasing the spirocyclic ring to a cyclopentane (32) gave a compound with good activity *in vitro*, but almost no activity *in vivo* (COX-1



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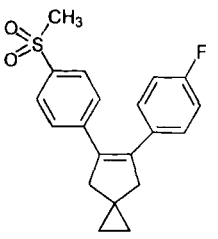

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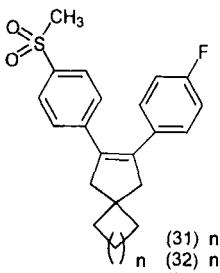
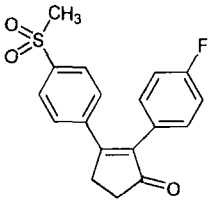


(31) n=1
(32) n-2
(33) n=3



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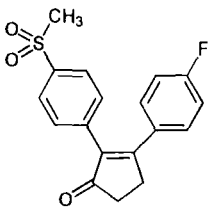
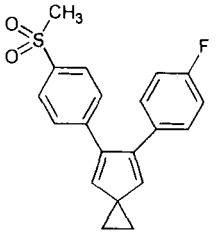


(35)

$IC_{50} \geqslant 100\mu M$ and COX-2 $IC_{50} = 0.062\ \mu M$). Finally, expanding the spirocyclic portion to a cyclohexane ring (33) resulted in a decrease of *in vitro* activity (COX-1 $IC_{50} \geqslant 100\ \mu M$; COX-2 $IC_{50} \geqslant 100\ \mu M$). Substitution of the 3-position with a ketone moiety, as in (34) or (35), was reported in the patent literature [163–165]. While both regioisomeric ketones have been claimed, only the isomer (34) has been exemplified.

Diaryl cyclopentadienes have also been described in a series of patents [166–168]. The *in vitro* activity of (36) against COX-2 appears to be comparable to its cyclopentene analogue (30). The potency of (36) against COX-1, however, was increased relative to (30). In the carrageenan induced hyperalgesia assay, (36) showed 94% inhibition at 10 mg/kg p.o.. Compared to their cyclopentene congeners, it appears that introducing a second double bond into the ring does increase the *in vivo* potency to some degree. Fusion of a benzene ring to the cyclopentene gives diarylindene derivatives. The methylsulphonyl substituted indene derivative (37) was a potent and highly selective inhibitor (COX-1 $IC_{50} \geqslant 100$ uM; COX-2 $IC_{50} = 0.011\mu M$). The corresponding sulphonamide analogue (38) was an extremely potent inhibitor of both enzymes (COX-1 $IC_{50} = 0.007\ \mu M$; COX-2 $IC_{50} = 0.005$ uM). No biological data were provided to determine whether diarylindenes possessed anti-inflammatory activity [169].



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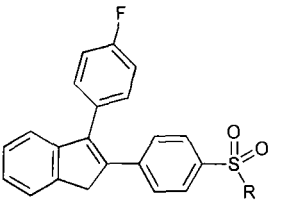
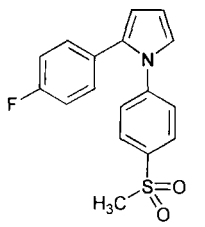


(37) R=CH_3
(38) R=NH_2

Derivatives with a central 5-membered heterocyclic ring

Pyrroles

A great deal of attention has been devoted to the synthesis and evaluation of diaryl 5-membered ring heterocycles and all the possible orientations of diarylpyrroles have now been reported. An extensive study of 1,2-diarylpyrroles showed that the two possible regioisomers, represented by (39) and (40), were both selective COX-2 inhibitors and active in the carrageenan paw oedema assay. Compound (39) had a COX-1 $IC_{50} \geqslant 100$ μM and a COX-2 $IC_{50} = 0.5$ μM, and gave 42% inhibition in the paw oedema assay at 10 mg/kg. Compound (40, R = H) had a COX-1 $IC_{50} \geqslant 100$ μM and a COX-2 $IC_{50} = 0.06$ μM, and produced a 25% inhibition of the inflammatory response in the paw oedema assay at 10 mg/kg. It would appear that when the 4-methylsulphonylphenyl group was in the 2-position there was about a tenfold enhancement of *in vitro* activity. Placement of a variety of substituents at the 4-position of (40, R other than H) did little to alter the *in vitro* or *in vivo* performance of these analogues [170]. 3,4-Diarylpyrroles were described in a patent application [171]. 2,3-Substituted analogues were the most extensively studied of the diarylpyrroles [172–174]. A very large num-



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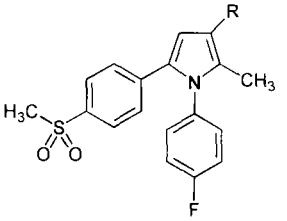

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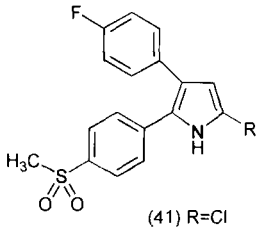
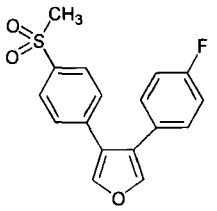


(41) R=Cl
(42) R=Br

ber of 5-substituted 2-(4-methylsulphonylphenyl)-3-(4-fluorophenyl) pyrroles (41) were prepared by the DuPont Merck group and evaluated *in vitro* and *in vivo*. In general, electron-withdrawing substituents in the 5-position afforded inhibitors with the greatest activity in the adjuvant induced arthritis assay. Specifically, in the adjuvant arthritis assay (41) and (42) had ED_{50}'s of 0.5 and 1.05 mg/kg, respectively.

Furans

The two possible regioisomeric 2,3-diarylfurans, unsubstituted at the 4 or 5-position, were reported in a recent patent [175]. 3,4-Diarylfuran derivatives bearing the appropriate substituents (43) are COX-2 selective inhibitors [171,175]. In general, the furan derivatives were found to be less potent *in vitro* than the corresponding thiophenes. There has been a good deal of literature on the preparation of 3,4-diarylfuranones [171,175] which may be either of the two possible regioisomers, (44) and (45) and both have been claimed. Placing small alkyl substituents at the 5-position of (44) provided analogues such as (46) that possess very high specificity for COX-2 [66]. Compound (46) possesses activity in all the standard animal models of inflammation (carrageenan paw oedema ED_{50} = 0.1 mg/kg p.o.) and was reported to not cause any gastric lesions in a five day twice daily dosing study at 100 mg/kg. A recent patent application describes hydroxy dihydrofuran (47) as a pro-drug for (46) [176].



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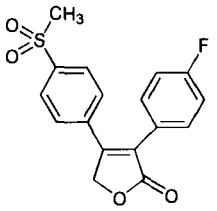

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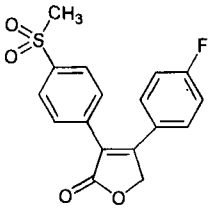

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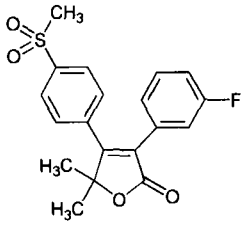

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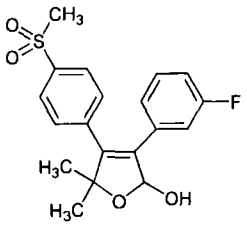

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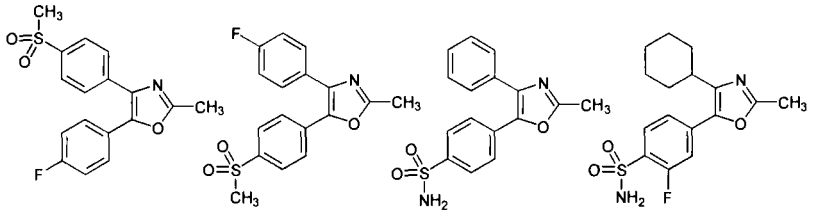


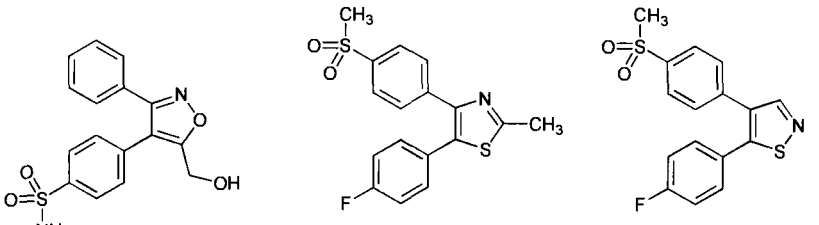
(48) (49) (50) (51)

Oxazoles

A large number of diaryl oxazoles have been prepared and evaluated as COX-2 inhibitors. It was found that the two possible regioisomers had considerably different *in vitro* activity [177]. For example, (48) had a COX-1 $IC_{50} \geqslant 100$ μM and a COX-2 $IC_{50} = 10$ μM, whereas, inexplicably, (49) had a COX-1 $IC_{50} \geqslant 100$ μM and a COX-2 $IC_{50} = 0.14$ μM. The sulphonamide analogues generally had greater potency against both COX-1 and COX-2. Compound (50) had a COX-1 $IC_{50} = 25$ μM and COX-2 $IC_{50} = 0.02$ μM. In the rat adjuvant arthritis assay (50) had an $ED_{50} = 0.2$ mg/kg [178]. Introduction of a fluorine atom adjacent to the sulphonamide (51, JTE-522) and saturation of the unsubstituted benzene was found to improve the selectivity and potency for COX-2 (carrageenan paw oedema $ED_{30} = 5.0$ mg/kg p.o.) [179–180]. The 2-positon of diaryloxazoles can be substituted with quite large substituents with retention of biological activity [181].

Isoxazole, thiazoles, isothiazole, thiadiazole and imidazole analogues

Diaryl isoxazole sulphonamide (52) has a high degree of specificity for COX-2 (COX-1 $IC_{50} \geqslant 1000$ μM; COX-2 $IC_{50} = 0.18$ μM) and in the carrageenan paw oedema assay had an $ED_{50} = 1.1$ mg/kg [182]. Thiazole based inhibitors such as (53) are potent and selective inhibitors of COX-2 [183]. Isothiazole analogue (54) and thiadiazole (55) are both selective inhibitors of COX-2, and (55) has activity *in vivo* (carrageenan paw oedema $ED_{50} \leqslant 1$



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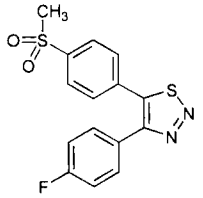

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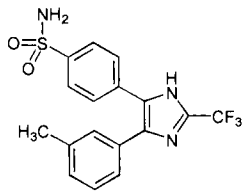

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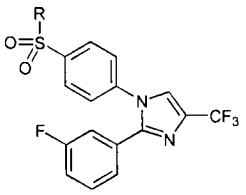
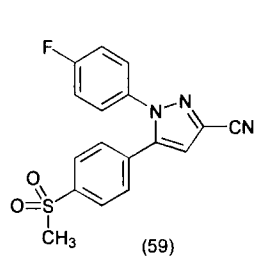


(57) R=CH_3
(58) R=NH_2

mg/kg) [155]. 4,5-Diarylimidazole sulphonamide derivative (56) had modest selectivity for COX-2 (COX-1 IC_{50} = 6.2 μM and a COX-2 IC_{50} = 0.1 μM) [184] in comparison with the 1,2-diaryimidazole (57) which had excellent selectivity (COX-1 $IC_{50} \geqslant 1000$ μM; COX-2 IC_{50} = 0.12 uM). In addition, (57) showed 88% inhibition at 2 mg/kg in a carrageenan-induced air pouch model [185]. The corresponding sulphonamide derivative (58) was more potent against both cyclooxygenase isoforms (COX-1 IC_{50} = 67.7 μM; COX-2 IC_{50} = 0.03 μM). In the carrageenan-induced air pouch model (58) exhibited 98% inhibition after a 2 mg/kg oral dose.

Pyrazoles

In the early 1990s researchers from Fujisawa disclosed a series of 1,5-diarylpyrazole inhibitors, typical of these compounds was (59) [186,187]. Recently, the *in vitro* activity of (59) against the two cyclooxygenase isoforms was reported (COX-1 $IC_{50} \geqslant 100$ μM; COX-2 IC_{50} = 0.24 μM) and showed 81% inhibition in the adjuvant-induced arthritis assay at 3.2 mg/kg after oral administration. Pyrazole (59) did not show any tendency to induce ulcers at concentrations well above the efficacious dose (UD_{50} > 10 mg/kg). SC-58125 (60) was one of the first well characterized COX-2 inhibitors, and served as useful tool for the elucidation of the pharmacology of such compounds *in vivo* [18]. The very long terminal half-life of (60), >200 h in



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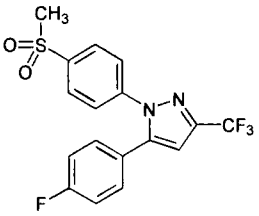

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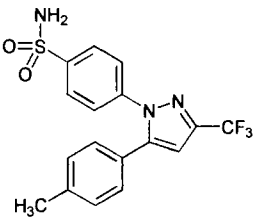
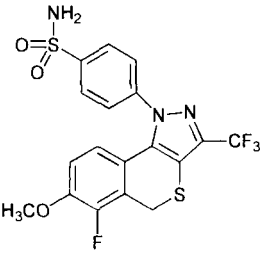


(61)

rodents, rendered it unacceptable for clinical evaluation. Changing the sulphone to a sulphonamide significantly improved the pharmacological profile of these compounds. In particular, sulphonamide containing analogues had substantially improved toxicological properties and much greater oral bioavailability than their sulphone congeners. An extensive pharmacological study of analogues in this series has been reported [189]. The 4-methyl substituted analogue, SC-58635 (celecoxib) (61), had an optimal *in vitro* and *in vivo* profile and was selected for clinical evaluation. The compound showed potent and selective *in vitro* activity (COX-1 IC_{50} = 13 μM; COX-2 IC_{50} = 0.04 μM) and marked anti-inflammatory activity in the rat adjuvant-induced arthritis assay (ED_{50} = 0.4 mg/kg) [189].

In certain instances it is possible to introduce conformational restraint by adding another ring and still retain specificity for COX 2, as in compounds (62) and (63). This demonstrates the active conformation for inhibiting COX 2, at least for this pattern of substitution. Analogue (63) was more selective *in vitro* (COX-1 $IC_{50} \geqslant$ 100 μM; COX-2 IC_{50} = 0.04 μM) than compound (62) (COX-1 IC_{50} = 11 μM; COX-2 IC_{50} = 0.016 μM) and they both exhibited comparable *in vivo* activity in the rat air pouch test at 2 mg/kg with 72% and 63% inhibition, respectively [193,192]. 3,4-Diarylpyrazoles have also been reported to be selective COX-2 inhibitors [190,191].



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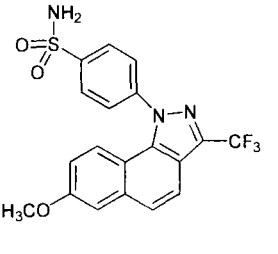
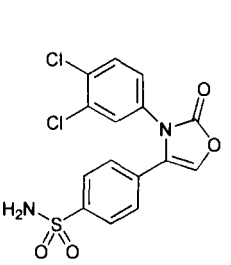


(63)

Other 5-membered ring derivatives

A recent patent application described the synthesis and biological evaluation of a number of 3,4-diaryloxazolone derivatives. For example, compound (64) was a potent COX-2 inhibitor but poorly selective *in vitro* (COX-1 IC_{50} = 3.5 μM ; COX-2 IC_{50} = 0.06 μM), and showed only 67% inhibition of swelling in the adjuvant arthritis model at 1 mg/kg p.o. [194]. In an interesting series of patent applications a group from Lab UPSA discovered that the two key aromatic rings could be attached to the same carbon



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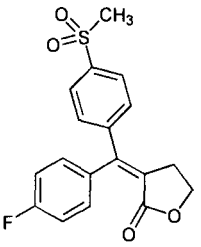
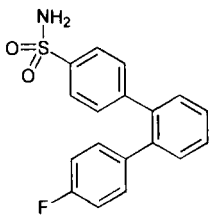


(65)

atom, as in (65). This compound produced a 60% reduction in paw swelling after dosing orally at 30 mg/kg [195,196].

Derivatives with a central 6-membered carbocyclic or heterocyclic ring

Several research groups have reported the preparation and evaluation of *ortho*-terphenyl or *ortho*-terpyridine analogues as COX-2 inhibitors. Most of the compounds that have been described are related to (66) [198–200]. This class of inhibitor generally performed very well in the adjuvant-induced arthritis and air pouch assays. Specifically, compound (66) had 300-fold selectivity over COX-1 *in vitro* (COX-1 IC_{50} = 19 μM; COX-2 IC_{50} = 0.06 μM) and showed 68% inhibition in the rat air pouch model. Insertion of an oxygen atom between one of the benzene rings as in (67) was found to be an acceptable structural modification in this series. These analogues possess selectivity for COX-2 and perform admirably in the adjuvant-induced arthritis assay, with ED_{50}'s of around 1 mg/kg [201]. The central ring may also be pyridine. 2,3-Diaryl [202, 203] and 3,4-diarylpyridine [204] isomers are possible and have been claimed in patent applications. Both possess considerable potency and selectivity for COX-2. A wide range of anti-inflammatory activity has been reported for different members of this series. Analogue (68) was reported to have exceptional anti-inflammatory activity, with an ED_{50} of 0.6 mg/kg in the carrageenan paw oedema assay [203].



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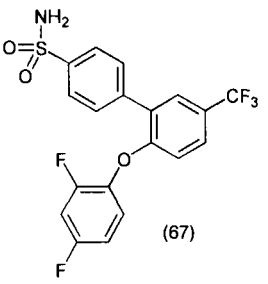

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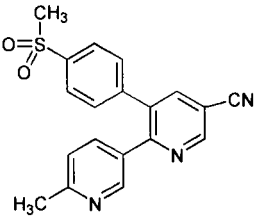
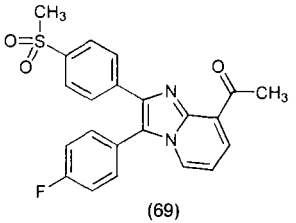


(68)

Derivatives with a central bicyclic ring

As was noted earlier, in certain instances it is possible to fuse an additional ring to the central carbocyclic or heterocyclic ring and maintain COX-2 activity, as represented by (37) and (38) [169, 200]. The variation of a bicyclic structure is being used increasingly by medicinal chemists to afford novel COX-2 structures. A Glaxo patent application describes a series of imidazo[1,2-a]pyridine derivatives with specificity for COX-2 [205] as exemplified by (69) which showed good potency and high selectivity for cyclooxygenases in cell culture (COX-1 $IC_{50} \geqslant 100\ \mu M$; COX-2 $IC_{50} = 0.15\ \mu M$). Fusion of a lactone to a central thiophene gave the selective COX-2 inhibitor (70) [206] which can exist in the carboxylate and hydroxy ring opened form at high pH, and in the lactone form at lower pH. The greater aqueous solubility of the ring opened pro-drug form enhances its oral absorption [38]. A recent patent application describes a wide variety of inhibitors with a central bicyclic ring [207]. 5,6-Diarylimidazo[2,1-b]thiazole (71, L-766,112) was found to be a highly potent and selective inhibitor in chinese hamster ovary cells transfected with human cyclooxygenases (COX-1 $IC_{50} \geqslant 50\ \mu M$; COX-2 $IC_{50} = 0.016\ \mu M$) [208–210]. In the carrageenan paw oedema assay, (71) had an ED_{50} of 2 mg/kg, but despite its selectivity still increased gastrointestinal permeability in rats at doses of 100 mg/kg twice daily for 10 days.



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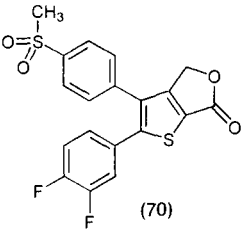

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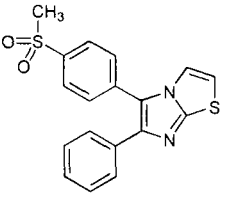
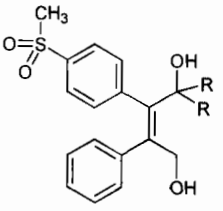


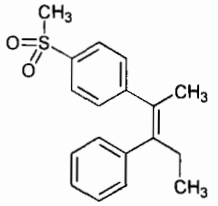
(71) L-766,112

1,2-DIARYLETHYLENE DERIVATIVES

Reduction of the furanone ring of (44) or (46) to the corresponding diol (72) or (73) was found to lead to active inhibitor candidates [211]. Even the *cis*-stilbene derivative (74) retained its ability to inhibit COX-2 [212]. This was the first example of a COX-2 inhibitor without a central ring. The compounds are selective for COX-2 and are anti-inflammatory in the standard inflammatory models.



(72) R=H
(73) R=CH_3

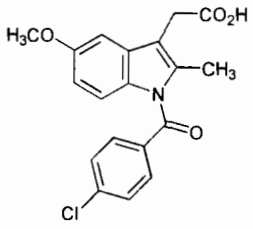


(74)

MODIFICATION OF KNOWN NSAIDS TO IMPROVE THEIR SPECIFICITY FOR COX-2

The larger active site volume of COX-2 suggested that structural modification of known NSAIDs might lead to compounds with improved specificity for the inducible enzyme [213–218]. The Merck Frosst group first reported improving the selectivity of indomethacin (75) for COX-2 by making the larger trichlorobenzoyl analogue (76) [219]. Enlarging the acetic acid side chain of (75) to a beta-branched butyric acid as in (77) improved the COX-2 activity. Removal of the benzoyl group at the 1-position and replacing it with a 4-bromobenzyl gave L-761,066, a compound with considerable potency and selectivity (COX-1 $IC_{50} \geqslant 10\ \mu M$; COX-2 $IC_{50} = 0.06\ \mu M$). The new analogue (77) had anti-inflammatory activity nearly identical to indomethacin. Unlike indomethacin however, (77) reportedly did not cause gastric permeability even with repeated administration of the effective dose [220].

More recently, exchanging the carboxylic acid moiety of indomethacin for a 4-bromophenyl thiazole group afforded the highly selective COX-2 inhibitor (78) [221]. It is not known how the 1-benzoylindole (79), lacking a side-chain at C-3 of the indole nucleus, binds to the active site in COX 2, but in a cell based assay (79) inhibited prostaglandin formation by COX-2 with an $IC_{50} = 0.04\ \mu M$ [197]. Following a similar strategy with zomiperac (80)



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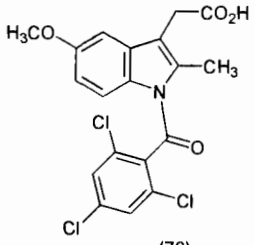

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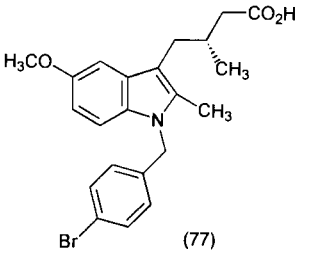

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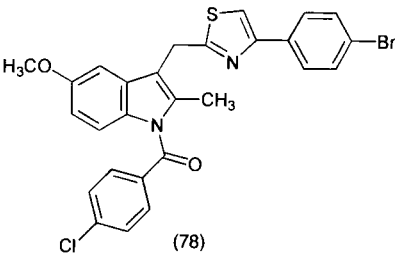

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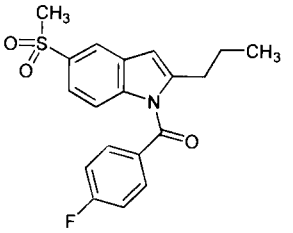
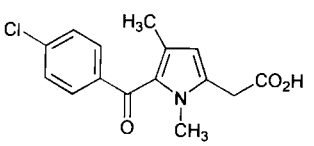


(79)

the carboxylic acid moiety was replaced by a pyridazinone residue to afford the highly COX-2 selective inhibitor (81) [222]. Structurally related pyrrole (82) was also reported recently as a COX-2 inhibitor [223]. Certain derivatives of piroxicam (83) such as meloxicam (84) have favourable selectivity for COX-2 [224]. Further structural modification of (83) resulted in quinolone (85) (COX-1 $IC_{50} \geqslant 1.4\ \mu M$; COX-2 $IC_{50} = 0.06\ \mu M$), which gave 40% inhibition in the carrageenan paw oedema assay after an oral dose of 30 mg/kg [225].



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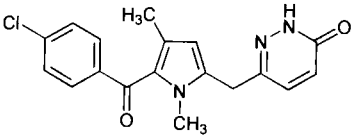

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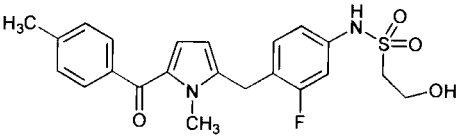

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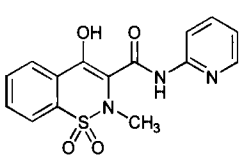

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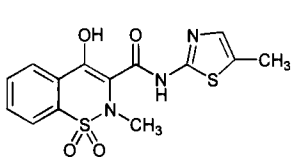

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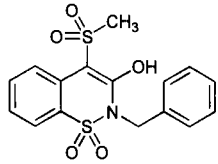

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CLINICAL FINDINGS WITH COX-2 INHIBITORS

Some early clinical findings with COX-2 inhibitors are beginning to be published. From the phase I clinical trial data, celecoxib (61) was found to have a half-life of 12 h and a T_{max} of approximately 2 h in man [226]. In a post-surgical study of dental pain, about 100 mg or 400 mg p.o. of celecoxib showed analgesic activity equivalent to 650 mg of aspirin, with the onset of action after 45 minutes [227]. Celecoxib does not induce ulcers or gastric erosion in humans after oral administration of 200 mg twice daily for 7 days. Platelet aggregation, which is mediated by COX-1, was unaffected by celexocib at an oral dose of 600 mg twice daily for 10 days [228]. Celecoxib was recently reported to show therapeutic benefit in a four week study in patients with rheumatoid arthritis [229]. MK-966 was reported to possess analgesic activity and excellent gastric tolerability in clinical trials at the reported dose of 25 mg once a day [230]. JTE-522 (51) was recently reported to be in clinical evaluation, but no clinical data have yet been published [231]. Nimesulide (3) was reported to be selective for COX-2 [232] and did not reduce serum thromboxane levels after two weeks at an anti-inflammatory dose of 100 mg twice daily. Meloxicam (84), has been reported to be a preferential inhibitor of COX-2 [233–236]. The pharmacokinetic profile of meloxicam has been discussed in detail [237]. Clinical studies in patients suffering from either osteoarthritis or rheumatoid arthritis showed that 7.5 mg or 15 mg doses of meloxicam possess anti-inflammatory activity equivalent to 100 mg of diclofenac, 750 mg of naproxen or 20 mg of piroxicam [238–240]. In a placebo controlled study 7.5 mg of meloxicam showed less gastrointestinal damage than 20 mg of piroxicam [241].

CONCLUSIONS

It has been estimated that more than 100,000 hospitalizations take place every year in the United States due to the complications of taking NSAIDs. COX-2 inhibitors offer the hope of relief from arthritic diseases and pain

without the complication of gastrointestinal side-effects. In addition, COX-2 inhibitors may be useful for the treatment or prevention of certain types of cancer and Alzheimer's disease. With the large number of NSAIDs that are currently available either over the counter or by prescription, it certainly seems likely that research will continue for the foreseeable future to find new types of COX-2 selective inhibitors. A few of the currently marketed drugs such as meloxicam (84), nimesulide (3), and etodolac show a degree of specificity for COX-2 and are reported to possess a greater gastric safety margin than conventional NSAIDs. Clinical results with celecoxib (61) and MK-966 are beginning to demonstrate the anticipated promise of selective COX-2 inhibition. Continuing clinical studies with COX-2 inhibitors will define the full potential of highly selective COX-2 inhibitors.

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6 Molecularly Imprinted Polymers – Preparation, Biomedical Applications and Technical Challenges

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INTRODUCTION

Molecular recognition is fundamental to the function of biological systems. Enzyme activity, receptor binding systems and the immune response require that the presence of one molecular species be perceived by another. Although biological systems are often very highly specific, the capacity for adaptation and apparent non-specificity is inherent. The polymeric system that provides the basis for these recognition events is the protein and nature's creation has been put to good use within the laboratory. However, protein based assays, immuno-affinity chromatography columns, protein-based high performance liquid chromatography (HPLC) columns and enzyme catalysed chemical re-

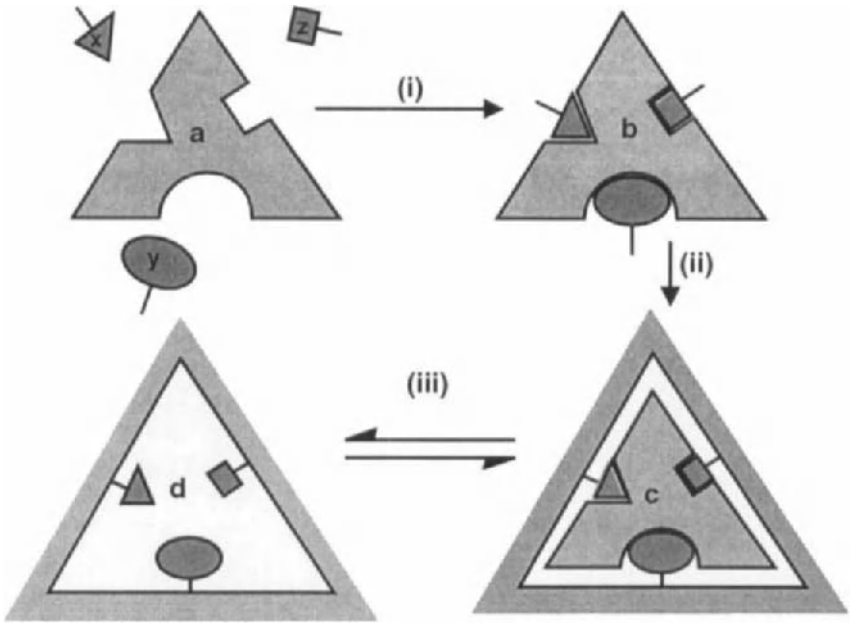


Figure 6.1 Schematic representation of a molecular imprinting process.
(i) formation of pre-polymerisable complex
(ii) polymerisation
(iii) template removal/rebinding

actions are notoriously sensitive to changes in conditions, intolerant to misuse and expensive. It is therefore not surprising that alternative, biomimetic systems have been sought which are capable of specific molecular recognition but possess innate durability, reliability and cost-effectiveness.

One approach has been to synthesize unique molecular structures designed to act as 'host' sites for a second moiety. This 'guest-host' approach has led to some elegant chemistry [1–4] utilising molecularly diverse structures such as crown ethers [5], cryptates [6] and cyclophanes [7]. However, a major drawback with this approach is that it is far from generic, each recognition problem requiring a novel solution. This often requires complex and expensive chemistry resulting in long development times. Other successful approaches have utilised naturally occurring macromolecules, such as cyclodextrins [8] and cellulose derivatives [9] which can act as general recognition sites. Each of these methods relies upon either a design/synthesis or a 'coincidental' fit approach.

As early as 1940, Linus Pauling postulated a mechanism for the formation

of artificial antibodies [10], although it was his student, F.H.Dickey, who first prepared an 'imprinted' adsorbent [11–12]. Since those very early days the concept received little attention until seminal investigations by Mosbach and Mosbach [13] led to what is now described as *molecular imprinting*. The process can be described generically as the 'specific organisation of polymerisable units about a template molecule that, on subsequent polymerisation, results in the formation of a template specific three dimensional cavity'. The product of this process has been termed a *molecularly imprinted polymer (MIP)* (*Figure 6.1*). The area has been the subject of intensive research over the last 5–10 years and several earlier reviews have been published [14–19].

The methodology has evolved along two routes, distinguishable in terms of the relationship between the template molecule and the polymerisable sub-units. In essence, the bonds between reciprocal functionalities on the template and the polymerisable units can be either covalent or non-covalent. The latter has become the method of choice in the majority of recently reported work and this review will therefore largely focus on the main concepts and mechanisms of this approach.

NON-COVALENT IMPRINTED POLYMERS

The fundamental process in the formation of all imprinted polymers is the formation of a stable non-covalent pre-polymerisation complex between the template molecule and functional polymerisable units [17]. In this approach, the template is non-covalently bound to the polymerisable moiety. The complex is *co*-polymerised in the presence of a cross-linking reagent to yield a rigid, macroporous product. The integrity of the pre-polymerisation complex is maintained entirely by non-covalent interactions. Typically, proton transfer events, giving rise to ionic species, and hydrogen bonds are central to the formation of the complex and its persistence. *Figure 6.2* shows the generalised imprinting process for an amphiphilic template where a pre-polymerisation complex is formed between the template and two monomers, the acidic methacrylic acid (MAA) and the basic 2-vinyl pyridine (2VPy). The pre-polymerisation complex represented in *Figure 6.2* is shown as stabilised by hydrogen bonds and ionic forces. Although the role of other interactions, such as hydrophobic, dipole-dipole, π-π coulombic and π-π Van der Waal forces, are not portrayed, it is thought that they can also play a significant role in template polymerisation [20, 21].

Recent advances in the area of non-covalent molecular imprinting have been largely associated with Mosbach's group in Lund, Sweden. Mosbach and Mosbach [13] reported a macromolecular 'entrapment' process and this led them to consider that the void left in the acrylamide polymers when

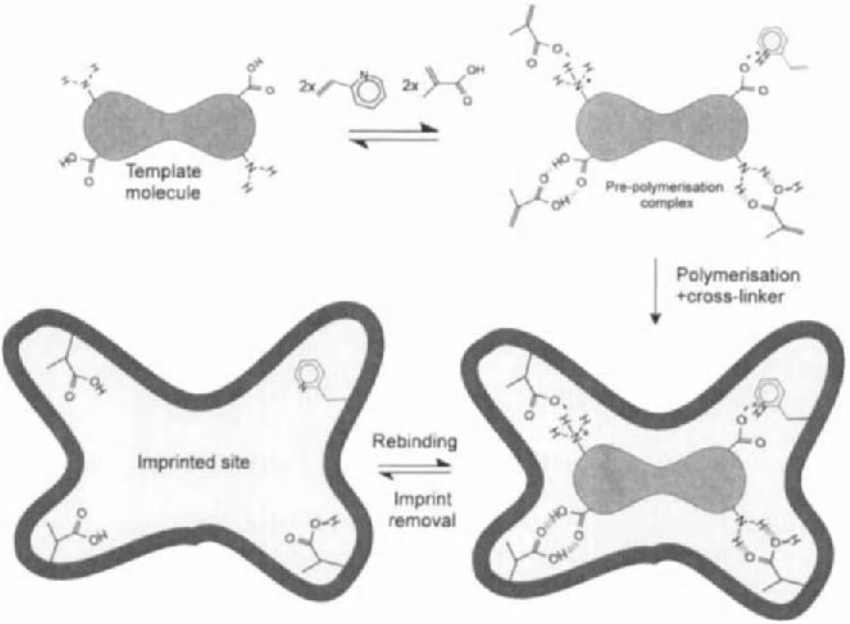


Figure 6.2 Non-covalent molecular imprinting of a generalised template molecule containing -NH_2 and -COOH groups using methacrylic acid and vinyl pyridine.

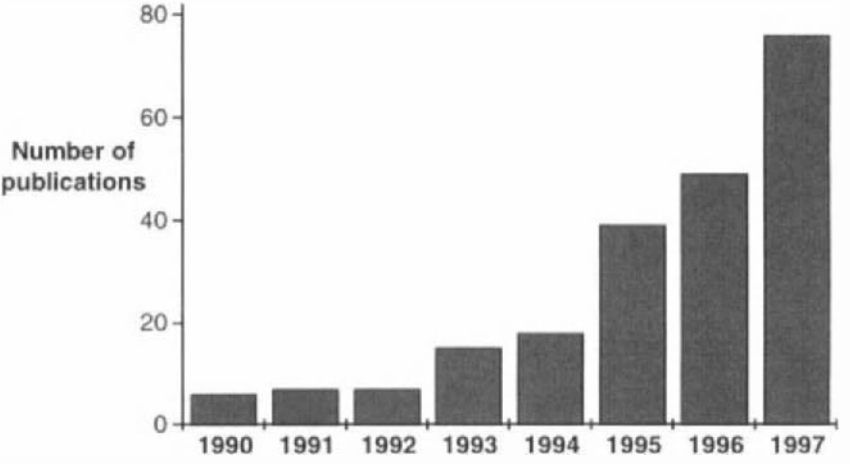


Figure 6.3 Growth of the research in non-covalent molecular imprinting.

the entrapped species was removed had the potential for molecular rebinding. In the early 1980s interest in non-covalent molecular imprinting increased world-wide after it was shown that non-covalent imprinting was capable of producing comparable results with those obtained using the covalent approach [22–25]. In addition, the versatility and practical ease of the non-covalent approach made it an attractive technology which gave it a definite advantage over covalent methods. The stimulation of interest in the field of non-covalent molecular imprinting in the late 1980s and 1990s has resulted in an explosion in published work (*Figure 6.3*).

At this point it seems suitable to reflect on the prescience of Linus Pauling who, almost 60 years ago [10], suggested that Dickey should attempt to prepare silica gel based selective sorbents using what was essentially an imprinting methodology [11, 12].

COVALENT IMPRINTED POLYMERS

The fundamental difference between the non-covalent and the covalent approach is that the latter involves template molecules which are covalently bound to monomer units prior to their addition to the reaction mixture. Subsequent *co*-polymerisation in the presence of a cross-linking reagent results in the incorporation of the template molecule within a polymer matrix. An important requirement is that the bond between the template and polymer is readily reversed as, to remove the template molecules, the covalent bonds between template and polymer must be cleaved. This cleavage step is very important and must be performed under conditions that will not profoundly alter the functionality or spatial arrangement at the imprinted site. The polymer is finally washed to leave the vacant imprinted sites.

Rebinding of the template to the binding site is achieved either by the reestablishment of the original bonds or by non-covalent interactions at the precisely positioned functional groups of the imprinted site [15]. The pioneering work in this area utilised the rapid and reversible nature of the boronic acid/diol reaction [15] (*Figure 6.4*).

Wulff *et al.* synthesised the boronate di-ester of phenyl-α-D-manno-pyra-

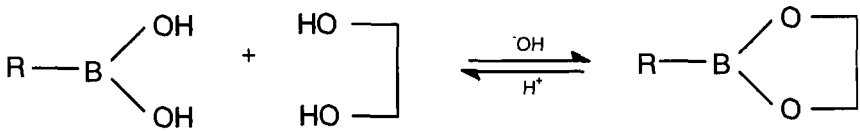


Figure 6.4 The reversible boronic acid/diol reaction.

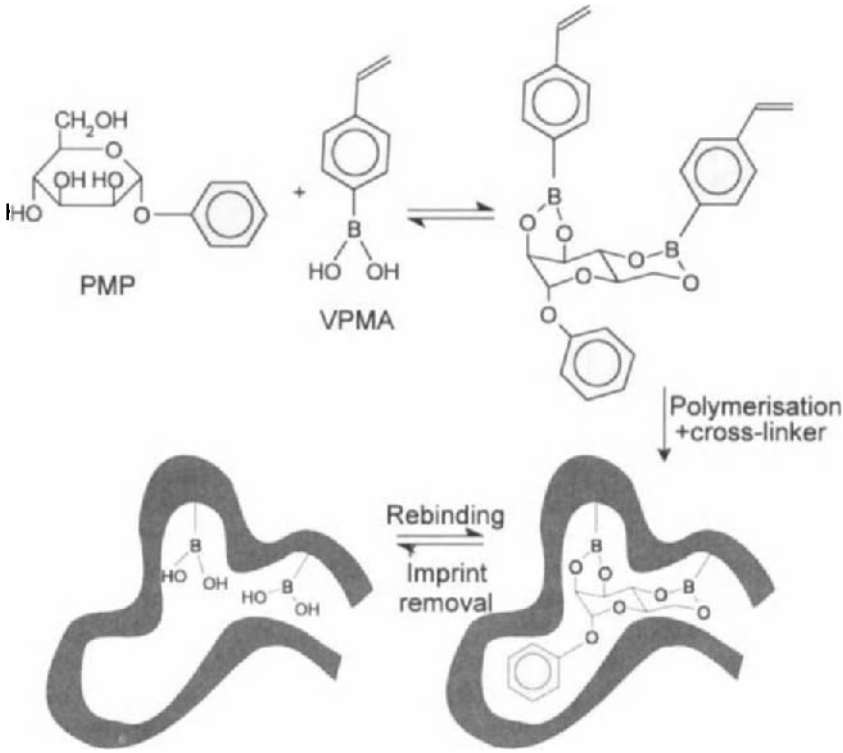


Figure 6.5 Boronic ester approach to covalent molecular imprinting of PMP using VPMA [26].

noside (PMP) using 4-vinylphenylboronic acid (VPBA) [26]. The boronate-sugar was then co-polymerised with a large amount of an acrylic cross-linking monomer to give a macroporous polymeric product (*Figure 6.5*). Under mildly hydrolytic conditions the template can be removed from the polymer to leave a closely fitting imprint site. Rebinding the template to the imprint site requires a change in conditions to those favouring the right side of the equilibrium (*Figure 6.5*).

An alternative approach to covalent imprinting was used by Shea and co-workers [27, 28]. Their tactic was similar, in that a template molecule was used to pre-position reciprocal functional groups within a polymeric cavity, but these studies used the reversibility of the reaction of ketones with 2-(p-vinylphenyl)-1,3-propanediol (VPD) to give ketals as a means of positioning and removing the template (*Figure 6.6*).

By selecting ketones of differing inter-carbonyl distances (*Figure 6.7*), and

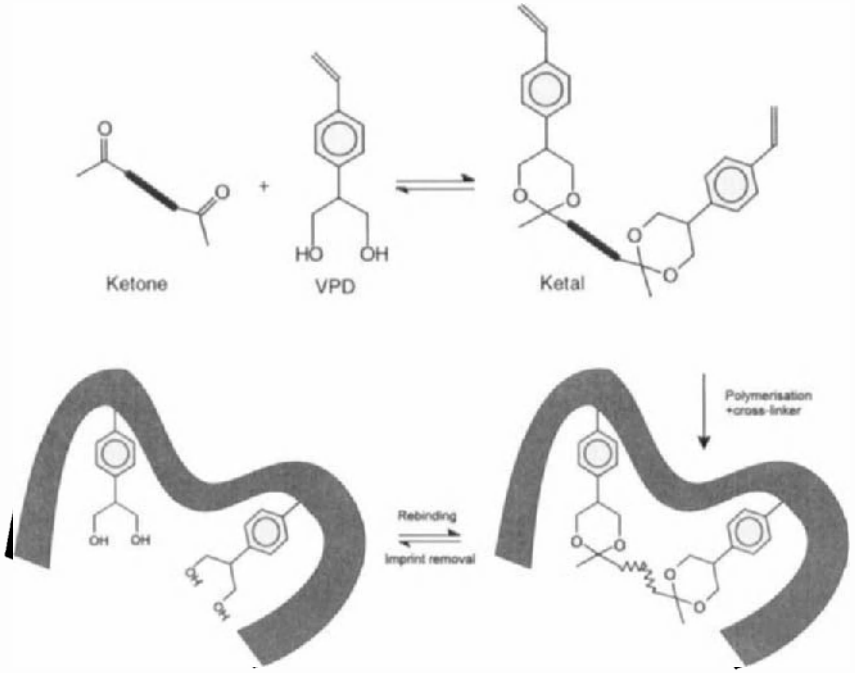


Figure 6.6 Ketone/ketal approach to covalent molecular imprinting using VPD [27].

rebinding them to a single template imprinted polymer, the effect of small changes in carbonyl separation on selectivity of the imprinted site was evaluated.

This study revealed that small changes in carbonyl separation, deviating from the initial template inter-carbonyl distances, resulted in significant changes in rebinding selectivity.

Schiff bases have also been investigated with regard to their suitability in covalent imprinting. However, it was found that, although the equilibrium

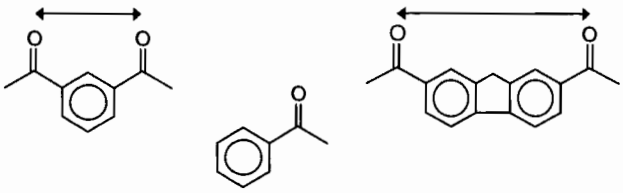


Figure 6.7 Ketones with different carbonyl separations.

position is favourable, the rate at which it is obtained is too slow for chromatographic applications [29]. This has proved to be a significant limitation for this approach, since much of the technology relies on chromatography as a means of assessing the selectivity of the imprint.

Covalent imprinting has been used to successfully produce selective sorbents for sugars, diketones, dialdehydes, aromatic ketones, transferrin, mandelic acid, amino acids and their derivatives, and bis-NAD [30].

COVALENT VS. NON-COVALENT IMPRINTING

The majority of recently reported work involves the non-covalent approach. The advantages of the non-covalent approach over covalent methodologies have been discussed [31] and are summarised in *Table 6.1*.

The most obvious advantage is that no specific covalent modification of the template molecule is required. Additionally the improved rebinding kinetics and practical flexibility appear to offer significant advantages over the covalent approach. In conclusion, the simplicity and elegance of non-covalent molecular imprinting makes it an attractive technology. Indeed, it allows specific molecular recognition approaches to be considered in areas where previously complexity has prevented their use.

Table 6.1 ADVANTAGES OF NON-COVALENT IMPRINTING OVER COVALENT IMPRINTING.

Non-covalent imprinting	*Covalent imprinting*
No chemical modification of the template molecule is required	Chemical modification of the template molecule is required
A general strategy can be identified	Templates must be individually considered
Good kinetics	Kinetics can be very poor
Protocols are simple	Chemistry can be complex
Simple washing to remove template	Chemical cleavage to remove template
Broad distribution of binding affinities	Narrower distribution of binding affinities

THE CHEMISTRY OF NON-COVALENT MOLECULAR IMPRINTING

AN OVERVIEW

The non-covalent imprinting process is perhaps best considered in terms of the sequential nature of the individual steps involved, which are summarised in *Figure 6.8*. The key to MIP formation is a series of regiospecific non-covalent interactions between the template and functional monomers to form a pre-polymerisation complex in the liquid phase.

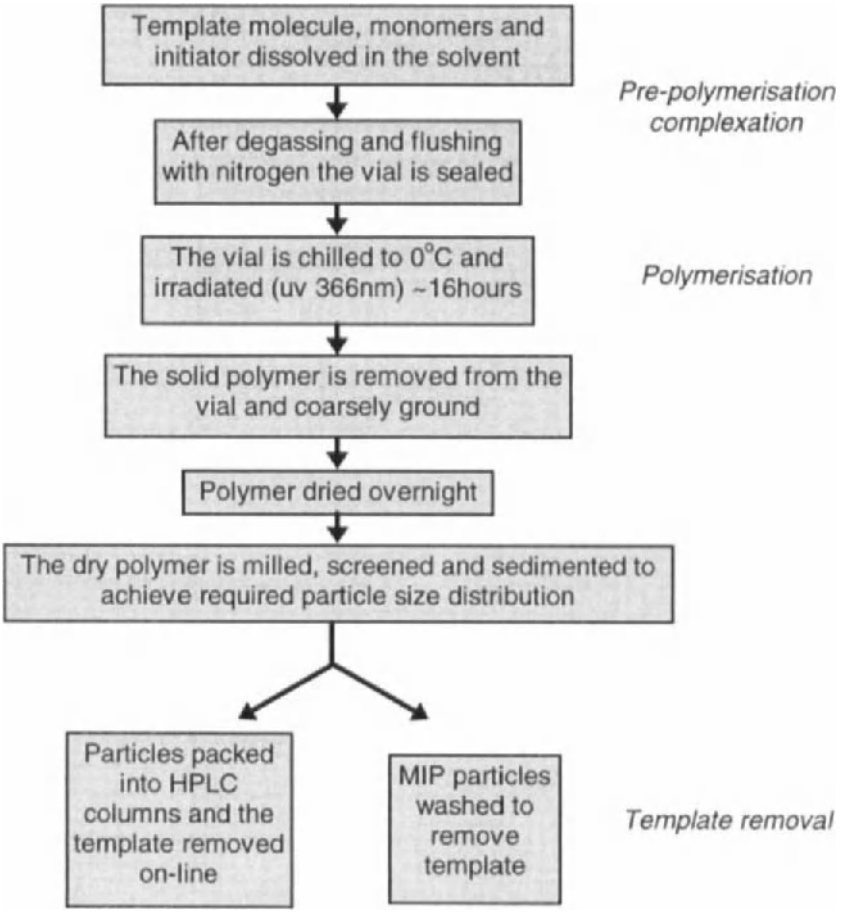


Figure 6.8 Overview of the preparation of a typical non-covalent molecular imprinted polymer.

THE TEMPLATE MOLECULE

To be successfully imprinted, a template must contain a certain level of functionality that can be paired with reciprocating moieties present on a polymerisable unit. Bowman *et al.* [32] observed that, in general, small, multi-functional, templates give rise to highly specific imprints whilst larger, monofunctional, templates produce imprinted sites which are less specific. In addition, a complex molecular backbone results in an imprint of more specific three dimensional structure, which is likely to lead to a more highly specific imprint than a template with simple linear geometry [31]. *Table 6.2* gives some examples of some successfully imprinted molecules, the functional monomers used, and the subsequent applications of the MIPs. (See also below)

For small, uncharged, solute molecules the strength of intermolecular non-covalent interactions is dependent upon the local environment which is, in turn, defined by the properties of the solvent [4, 33, 34]. In general terms, the stability of the pre-polymerisation complex is favoured in non-polar solvents. Therefore solubility in potential porogens must be considered when assessing the suitability of a compound for molecular imprinting. For polar template molecules, there is usually a trade off between imprint specificity and solubility. If the solubility of the template is such that a polar solvent is required, then the complex will be less stable and the imprint less specific [17].

NON-COVALENT INTERACTIONS

Non-covalent interactions can be classified into two major types:

(a) directional, induction and dispersion forces

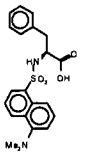
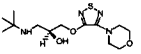
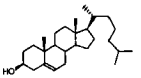
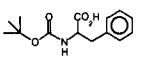
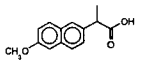
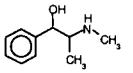
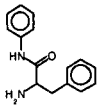
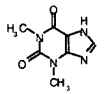
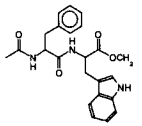
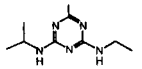
(b) hydrogen bonding and charge transfer forces

The former are non-specific and cannot be saturated, whilst the latter are directional, specific and can be saturated [34].

Directional, induction and dispersion forces

In a molecule with no overall net charge, these forces exist due to the interaction between a dipole and a system containing an opposite charge. The dipoles can be permanent, induced or temporary. Permanent dipoles (μ) arise from unsymmetrical charge distribution within a molecule, whilst temporary dipoles originate due to chance imbalance of charge resulting from random electron movement. Both permanent and temporary dipoles can affect neighbouring molecules. The existence of dipoles can give rise to various at-

Table 6.2 TEMPLATE MOLECULES.

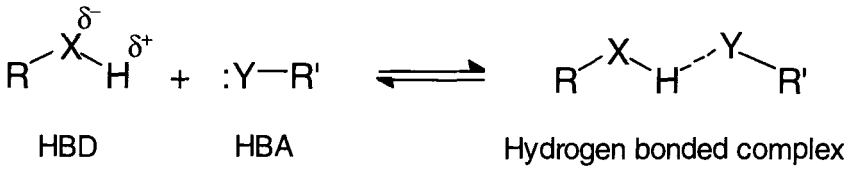
Template	*Structure*	*Monomers*	*Application*	*Reference*
Dansyl-phenylalanine		2VPy MAA EGMA	Sensor	[48]
Timolol		MAA EGMA	Chiral stationary phase	[49]
Cholesterol		Cross-linked cyclodextrins	Polymeric receptor sites	[146]
Boc-phenylalanine		MAA EGMA	Chiral stationary phase	[70]
Naproxen		2VPy EGMA	Chiral stationary phase	[147]
Ephedrine		MAA EGMA	Chiral stationary phase	[102]
Phenylalanine anilide		MAA EGMA	Chiral stationary phase	[59]
Theophylline		MAA EGMA	Immunoassay	[104]
Ac-Trp-Phe-OMe		MAA EGMA	Chiral stationary phase	[100]
Atrazine		MAA EGMA	Binding assay	[148]

MAA = methacrylic acid
EGMA = ethyleneglycol dimethacrylate
2VPy = 2-vinyl pyridine

tractive forces: dipole-dipole, ion-dipole, dipole-induced dipole, instantaneous dipole-induced dipole (dispersion interaction) [34–36].

Hydrogen bonding and charge transfer forces

A general definition of a hydrogen bond is: *when a covalently bound hydrogen atom forms a second bond to another atom, the second bond is referred to as a hydrogen bond* [37]. The bond consists of a hydrogen bond donating moiety (HBD) or electron acceptor and a hydrogen bond accepting group (HBA) or electron donator (*Figure 6.9*).



X represents a more electronegative atom than hydrogen
:Y provides the electron pair for the bond.

Figure 6.9 Formation of the hydrogen bond.

The traditional view of the hydrogen bond is that the forces involved are a result of an exaggerated dipole-dipole effect. The fact that the strongest hydrogen bonds are associated with the most electronegative atoms supports this view. However, hydrogen bond lengths suggest an overlap of van der Waals radii, which should lead to repulsion and, in addition, symmetrical hydrogen bonds cannot be explained in terms of a pure dipole-dipole interaction. It is thought that protomeric resonance forms exist as a result of overlap of the orbitals of the X-H bond with those of the donating electron pair [34]. The result is a resonant covalent structure (*Figure 6.10*).

X and Y are usually oxygen, nitrogen or sulphur atoms, although the π-electrons of aromatic groups can also act as hydrogen bond acceptors. Although bond energies for the amide-aromatic hydrogen bond are less than for other hydrogen bonds, it is suggested that they may be important in molecular recognition processes under certain conditions [38, 39]. *Figure 6.11* includes some common hydrogen bonding groups.

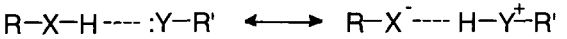


Figure 6.10 Protomeric resonance view of a hydrogen bond.

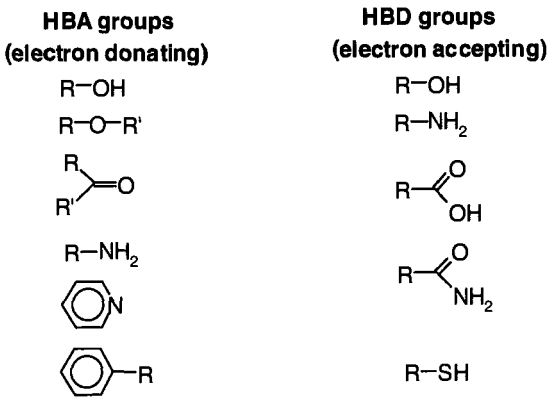


Figure 6.11 Hydrogen bond accepting (HBA) and donating groups (HBD).

For organic molecules, hydrogen bond dissociation energies vary over an approximate range of 13–42kJmol^{-1}, although the H-F$\cdots$ H-F bond has an energy of 155kJmol^{-1} [34]. The energy of a particular hydrogen bond can be estimated using tables of calculated HBA and HBD values [40]. This can be a useful method of predicting the effects of changes of solvent on the stability of the pre-polymerisation complex.

Charge transfer forces involve the movement of electrons or protons from one molecule to another. Electron pair donor - electron pair acceptor complexes (EPD-EPA) result from the donation of a pair of electrons giving rise to electrostatic attraction between two charged species. The difference between this type of bond and a normal chemical bond is that both bonding electrons are derived from the same molecule (the EPD), the role of the EPA being to provide an empty orbital. It is important not to confuse the EPD-EPA complex with ion pair formation resulting from proton transfer [34].

EPD-EPA can be classified according to the type of orbitals involved in the bond. Since, in theory, any of the three orbital types associated with EPD molecules (n, σ and π) can be involved with any of the three types associated with EPA molecules then nine possible EPD-EPA interactions exist. The most important with regard to host-guest interactions at the pre-polymerisation stage of molecular imprinting, are π-π and π-σ. An important example is the 'π-acid/π-base' π-π complex between electron rich and electron deficient aromatic systems, for π-π complexes bond dissociation energies are in range of 11–42kJmol^{-1} [41]. *Figure 6.12* shows two examples of π-EPD-π-EPA interactions.

Ihara *et al.* [42] showed that specific selectivity can be detected with linear

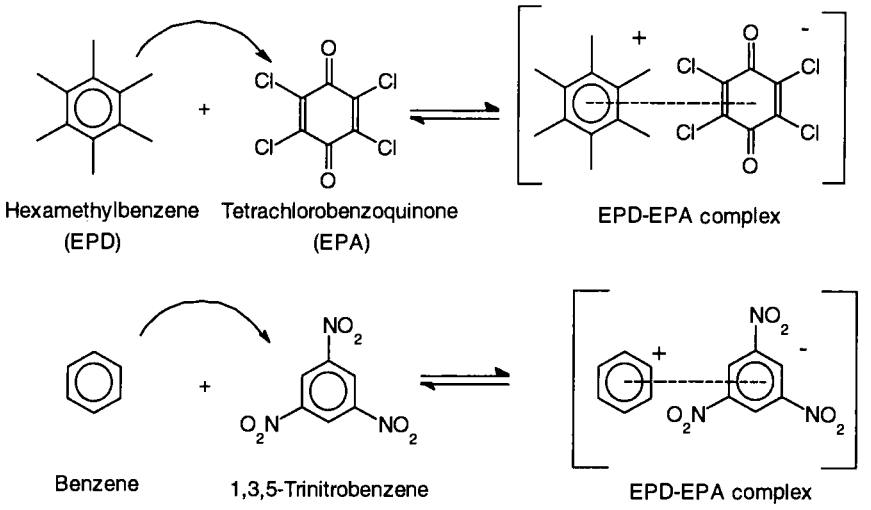


Figure 6.12 π-EPD/π-EPA (π-acid/π-base) complex formation.

poly(methyl acrylate), where π-electron and molecular shape are recognised. Polymerisable units containing π-acid moieties have recently been synthesized in these laboratories [43].

Ionic interactions

In the context of non-covalent molecular imprinting, the most important ionic interaction is the Brønsted acid-base or proton transfer process where the removal of an acidic proton by a base results in electrostatic attraction between the resulting ions. The process is governed by the dissociation/association constants of the acidic and basic moieties, the relative acidity or basicity of the solvent and the differential solvating power of the solvent for the intermediate complexes of proton transfer (*Figure 6.13*).

Whether the proton transfer process proceeds through to fully disso-

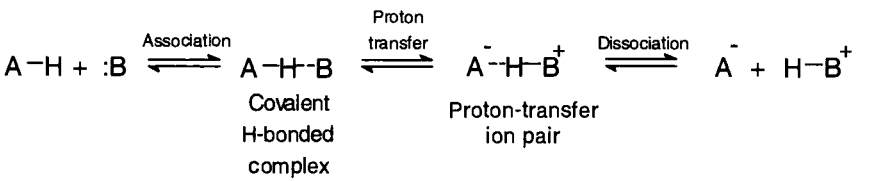


Figure 6.13 The proton transfer process.

ciated/solvated ions, or remains at the proton transfer ion-pair stage, depends upon the dielectric constant of the solvent. In addition, varying the polarity of the solvent determines whether the complex is maintained as a covalent hydrogen bound structure or as an ionic-pair. If the dielectric constant of a solvent is >40 then ion-pairs are rarely seen whereas, when dielectric constants are <10, no free ions are observed [44]. In terms of the formation of a stable pre-polymerisation complex it is important that spontaneous dissociation does not take place.

POLYMER MATRIX

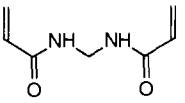
The structure of the polymer matrix is fundamental to MIP function. A high degree of cross-linking is required to fix the spatial orientation of the pre-polymerisation complex permanently within the polymer as, without this rigidity, specificity is greatly reduced [31]. On the other hand, it is essential that the imprinted recognition sites are freely accessible to the rebinding molecules during MIP-ligand interaction and the polymer must therefore be accessible and flexible [15, 45]. In addition, the polymer matrix must provide sufficient mechanical strength and a degree of thermal stability. The nature of the cross-linking process has a major effect on all of these aspects [46].

Cross-linker

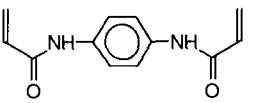
Although ethylene glycol dimethacrylate (EGMA) has been by far the most commonly used cross-linker in MIP systems [15, 30], alternative cross-linkers have been investigated (*Figure 6.14*).

Since, ligand accessibility and template definition are maximal in stiff polymers, whilst good kinetics and rapid equilibration are favoured in flexible polymers, the choice of cross-linker must inevitably be a compromise [47]. It should be pointed out that, in addition to the type and proportion of cross-linker involved, the porogenic solvent and method of polymerisation both also affect the macro structure of the polymer in terms of porosity and internal surface area.

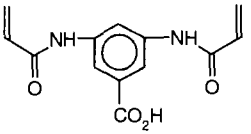
Wulff *et al.* [46] observed that, for EGMA, tetramethylene dimethacrylate and divinylbenzene, selectivity was not observed with cross-linker proportions from 0% to ~10%. An increase towards 50% gave a gradual rise in selectivity and, between 50% and 70%, a more rapid improvement was noted. In general, the higher the proportion of cross-linker the greater the selectivity. In terms of structural variation of the cross-linker, they concluded that 'although many different cross-linkers were evaluated, including some synthesized for the first time, EGMA was the most suitable for most imprint-



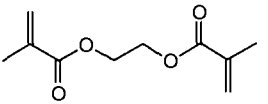
N,N-methylenediacrylamide



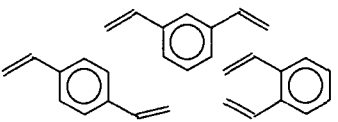
N,N'-1,4-phenylenediacrylamide



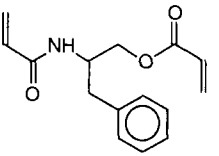
3,5-bis(acryloylamido)benzoic acid



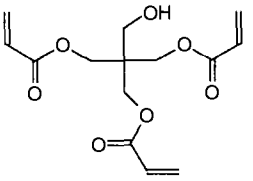
ethylene glycol dimethacrylate



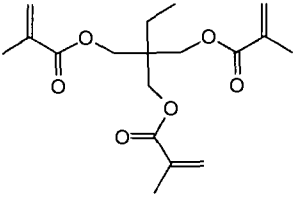
divinylbenzenes



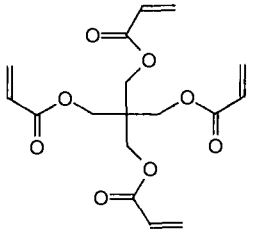
N,O-bisacryloyl-L-phenylalaninol



pentaerythritol triacrylate



trimethylolpropane trimethacrylate



Pentaerythritol tetraacrylate

Figure 6.14 Cross-linking monomers used in molecular imprinting [30].

ing processes' [15]. He also stated that EGMA gave better selectivity and resolution than some new optically active cross-linkers. Importantly, EGMA was also the cheapest and most easily purified.

Functional monomers

The purpose of the functional monomer is twofold. Firstly, it undergoes regiospecific, weak, complementary, interaction with a particular moiety of the template molecule. Secondly, it contains a polymerisable unit. The choice of functional monomer should therefore be based upon the functionality of the molecule to be imprinted.

Methacrylic acid (MAA) has been used extensively as a functional monomer as it fulfils the essential criteria of possessing a polymerisable moiety and functionalities that can interact with opposite moieties in a reciprocating arrangement. The carboxylic acid group is capable of acting as both a hydrogen bond donor and receptor. Although liquid phase dimerisation of MAA might be anticipated, it does not appear to be a significant practical problem [48]. Using a tertiary MIP, containing methacrylic acid, vinyl pyridine and ethyleneglycol dimethacrylate, enhanced selectivity was demonstrated for amino acid derivative templates.

Itaconic acid has been used [49], although its performance was inferior to that of MAA. Fluoro-functionalised derivatives have also been successfully employed. Matsui *et al.* [50] used 2-trifluoromethyl acrylic acid to imprint the herbicide prometryn. The acidity of this monomer is higher than MAA, due to the strong electron-withdrawing effect of the trifluoromethyl group, and it therefore should interact more strongly with basic template molecules. In chromatographic studies, the polymers based on this material exhibited higher affinity and selectivity for prometryn than those based on methacrylic acid. Apparently, steric and electronic influences of the bulky CF_3 group did not adversely affect the polymer. Diethylamino-ethyl methacrylate was recently used in the HPLC analysis of chloramphenicol [51]. In addition, acrylic acid [52], *p*-vinylbenzoic acid [53] and 2-acrylamido-2-methyl-1-propane-sulphonic acid [54] have been used for basic templates. For acidic templates, 2-vinylpyridine [55], 4-vinylpyridine [29], 1-vinylimidazole [56] have been employed. Methyl methacrylate [57] and acrylamide [58] have also been used. The structures of some functional monomers employed are shown in *Figure 6.15*.

Terpolymers have been reported. Ramstrom *et al.* [48] described the simultaneous use of MAA and vinyl pyridine in the reaction mixture to interact with the basic and acidic moieties of amino acid derivative template molecules. The co-operativity produced polymers had improved performance

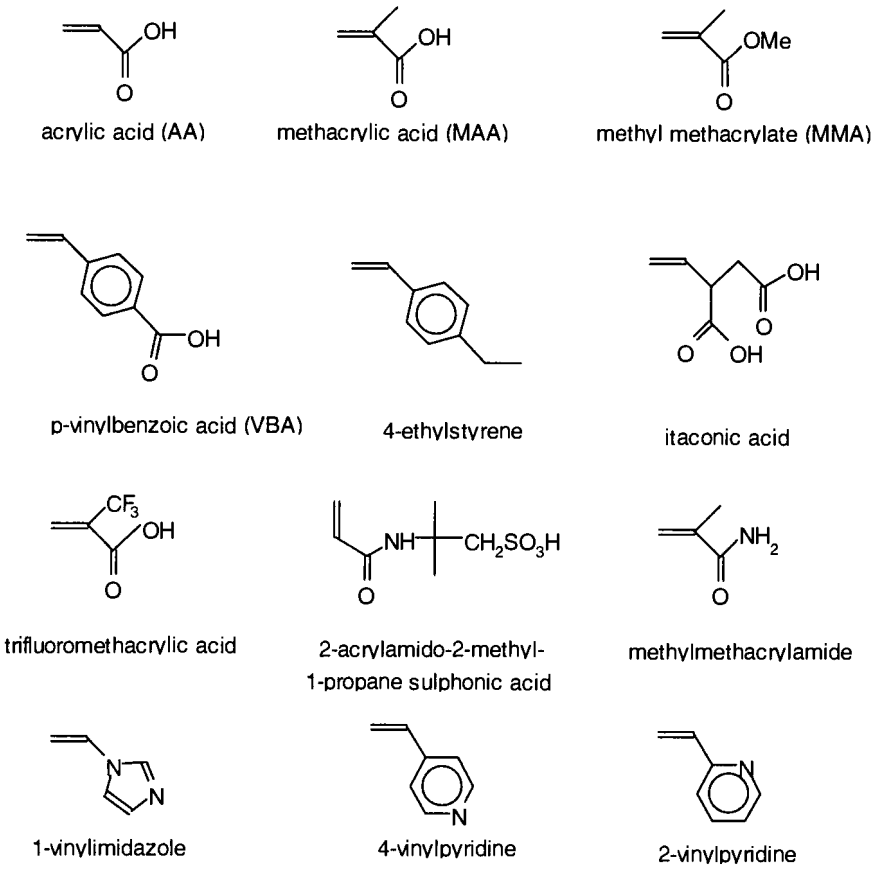


Figure 6.15 Functional monomers used in non-covalent molecular imprinting.

over polymers based solely on MAA. Acid-base pairing of the monomers in solution was not a problem. This is an area worthy of further investigation, as it demonstrates that monomer-monomer interactions are not necessarily detrimental to the process and that reactivity ratios are comparable.

Solvent/porogen

Macroporous polymers are formed if polymerisation is carried out with a high proportion of cross-linker in the presence of a solvent. It is the formation of the polymer around these solvent molecules that produces the pores.

The solvent is therefore termed a *porogen*. Sellergren and Shea [59] showed that the porogen has a large influence on polymer morphology, but concluded that there was no obvious relationship between morphology and MIP selectivity. They concluded that it was the hydrogen bonding capacity of the porogen that was the main influence on selectivity.

The solvent also has a significant effect on the nature of the non-covalent interactions that stabilise the pre-polymerisation complex. In general, it is thought that the significant interactions associated with the pre-polymerisation complex are polar and, since the strengths of polar interactions are maximal in apolar solvents, the more apolar the solvent the more stable the pre-polymerisation complex [21, 25, 60]. Since there is a positive correlation between stability of the pre-polymerisation complex and the recognition properties of the resultant MIP, polymerisation has generally been carried out in non-polar solvents such as chloroform, toluene, dichloromethane and benzene. However, useful MIPs have also been prepared using acetonitrile, where it is thought that the negative influence of increased polarity is countered by the positive influence of increased porosity and internal surface area [59].

Smithrud and Diederich [33] reported on the effects of varying solvent conditions on apolar complexation and showed that apolar binding is promoted in polar protic solvents such as water. These solvents are characterised by high cohesive interactions and low molecular polarisabilities. It is a useful observation that, although apolar interactions can be maximised in polar solvents, it is unlikely that they, alone, would result in a stable enough pre-polymerisation complex to produce a valid imprint. However, it is probable that apolar interactions play a significant role in the recognition process [20, 21].

The MIP-ligand rebinding process can be considered thermodynamically. Solvation of an organic molecule in an organic solvent is usually associated with a negative solvation energy [34]. Non-covalent binding to a MIP results in a loss of solvation and, for binding to be favoured, binding energy must be negative and larger than the increase in free energy associated with the loss of solvation prior to binding.

In the paper entitled 'A remarkable effect of solvent size on the stability of a molecular complex', a strong correlation was shown between binding energy and surface area of the solvent molecule [61]. This study compared closely related families of solvents to determine the effect of solvent surface area on complex formation. Correlation was also shown between binding energy and solvation energies for the complexation of two families of solvents. It was proposed that the increase in solvation energy (positive values) that occurs as solvent size increases makes it thermodynamically favourable

for binding to occur, i.e. larger solvents result in more stable complexes. Chapman also suggested that larger solvent molecules find it more difficult to solvate internal binding cavities. Therefore a binding site need not be extensively desolvated, with a corresponding increase in free energy, for binding to occur. Assuming that the 'assembly' of a MIP binding site is a dynamic process, formation will only occur if assembly is energetically favoured, i.e. the pre-polymerisation arrangement of molecules is lower in energy than the fully solvated arrangement. In practical terms the choice of solvent is often based on solubility considerations, the aim being to use a solvent that is as non-polar as possible yet still provide a system in which monomers and template are freely soluble.

Proportions of Monomer:Template:Porogen

The relative proportions of the constituents of a MIP affect both the morphology and the function of the polymer, although most recent studies have used an empirically optimised set of proportions. The majority of studies have used 80–85% of cross-linker, as higher proportions lead to problems with subsequent mechanical processing.

The effect of the ratio of porogen to monomer was investigated extensively by Wulff's group who concluded that an approximate ratio of 1:1 (ml:g) gave rise to optimal selectivity and affinity [47]. Other studies have broadly agreed, although ratios are often a little higher (1.3:1 – 1.5:1) [31, 49, 62]. The flexibility of the MIP system was demonstrated by Matsui *et al.* [63] who used a ratio of 2.4:1 but still obtained high affinity polymers.

The quantity of cross-linker is fundamentally important for high selectivity [15, 46, 64]. Below 10% cross-linker selectivity was not observed although, as the proportion was increased from 10% to 95%, a corresponding increase in selectivity was observed. Sellergren showed that the internal surface area of the polymer was proportional to the percentage of cross-linker, which correlated well with selectivity [59, 64].

The final important ratio to consider is that of template:functional monomer. Sellergren [64] demonstrated that selectivity of a L-phenylalanine anilide (L-PheNHPh) for its imprint molecule was maximal when the mol-% of MAA was ~25%, a value that corresponds to a template functional monomer ratio of 3:1. However, the maximum capacity factor was not obtained until the mol-% MAA was ~50%. This was important in that maximum values for affinity and selectivity were not obtained with the same MIP composition. Sellergren [31] proposed a 2:1 model for the MAA:L-PheNHPh complex, but suggested a 4:1 ratio should be used to maximise the number of interactions at a given time. Subsequent studies have generally used a ratio of

functional monomer to template of approximately twice the stoichiometric ratio to one.

To obtain a series of L-phenylalanine anilide (L-PheNHPh) MIPs with varying MAA: L-PheNHPh ratios, Sellergren [64] kept the template and the total monomer concentrations constant and changed the amount of MAA and therefore the amount of cross-linker must also have changed. Using a chromatographic system to assess these polymers he found that maximum selectivity occurred at a different concentration of MAA to that of maximum capacity. However, it has already been observed that varying the amount of cross-linker affects the selectivity of the polymer and hence the observed effects are difficult to interpret.

THE IMPRINTING PROCESS

Pre-polymerisation complexation

It is generally accepted that hydrogen bonding, steric effects and acid/base proton transfer equilibria are the prime interactions involved in the formation of stable pre-polymerisation complexes. In addition, it is reasonable to assume that the other types of non-covalent interaction may contribute, although their role is more difficult to quantify (*Figure 6.16*) [15, 17].

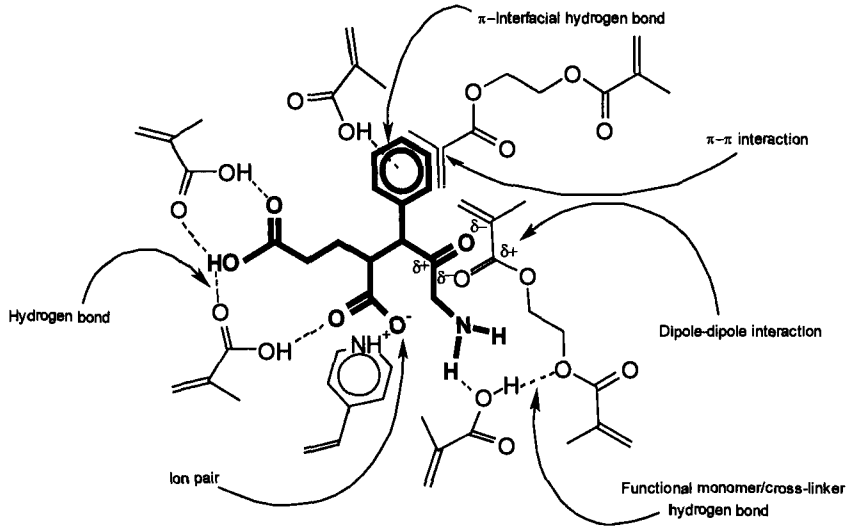


Figure 6.16 Schematic representation of an example of pre-polymerisation complex showing a range of regiospecific solvation interactions. In bold is a hypothetical template molecule, functional monomers are 4-VPy and MAA whilst the cross-linker is EGMA.

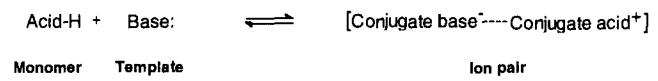


Figure 6.17 Ion-pair formation.

Furthermore, as the cross-linking reagents commonly used also possess functionality, they may also be involved in the formation of the pre-polymerisation complex.

It should be re-emphasised that the magnitude of a non-covalent interaction between reciprocal groups is not fixed and the strength of a particular interaction is a function of the environment [34]. To generalise, non-polar interactions are favoured in polar environments and vice versa. However, this statement provides only a guide to the effect of a solvent on the stability of a pre-polymerisation complex. For instance, ion-pair formation, as a result of an acid/base process, is a two stage event. The first stage, proton transfer, is favoured in polar (high dielectric constant), hydrogen bonding solvents whilst the second stage, ion-pairing, is favoured in apolar (low dielectric constant), non-hydrogen bonding solvents. In a solvent with a dielectric constant greater than 40 (e.g. water, formic acid, formamide), assuming proton transfer has occurred, ion-pairing barely exists, whilst for values less than 15 (e.g. hexane, chloroform, acetic acid) free ions exist only at very low concentrations (*Figure 6.17*) [34].

Polymerisation

Reported MIP systems have been random *co-* or *ter-* acrylic addition polymers based upon the classification of Carothers [65]. An addition polymer has a structural unit with the same molecular formula as the monomer (*Figure 6.18*)

The polymerisation process consists of three stages: initiation, propagation and termination. During the initiation stage a reactive species is formed that starts the polymerisation of the relatively unreactive vinyl compounds. Propagation results in the formation of a high molecular weight polymer. The termination stage is a deactivation process which results in the forma-

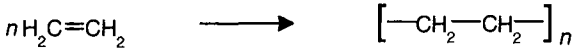


Figure 6.18 An addition polymer has a structural unit with the same molecular formula as the monomer.

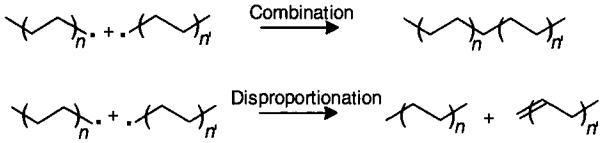


Figure 6.19 Termination of addition polymerisation.

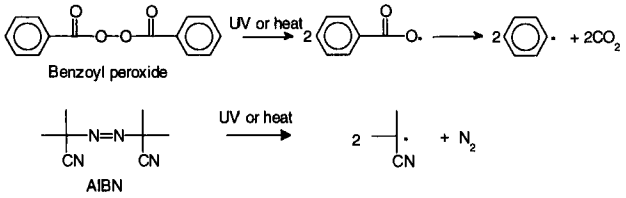


Figure 6.20 The formation of radical species from the decomposition of benzoyl peroxide and AIBN.

tion of the stable polymeric product. This arises when two radicals combine either by combination or by disproportionation (*Figure 6.19*).

Only free radical polymerisation, which requires the formation of reactive free radical species to initiate polymerisation, appears to have been used to form MIPs. Free radicals are produced by the decomposition of an initiator species by the action of heat or light. Commonly used initiators are benzoyl peroxide and azobis compounds such as azobisisobutyronitrile (AIBN) or 2,2'-azobis(2,4-dimethylvaleronitrile) (ABDV) (*Figure 6.20*).

Once formed, the free radical species (R') attack the double bonds of the monomer to give rise to an initiated monomer radical. This, in turn, rapidly attacks further monomers to form a polymer chain (*Figure 6.21*).

AIBN can be decomposed by heating (~60°C) or by irradiation (uv 366nm). It has been shown that polymerisation temperature has a large influence on the selectivity and binding affinity of MIPs. Using an L-PheNHPh MIP, and comparing the affinity and selectivity for the D- and L- enantiomers, O'Shannessey [66] demonstrated optimal selectivity occurred when polymerisation was carried out at 0°C (AIBN/uv) whilst maximum retention was observed at 60°C. The same study measured average pore diameter,

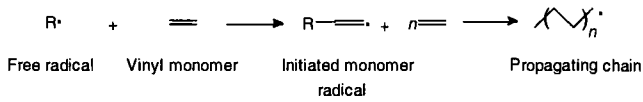


Figure 6.21 Radical initiation and chain propagation.

Table 6.3 COMPARISON OF PROPERTIES AND CHROMATOGRAPHIC PERFORMANCE OF L-PheNHPh MOLECULAR IMPRINTS PREPARED UNDER DIFFERENT CONDITIONS (O'SHANNESSY,1989). K'_D AND K'_L ARE CAPACITY FACTORS FOR D- AND L-PheNHPh.

Polymer preparation	K'_D	K'_L	α	*Average pore diameter (Å)*	*Pore volume (cm^3/g)*	*Inner surface area (cm^2/g)*
Acetonitrile 60°C	1.1	1.7	1.6	270	0.81	272
Chloroform 60°C	2.4	4.8	2.0	140	0.19	151
Acetonitrile 0°C	1.1	2.5	2.3	150	0.23	53
Chloroform 0°C	1.4	3.2	2.3	100	0.04	3

pore volume and inner surface area and showed that at lower temperatures, especially when the porogen was chloroform, all of these parameters were greatly reduced (*Table 6.3*).

Change in polymer morphology from macroporous to microporous resulted in improved selectivity but a decrease in overall retention, which is desirable in terms of chromatographic performance. For some MIPs uv initiation is not suitable, e.g. when the template is uv sensitive, and thermal initiation is required. In this situation ABDV is favoured since it thermally decomposes at a lower temperature than AIBN (45°C).

Post-polymerisation processing

MIPs are generally produced as opaque, vitreous, brittle, plastics. To be of practical use MIPs need to be reduced to a fine particulate material of uniform particle size. Typically, this is achieved by grinding processes, either by hand in a mortar and pestle or by mechanical means. The material is then sieved to give a powder of fixed upper particle size (~25–45μm). Depending upon the final application, this material is then sedimented over a pre-determined time period to remove material that is too fine. The sedimentation step is particularly important if the MIP is to be used for HPLC applications where fine material causes high back-pressure and column blockage.

Recovery of MIP particles of controlled particle size after sedimentation can be as low as 40%.

The size reduction step can be the source of problems. Firstly, it is often difficult to replicate the milling and sizing processes from batch to batch which, in turn, leads to poor reproducibility. We have found that a combination of wet ball-milling, using a ceramic system, and laser particle sizing allows the production of higher quality material [67]. However, since the MIP particles are not uniform in shape, MIP columns still tend to demonstrate relatively poor flow dynamics, which result in poor chromatographic performance. In spite of this problem, the exceptional selectivity of the polymers has still made them useful.

Template recovery

The final step in the preparation of a MIP is the removal of the template molecule. This is achieved by washing the polymer with a sufficiently polar solvent system to remove the template from the imprinted site. Methanol or acetonitrile, with the addition of acetic acid, is commonly used to create an environment unfavourable for template-MIP hydrogen bonding and ion pairing. This has been carried out using Soxhlet exhaustive hot extraction or by simple stirring, filtering and resuspending protocols. For chromatographic purposes the MIP is packed into a column and the template is removed by washing 'on-line'. Estimations of the amount of template recovered have commonly indicated >95%. The remainder is termed non-recoverable and, for most applications, this has proved to be no problem. However, Venn [68] reported difficulty in determining ultra low levels of analyte, using a MIP-solid phase extraction (SPE) method, and concluded that extremely low levels of template 'seepage' was responsible for the observed variability in results.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF MIPS

The end product of the MIP production process, in the vast majority of cases, is a fine powder containing a population of vacant binding sites with a small proportion of non-recoverable template trapped deep within the particle matrix. For monolithic polymers binding sites are of two basic types; internal and external. External sites are the product of random cleavage of the cage structure of the total 3-dimensional site. Their geometry depends upon the orientation of the cavity constituents relative to the fracture plane. These are believed to contribute only a small amount of the total binding

sites, but may be of greater significance in terms of site heterogeneity. Of more importance are the internal binding sites which are both entire and in much higher abundance.

Since MIPs are acrylic *co*-polymers they possess many of the attributes that make this type of polymer such a useful material. They are highly resistant to physical factors such as temperature and pressure extremes, they cope well with mechanical stress and they are chemically inert, coping with acids, bases and most organic solvents without loss of selectivity. Moreover, shelf life at room temperature is measured in years and with minimal care MIPs can cope with long periods of continual use [17].

BINDING SITE RECOGNITION AND SPECIFICITY

The interactions that govern ligand rebinding to a MIP are the same as those that are responsible for the pre-polymerisation complex [17]. Under the same conditions, the non-covalent processes that stabilised the pre-polymerisation complex bring about rebinding and recognition of the template. It is therefore a reasonable assumption that optimal conditions for rebinding template to MIP will be those prevailing at the pre-polymerisation complex stage [31]. Interactions between template and function monomer have been studied using NMR and the information used to provide an insight into the events that occur during rebinding [31, 69].

As previously discussed, hydrophilic interactions (hydrogen bonding and ionic) are the significant interactions promoting rebinding and recognition. An enantiomeric template molecule with less than two spatially separated hydrogen bonding/ionisable groups does not give rise to an imprinted polymer capable of differentiating the two enantiomeric forms. Assuming a three point interaction model for enantio-differentiation, this suggests that other non-covalent interactions are of secondary importance. Furthermore, Nicholls [21], using a protected-dipeptide imprinted polymer, concluded that hydrogen bonding was the major factor controlling enantio-resolution, whilst hydrophobic interactions, which were common to both enantiomers, were non-specific.

The strength of a hydrogen bond is dependent upon the electronic environment of the polarisable group [40] and the environment in which it exists. Therefore, the affinity of the MIP for the template can be maximised in a hydrophobic environment, using a strongly hydrogen bonding functional monomer. Andersson *et al.* [62] demonstrated that, using a chromatographic approach (HPLC), enantiomeric resolution could be achieved using non-covalent and non-ionic interactions. MIPs prepared using poorly basic *N*-protected amino acids were shown to be capable of resolving racemates

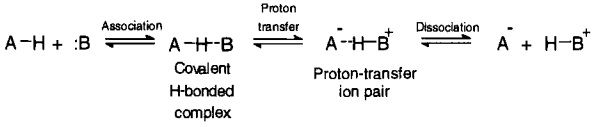


Figure 6.22 The proton transfer process [34].

with enantiomer separation factors (α) of 2.5. Other studies using hydrogen bond reliant processes have demonstrated similar results [30, 67, 70].

In the context of molecular imprinting, ionic interactions occur as a result of a proton transfer in a Brønsted acid/base type process. Alternatively, this type of ionic interaction can be viewed as an extension of the hydrogen bonding process. *Figure 6.22* illustrates that the proton transfer ion-pair is in equilibrium with the covalent H-bonded complex, the position of the equilibrium being dependent upon the pK_as of the acidic and basic groups and the solvent environment [34].

When conditions are such that the formation of ion-pairs is favoured, it is generally observed that interactions between MIP and ligand are stronger than for complexes stabilised only by hydrogen bonds [60]. MIPs with high rebinding affinity for their template are also highly selective, which can be explained simply in terms of the numbers of non-covalent interactions occurring between ligand and polymer, since the greater the number of individual directional interactions, the greater will be the predicted selectivity (an extension of the three point rule for enantio-differentiation). In addition, Nicholls [21] demonstrated that hydrophobic interactions tended to be non-specific, which suggests that the more abundant the interaction the greater the potential specificity. Since, under hydrophobic conditions, hydrogen bond / ionic interactions are the 'strong' interactions, then a direct correlation should exist between specificity and affinity. This effect is particularly evident in chromatographic data obtained from MAA-*co*-EGMA imprinted polymers for a series of L-phenylalanine derivatives (*Figure 6.23*).

For each of these templates, selectivity, in terms of their ability to resolve the racemate (α), was determined and shown to correlate with the number of theoretical hydrophilic interactions between the template and MAA (*Table 6.4*).

The 'number of ionic interactions' quoted in *Table 6.4* are the theoretical maxima and, in a 'real' situation, proton transfer is dependent upon pK_a and local environment. Dauwe and Sellergren [71] observed that, in a hydrophobic environment, the pK_a values of a closely related series of template molecules had a direct bearing on their chromatographic retention on complementary EGMA-*co*-MAA MIP stationary phases. In the same study,

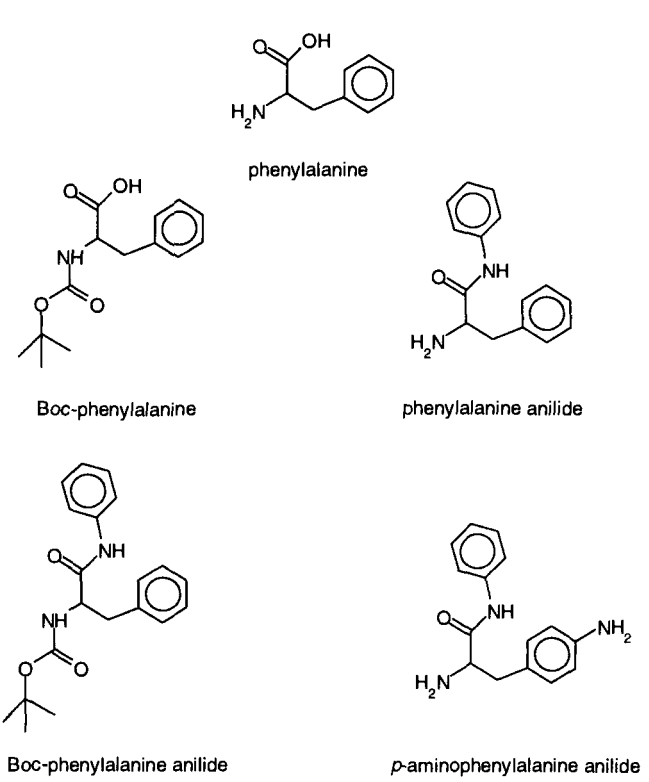


Figure 6.23 Phenylalanine derivatives.

Table 6.4 CORRELATION BETWEEN NUMBER OF HYDROPHILIC INTERACTIONS AND ENANTIO-SEPARATION FACTOR α. HBD IS HYDROGEN BOND DONATOR; HBA IS HYDROGEN BOND ACCEPTOR

	Number of HBD Groups[‡]	*Number of HBA Groups*[‡]	*Number of Ionic bonds*[*]	α	*Reference*
Boc-phenylalanine	2	6	0	2.3	[67]
Boc-phenylalanine anilide	2	7	0	2.9	[149]
Phenylalanine anilide	1	4	1	4.5	[64]
p-Aminophenylalanine anilide	1	4	2	8.3	[150]

[‡]potential maximum number of hydrogen bond contributing groups (it is assumed that a particular group cannot be involved in a hydrogen bond and an ionic bond at the same time)
[*]theoretical maximum number of ionic interactions

using an aqueous-rich mobile phase, a further direct correlation was observed between hydrophobicity (in terms of log P_{ow}, the octanol/water partition coefficient) and retention. These two trends demonstrated the complexities of MIP-ligand rebinding mechanisms and the subsequent difficulties involved in predicting retention behaviour on MIP HPLC columns. In the former, hydrophilic interactions dominated retention in an hydrophobic environment, whilst in the latter, the opposite was true. In a further study, a similar effect was demonstrated by increasing the acidity of the functional monomer. Matsui *et al.* [50] showed that, by replacing MAA with trifluoromethacrylic acid (TFMAA), to give EGMA-*co*-TFMAA imprinted polymers, significant improvements in selectivity could be achieved.

Andersson *et al.* [72] investigated the recognition process using a series of related structures containing pyridyl nitrogens (*Figure 6.24*).

Aromatic nitrogens in the 4-position were more favourable for interactions than those in the 2-position which demonstrated that steric hindrance of non-covalently interacting moieties can affect the affinity of the imprinted site. Furthermore, it was suggested that a probable explanation for the surprising lack of a template effect with terpyridine was the result of steric hindrance of the pyridinyl nitrogens by the aromatic rings. This work again emphasises the dangers of overly simple predictive models for template

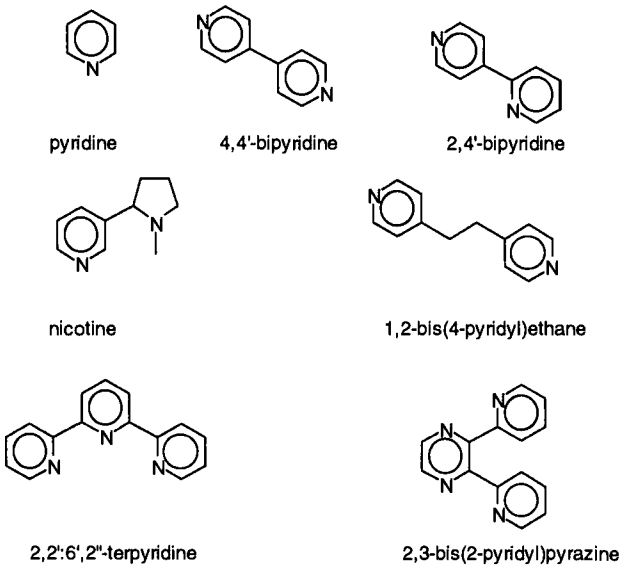


Figure 6.24 Pyridyl-containing template molecules [72].

formation and subsequent molecular recognition, and demonstrates that changes in polymer morphology associated with different template molecules may also be of significance.

The general approach to the control of template-MIP rebinding affinity has been through changes in composition of the rebinding solvent. Such variations have been routinely studied using chiral HPLC. It is generally accepted that increase in hydrogen bonding potential of the solvent/mobile phase results in attenuation of affinity and selectivity [21]. This effect is due to disruption of template-MIP hydrogen bonding induced as a result of an increase in competition for MIP and template hydrogen bonding sites by the hydrogen bonding functional groups of the solvent molecules. Although a simplification, this model works well for non-ionic binding systems. However, Sellergren and Shea [73] demonstrated that, when ionic interactions occurred, chromatographic retention behaviour of MIP stationary phases could be explained more fully by applying an ion-exchange component to the simple hydrogen bonding model.

In addition to direct non-covalent interactive effects, it is thought that the overall shape and rigidity of the template is significant [31]. For a template molecule possessing a bulky aliphatic side chain, it can be envisaged that the resultant imprinted site has a hydrophobic pocket positioned reciprocal to the bulky apolar group. Results have indicated that, for rebinding ligands differing from the template only in the size of this side chain, affinity is related to the relative dimension of the group involved [74]. For ligands with aliphatic side chains larger than that of the template there was a reduction in binding affinity, whilst for aliphatic groups smaller than the template affinity approached that for the imprint molecule (*Figure 6.25*).

This effect has particular relevance for the specificity of the polymer. *Figure 6.25* illustrates that, for a non-template ligand, provided one or more reciprocal functional groups are present and steric restraints are minimal, cross-reactive binding can occur.

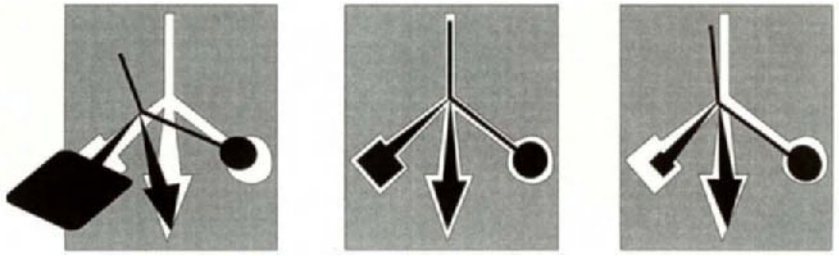


Figure 6.25 Effect of side group size on ligand fit into imprinted site.

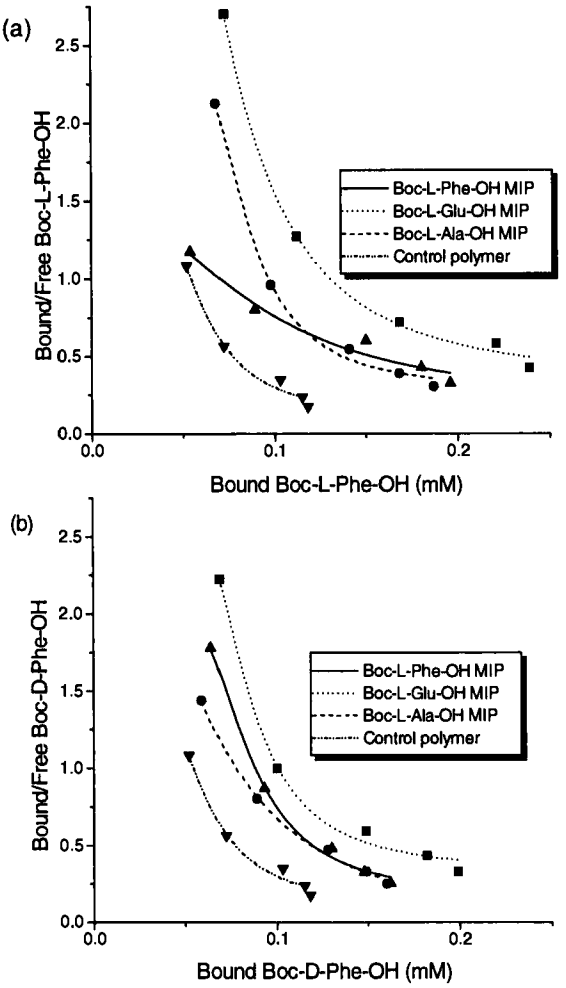


Figure 6.26 Curved Scatchard plots for (a) the binding of Boc-L-Phe-OH to Boc-L-Phe-OH MIP, Boc-L-ala-OH MIP, Boc-L-Glu-OH MIP and non-imprinted control. (b) the binding of Boc-D-Phe-OH to the same polymers [109].

Quantitative evaluation of template-binding site affinity has been carried out using two approaches: frontal chromatography and batch rebinding. Frontal chromatography, developed as a method for determining ligand affinity in traditional affinity chromatography [75, 76], has been applied to MIP chromatographic systems to estimate the dissociation constant (K_d)

Table 6.5 ESTIMATIONS OF K_d AND N_b FOR SOME MOLECULARLY IMPRINTED POLYMERS.

Ligand	*MIP*	K_d *(mM)*	N_b *($\mu molg^{-1}$)*	*Reference*
Boc-L-phenylalanine	Boc-L-phenylalanine	6.3	28	[70]
Boc-D-phenylalanine	Boc-L-phenylalanine	8.1	28	[70]
Atrazine	Atrazine	0.8	7.7	[148]
4,4'-Bipyridyl	4,4'-Bipyridyl	20	70	[72]

for MIP-ligand complexes and active binding site number (N_b). The second approach, batch rebinding, relies on the determination of equilibrium ligand concentration to provide data for Scatchard or related plots from which K_d and N_b can be determined. Scatchard plots are commonly employed to determine binding parameters from batch binding type data (*Figure 6.26*).

A straight line will only be obtained for a system with a single type of receptor site, represented by a single binding constant. However, for MIPs, this is not the case since a heterogeneous distribution of binding sites exists, so that a curved Scatchard plot should be expected and. *Table 6.5* contains some binding constants for MIP systems.

BIOMEDICAL APPLICATIONS

Synthetic materials capable of predictable and specific molecular recognition are of potentially high value in a diversity of fields. The fact that MIPs have the power to differentiate between structural and spatial minutiae has been instrumental in bringing MIP methodology to the attention of the scientific community. There has already been significant work which impacts upon the chemical industry, research community, health care and environmental control areas. A non-exhaustive list of potential applications for such technology includes downstream processing, biochemical monitoring, affinity chromatography phases, sample clean up, specific drug/analyte selection and environmental monitoring. Recent developments in MIP technology have been accompanied by a concurrent expansion in the catalogue of potential applications, which are outlined below. For convenience, applications have been classified in terms of the conditions under which the major binding process occurs, although there is much practical overlap.

TRANSIENT BINDING

Applications in this category are those that are generally described as chromatographic in nature. The analyte molecule is eluted through the MIP stationary phase and interacts with the binding sites in a series of discrete transitory interactions. The system is highly dependent upon the chemical environment and these mobile phase effects are discussed in depth below.

HPLC

Chromatography has featured very prominently in MIP development [30], largely as a consequence of the common reliance on chromatographic methodology to demonstrate recognition in MIP systems. A wide range of compounds can be succesfully imprinted, subject largely to solubility constraints.

Chiral HPLC

The chromatographic separation of enantiomers has become a large subject area over the last decade. As a result of the proliferation in the types of chiral stationary phases (CSP) presently available, the majority of racemic mixtures can ultimately be resolved. However, there are still major difficulties in the rapid identification of a suitable CSP and conditions for undocumented separations, which are compounded by the high initial cost of such CSPs. On the other hand, chiral discrimination can, in principle, be easily introduced into a 'tailor-made' MIP designed for a specific separation by using an optically pure template. Thus, by preparing a polymer imprinted with an optically pure single enantiomer, the processed material can be used to resolve the racemic mixture. The relative simplicity of CSPs based on MIPs has led to a flurry of research in this area and the literature is rich in highly successful, indeed sometimes verging on remarkable, chiral separations [30]. MIP HPLC columns have the added advantage of always separating racemates in a defined manner, in that the elution order is always predictable, the non-imprinted enantiomer preceding the imprinted.

Figure 6.27 illustrates a sample chromatogram for racemic resolution on a MIP stationary phase, whilst *Table 6.6* summarises the developments in the field of molecularly imprinted chiral separation technology.

Classical chiral recognition theory requires that one enantiomer interacts with the CSP at 3 points whilst the other enantiomer interacts at a maximum of 2 points. It is easy to extend this analogy to explain the enantioselectivity of enantiomer-imprinted polymers in terms of 3-dimensional binding sites.

The pattern of peak shape shown in *Figure 6.27a* is typical of that achieved

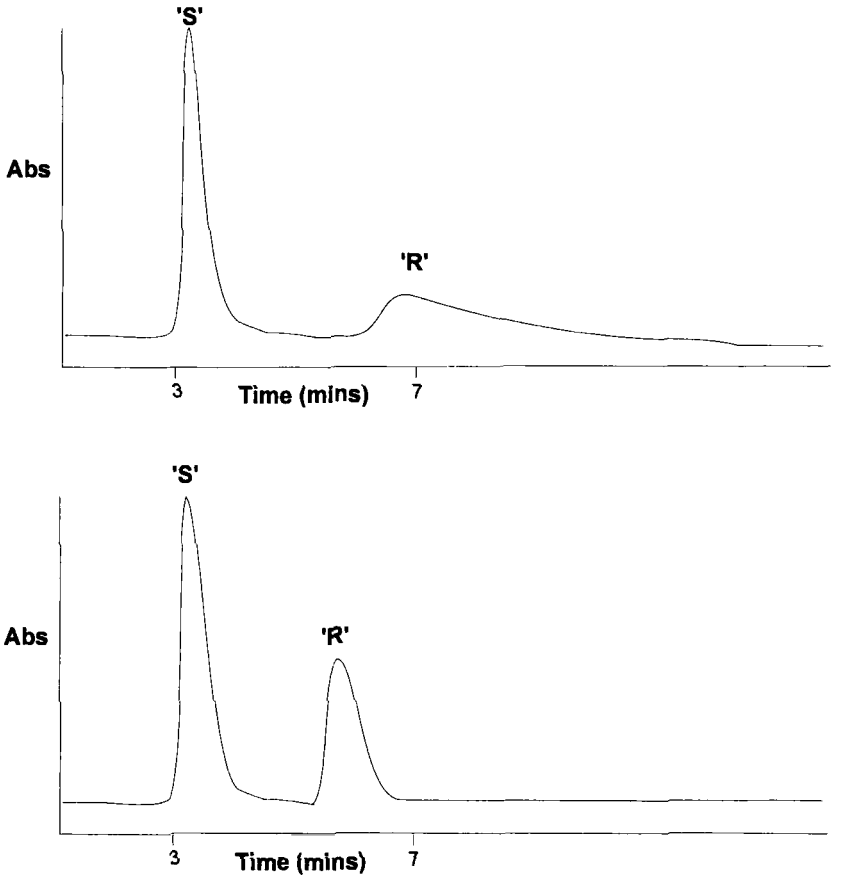


Figure 6.27 Typical separation of a racemate (RS) on an 'R' imprinted MIP using (a) isocratic elution and (b) gradient elution.

using MIP CSPs. The relatively poor peak shape of the second peak can be the result of the heterogeneity of binding sites and the resulting variety of binding constants, as well as non-specific binding and the generally poor kinetics of the systems [77]. The symmetry of the second, imprinted, peak can usually be improved by raising the temperature and decreasing the flow rate, as both of these changes beneficially affect the kinetics of binding-rebinding [15, 77]. Further improvement can be achieved by using gradient elution (*Figure 6.27b*). In addition, the ion-exchange approach to MIP chiral chromatography [73] demonstrated clear improvements in α and resolution (R_S) values when acetonitrile-aqueous buffer mobile phases replaced the

Table 6.6 CHIRAL SEPARATIONS ACHIEVED USING MOLECULARLY IMPRINTED POLYMERS.

Year	*Imprint molecule*	*Polymer type*	α	K'_L	K'_D	*Ref.*
1985	L-phenylalanine ethyl ester	EGMA/AA/AIBNΔH	1.3	0.48	0.37	[25]
1989	L-phenylalanine anilide	EGMA/MAA/AIBNuv0°C	2.25	3.15	1.4	[151]
1990	Cbz-L-glutamic acid	EGMA/MAA/AIBNuv0°C	2.5	1.90	0.75	[60]
1991	(*S*)-(-)-Timolol	EGMA/MAA or ITT/AIBN0°C	2.9	5.8	2.00	[49]
1991	Boc-L-phenylalanine	EGMA/MAA/AIBNuv0°C	2.1	3.12	1.46	[70]
1993	L-Phenylalanine anilide	EGMA/MAA/AIBNuv15°C	5.4	9.3	1.7	[59]
1993	Boc-L-tryptophan	EGMA/2VPy/MAA/AIBN0°C	4.4	–	–	[48]
1994	N-Ac-L-Phe-L-Try-OMe	EGMA/MAA/AIBNuv0°C	17.8	6.40	0.36	[100]
1994	(*S*)-Naproxen	EGMA/2VPy/AIBNuv0°C	1.7	3.58	2.17	[147]

commonly used acetonitrile-acetic acid systems. In that study, strong retention and high selectivity were favoured by buffers of low pH and vice versa. They proposed that small variations in pKa of the acid residues within the selective sites, as compared to those of the non-selective sites, explained these pH dependent features. They considered that the poor peak symmetry and peak splitting that is often encountered when using MIPs may, in part, be associated with the poor performance of the acetonitrile-acetic acid mobile phase and not entirely due to shortfalls of the stationary phase. This was further supported by a later study [77] from which it was concluded that the strong dependence of peak symmetry on sample load, and weak dependence on flow rate, implied that a non-linear adsorption isotherm, and not simply slow kinetics, was the main reason for poor peak shape.

The majority of chiral separations have been achieved using imprinted MAA-*co*-EGMA or 2VPy-*co*-EGMA polymers and, in general, the relatively easy success achieved with these phases appears to have been a disincentive to the investigation of alternative polymeric systems. One exception is the work of Yu and Mosbach [58], where attempts to develop an aqueous polymerisation system led to the use of acrylamide as the functional monomer. The resulting polymers demonstrated increases in both enantioselectivities and capacities, together with an improvement in peak symmetry.

Table 6.7 THE RETENTION BEHAVIOUR (k') ON A SERIES OF β-BLOCKERS ON NON-IMPRINTED, ATENOLOL-MIP, PROPRANOLOL-MIP AND *S*-TIMOLOL-MIP

Analyte	*Non-imprinted*	*Atenolol*	*Propranolol*	*S-Timolol*
± Acebutolol	0.38	0.25	1.50	0.42, 0.92 *(2.19)*
± Atenolol	0.50	9.67	2.50	1.42, 3.25 *(2.29)*
± Labetolol	0.60	0.50	2.25	1.33, 2.67 *(2.01)*
± Metoprolol	0.38	1.33	2.45	1.25, 1.92 *(1.54)*
± Nadolol	0.50	0.33	1.50	0.92, 3.42 *(3.71)*
± Oxprenolol	0.38	0.33	1.40	0.33, 0.75 *(2.27)*
± Pindolol	0.40	0.33, 0.67	3.00	2.08, 3.67 *(1.76)*
± Propranolol	0.40	0.33	4.70	0.67, 3.00 *(4.48)*
s-Timolol	n/a	0.32	n/a	6.33

Note: all racemic β-blockers were resolved. The *S*-timolol values are for k'_R, k'_S and separation factor, α, in parentheses.

An *S*-timolol MIP has been noted several times for a high ability to resolve the enantiomers of other β-blockers [32, 49]. For example, *Table 6.7* includes data obtained for such a material in this laboratory.

Capillary electrophoresis

Imprinted polymers have been shown to be a suitable medium for electrophoretic separations. Bruggemann *et al.* [78] successfully resolved racemic 2-phenylpropionic acid using an electrophoretic capillary internally coated with a *S*-2-phenylpropionic acid MIP. Another study [79] used an *N*-acryloyl-alanine polymer imprinted with *S*-propranolol as a chiral additive to resolve the racemate.

Electrochromatography

Electrochromatography (EC) is a hybrid separation technique combining the stationary phase of liquid chromatography with the electrically driven mobile-phase transport of electrophoresis [80–82]. Flow through the column

is *electroosmotic* and is electrically driven. The system therefore does not generate the back pressure that limits the minimum stationary phase particle size that can be used in conventional hydraulically driven liquid chromatography. Since this limiting factor is removed in EC very small particle sizes can be employed, with resultant increases in efficiency, which can be important in MIP applications. The two techniques have been successfully combined to demonstrate enantiomeric resolution of amino acids [83–86] and a comparative study [87] showed that a MIP-EC system compared favourably with cyclodextrin-EC and cellobiohydrolase-EC for the separation of β-adrenergic antagonist enantiomers. Racemic samples of ropivacaine, mepivacaine and bupivacaine were resolved using a ropivacaine imprinted MIP-EC approach [88].

Combinatorial screening

The phenomenon of cross reactivity inherent in the current generation of MIPs was the basis of a novel method currently under investigation in these laboratories for the high throughput screening of complex mixtures, particularly combinatorial libraries. Bowman *et al.* [32] found that it was possible to identify β-blocking drugs from a 'bookshelf' of diverse compounds (*Table 6.7*). This phenomenon has been investigated further by Richardson *et al.* [89]. A potential advantage of such a system is it can be self-optimising (*Figure 6.28*).

Berglund *et al.* have recently described selection of phage display combinatorial library peptides with affinity for a yohimbine imprinted methacrylate polymer [90].

Thin layer chromatography

A novel approach to racemic resolution involved the preparation of thin-layer chromatography plates using a polymer imprinted with L-phenylalanine anilide, which was coated onto a sanded glass slide using plaster of paris as adhesive. Rapid chiral separation of L- and D-phenylalanine anilide was achieved, together with resolution of the dansyl, ester, and amide derivatives [91]. A chiral separation factor of 3.5 was reported for the anilide. Although there have been no further reports of TLC chiral separations using MIPs the subject certainly warrants further investigation.

Supercritical fluid chromatography

MIP columns were eluted with supercritical carbon dioxide and a range of

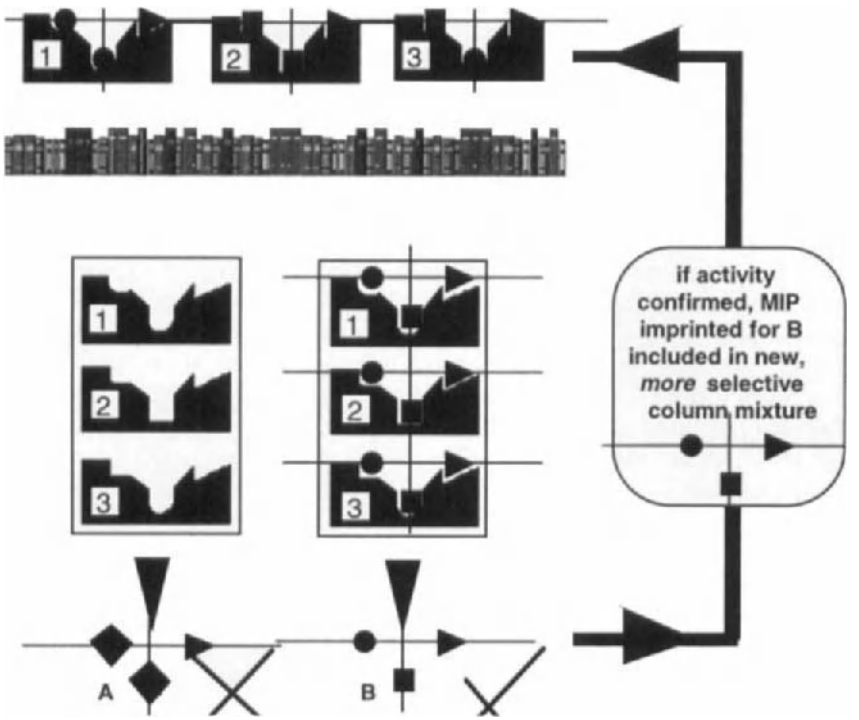


Figure 6.28 High throughput screening using a mixed-MIP stationary phase. Complex mixture (combinatorial library) eluted through column comprised of MIPs prepared for several (in this case 3) molecules active at target receptor. Molecule B binds to none of the 3 MIPs, is not retained and discarded. Molecule B binds to all 3 MIPs, is well retained and therefore a candidate for further testing. Self-optimisation by incorporation of B-MIP.

modifiers [92] with two major conclusions. Firstly, MAA-based MIPs are unstable under these conditions, the particles apparently rapidly breaking down and passing through the system. Secondly, an unexpected upward baseline drift on elution suggested the removal of 'irrecoverable' template from deep within particles. It is possible that rapid washing with a supercritical fluid might be a practical method of removing such deeply embedded template.

Mobile phase effects

The influence of varying of the mobile phase composition on the retention behaviour of a chiral solute on MIP HPLC stationary phases has been stud-

ied [67, 77]. It is generally held that, for non-ionised analytes, the most important interaction governing the separation of enantiomers on such materials is H-bonding, and that retention times increase with decreasing H-bonding potential of the mobile phase. Studies have largely concerned mobile phases containing chloroform with acetic acid as a polar modifier. Boc-L-Phenylalanine (Boc-L-Phe-OH) MIPs were prepared, processed and packed into HPLC columns, which were used to investigate the retention characteristics of Boc-L-Phe-OH and Boc-D-Phe-OH using a range of mobile phases [67]. In chloroform based mobile phases there was generally a linear relationship between the H-bond donator factor of the polar modifier and capacity (K'). Results also indicated a hydrogen bond donor parameter value for a polar modifier at which retention became concentration independent. For given values of K'_L, K'_D varied depending upon the polar modifier, indicating that enantiomer resolution was solvent dependent. Using mobile phases based on solvents of lower polarity/H-bonding potential than chloroform, substantial increases in K' were observed, although enantioselectivity was greatly reduced.

The use of a mobile phase other than the solvent used during the polymerisation process could be of significance. Firstly, the solvation shell around the ligand will be different to that which existed during the polymerisation process, which will affect docking with the imprint site. Secondly, the differential swelling observed in various solvents [59] infers a change in the accessibility and geometry of the imprinted site.

Stationary phase stability

The stability of MIP stationary phases has not been accurately determined, although authors allude to a lifetime of 'many 100s of injections'. It is likely that they are, in fact, as susceptible to degradation in performance due to sample impurities/poisoning as other types of stationary phase. However, as the mechanical and chemical stability of MIPs allows their use under a wide range of temperatures and mobile phases, with the possible exception of supercritical fluids [92], cleaning procedures are likely to be successful.

STATIC BINDING

Static binding processes are those where the analyte is under conditions that allow the establishment of a binding equilibrium. Such processes are often quantified in terms of Scatchard plots, where association constants and both the number and heterogeneity of binding sites can be determined. How-

ever, the generally inverse relationship between solubility and adsorption (Lundelius' rule) needs to be considered when examining binding behaviour.

Solid phase extraction (SPE)

Although solid phase extraction can be considered an 'immuno'-chromatographic process, the subject is considered here as the crucial step in a static binding process. MIPs have been used as solid phase sorbents in several successful recent studies and this is an area in which MIPs have great potential. Martin *et al.* [93] used a MIP solid phase system to selectively extract propranolol from urine and plasma and highlighted the importance of the eluent in limiting non-specific binding. Abd El Ghafar *et al.* [94] used a similar system to assess cross-reactivity between a range of β-blocker drugs. Rashid *et al.* [95] used a tamoxifen-imprinted polymer for SPE of tamoxifen from biological samples and reported that clean traces were obtained for HPLC, with UV detection, using this type of protocol. Muldoon and Stanker [96] used a MIP to extract atrazine from a solvent extract of beef liver. In these studies, MIP-SPE systems were shown to be sufficiently robust to be able to deal with selective extraction from biological samples. Andersson *et al.* [97] used a semeridine MIP to pre-concentrate the analyte prior to analysis by gas chromatography (GC) and Walshe *et al.* [98] used a MIP-SPE for 7-hydroxycoumarin to extract this from urine. It was observed that, because of the high specificity, sample preparation was extremely efficient, which lead to cleaner analytical performance, resulting in better levels of sensitivity. Full recovery of nicotine and several metabolites was reported from patients using nicotine containing chewing gum [99].

A limitation of the use of precisely imprinted MIPs in SPE is that seepage of non-recovered template may become significant at low concentrations [68]. A potential solution to this problem is to imprint with a close analogue of similar structure which can be distinguished by the quantitation procedure.

Receptor mimics

There has been much recent interest in 'biomimetic' systems , i.e. those that mimic the degree of selectivity observed in biological systems. The formation of a molecular imprint of either an agonist or an antagonist of a particular receptor gives rise to a site that can be considered akin to the receptor, or part of the receptor. Such imprints have been termed 'receptor mimics'. Synthetic peptide receptor mimics [100] were highly stereoselective when used as HPLC stationary phases. Andersson *et al.* [101] used imprints of the en-

dogenous neuropeptide Leu-enkephalin and morphine to mimic opioid receptors. They found that recognition was not limited to organic environments but demonstrated high affinity binding in aqueous media, although the specificity of the opioid receptor mimic was lower in aqueous conditions. A pH dependency was observed for both affinity and specificity.

Mimics of α_2-adrenergic [90] and β-adrenergic [102] receptors have been prepared. Berglund *et al.* [90] prepared imprinted polymers against two α_2-adrenergic antagonists (the alkaloids yohimbine and its diastereoisomer corynanthine). They observed pronounced stereospecificity with a K_d for the binding of yohimbine MIP to its template of 60nM which compared very well with the K_d value of 8nM for the binding of yohimbine to endogenous receptors. The β-adrenergic mimics were prepared as imprints against the adrenomimetic moieties ephedrine and pseudoephedrine and the results again suggested that adrenergic receptor mimics were good models for the endogenous receptors. A further study [103] described the optimisation of a molecular imprinting strategy for the preparation of testosterone receptor mimics. Excellent selectivity among closely related steroids was reported and testosterone affinity for the imprint was 4-fold higher than that for a non-imprinted control polymer. Bowman *et al.* [32] extended this concept to directly examine MIPs as potential preliminary screens for β-adrenergic antagonistic agents from libraries of compounds.

Molecular imprinting has also been used in these laboratories to create a histamine receptor mimic through which the binding of receptor antagonists could be studied [89]. The binding behaviour of a range of compounds illustrated the requisites of antagonistic behaviour, demonstrating the importance of exact molecular conformation and composition. This receptor mimic, being an agonist imprint, could effectively recognise moieties for which it was imprinted, although it failed to respond to variations in side-chains of antagonists. These side-chains play an essential role in antagonism of the endogenous receptor by stabilising the interaction complex through binding to accessory sites which were not present on the MIP mimic. Hence this MIP mimic could only provide a generalised picture of their interactive capability. The polymer binding site also appeared to rely heavily on a molecular conformation similar to histamine for binding interaction. The actual biological receptor site, although very specific in binding agonists, can also bind compounds structurally distinct from histamine.

Biomimetic assay

It was quickly recognised that some elements involved in the molecular imprinting process are strongly analogous to the *in vivo* formation of antibod-

ies. The term 'plastibodies' has been used in recognition of this analogy. In this context, morphine MIPs have been described as anti-morphine. One practical manifestation of this has been in the attempted use of MIPs as antibody substitutes in 'immuno'-assays.

Vlatakis *et al.* [104] demonstrated that MIPs could replace antibodies as the selective binding moiety in a competitive 'Molecularly Imprinted sorbent Assay' (MIA). MIPs prepared for theophylline and diazepam were shown to possess high affinity and to demonstrate cross-reactivity akin to antibody systems. In general, MIPs have been shown to be analogous to polyclonal antibodies, although, in some cases, their affinity and specificity can approach that of monoclonal antibodies [18, 19, 105–107].

Initial MIP binding assays were complicated by the need to extract the analyte into an organic solvent in order to facilitate MIP-ligand binding [104]. However, improvements in recognition properties have lead to more efficient systems that are capable of recognition in aqueous environments. Synthetic opioid receptors prepared by imprinting Leu-enkephalin and morphine [101] were shown to be capable of specific binding in aqueous conditions and showed an affinity approaching one tenth that obtained in an optimised organic environment [K_d(organic) 92 ± 52nM, $8.9 \pm 2.1\mu$M; K_d(aqueous) $1.2 \pm 2.1\mu$M, $24 \pm 4.9\mu$M]. In addition, cross-reactivity of the *anti*-morphine MIP with codeine, a notoriously difficult cross-reactant in conventional immunoassays, was shown to be lower than for a morphine monoclonal system.

Due to binding site heterogeneity, the Scatchard plots for the *anti*-morphine MIPs were curved and two dissociation constants (low and high affinity) were derived from extrapolations of the shallow and steep portions of the curve. Although this treatment of binding data provides an indication of relative affinities and is commonly applied, this approach is mathematically questionable [108, 109]. MIP antibody mimics prepared against the corticosteroids were found to be highly selective for their template molecule and demonstrated low cross-reactivities with structurally related compounds [110]. Haupt *et al.* [111] reported imprinting with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) could be achieved in the presence of the polar solvents methanol and water. Formation of the prearranged complex relied on hydrophobic and ionic interactions between the template and the functional monomer 4-vinylpyridine. The potential use of micrometer-sized imprinted polymer particles as the recognition element in a radioligand binding assay for 2,4-D was demonstrated.

Catalysis and selective reactions

The formation of a spatially and functionally specific cavity has an obvious application in enzyme type catalysis [112]. Furthermore, in the light of interest in catalytic antibodies [113], the analogy between antibody and MIP suggests the concept of 'catalytic plastibodies'. Robinson and Mosbach [114] demonstrated the potential for catalytic activity by imprinting a transition state analogue (*p*-nitrophenylmethyl-phosphonate) of the hydrolysis of *p*-nitrophenylacetate (*Figure 6.29*)

The 1.7-fold increase in reaction rate was specifically inhibited by the addition of *p*-nitrophenylmethylphosphonate, demonstrating that the imprinting process had given rise to specific catalytic sites.

The role of metal ions in catalysis is well documented. Matsui *et al.* [115] used a co-ordinating Co^{2+} ion in a transition state analogue imprinting procedure to prepare synthetic 'class II aldolase' mimics as catalysts of the aldol condensation (*Figure 6.30*).

Their approach was to from a pre-polymerisation complex with the transition state analogue Co^{2+} co-ordinated dibenzoylmethane and the monomers vinylpyridine, styrene and divinylbenzene. The procedure resulted in an 8-fold increase in reaction rate and high substrate specificity (*Figure 6.31*).

Other studies have attempted to utilise the properties of MIPs as selective catalytic materials for the hydrolysis of esters [116–118], whilst others [119–124] have used a 'footprint' technique to successfully imprint catalytic sites onto inorganic surfaces.

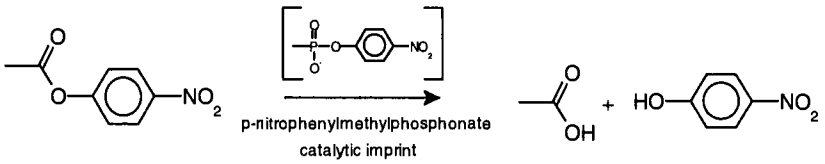


Figure 6.29 Catalysed hydrolysis of p-nitrophenylacetate by anti p-nitrophenyl methylphosphonate imprinted polymer.

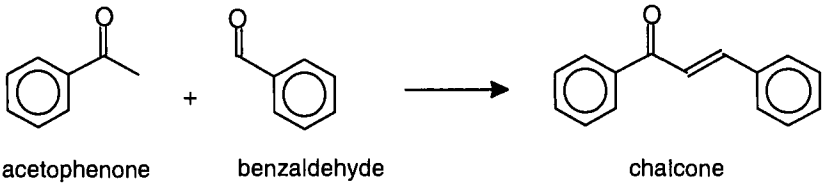


Figure 6.30 Aldol condensation of acetophenone and benzaldehyde to yield chalcone.

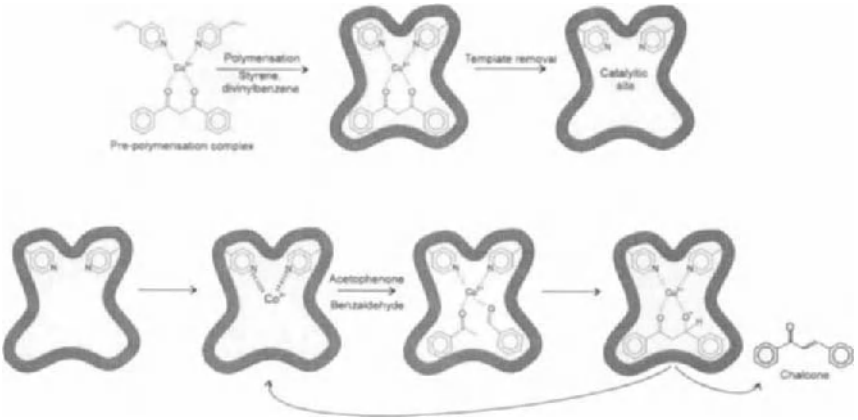


Figure 6.31 Preparation of Class II aldolase and catalysis of aldol reaction.

Sensors

One of the reasons why biosensors have had only limited commercial success is the relative fragility of the recognition elements. Generally, they lack the robustness demanded by the market. The replacement of enzymes and antibodies with highly stable and robust MIPs could help overcome this problem. *Figure 6.32* illustrates a general MIP biomimetic sensor where the MIP is bound to a transducing element via an interface.

Binding of the analyte to the MIP is detected by the transducer and this results in an output signal. Typically, the system can be calibrated to provide quantitative information. MIPs have been used as sensor recognition elements for a variety of analytes using transduction methodologies based on

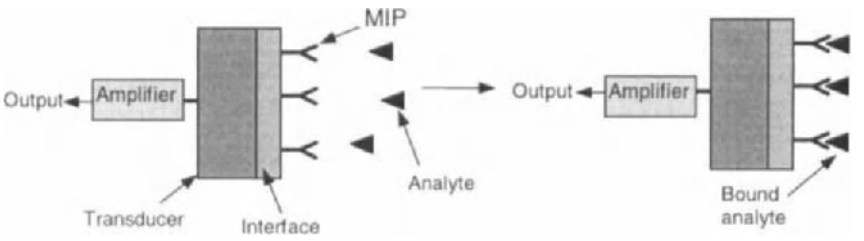


Figure 6.32 Generalised structure of biomimetic sensor using MIPs.

Table 6.8 EXAMPLES OF BIOMIMETIC MIP SENSORS.

Analyte	*Range (μg/ml)*	*Transducer*	*Reference*
Vitamin K_1	0–4	Ellipsometry	[152]
Phenylalanine anilide	33–3300	Potentiometry	[62]
Morphine	0–10	Amperometry	[153]
Dansyl-L-phenylalanine	0–30	Fibre-optic	[154]
Atrazine	0–0.5	Conductometry	[125]
Benzyltriphenylphosphonium ions	0–400	Conductometry	[156]
EtOH, EtAc, THF, $CHCl_3$	–	Optochemical	[157]
Sialic	0–3	Fluorescence	[155]

capacitance, potentiometry, amperometry, conductometry, fibre-optic fluorescence and optical fluorescence. *Table 6.8* provides a summary.

Molecularly imprinted membranes

Selectively permeable membranes can be produced using a molecular imprinting approach. Using a phase inversion method, a poly(acrylonitrile-co-acrylic acid) ultrafiltration membrane was imprinted with theophylline [52]. When solutions of theophylline and caffeine were filtered, a significantly greater amount of theophylline was retained within the membrane (*Figure 6.33*).

Using diethyl aminoethylmethacrylate and ethylene glycol dimethacrylate, atrazine selective membranes were produced and incorporated into an atrazine sensor [125]. Atrazine could be detected over the range 0.01–0.50 mg/L with a response time of ~30 mins and, most importantly, the sensors did not show loss of sensitivity over a four month period. Selective membrane diffusion of adenosine over guanosine was achieved using a membrane imprinted with 9-ethyladenine [126] and other selective membranes have also been prepared [127, 128].

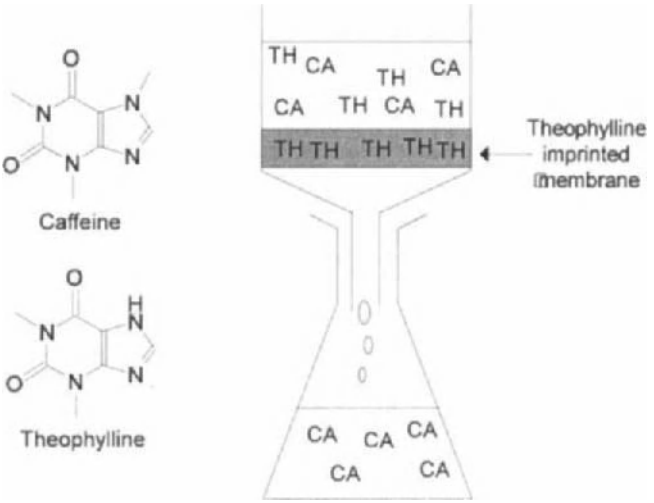


Figure 6.33 Selective filtration through a theophylline imprinted membrane. TH = theophylline, CA = caffeine [52]

Controlled release excipients

The applications previously described all make use of the selective *rebinding* properties of MIPs. On the other hand MIP-based controlled release systems are pre-loaded with the template and then utilise the *release* characteristics of the polymer to obtain an advantage in terms of rate or selectivity of release.

It may be useful to selectively retard the release of one stereoisomer in the presence of the other. Stereospecific release of cinchonine and the diastereoisomer cinchonidine was demonstrated from a cinchonidine MIP under dissolution conditions [129]. In an alternative approach, Allender *et al.* [130] incorporated a propranolol MIP pre-loaded with template within a transdermal adhesive. By modifying the drug:MIP ratio and adding hydrogen bonding excipients to the system controllable rates of delivery were achieved. Sreenivasan [131] demonstrated time-dependent release of hydrocortisone from a hydrocortisone imprinted polymer, whilst Karmalkar [132] developed a controlled release device incorporating a MIP catalyst that converted pre-drug into the active moiety within a hydrogel matrix. We are currently investigating the potential use of MIPs for controlled release from a range of delivery systems.

Cell surface imprinting

A 'lithographic' approach to the molecular imprinting of bacterial cell surfaces has been reported [133]. This 'indirect' method resulted in the formation of polyamide beads with 'spatially-defined, highly functional patches, of size and shape corresponding exactly to those of the bacteria'. Although only limited evaluation of the bacterial recognition properties have been carried out, the initial results suggest that discrimination between bacteria can be observed.

TECHNICAL CHALLENGES

In a relatively short period of time molecular imprinting has been transformed from little more than a laboratory curio into a technique that is potentially of great importance in a wide gamut of analytical and biomedical applications. The universal message from recent literature is that these materials have *great potential for the future.* However, although the literature base records an ever-expanding list of potential applications, the current state of development of the technique retains some major shortfalls that prevent the use of MIPs on a much wider scale. These are discussed in detail below.

The term 'plastibodies' is sometimes used in recognition of a perceived analogy between MIPs and antigen-antibody systems. For current systems, however, this analogy with biological antibodies does not bear close scrutiny for a number of reasons. Firstly, one of the ways in which biochemical processes achieve high levels of specificity and chiral recognition is through the use of a series of different functionalised chiral building blocks (amino acids) which are assembled in a precise manner complementary to the dimensions and functionality of the ligand. This contrasts sharply with conventional MIP formation, which involves the regiospecific solvation of a template molecule with, most commonly, only a *single* type of functionalised monomer prior to polymerisation. This limits the range of potential template molecules that can imprinted with highly specific sites. For example, it has not proved possible to produce an enantioselective MIP using *S*-ibuprofen and vinylpyridine/EGMA, presumably due to the limited template functionality [92, 134]. Rather more surprising was the fact that it was not possible to produce an enantioselective MIP using the functionally diverse L-hyoscyamine and MAA/EGMA [92]. In the latter case, it was demonstrated that neither racemisation nor hydrolysis during polymerisation could explain this phe-

nomenon. The limited range of interactions in most MIPs also severely increases the potential for introduction of cross-reactivity into the matrix.

Secondly, particularly where bioassays are concerned, perhaps the greatest problem with MIPs is their low aqueous compatibility. Antibodies are hydrophilic entities that operate primarily in aqueous or polar environments, unlike most MIPs which are formed under non-polar conditions and are hydrophobic in nature.

AVAILABILITY OF TEMPLATE MOLECULES

A common problem encountered, particularly in the development of CSPs, is the considerable amount of the template needed to prepare a MIP (normally in the range of 50–500μmol per gram of polymer). Therefore, non-availability or great expense of a template molecule can make the development of a MIP non-viable. It may be possible to imprint with a more readily available analogue of the template and then rely on cross-reactivity to bind the molecule of real interest, although this option may not be appropriate in all cases. The problem of template cost can be lessened to a large degree by recycling, since recoveries of up to 99% of the original template molecule are possible. Another significant problem is the question of solubility. Since many potential template molecules are soluble only in polar solvents, their ability to give rise to high affinity/selectivity imprints is limited. Alternative solvents, including acetonitrile, dichloromethane and THF, have also been successfully used in imprinting.

BINDING CAPACITY

The binding capacities of MIPs are dependent upon the concentration of template used and are usually relatively low per unit mass of polymer. Estimations of the number of binding sites per unit weight of MIP, obtained using frontal chromatographic or batch rebinding procedures, have shown that the ratio of [template molecule used]:[imprint site obtained] is quite high [17]. The reason for this is thought to be the presence of alternative complexes, other than the desired template-functional monomer complex, at the pre-polymerisation stage. The formation of MAA-MAA dimers and various aggregates of the template molecule would lead to a reduction in the number of binding sites. It has been shown that improved levels of capacity can be obtained using novel branched cross-linkers and MIPs have now been made with capacities approaching those demonstrated by other adsorbent materials [30].

It is worth reconsidering the processes that occur during polymerisation.

In the liquid phase the regiospecific solvation cage is optimally organised about the template and the double bond carbon atoms of the monomer units are sp^2 hybridised. Polymerisation causes the atoms to convert to sp^3 hybridisation and thus the planar moiety becomes tetrahedral and part of a chain. This intramolecular rearrangement will inevitably cause the previously optimised distances between interacting moieties to change. Although each individual change may be small, when these are repeated over the entire complex, the result will render the system much less optimised. This may explain unexpectedly low capacity and lack of selectivity. This feature is exacerbated by the probablility that the pre-polymerisation process involves more than one conformation of the species involved, in particular the template.

BINDING SITE HETEROGENEITY

The binding sites of MIPs are principally heterogeneous in nature and extension of the plastibodies analogy places MIPs in the polyclonal variety. According to Sellergren [64] and Vlatakis *et al.* [104], the variations in pre-polymerisation complexes and resultant polymerisation results in a population of binding sites with binding affinities varying between zero and a maximum value. The source of this polyclonality is difficult to pinpoint, but probably involves a combination of factors including high non-specific binding, irregularity of surface binding sites, and the imprinting of different conformations of a single type of molecule. It has been observed that conformationally rigid template molecules lead to highly selective MIPs [135].

Binding site heterogeneity can contribute to a number of practical problems, including poor peak symmetry in chromatographic applications and non-linearity in immunoassays. To produce a MIP with a population of binding sites that can be described by a single binding constant would require that every pre-polymerisation complex was identical and that all underwent subsequent polymerisation in the same manner. To achieve this utopian state is particularly challenging!

Whitcombe described a hybrid method of imprinting where a covalent bond conformationally limited the template prior to a non-covalent type pre-organisation and subsequent polymerisation [136]. It was claimed that this type of approach reduced heterogeneity in the binding site population.

CROSS-REACTIVITY

Like true biological antibodies, MIPs have the innate capacity to bind chemical structures other than the template around which they were formed.

Cross-reactivity is a well recognised and much studied phenomenon in the field of immunoassay, although the literature contains relatively few accounts involving MIPs. Levels of cross-reactivity depend greatly upon the precise nature of the system. Despite the general acceptance of heterogeneity of MIP binding sites, many studies have reported linear Scatchard plots which suggests a surprising level of binding site homogeneity in these cases. Vlatakis *et al.* [104] studied cross-reactivity in a theophylline MIP. Allender *et al.* [109] examined MIPs imprinted with Boc-L-phenylalanine, Boc-L-alanine, Boc-L-glutamic acid (plus a non-imprinted control) for their ability to differentially bind the enantiomers of Boc-protected phenylalanine. Batch rebinding studies showed a degree of predictability for a number of MIP-ligand pairs, although other combinations showed unexpectedly high levels of cross-reactivity. This study produced curved Scatchard plots which clearly demonstrated site heterogeneity (*Figure 6.26*). Although cross-reactivity (or non-specificity) has been generally accepted as a source of significant potential problems in MIP use, the phenomenon can be of some value. For example, we have exploited the cross-reactivity of MIPs imprinted with β-blockers in the development of a system for the screening of combinatorial libraries [32].

The prospects for reducing cross-reactivity (or increasing specificity) will depend upon investigations which address the basic science of imprint formation. One possible answer, currently under investigation in these laboratories, involves a more biomimetic approach where multiple regiospecific interactions combine to yield cavities of greater specificity. Such cooperativity is, of course, dependent upon the the formation of the required complex in the liquid phase, followed by retention of the complex during the polymerisation step.

NON-SPECIFIC INTERACTIONS

Non-specific binding is a problem associated with all selective materials and can lead to a reduction in selectivity, binding capacity and poor chromatographic resolution. In MIPs it arises as a consequence of extraneous functionalities within the reagents present during the liquid phase. For example, the ratio of functionalised monomer to template units is often 4:1. Therefore, unless each carboxyl functional group of MAA is involved in the regiospecific complexation of the template, the excess will be available to interact in a non-specific manner. In addition, commonly used cross-linking reagents also contain excess functionality (e.g. hydroxyl groups) that can also contribute to non-specific binding.

Although it is not possible to completely eradicate non-specific binding

from a system, two approaches can be employed to ameliorate this problem. Firstly, end-capping is commonly used to reduce peak tailing effects in reverse phase HPLC columns by blocking residual underivatised silanol groups. A similar approach may be applicable to MIPs. McNiven *et al.* [137] reported that the selectivity of a MIP could be substantially enhanced by treatment of the polymer with varying amounts of methyl iodide in the presence of the template molecule.

A second approach might be to reduce non-specific binding by addition of a polar modifier to the solvent environment. This assumes that the binding affinity of the analyte to the specific sites is higher than that to the non-specific sites. Hence, it may be possible to reduce the affinity for non-specific binding sites to a critical level before there is a significant reduction in specific binding. This may explain why small amounts of acetic acid are commonly useful in MIP systems [101].

AQUEOUS MOLECULAR RECOGNITION

MIPs generally exhibit poor recognition in aqueous systems due to two factors. Firstly, MIPs are overall very hydrophobic, due to the high levels of apolar cross-linking. In practice, this limits the ability of an aqueous polar medium to wet the polymer surface and makes the transfer and uptake of analyte molecules thermodynamically unfavourable. A further problem is that, even if the analyte overcomes the wetting barrier, the polar interactions which were essential to pre-polymerisation complexation are readily overwhelmed under aqeous conditions. In spite of the difficulties, several workers have reported some success using aqueous optimisation procedures [73, 101, 138–140].

PARTICLE SIZE AND SHAPE CONTROL

The usual approach to molecular imprinting results in the formation of a polymer in the form of a monolithic block. The process of grinding this block into a particulate product of controlled size is both time consuming and wasteful (~40% lost). Another consequence of this approach is that the resultant particles are irregularly shaped. This physical feature contributes to poor chromatographic performance. The future development of improved MIP applications may depend upon the development of improved polymerisation procedures that reproducibily yield a spherical product of pre-determined particle size. Several studies have achieved some success in this area. Sellergren [141–142] described a non-stabilizing dispersion polymerization method for preparing pentamidine MIP particles, *in situ*, within a HPLC

column. Dispersion polymerization, using perfluorocarbon as the dispersant, yielded MIPs of controlled particle size and good selectivity [143]. Although such approaches may be a way forward for MIP technology, drawbacks with these newer methods include solubility limitations and increases in complexity.

CONCLUDING REMARKS

The basic methodology involved in MIP preparation and application is highly attractive in its simplicity and the range of potential uses is very large and diverse. However, in view of the range of limitations discussed above, it is unlikely that their full potential will be realised unless the science of the interaction processes is tackled head on. If effective progress is to be made over the next few years it is essential that there is investment in the examination of this technology at the fundamental level.

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Cumulative Index of Subjects for Volumes 1–36

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