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Review

Strategies for the inhibition of serine proteases

B. Walker* and J. F. Lynas

Division of Biomedicinal Chemistry, School of Pharmacy, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland (United Kingdom), Fax +44 (0) 28 90247794, e-mail: brian.walker@qub.ac.uk

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Abstract. Serine proteases have been shown to play a multifarious role in health and disease. As a result, there has been considerable interest in the design and development of synthetic inhibitors of these enzymes. In view of their diverse roles in biological processing events, one of the great challenges in such endeavours has been the need to produce compounds with exquisite selectivity. Inhibitor design has been broadly guided by the use of either peptide- or heterocyclic-based compounds, designed to exploit the known substrate specificity characteristics of individual enzymes. This review describes the thinking and strategies employed in such efforts.

Key words: Serine proteases; synthetic inhibitors; peptide-based inhibitors; heterocyclic inhibitors; inhibitor design.

Introduction

Serine proteases are the most widely studied group of proteins in biology. Interest in this remarkable family of enzymes is generally due to their well-characterized, widespread and spectacularly diverse role in a host of physiological and pathological processes. Many pathological disorders are caused by deficiencies in the normally exquisite regulation of the activity of proteolytic enzymes, resulting in abnormal tissue destruction and/or the aberrant processing of other proteins and peptides. For example, in addition to their role in clot dissolution, the urokinase- and tissue-type plasminogen activators (uPA and tPA) play a critical role in a number of processes including extracellular matrix (ECM) remodelling [1], angiogenesis [2], wound healing [3], tumour invasion and metastasis [4–6]. Cytotoxic T cells contain cytolytic granules filled with multiple serine proteases termed granzymes [7, 8], some of which are involved in apoptosis [9]. Mast cells contain chymase and tryptase, which are produced in response to mast cell activation [10] and play a key role in mediating inflammatory responses, are also important factors in allergic conditions, such as asthma and rhinitis [11]. Neutrophils contain a battery of proteolytic enzymes, including the serine proteases human neutrophil elastase (HNE), cathepsin G and protease 3, all of which are involved in the catabolism of phagocytosed protein [12]; however, the abnormal regulation of these enzymes is known to be a crucial factor in the aetiology of emphysema and chronic bronchitis [13]. Perhaps the most widely studied group of serine proteases has been those involved in the coagulation cascade (thrombin, protein C, factor VII, IX, X and XII), complement system (C1r, C1s, C3 convertase, C5 convertase and factor D) and fibrinolysis (tPA, uPA and plasmin) [14–17]. Most clotting abnormalities, and associated diseases, are mediated by inappropriate activity of these enzymes. Additionally, the important role of serine proteases has also been elucidated in the pathology of viral infections, including hepatitis C and herpes [18, 19].

* Corresponding author.

The mechanism of serine proteases

The availability of relatively large amounts of serine proteases from a variety of sources has provided numerous research groups with a useful means to undertake detailed studies of their physiocochemical properties and catalytic specificity against a wide range of both endogenous protein/peptide and synthetic substrates. Many of the most valuable insights into structure, function and specificity have been gained through the use of site-directed mutagenesis studies (extensively reviewed in [20]).

Serine proteases are characterized chiefly by the presence of an active site serine residue [Ser 195 (chymotrypsin numbering)], the γ hydroxyl group of which acts as a nucleophile during the hydrolytic process. Two other amino acid residues are directly involved in the catalytic mechanism, in addition to this serine residue. These are His 57, a general base/acid catalyst, and Asp 102, which is believed to orientate the catalytic histidine. In addition to this so-called catalytic triad, the enzyme possesses an oxyanion binding site. This is made from the backbone amide NH groups of Ser 195 and Gly 193. The mechanism is outlined in figure 1.

After noncovalent binding of the substrate to the enzyme active-site region, the OH group of Ser 195 attacks the carbonyl carbon of the scissile bond via general base catalysis by His 57. This action leads to formation of the tetrahedral intermediate. The structure of the tetrahedral intermediate is stabilized through the newly formed oxyanion component by forming hydrogen bonds to the backbone NH groups of Ser 195 and Gly 193 residues.

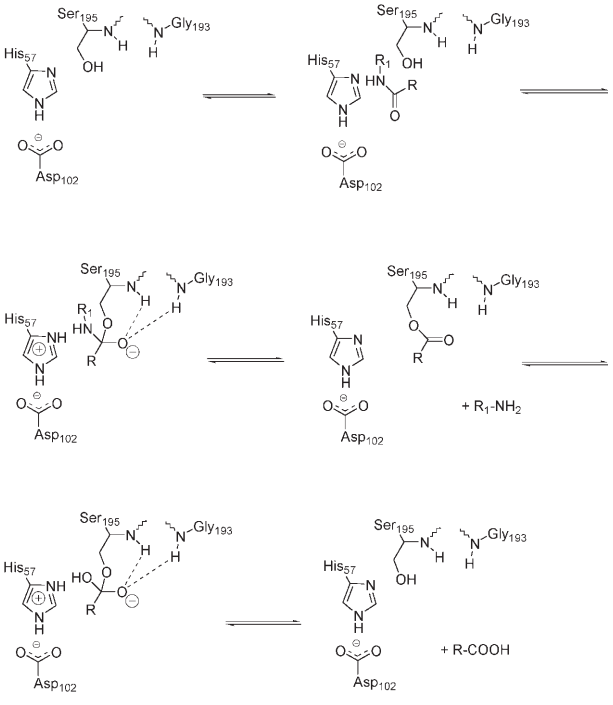


Figure 1. The mechanism of action of the serine proteases.

His 57, acting now as a general acid, transfers a proton to the amine of the tetrahedral intermediate; this subsequently breaks down, a product amine leaves and the covalent acyl enzyme intermediate is formed.

The acyl enzyme complex is attacked by a water molecule to generate a new tetrahedral intermediate. This intermediate collapses, assisted by general acid catalysis from His 57, resulting in the formation of product acid and the regenerated Ser 195 OH leaving group.

Whilst Barrett and Rawlings have revolutionized the nomenclature for the classification of all hydrolytic enzymes, describing species in so-called families and clans [21], for the purposes of this review we shall simply distinguish serine proteases on their differences in substrate specificity. The topology of the enzyme active site discriminates between peptides with defined amino acids sequences, particularly at the P_1 specificity subsite [22]. Serine proteases are generally classified according to their broad substrate specificity, and while there are notable exceptions (such as granzyme B, which cleaves after Asp residues), there are three main categories: trypsin-like (cleaving after basic amino acids), chymotrypsin-like (cleaving after bulky hydrophobic residues) and elastase-like (cleaving after small, aliphatic residues). Almost all synthetic inhibitor development has been targeted against serine proteases with these broad substrate characteristics.

Serine protease inhibitors

A tremendous amount of work has been undertaken into the discovery and elucidation of the mechanisms of natural proteinaceous inhibitors of the serine proteases. A discussion of such efforts is beyond the scope of this review; however, some of the mechanisms of action of proteinaceous inhibitors have provided inspiration for the design of synthetic inhibitors. As such, for detailed descriptions of the nature of endogenous protease inhibitor action, we would draw the reader to the excellent reviews by Bode and Huber [23] and with more specific references to the serpins [24, 25].

This review will discuss the design and biological properties of wide range of synthetic serine protease inhibitors which have been described in the literature. A crucial point has to be made at this juncture. Over the last 40 years, biologists and biochemists have often (in good faith) utilized inhibitors for the tentative classification of proteolytic enzymes involved in various pathophysiological processes. These inhibitors often suffer from two main drawbacks:

1) Many synthetic serine protease inhibitors often have poor selectivity and specificity for serine proteases. For example, blockage of a biological process by the commonly used chloromethyl ketone-based reagents *N*-α-tosyl lysine chloromethyl ketone (TLCK) (**1**, scheme 1)

and its phenylalanine analogue, TPCK **(2)**, have often incorrectly been used to announce that trypsin-like or chymotrypsin-like serine proteases are involved in that process. With the development of new, more specific agents, we believe that such conclusions are no longer acceptable. Both these reagents are not only inhibitors of serine proteases but also cysteine proteases and some protein tyrosine kinases. Thus conclusions drawn solely from the activity of such agents in a system should be subjected to the utmost scrutiny.

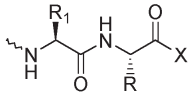
2) Inhibitors may have poor cell permeability and/or stability and thus bioavailability in model systems, leading to spurious results. This is a particular problem with studies on intracellular serine protease action. For example, the commonly used aldehyde-based inhibitor leupeptin **(3)** has often been used in whole-cell studies. This inhibitor suffers from two main drawbacks. First, the presence of an arginine residue in the inhibitor structure greatly reduces its propensity to cross cell membranes, and second, hydration of the aldehyde group reduces the activity of this inhibitor in vitro.

With these two points in mind, great efforts have been made to develop inhibitors with high specificity and selectivity for the serine proteases alone. Biological and medicinal chemists have undertaken a number of approaches for the development of peptide- and heterocycle-based agents for use in the study of the role of serine proteases in interesting biological systems both in vivo and in vitro. An enormous number of synthetic serine protease inhibitors have been reported in the literature, including peptide-based aldehydes [26, 27], chloromethyl ketones [28], fluoromethyl ketones [29], dimethyl sulphonium salts [30], α-keto-acids and amides [31], α-keto-esters [32] and α-keto-aldehydes (glyoxals) [33] (fig. 2). Judicious use of peptide targeting sequences has enabled researchers to achieve high selectivity and potency for specific serine proteases; however, compounds based on the above have also been shown to be superb inactivators of the cysteine proteases; (for a review see [34]) and therefore, in cases where there is some substrate overlap, selectivity is poor. For this reason we do not propose to discuss any of the above in this review, and instead will concentrate on those inhibitors which have been developed mainly as inhibitors of the serine proteases alone. We would also draw readers attention to a number of highly specific serine protease inhibitors developed by medicinal chemists as potential therapeutic agents, for example the factor Xa inhibitors DX9065a **(4)** (K_i = 41 nM) [35] and ZK-807191 **(5)** (K_i = 0.1 nM) [36], a number of thrombin inhibitors, including the (D)Phe-Pro-Arg-Pro- analogues argatroban **(6)** (K_i = 39 nM) [37], napsagatran **(7)** (K_i = 0.3 nM) [38], inogatran **(8)** (K_i = 15 nM [39] and melagratran **(9)** (K_i = 2 nM) [40] and the tryptase inhibitor APC-366 **(10)** (K_i = 0.84 μM) [41]. All these compounds are at some stage of clinical trials (for a wide-ranging review into serine protease inhibitors with therapeutic potential, see [42]). Whilst the design and development of each of these compounds present a fascinating study into drug design and development, this review will concentrate on agents with wider applicability.

Selective peptide-based serine protease inhibitors

Phosphorus-based inhibitors

Organophosphorus-based synthetic serine protease inhibitors represent an excellent model for the evolution of inhibitors with enhanced chemical and biological properties for the study of serine proteases in a variety of biological systems and as potential lead compounds for therapeutic use. These agents were identified as one of the first inhibitors exhibiting selectivity of action for the serine proteases. Diisopropylphosphofluoridate (DFP) **(11)** is still one of the most widely used broad – spectrum inhibitors of these enzymes [43]. Its mode of action involves the formation of a single covalent bond between the



Where X =

-H (aldehyde)

-CH_2F (fluoromethyl ketone)

-CH_2Cl (chloromethyl ketone)

-$CH_2S(Me)_2^+$ (dimethylsulphonium salts)

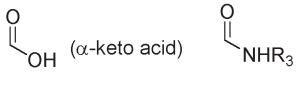


Figure 2. Peptide-based inhibitors active against both the serine and cysteine proteases.

phosphorus atom of the inhibitor and the Oγ atom of the active-site serine hydroxyl [44, 45]. Crystal structures and neutron diffraction experiments of DFP-trypsin illustrate the presence of a monoisopropyl group, thus leading to the conclusion that inhibition involves phosphonylation of Ser 195, preceding hydrolysis of a single isopropoxy group to give a final charged structure. The remaining isopropyl group interacts with the entrance to the primary specificity subsite (S_1). The tetrahedral configuration of the pentavalent phosphorus atom in this compound is postulated to be an excellent transition state analogue for amide bond hydrolysis [45, 46]. The major drawback with DFP is its extreme toxicity. It will also irreversibly modify acetylcholinesterase, and this property has been used in the development of chemical warfare agents. From a more mundane point of view, it has limited use in biological studies because of low stability in physiological buffers and a total inability to discriminate between different subclasses of serine protease, as it lacks the structural components necessary for enzyme-substrate interactions.

In order to circumvent these selectivity problems, Lambden and Bartlett prepared N-protected analogues of phenylalanine with a phosphonyl fluoride group **(12)** [47]. These were shown to be excellent, selective inhibitors of chymotrypsin, but the presence of the highly labile P–F bond renders the inhibitor unstable, being rapidly hydrolysed in physiological buffers.

A major breakthrough in organophosphorus protease inhibitors over the last few years was the development of α-aminoalkyl diphenylphosphonate esters. These have been shown to be superb inhibitors of serine proteases, with no activity against the cysteine proteases ([48]; B. Walker, unpublished data). These agents are analogues of normal amino acids involved in peptidyl recognition sequences, for instance Cbz-Phe^{P}(OPh)$_2$ **(13)**. Examples of the efficiency and discriminatory capabilities of these inhibitors are shown in Table 1. The diphenyl phosphonate esters are also completely stable for prolonged periods of time in plasma [48].

Mechanistically, the peptidyl portion of the inhibitor targets the diphenyl phosphonate ester into the protease active site, whereupon the inhibitor forms an irreversible complex with the enzyme. The catalytic mechanism of the protease is a means to activate the phosphorous atom for a substitution reaction to occur with the catalytic serine residue (fig. 3).

Table 1. Kinetic constants for the inhibition of serine proteases by diphenyl phosphonate ester analogues [48].

Inhibitor	Chymotrypsin	PPE	HNE
	k_{obs}/[I] ($M^{-1}s^{-1}$)		
Z-Val^{P}(OPh)$_2$	N.I.	9[a]	280
Z-Phe^{P}(OPh)$_2$	1200	N.I.	N.I.
Z-Pro-Phe^{P}(OPh)$_2$	230	N.I.	–
Boc-Val-Pro-Val^{P}(OPh)$_2$	N.I.	11,000	27,000
Suc-Val-Pro-Phe^{P}(OPh)$_2$	44,000	N.I.	N.I.

N.I., No Inhibition.
a-20% Inhibition at start of reaction, then no further inactivation.

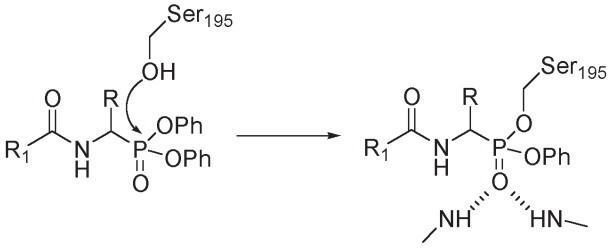


Figure 3. Proposed mechanism of action of diphenyl phosphonate esters [48].

The electronegative phenoxy leaving group is also necessary to activate the phosphorus atom; indeed, the addition of electronegative substituents on the phenoxy ring have been shown to increase inhibitor reactivity [49]. Increasing the length of the peptide chain on the N-terminus to di- and tripeptides results in better inhibition, as would be expected. Peptidyl diphenylphosphonate esters based on sequences of known substrates for various serine proteases were shown by Oleksyzyn and Powers [48] to be excellent inhibitors. Elastases inactivated by diphenylphosphonate esters have no observable reactivation, even after several days; however, chymotrypsin has a half-life of reactivation of between 7.5 and 26 h. Interestingly, chymotrypsin inhibited by Cbz-Phe^{P}(OPh)$_2$ regained 50% of initial activity after approximately 10 h, but no further dephosphonylation was observed, suggesting the possibility of different binding modes or mechanisms for the hydrolysis reaction. Diphenylphosphonate esters of basic amino acids such as Lys and Arg have been incorporated into peptide sequences selective for trypsin-like serine proteases, including thrombin [50, 51], and shown to be excellent inhibitors. Continuing this theme, Oleksyzyn et al. [52] have described peptidyl-(α-aminoalkyl)phosphonate diphenyl esters containing P_1 4-amidinophenyl groups. The best inhibitor reported in this series was Boc-(D)Phe-Pro-(4-AmPhGly)^{P}(OPh)$_2$ **(14)**, with k_{obs}/[I] values of 11000 $M^{-1}s^{-1}$ (vs. thrombin) and 2200 $M^{-1}s^{-1}$ (vs. trypsin) respectively. The single amino acid analogue Cbz-(4-AmPhGly)^{P}(OPh)$_2$ **(15)** was essentially equipotent with trypsin (k_{obs}/[I] = 2000 $M^{-1}s^{-1}$) and was also found to be an excellent inhibitor of plasma kallikrein (k_{obs}/[I] = 18000 $M^{-1}s^{-1}$). More recently, Powers' group have reported peptide-based 4-amidinophenylglycine analogues as potent inhibitors of granzyme A and K [53], and inhibition of *Staphylococcus aureus* V8 protease by Cbz-Glu-^{P}(OPh)$_2$ **(16)** and Cbz-Asp-^{P}(OPh)$_2$ **(17)** has been reported by our laboratories. Interestingly, neither compound nor the peptide analogue Cbz-Ala-Ala-Asp-

$^{P}(OPh)_2$ inhibit the serine protease granzyme B, which has specificity for Asp at P_1 ([54]; J. Lynas, unpublished data). Cbz-(4-AmPhGly)$^{P}(OPh)_2$ was the first diphenyl phosphonate to be used for X-ray crystallographic studies. The crystal structure of trypsin inactivated with this compound at 1.8 Å revealed the formation of a tetrahedral phosphorus atom bound covalently to the active site serine. The formation of the covalent bond is actually accompanied by loss of both phenoxy groups. In addition, it was found that only the L configuration of the inhibitor binds. One phosphonate oxygen atom interacts directly with the oxyanion binding site of trypsin; the second H-bonds to the Nε2 atom in the imidazole ring of His 57. The structure obtained was subsequently utilized to model the inhibitors into the active site of thrombin [55].

Powers' group have also described a series of dipeptide phosphonates with a proline or homoproline at the P_1 position, as irreversible inhibitors of the serine protease dipeptidyl peptidase IV (DPP-IV) [56]. This enzyme has been receiving increasing research attention to elucidate its biological role, particularly in the immune system [57–59]. These compounds were found to be moderate inhibitors of DPP-IV, the best being the Ala-Pip analogue **(18)** (k_{obs}/[I] = 1300 $M^{-1}s^{-1}$). More recently, a further series of DPP-IV inhibitors have been described with more elaborate substitutions at the 4-position of the phenoxy groups [60]. The Pro-Pro analogues **(19)** and **(20)** were shown to be amongst the most potent of the series, with $k_{calc} = 4.8 \times 10^4$ $M^{-1}s^{-1}$ and 3.3×10^4 $M^{-1}s^{-1}$.

The crystal structure of the serine protease cathepsin G has been elucidated with the aid of diphenyl phosphonate esters, illustrating their utility in biological studies [61]. As a consequence of our ongoing development of serine, cysteine and metalloprotease affinity labels containing biological reporter groups [62–66], we and others have incorporated biotin or fluorescent reporter groups on the peptidyl portion of a number of these diphenylphosphonate esters. Incubation of biological samples with the inhibitors and subsequent Western blotting analysis has enabled the selective disclosure of serine proteases in a number of biological systems [67–70].

In another strategy, Bartlett's group have also reported inhibitors of α-lytic protease based on mixed phosphonate esters containing C-terminal groups which mimic extended peptide sequences [71, 72]. Analogues such as **(21)** were prepared with the aim of fulfilling additional subsite interactions in the S′ subsites. As with the diphenyl phosphonates, these inhibitors were found to form a formal covalent bond between Ser 195 and the phosphorus atom. The resulting transition state adduct makes extensive interactions with the oxyanion binding site region, particularly with backbone amide nitrogens of Ser 195 and Gly 193. The advantage of designing inhibitors with extended amino acid sequences is that they can form extensive interactions with the extended binding sites of the protease; however, experimental evidence suggested that the S1′ and S2′ sites were not occupied by these inhibitors [71]. In addition, configuration of the phosphorus atom was shown to be of little consequence in these compounds. Subsequent X-ray crystallographic studies were carried out which revealed interesting aspects concerning the structure of the irreversibly bound phosphonate occupying the active site after hydrolysis. This was found to be influenced by the stereochemical configuration of the initial inhibitor. The complex observed with isomer **I** shows the phenoxy group displaced by Ser 195. With isomer **II**, this structure is also observed, as well as another adduct where both phenoxy groups appear to be hydrolysed, similar to the phenomenon reported by Bertrand et al. [55]. This led to the conclusion that since the inhibitors exist as diastereoisomers and both lead to a similar complex formed with the protease, then one isomer must interact with inversion of configuration, whilst the other reacts with retention of configuration at the phosphorus atom. This may explain in part the unusual mode of dephosphonylation observed with the diphenyl phosphonates described above. In a similar strategy, we have recently reported the design and synthesis of *N*-α-protected monophenyl phosphonate alkyl ester analogues of simple amino acids as potent, new inhibitors of serine proteases [73].

Fluorine-containing serine protease inhibitors

In order to prepare metabolically stable analogues of inhibitors containing electrophilic carbonyl groups, researchers at ICI (now AstraZeneca) Pharmaceuticals, prepared trifluoromethyl ketones (TFMKs) as potential serine protease inhibitors [74]. These researchers and another independent group showed that such agents were excellent inhibitors of their target enzymes, in this instance chymotrypsin and elastase [75–77]. In a seminal paper on the mechanism of inhibition of HNE by TFMKs, the ICI group provided a number of lines of evidence for the formation of a hemiketal intermediate between the electrophilic ketone group of the inhibitor with the Oγ atom of the active site serine residue. It is proposed that the trifluoromethyl group facilitates nucleophilic attack by the serine residue, on the carbonyl group of the inhibitor [78]. Crystallographic studies tend to confirm this mechanism [79], which is outlined in figure 4.

^{13}C NMR studies suggest that the TFMKs are hydrated in the presence of water, due to their high susceptibility to

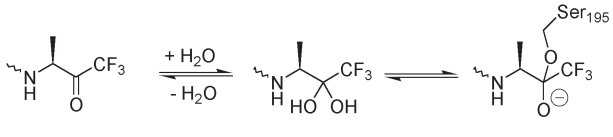


Figure 4. Proposed mechanism of action of peptide trifluoromethyl ketones [78]

nucleophilic attack. This does not inactivate the inhibitor, although the nonhydrated form is the more effective inhibitor species [80].

Since these initial studies, there has been enormous interest in the preparation and potential therapeutic usage of TFMKs, due to their stability and pharmacological properties, for the treatment of diseases in which serine proteases, particularly HNE, play a destructive role [80–84]. While the majority of TFMKs prepared in these studies, such as ICI 200 880 (X = Cl; K_i = 0.5 nM) **(22)** and ICI 200 355 (X = Br; K_i = 0.6 nM) **(23)**, are peptidic in nature, pharmacological criteria necessarily dictate a desirability to reduce such character for optimal biological activity. With this in mind, a number of analogues containing the TFMK moiety with lower peptidic characteristics have been studied and evaluated against HNE [85–88]. Inhibitors containing the peptidomimetic pyridone moiety, such as **(25)** (K_i = 4.5 nM) [89] and **(26)** (K_i = 8.4 nM) [90], designed by the use of computer modelling, X-ray crystallographic and extensive structure activity relationship (SAR) studies, have been prepared with in vivo activity via tracheal and oral administration. This work is an excellent example of the development of non-peptide – based serine protease inhibitors as potential therapeutics using peptide-based lead structures as the starting point, demonstrating the evolution of inhibitors, from the simple Cbz-Val-Pro-Val-CF_3 **(24)** (K_i = 1.6 nM) [78] to agents with improved pharmacological characteristics. Compound **26** was developed to extend interactions within the S_5 and S_4 subsites. The presence of the heteroatoms on the pyrimidine ring also enhances aqueous solubility.

Whilst the most extensive development of TFMKs has been directed against HNE, these compounds have also been shown, with suitable use of peptide – targeting portions, to be effective inhibitors of cathepsin G [80] and thrombin [91]. Interestingly, the thrombin-directed inhibitor Me(D)Phe-Pro-Arg-CF_3 appears to behave as an irreversible inhibitor. This latter group have also introduced difluoromethyl and pentafluoroethyl ketones as excellent thrombin inhibitors [91].

In an attempt to exploit the success achieved with TFMKs against mammalian serine proteases, Ogilvie et al. [92] have recently employed similar strategies for the development of inhibitors of human cytomegalovirus (HCMV) protease to produce lead compounds.

As well as the TFMKs, a number of other fluorine-containing inhibitors of serine proteases have been described. While most serine protease inhibitors are designed to occupy the S_{1-n} subsites, a large body of evidence, particularly from crystallography studies, suggests that the $S_{1'-3'}$ subsites form favourable interactions with proteinaceous protease inhibitors. This is perhaps most clearly illustrated in the crystal structure of the complex between HNE and the third domain of turkey ovomucoid inhibitor (OMTKY3) [93]. In attempts to exploit such observations into inhibitor design, Imperiali and Abeles [94] prepared putative inhibitors of chymotrypsin based on fluoromethyl ketones, which were designed to interact with the S′ subsites. Systematic modification of the difluoro ketone, Ac-Leu-Phe-CF_2H [K_i (vs. chymotrypsin) = 25,000 nM)], involving replacement of the hydrogen group with moieties increasingly peptidic in nature led to the discovery of inhibitors **27** and **28** with enhanced potency (K_i = 7800 and 14 nM, respectively), thus illustrating the substantial contribution made by S′ subsite interactions in this series of compounds.

This principle was applied to the design of new inhibitors of HNE. Skiles et al. [95] have described tripeptide α,α-difluoromethyl ketones such as **29** (IC_{50} = 0.635 μM), which interacts with the $S_{1'}$ subsite and α,α-difluorostatones such as **30** (IC_{50} = 0.057 μM), which interacts with the S_3–$S_{1'}$ subsites (IC_{50} = half maximal inhibitory concentration).

In common with TFMKs, it is believed that these agents act via hemiketal formation with the active-site serine residue. The electron-withdrawing capability of the α,α-difluoromethylene functionality enhances nucleophilicity of the ketonic group. Abeles' group have described β,β-difluoro-α-keto esters such as **33** and acids as serine protease inhibitors which are more potent than analogous amino acid-based TFMKs **(31)** and α-keto ester **(32)** analogues [96]. Addition of Ala-Leu-Arg-OMe, replacing the ethyl ester, results in a slow, tight-binding inhibitor of chymotrypsin.

Pentafluoroethyl ketones such as **34** have also been shown to be excellent inhibitors of serine proteases. Indeed, there is evidence that the PFEKs have improved pharmacological activity against HNE [97, 98] and thrombin [91].

Peptidyl boronic acids

Chymotrypsin and subtilisin were the first serine proteases shown to be inhibited by aromatic boronic acid derivatives [99, 100].The crystal structure of subtilisin, complexed with phenylethane boronic acid was determined by Matthews et al. [101], clearly showing that binding and interaction of the trigonal boronic acid moiety, with the active site serine residue leads to the formation of a tetrahedral adduct in complex with the enzyme, mimicking the putative transition state. In this structure, one –OH group interacts with the oxyanion binding site, whereas the other occupies a position analogous to the –NH– leaving group found during normal peptide bond hydrolysis (fig. 5).

(*R*)-acetamidophenylethane boronic acid **(35)**, a phenylalanine analogue, was shown to be several times more effective as a chymotrypsin inhibitor than aromatic boronates, being active in the micromolar range [102]. Kettner

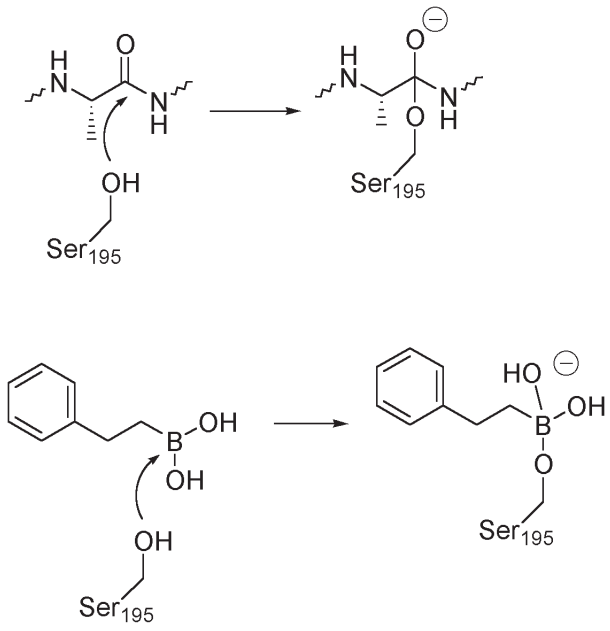


Figure 5. Comparison of amide bond hydrolysis with the proposed mechanism of action of boronic acids.

and Shevni [103] prepared the first α-amino boronate-containing peptides in an effort to improve their selectivity and potency as inhibitors of chymotrypsin- and elastase-like enzymes. Their strategy was based on the use of selected peptide-targeting motifs based on known substrate sequences, including methoxysuccinyl-Ala-Ala-Pro- with a P_1 boroPhe-OH residue **(36)** (R = Ph) (for chymotrypsin-like serine proteases) and P_1 boroVal-OH **(37)** (R = isobutyl) or boroAla-OH **(38)** (R = Me) (for elastase-like species). The inhibitors were shown to be effective inhibitors of chymotrypsin, HNE and PPE at nanomolar concentrations (table 2), with inhibitors generally displaying slow, tight-binding kinetics. This observation is generally associated with inhibitors acting as transition-state mimics; however, for peptidyl boronic acids to exhibit this mode of inhibition, a number of structural requirements must be fulfilled.

Table 2. Inhibition of serine proteases by peptidyl boronic acids [103].

Enzyme	Inhibitor MeOSuc-AAP	K_i (nM)	
		(initial)	(final)
Chymotrypsin	Ac-bPhe-OH	2100	–
	R-bPhe-OH	3.5 ± 1.1	0.16 ± 0.03
	R-(D)bPhe-OH	11.0 ± 2.3	–
	R-bVal-OH	1200 ± 50	–
	R-bAla-OH	9100 ± 900	–
PPE	R-bPhe-OH	270 ± 50	
	R-bVal-OH	30 ± 13	0.25 ± 0.02
	R-bAla-OH	18 ± 2.5	0.32 ± 0.04
HNE	R-bPhe-OH	350 ± 80	–
	R-bVal-OH	15 ± 6	0.57 ± 0.12

First, binding in the P_1 site is insufficient by itself to achieve slow, tight-binding characteristics, as **35** was found to be a simple competitive, reversible inhibitor. Therefore, to observe slow, tight-binding characteristics, interactions between inhibitor and enzyme secondary subsites are necessary.

Second, the stereochemistry of the P_1 aminoboronate also influences activity Methoxysuccinyl-Ala-Ala-Pro-(L)boroPhe-OH is a potent, slow, tight-binding inhibitor of chymotrypsin, whereas the analogous sequence containing (D)boroPhe-OH is a simple, competitive inhibitor with 200-fold less efficacy (table 2).

Third, in common with virtually all inhibitors, specificity at the S_1 site of the target enzyme must be fulfilled. Against chymotrypsin, the elastase-directed sequences described above with P_1 boroAla-OH or boroVal-OH act as simple, competitive inhibitors (with greatly reduced potency) rather than slow, tight binders (as observed with the boroPhe-containing analogue). Additionally, it is remarkable to note that inhibitors containing the bulky amino acid analogue boroPhe at the P_1 position, which would normally preclude activity against elastases, are still reasonably effective inhibitors, unlike virtually all extended peptide-based inhibitors.

Crystallographic analysis of the interaction of peptide boronic acids with α-lytic protease demonstrated some interesting structural features of the enzyme-inhibitor complex [104, 105]. The structure indicated that the boron atom of the peptide inhibitor covalently links to the active site serine residue, but not His 57. This has been subsequently confirmed by further studies, for example Takahasi et al. [106] and Bachovchin et al. [107]. Further crystallographic studies, in this instance of the complex between Cbz-Ala-bIle-OH **(39)** and PPE, illustrated the presence of an unusual coordinate covalent bond between the boron atom and the nitrogen of the imidazole ring of His 57 in PPE and proposed the mechanism outlined in figure 6.

Inhibitor and enzyme form a noncovalent Michaelis complex **(I)**, whereupon the nucleophilic Oγ atom of the catalytic serine attacks the boron atom of the inhibitor **(II)** [the boron atom in the trigonal planar configuration has sp_2 hybridization with empty 2p orbitals. This results in the boron being electron deficient and thus capable of acquiring a fourth ligand to become tetrahedral (with sp_3 hybridization)]. His 57 is very close to one of the oxygen atoms of the inhibitor and donates a proton to this atom to produce a water molecule. The His 57 Nε atom then proceeds to make a coordinate covalent bond with the boron atom of the inhibitor, stabilizing the tetrahedral configuration **(III)**.

It was intriguing to note that in the PPE-**(39)** complex, the –OH group of the boron atom is not situated in the oxy-

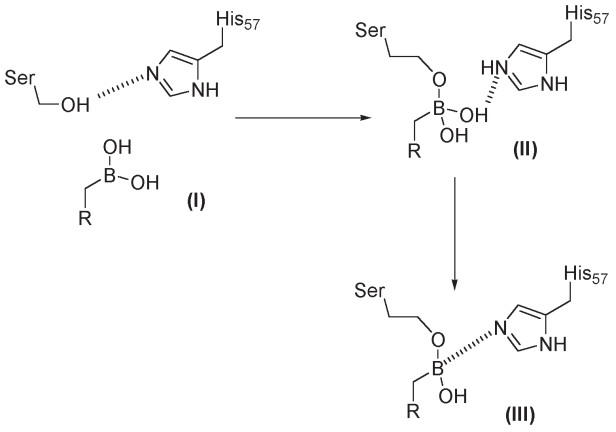


Figure 6. Postulated active site interactions of boronic acids [109].

anion binding site, as one would expect. The authors suggest that this is due to notable displacement of the boron atom caused by the steric hindrance afforded by the interaction between the bulky aliphatic side chain of Ile and the S_1 site residues. Such contacts influence coordinate bond formation between the boron atom and the Nε of His 57. This interesting observation has prompted further work to determine the action of different boronic acid-based inhibitors against a variety of serine proteases, particularly with reference to the different modes of inhibition observed in earlier studies, modulated by stereochemical and sequence constraints [108–112].

Analogous di- and tripeptidyl boronate-containing inhibitors of chymotrypsin- and elastase-like serine proteases have been shown to function as antimetastatic agents against B16BL6 melanoma in rat models, supporting a role for these enzymes in the invasion/metastasis process [113].

Kettner's group at DuPont Merck synthesized the first analogues of boroarginine, and the inclusion of this residue in the P_1 site of peptide sequences specific for thrombin resulted in highly effective, slow, tight-binding inhibitors of this enzyme [114]. These compounds **(40–42)** had between 2–40-fold greater affinity for thrombin [(K_i = 0.04 nM **(40)**, 4 nM **(41)** and < 4 nM **(42)**], compared with factor Xa [K_i = 9000 nM **(40)**, 940 nM **(41)** and 8200 nM **(42)**] and plasmin [K_i = 5100 nM **(40)**, 460 nM **(41)** and 3200 nM **(42)**] (dependent on group at the N-terminus), and significantly, prolonged activated partial thromboplastin time in vivo, thus indicating their potential as antithrombotic agents.

More recently, the same group have described the crystal structure of thrombin complexed with Ac-(D)Phe-Pro-boroArg-OH (DuP714) **(40)** [115]. This structure provides further evidence for covalent bond formation between the boron atom of the inhibitor and the Oγ atom of the Ser 195 residue. Whilst this result has been shown before with other serine proteases, this study also explored the contribution and interaction of a number of S_1 substituents. In addition to boroarginine, boronate analogues of *N*-δ-amidino ornithine, lysine, ornithine and homolysine were prepared and studied in complex with the enzyme, providing some of the most comprehensive and detailed structural information on interactions within the active site of thrombin to date.

Interestingly, Lim et al. [116] have shown that DuP714 has secondary structure in aqueous solution which is remarkably similar in the (D)Phe-Pro- portion to the conformation of (D)Phe-Pro-Arg-CH_2Cl bound to thrombin [117]. The authors attribute this to hydrophobic interactions between the phenyl ring [of (D)Phe] and the amide bond between (D)Phe-Pro-. With this in mind, a series of ring-constrained and conformationally restricted peptide boronates were prepared as thrombin inhibitors in an attempt to exploit any advantages conferred by such structural restrictions [118, 119]. These inhibitors are notable by their replacement of boroArg with boroLys. The guanidino side chain of the arginine analogue was shown to confer an order of magnitude greater potency than the amino group in the lysine analogue [115], but suitable replacement of the (D)Phe moiety by a constrained mimetic led to inhibitors such as **43** [K_i (vs. thrombin) = 0.32 nM)] and **44** (K_i = 0.07 nM), having potency almost equal to DuP714 with substantially less peptidic character.

Modulation of the N-terminal group of DuP714 and replacement of the P_2-constrained proline residue have been recently shown to improve selectivity for thrombin over complement factor I [120]. Inhibition of this enzyme by DuP714 (IC_{50} = 10 nM) may be the explanation for its well-documented toxicity problems, including hypotension induction (via amplification of alternate pathway of the complement cascade) [121, 122]. Compound **45** has similar potency against thrombin (K_i = 0.04 nM) but is substantially less active against complement factor I. More important, it did not affect liver function tests or induce hypotension after administration of an IV bolus dose in rat models.

Another approach undertaken to achieve selectivity for thrombin over other trypsin-like serine proteases has been to replace the charged P_1 residue employed in most inhibitors with a neutral group. A number of groups have described the replacement of boroArg by boro-methoxypropylglycine (boroMpg) [123, 124]. Cbz-(D)Phe-Pro-boroMpg-OH **(46)** was ~ 5–10-fold less effective than its boroArg counterpart (K_i = 22 nM); however, it had much greater selectivity for this enzyme compared with factor Xa (K_i = 12,000 nM), kallikrein (K_i = 77,000 nM), uPA (K_i = 28,000 nM) and plasmin (K_i = 2500 nM). Deadman et al. [125] have synthesized a number of analogues of Cbz-(D)Phe-Pro-bMpg-OH with different substitutions and lengths of the alkyl side chain. A number were found to be good thrombin inhibitors with good specificity for

this enzyme, compared with other serine proteases. However, compounds with the methoxypropyl side chain at P_1 were still shown to be the best inhibitors.

Where the basic side chain is replaced by a *m*-cyano-substituted phenylalanine [126], the cyano group was shown to increase binding by several orders of magnitude, for example Ac-(D)Phe-Pro-boroPhe-OH binds with a K_i of 320 nM, whilst the K_i of the analogous compound with the boro(*m*-CN)Phe-OH substituent is 0.79 nM. The crystal structure of the latter complexed with thrombin shows that the aromatic group is bound in the S_1 site, and the cyano group acts as a hydrogen bond accceptor for an amide proton from Gly 219.

Flentke et al. [127] reported the first boroPro-containing dipeptides as inhibitors of CD 26. H-Ala-bPro-OH (K_i = 15 nM) and H-Pro-bPro-OH **(47)** (K_i = 16 pM) were found to be potent inactivators of the enzyme, acting in a slow, tight-binding manner [128]. It is thought that such compounds may have potential as immunosuppressive drugs, as inhibitors have been shown to block T-cell-mediated immune responses both in vitro and in vivo [57–59]. Unfortunately, these dipeptides were shown to lose activity in a time-dependent manner in physiological buffer systems, thus indicating inherent instability. Subsequent structural studies determined the instability to be due to intramolecular cyclization of the compound through formation of a B–N bond. The group showed that the ability to inhibit dipeptidyl peptidase IV relies on an equilibrium formed between both conformations [129, 130]. The same group have performed SAR analysis with the aim of improving the biological activity of these compounds [131]. Slight improvements in potency were achieved by modulating the P_2 amino acid residues. Several substitutions are tolerated at this position, consistent with the known substrate specificity of CD 26 [132]. In addition to their activity as immunosuppressants, peptidyl prolyl boronates have also been shown to have activity against IgA1 proteases from *Neisseria gonorrhoeae* and *Haemophilius influenzae*, both important microbial pathogens [133].

Katz et al. [134] showed that tripeptide boronic acid esters retained their ability to inhibit serine proteases. The ester groups were postulated to be useful as an additional means of achieving selectivity for specific enzyme species. Enzyme (trypsin) was reacted with a peptidyl boronate pinanediol ester in the presence of an excess amount of various alcohols. Selection and incorporation of the alcohol derivative yielded mono- or disubstituted ternary boronate esters at the active site of the enzyme, with additional binding substituents (fig. 7).

This so-called epitaxial selection has been applied in attempts to prepare new inhibitors of serine proteases act-

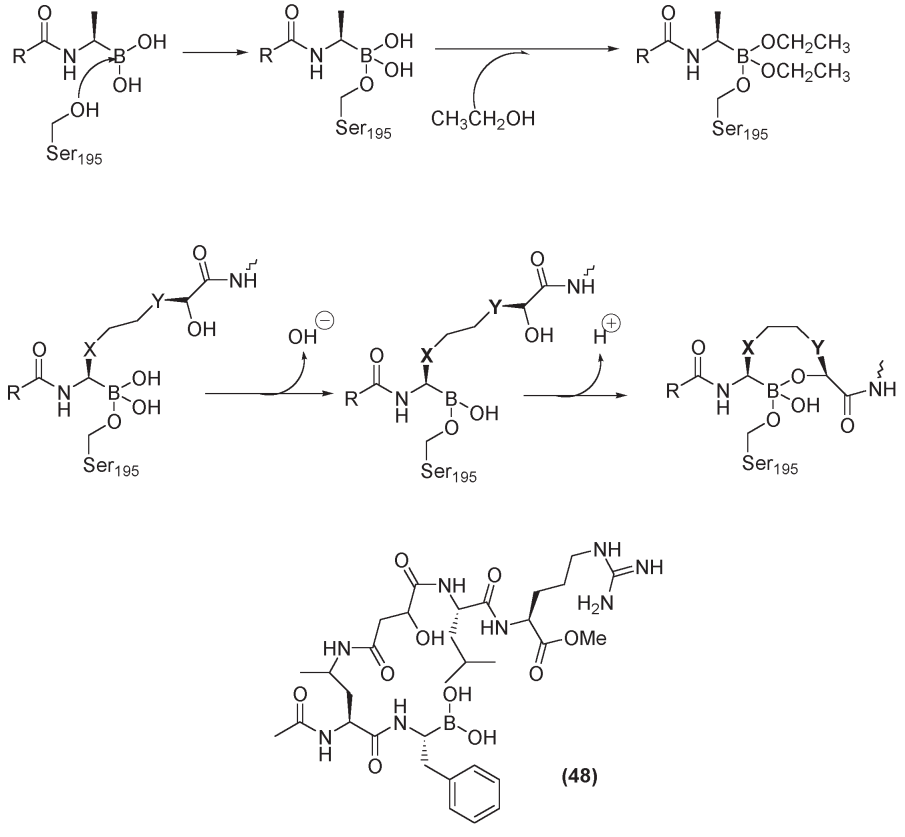


Figure 7. Epitaxial selection of enzyme-bound boronates [134].

Table 3. Selectivity of peptide-based boroArg analogues [114].

R	K_i (nM)		
	Thrombin	Factor Xa	Plasmin
(40) Ac-	41	9000	5100
(41) Boc-	4	940	460
(42) H-	< 4	8200	2300

ing through an intramolecular process [135]. The resultant complex formed by the action of such agents would be expected to mimic the first tetrahedral intermediate formed during peptide bond hydrolysis.

Compounds were prepared in which the P_1 and P_2 structures and the P_1' and P_3' residues are connected through the P_2 and P_1' side chains, thus attempting to encourage formation of a diester or amide ester adduct through macrocyclization (fig. 7).

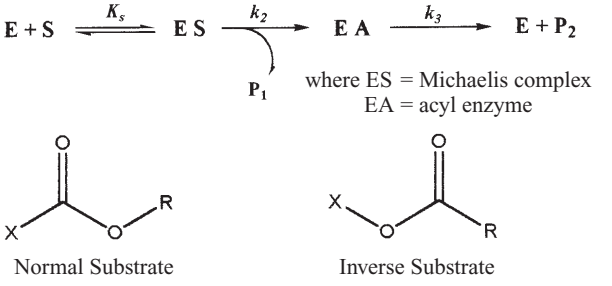
Using a boronate analogue of active site hexapeptide sequence of secretory leukocyte protease inhibitor (SLPI), which extends from the S_3–S_3' subsites **(48)**. Whilst the extended inhibitors were found to be more potent than their analogous dipeptide boronic acids, the difference was less than anticipated. The authors suggest a number of reasons for this: (i) the inhibitors simply do not cyclize as predicted, and (ii) the occupancy of the S′ subsites and formation of the diester do not enhance binding sufficiently to overcome the dynamic chain movement. As Katz and colleagues had previously shown crystallographic evidence for enzyme-boronate-alcohol ternary complexes, it was suggested that the disappointing results may be due to use of an inappropriate linker group. It will be interesting to follow the future progress of this work, as it is certainly one of the most ingenious strategies developed in recent years for inhibition of proteolytic enzymes.

Inverse substrates

In an extension of early work investigating the use of esters of *p*-amidinobenzoic acid such as **49** as potential substrates for the trypsin-like serine proteases [136, 137], Tanizawa's group prepared a series of closely analogous 'inverse substrates', in which the scissile ester bond is effectively reversed **(50)**.

After mixing of a *p*-amidinophenyl based inverse substrate with trypsin, rapid acylation of the enzyme occurs, followed by slow deacylation. This steady-state hydrolysis reaction results in accumulation of an acyl-enzyme intermediate. The unusual feature of such compounds is that the amidinium ion is both the targeting and leaving group of these compounds, and thus the exact opposite of traditional substrates. Tanizawa has shown that the acylation rate of inverse substrates is comparable to that of their analogous ester counterparts. The binding constants

Table 4. Kinetic constants for cleavage of normal and inverse substrates [138]



where X = targeting portion of the substrate (i.e. amidinophenyl or peptide alcohol)

Substrate	K_s (μM)	k_2 (s^{-1})	k_3 ($s^{-1} \times 10^2$)	k_2/K_S (s^{-1} μM)
X = CH_3	38.7	17.0	0.926	0.44
X = 4-NP	5.03	30.4	6.53	6.04
4-NP-acetate	21,000	1.5	1.3	7.1×10^5

(referred to as K_s values) were shown to be larger with the inverse substrates, and the observation that the nonspecific agent *p*-nitrophenyl acetate binds very poorly to trypsin with a slow acylation rate (k_2) demonstrated that the *p*-amidinophenyl moiety is an effective targeting group for trypsin (table 4; [138]).

Figure 8 illustrates the postulated reaction mechanism for *p*-amidinophenyl-based inverse esters. It can be seen that the major difference between the normal and inverted esters occurs after formation of the Michaelis complex. With inverse esters, the *p*-amidinophenol group departs as a leaving group, and by manipulating the acyl group, the resultant acyl-enzyme may be highly stabilized.

This early work burgeoned into a wealth of studies with a series of novel acyl groups being introduced into the active site of different trypsin-like serine proteases. For example, *p*-amidinophenyl esters carrying a stable free radical **(51)** [139], fluorophores **(52)** [140] and an optically active chromophore **(53)** [141] were prepared, with the goal of obtaining detailed information of the micromilieu of the active site of serine proteases. After introduction of these 'reporter groups', acyl-trypsins were easily isolated by gel filtration, and the resultant macromolecule subjected to the appropriate spectroscopic analysis. Perhaps the most important use of the inverse substrates has been the generation of novel pro-drugs for the dissolution of fibrin clots. Smith et al. [142] first reported a new approach to thrombosis therapy using in vitro acylated plasmin derivatives.

Plasmin is the major component in the dissolution of fibrin clots in vivo. For pharmacological and toxicological reasons, however, it is not practical to administer this enzyme parenterally to patients directly. The therapeutic index is so low that any potential advantages of its administration are nullified. The development of acyl-plas-

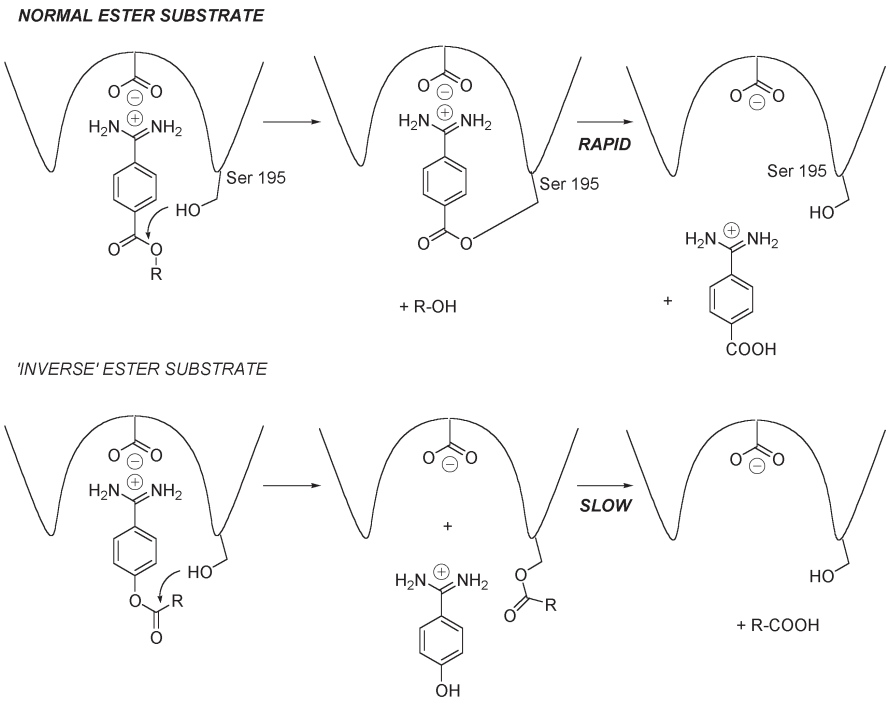


Figure 8. Comparison of the cleavage of 'normal' and 'inverse' substrates [138].

mins has largely overcome these drawbacks. In this procedure, plasmin is inactivated with *p*-amidinophenyl-*p*-methoxybenzoate **(54)**, followed by chromatographic purification of the resultant long-lived acyl-enzyme intermediate, prior to administration. The catalytic serine is thereby blocked, rendering the enzyme catalytically inert. The enzyme retains its ability to bind to fibrin clots through its kringle domains, and the subsequent deacylation step generates an active plasmin-fibrin complex that will undergo fibrinolysis slowly.

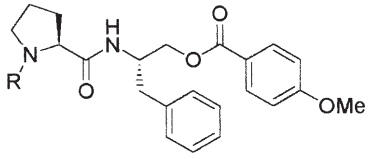
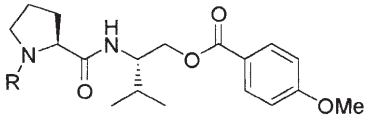
Using *p*-methoxybenzoyl-plasmin, Smith et al. demonstrated effective fibrinolysis in dogs [142] and rabbits [143]. *p*-methoxybenzoyl-plasmin had been previously shown to have a suitably long half-life (15 h) for effective therapeutic administration [144, 145].

Unfortunately, the major drawback to the use of inverse substrates as therapeutic inhibitors in vivo with these early agents is their comparative lack of selectivity for enzymes within the same serine protease subclass. The *p*-amidinophenyl esters will readily inactivate trypsin-like serine proteases such as thrombin, plasmin, kallikrein and uPA [146]. Whereas the deacylation rates for these respective acyl-protease complexes have been shown to vary considerably, *p*-amidinophenyl inverse esters will inactivate all these species with similar binding and acylation rate constants [146] (table 4). Additionally, no effective, selective inverse substrate-based inhibitors for other biologically important serine protease subclasses such as the chymotrypsin- and elastase-like enzymes were reported. With these problems in mind we decided to prepare inverse substrates of peptides with C-terminal β-amino alcohols esterified with *p*-methoxybenzoic acid. We have reported the preparation of such compounds, incorporating enzyme-specific substrate sequences with high selectivity for neutrophil elastase (Ac-Val-Pro-Val-*p*MOBA) and chymotrypsin (Ac-Val-Pro-Phe-*p*MOBA) **(55)** [147] and trypsin-like serine proteases [Boc-Ile-Glu-Gly-Arg-*p*MOBA **(56)**, Boc-(D)Phe-Pro-Arg-*p*MOBA) **(57)**] [148]. This work followed detailed kinetic mechanistic studies of the inactivation of serine proteases by these inhibitors, and we hope will be further developed into agents with biological and medicinal properties. Some of the kinetic constants for these compounds are shown in table 5. Attempts by our group to utilize other C-terminal acylating functions was summarily unsuccessful, suggesting that the *p*-methoxybenzoate function is optimal when coupled to a peptide targeting sequence (J. Lynas, unpublished data).

Other peptide-based serine protease inhibitors

Commercial necessities for novelty and patentability of compounds has led biological and medicinal chemists to develop new peptide-based inhibitors with novel functional groups. A number of groups have described agents with novel C-terminal electron-withdrawing groups, based on α-keto heterocycles. These have been developed to enhance susceptibility of the modified C-terminal carbonyl group towards nucleophilic attack by the active site serine moiety of the target serine protease. Peptide α-keto

Table 5. Inhibition of serine proteases by peptidyl inverse substrates [148].

Protease	Inhibitor	k_i/K_i ($M^{-1} \cdot min^{-1}$)
		
Chymotrypsin	R = Ac-Val-	$4.0 \pm 0.5 \times 10^8$
	R = Boc-Val-	$1.1 \pm 0.2 \times 10^8$
	R = Ac-Ala-	$6.6 \pm 0.8 \times 10^6$
		
Neutrophil Elastase	R = Ac-Val-	$8.0 \pm 4.0 \times 10^8$
	R = Boc-Val-	$8.4 \pm 3.0 \times 10^8$

Values are S.E.M. for four determinations.

benzoxazoles such as **58** and **59** were shown to be potent, competitive, reversible inhibitors of both pancreatic and neutrophil elastase [K_i (vs. HNE)] = 3.0 nM and 37 nM, respectively), in contrast to the analogous TFMKs and aldehydes, which behave as slow, tight-binding inhibitors of the target enzymes.

Crystallographic studies by Edwards et al. [149] showed the benzoxazole ring making unique contributions to the enzyme-inhibitor complex through binding interactions with both the active site serine and the imidazole group of the His 57. The carbonyl oxygen of the P_1 residue, valine, is orientated towards the oxyanion binding site, forming hydrogen bonds with the backbone nitrogens of Gly 193 and Ser 195. The nitrogen atom of the benzoxazole ring and the Ser Oγ atom H-bond with the Nε2 atom of the imidazole ring of His 57. The authors postulate that this latter set of interactions will stabilize the tetrahedral hemiketal intermediate (fig. 9).

Consistent with this hypothesis, the α-hydroxy benzoxazole **(60)** has little activity, presumably because it lacks the ketone with the ability to form covalent attachment to the active site serine. The X-ray crystal structure of the **(59)**-PPE complex confirms this, showing a direct covalent attachment between Ser 195 and the α-keto carbonyl.

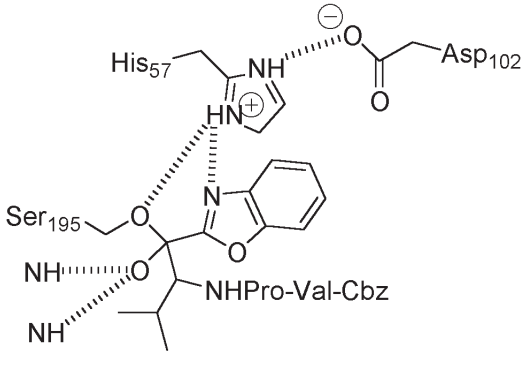


Figure 9. Postulated active site interactions of peptide α-keto benzoxazoles [149].

In subsequent studies, the same group have performed extensive SAR studies, mainly by variation of the heterocycle to modulate in vivo potency [150, 151]. A number of heterocyclic groups were evaluated, with the 2-oxazoline **(61)** having the most potent in vitro activity ($K_i = 6.0 \times 10^{-10}$ M) and is a better inhibitor than the analogous TFMKs (K_i = 1.6 nM) and aldehyde (K_i = 41 nM).

The K_i values observed for **58** (3.0 nM) and **62** (49.0 nM) illustrate that properties other than the electron-withdrawing capabilities are important factors in the potency of these α-keto heterocycles, possibly, in this case steric influences. This type of phenomena has been reported before, to account for the unexpected, relative potencies of α-keto acids, amides and esters [152]. Another important observation in this report was that the available data supported the importance of the H-bond between the heterocyclic substituents and the imidazole of His 57. A follow-up study aimed at improving the pharmacological profile of α-keto benzoxazoles illustrated that variance of the substituents on the benzoxazole ring system has a major effect on in vivo potency [151]. And indeed, the desired pharmacological profile was shown to be obtained by altering this area in conjunction with modifications of the N-terminus to provide agents, including the development compound **63** (K_i = 0.2 nM), with excellent in vivo activity, after intratracheal administration, against HNE-induced lung injury in hamsters.

A number of groups have extended these studies to develop inhibitors of other important serine proteases. Tsut-

sumi's group have described the submicromolar activity of α-keto thiazoles [153] and general α-keto heterocycles [154], based on N-protected -Pro-Pro- motifs against prolyl endopeptidase (PEP), a serine protease implicated in a number of neurological disorders. Results from this work showed that PEP displays a much greater tolerance for variation in the heterocyclic group, for example, α-keto thiazoles and benzoxazoles had similar activity (in contrast to results obtained with PPE and HNE [151]); however, it was shown that the critical requirement for the β-nitrogen in the heterocyclic ring, observed with HNE, was also necessary for PEP.

The same group has also shown that α-keto thiazoles had excellent activity against thrombin and trypsin [155]. More extensive SAR studies have shown that variation of the P_1 basic residue (Arg, Lys or homoLys) resulted in a reduction in potency for thrombin in comparison with trypsin. Selectivity for thrombin could be achieved by modification of the P_2 residue (Pro) to pipecolinic acid (Pip) (56-fold greater selectivity for thrombin) or valine (21-fold greater), although both were poorer inhibitors of thrombin, in comparison with the (D)Phe-Pro-Arg- analogue. These studies have been elaborated to explore the activity of α-keto heterocycles against the trypsin-like serine proteases. Tamura et al. [156] have described arginine analogues of α-keto heterocycles, as inhibitors of factor Xa, trypsin, plasmin and thrombin, with intriguing results in respect of comparisons of inhibitors containing either an α-keto benzoxazole **(64)** or α-keto imidazole **(65)** group (table 6). Comparative modelling of these compounds in the active site of trypsin illustrated unfavourable steric interactions in the active site which reduce the potency of the imidazole analogue. Thus, modulation of the ring substituents may be utilized to achieve discrimination between different enzymes of the same subclass.

Constanzo et al. [157] have performed crystallographic studies on α-keto benzothiazoles which showed favourable hydrophobic interactions between the heterocyclic aromatic moiety and residues in the S′ subsite of thrombin (Trp60D and Tyr60A), Trp60D stacks in a perpendicular fashion with the aromatic ring of the benzothiazole, whilst the nitrogen atom of the benzothiazole moiety is shown to H-bond to the imidazole nitrogen of His 57 (analogous to that reported by Edwards et al. [149]). The hydrophobic interactions are believed to confer selectvity for thrombin in comparison with trypsin and plasmin. The *N*-methyl (D)Phe-Pro-Arg α-keto thiazole analogue **(66)** also inhibited thrombin-induced platelet aggregation (IC_{50} = 23 nM) and exhibited excellent anti-thrombotic activity in several animal models after IV administration.

Table 6. Inhibition of trypsin-like enzymes by α-keto imidazoles and benzoxazoles [156].

Inhibitor	Thrombin	Factor Xa	Trypsin	Plasmin
	K_i (μM)			
63	0.068	0.11	0.0023	1.23
64	0.0921	> 2.5	1.01	> 2.5

Natural product inhibitors of serine proteases

Natural products have been the focus of attention for protease researchers for many years. Since the discovery of the peptide aldehydes such as leupeptin and antipain by Umezawa's group in the late sixties and early seventies (for a review, see [158]), there has been enormous interest in the development of modified and synthetic analogues of these compounds as inhibitors of serine proteases (see, for example, [159]). Whilst a number of groups have shown that judicious use of peptide-targeting sequences can achieve selectivity for individual enzymes, their nonclass specificity puts aldehydes beyond the scope of further discussion in this work. We shall, however, discuss a number of relatively recent discoveries of important natural product serine protease inhibitors and their development as potentially useful biological agents.

The most significant discovery in this field over the last number of years has been the cyclotheonamides, derived from the Japanese marine sponge *Theonella* sp. These are a series of macrocyclic pentapeptides analogues that inhibit various trypsin-like serine proteases [160–162]. To date, five cyclotheonamides, termed A–E **(67–71)**, respectively, have been discovered. All are orthologous to the same molecular template, in particular an α-keto amide bond, with variations in composition of the amino acids or the substitution of the side-chain groupings [163]. All analogues have been tested and found to have excellent inhibitory properties. The total chemical synthesis of these compounds has been achieved, and significant structural data obtained from nuclear magnetic resonance (NMR) studies [164, 165], which has enabled SAR studies to be carried out, particularly against thrombin.

Studies screening natural products from blue-green algae have also led to the discovery of a number of interesting serine protease inhibitors. Bonjoulikan et al. [166] have reported the isolation and structural elucidation of a nonspecific serine protease inhibitor, designated A90720A **(72)**, from *Microchaete lokatensis*. This compound, structurally related to a number of other blue-green algal-derived agents such as aeruginopeptins, cyanopeptolins and micropeptins, was active against thrombin (IC_{50} = 270 ng/ml), trypsin (IC_{50} = 10 ng/ml) and plasmin (IC_{50} = 30 ng/ml). The crystallographic studies of the complex between bovine trypsin and A90720A illustrated that the NMR-determined solution conformation and enzyme-bound conformation of this compound were remarkably similar.

Microcystin species, such as *M. aeruginosa* and *M. viridis* have been shown to contain a number of interesting biological compounds, not least the aeruginosins, which have been investigated as inhibitors of trypsin-like serine proteases. Matsuda et al. [167] described the aeruginosins 102-A [(L-Arg)] and 102-B [(D)Arg] **(73)** as inhibitors of trypsin, thrombin and plasmin. A number of other aeruginosins with inhibitory activity have subsequently been discovered [168, 169], and recently their crystal structures in complex with target proteases have been reported.

Aeruginosin 298-A **(74)**, which contains a C-terminal alcohol analogue, was shown to inhibit thrombin and trypsin (IC_{50}s 0.5 μM and 1.7 μM, respectively), but it has little or no activity against chymotrypsin, elastase or plasmin. The crystal structure of aeruginosin 298-A bound to thrombin-hirugen revealed a number of interesting features [170]: (i) the C-terminal β-hydroxyl group of the argininol residue hydrogen bonds with the Nε2 atom of His 57, thus disrupting the normal hydrogen bond network of the catalytic triad, and (ii) the leucine and 4-hydroxyphenyllactic acid (Hpla) residues interact with the S_3 subsite of thrombin. This site (the D-enantiomorphic subsite) is generally regarded as being hydrophobic and preferably binds D-amino acids. The L-leucine group is orientated in such a way that it is partially accepted into the site, whilst the Hpla residue, rather than interacting with the P_4 site as one would expect (due to its spatial position), interacts with an S_3 subsite residue, Gly 219, via a hydrogen bond between its aliphatic hydroxyl and the backbone amide.

It is worth noting that we and others have shown that amino and peptide alcohols are generally poor inhibitors of the serine proteases [147, 171], Steiner et al. make an important contribution that agrees with a previous observation: thrombin is generally very tolerant of imprecision in substrate/inhibitor recognition, compared with other serine proteases. This may explain the unusual mode of inhibition observed in the aeruginosin 298-A-thrombin interaction.

Aeruginosin 98-B **(75)**, also initially described by Murakami's group, which contains a C-terminal agmatine moiety, inhibits trypsin (IC_{50} = 0.9 μM), plasmin (IC_{50} = 10 μM) and thrombin (IC_{50} = 15 μM). Interestingly, this occurs without the inhibitor making any formal contact with the catalytic triad residues. Inhibition is primarily mediated through interactions with specificity elements within the substrate recognition/binding region [172]. Although the authors quite rightly state that this mode of inhibition is unique to a natural product, this interaction closely resembles that previously observed with some synthetic compounds, including (D)Phe-Pro-agmatine [173], argatroban **(6)** and napsagratran **(7)**.

This prolific research group have also described radiosumin **(76)**, isolated from *Plectonema* spp., which was found to inhibit trypsin, thrombin and plasmin, with highest potency against trypsin (IC_{50} = 0.14 μg/ml). The structure and total synthesis of this agent has recently been described, and modifications may lead to enhanced selectivity for other enzymes [174].

Serine protease inhibitors based on heterocyclic structures

Whilst some peptide-based compounds have had considerable utility as agents for the study of the role of particular serine proteases in biological systems, and a few have progressed to late-stage clinical trials, the utilization of any compounds based on peptides has a number of well-documented pharmacological drawbacks, including poor oral bioavailability, solubility, stability and toxicological effects. Dramatic advances in the development of new drug delivery and formulation strategies may eventually circumvent many of these drawbacks; however, strategically, medicinal chemists continue to pursue heterocyclic and other nonpeptide scaffolds as the basis for the design of new protease inhibitors. Some of the seminal work in this area has been carried out on the development of mechanism-based serine protease inhibitors, and the following section deals with some of the most important examples, including some recent progress on entirely novel strategies for inhibitor design.

N-hydroxysuccinimide heterocycles and related compounds

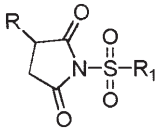
One of the most prolific groups, led by William Groutas, specializing in the design and synthesis of irreversible heterocyclic serine protease inhibitors, have reported a number of mechanism-based inactivators containing an *N*-hydroxysuccinimide moiety or closely related analogue (such as saccharins).

Early work from this author [175] was based on the design of mechanism-based inhibitors of PPE of general structure **(77)**. It was proposed that abstraction of a proton from the R–NHCO– portion of the inhibitor by the imidazole group of His 57 causes decomposition, yielding an alkyl isocyanate (R–N=C=O) which covalently modifies the active site serine. We expanded this hypothesis to prepare a series of inhibitors of trypsin-like serine proteases by replacing the alkyl R group by NH_2–$(CH_2)_6$– to fulfil primary specificity requirements at the S_1 subsite **(78)** [176].

Groutas' group have since reported that HNE and chymotrypsin are inactivated by *dl*-3-benzyl-*N*-[(methylsulphonyl)oxy]succinimide **(79)** [177]. A series of SAR studies to achieve optimal activity of these agents against HNE led to the preparation of compounds with enhanced activity and interesting mechanistic features [178]. Re-

sults obtained were in agreement with previous specificity studies for HNE, illustrating a preference for small, aliphatic side chains at the 3-position within the inhibitor ring structure, and occupying the S_1-specificity pocket. It should be noted at this point, however, that studies have shown that HNE can accommodate bulkier substituents at the P_1 position if the substrate is monomeric in nature (as opposed to an extended structure such as peptides) [179, 180]. This explains accommodation of the bulky benzyl moiety at S_1 in the early series of inhibitors. Alteration/substitution of the sulphone derivative had little effect on the potency of these compounds against HNE. The one exception was if the substituent was a *trans*-styryl moiety **(80)**. This compound displayed a second-order rate constant of over 100,000 $M^{-1}\,min^{-1}$ (table 7). It is believed that favourable interactions between the *trans* styryl group and the S′ subsites of the enzyme account for the enhanced reactivity (in the presence of a suitable P_1 residue). The authors suggest that, since previous studies by Renaud et al. [181] had shown that the S_2' subsite is capable of providing favourable, additional interactions with substrates, it is likely that this is the site of *trans*-styryl binding.

Table 7. Substituent effects on *N*-hydroxysuccinimide sulphone analogues [178].

R	R_1	$K_{obs}/[I]$ ($M^{-1}s^{-1}$)
		
Isopropyl	Me	3,817
Isobutyl	Me	49,338
Isopropyl	*trans*-styryl	100,000
Isopropyl	*trans*-styryl	100,000
Benzyl	*trans*-styryl	9,428

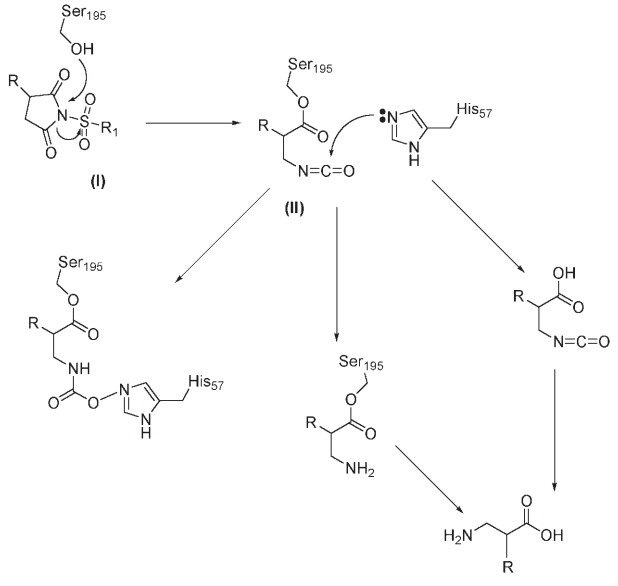


Figure 10. Mechanism of action of *N*-hydroxysuccinimide derivatives [177].

The mechanism of action of the NHS compounds was proposed to proceed through enzymatic activation of the compound (fig. 10). Serine 195 is acylated, generating an isocyanate moiety which may undergo further reaction at the active site, leading to formation of an irreversibly modified enzyme; alternatively, initial loss of activity may be followed by slow regeneration of activity.

A further series of 3-alkyl-*N*-hydroxysuccinimides were prepared with the aim of improving the pharmacological profile. This strategy led to inhibitors containing pyridinium **(81)** and phenyl carboxylate **(82)** ionic groups attached to the sulphone, substitutions known to improve solubility and bioavailability. These were found to retain their capacity to be inhibitors of HNE and cathepsin G [182]. It is interesting to note that the pyridinium moiety was included as an isostere of the amino acid desmosine, which is found abundantly in elastin, the major physiological substrate of HNE [183]. This work has been part of an ongoing programme aimed at improving the selectivity and potency of the 3-alkyl series of NHS-based compounds against neutrophil proteases through structural modification of functional groups on the core template [184–189].

One interesting aspect of this research has come as a consequence of the use of 3-(alkylthio)-*N*-hydroxysuccinimides as HNE inhibitors. Oxidation of the sulphur atom in this series does not adversely affect the biological activity of these compounds, thus illustrating one of the first examples of compounds with protease inhibitory and anti-oxidant activity (a common problem in the inactivation of the natural proteinaceous HNE inhibitor α_1-PI in various pathological states [190]). An exciting expansion of this concept has been the development of compounds with protease inhibitor and antioxidant functionality. This concept has been postulated as a potential therapeutic strategy in diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) and led to the design and synthesis of compounds based on oxidation-sensitive and -insensitive-aromatic ester inhibitors of HNE **(83)**, thiazolidines **(84)** and azabicyclo-(2,2,2)-octane and perhydroindole derivatives of TFMKs **(85)** [191–194].

SAR studies on modified analogues containing the isothiazolidin-3-one ring structure **(86)** (where X = leaving group) [194] showed that these compounds are also irreversible inactivators of the target enzymes. Hlasta et al. at Sterling-Winthrop/Eastman Kodak have also reported potent and selective inhibitors based on benzoisothiazolone templates such as **87** and **88** as HNE inhibitors [195, 196]. Activity in this series of compounds was clearly shown to be dependent on two factors, first, the R_4

substituent on the ring and, second, the nature of the leaving group on the nitrogen of the benzoisothiazolone nucleus. Replacement of the phenylmercaptotetrazole leaving group with 2,6-dichlorobenzoate enhanced binding approximately 10-fold (K_i = 0.023 nM as opposed to 0.27 nM). One such derivative, WIN 63759 **(89)** (K_i = 0.013 nM) has been shown to possess promising pharmacological properties in animal models. In further attempts to improve the pharmacological profile of these agents, a novel class of leaving groups was introduced into the benzoisothiazolones, with a common structural cyclic β-dicarbonyl structure, leading to the discovery of a promising lead compound, WIN 65936 **(90)** (K_i = 0.066 nM) as a potent and stable inhibitor of HNE [197].

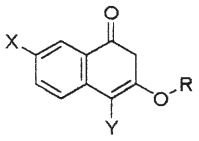
Isocoumarins

Compounds based on the isocoumarin scaffold have been developed by Powers' group at Georgia Tech. The original derivative, 3,4-dichloroisocoumarin **(91)** has been shown to act as a broad-spectrum inhibitor of a number of serine proteases, including chymotrypsin-, trypsin- and elastase-like enzymes [198]. This compound does not generally inhibit other cysteine, or indeed metallo- or aspartyl proteases, and as such has often been utilized to classify the action of serine proteases in biological systems. It should be noted, however, that this compound has been shown to be an inhibitor of the intracellular cysteine protease calpain [199], and more recently proteasome [200]. In attempts to enhance selectivity of isocoumarins for specific subclasses of serine protease, a large number of analogues have been prepared, particularly based on 3-alkoxy-4-chloro-7-substituted analogues. Generally, isocoumarins with 3-methoxy- or -ethoxy **(92)** substitution confers selectivity for elastases, 3-benzyloxy- **(93)** or 3-phenylethoxy substituents inhibit chymotrypsin-like enzymes and incorporation of a basic group **(94)** enhances activity against trypsin-like enzymes (for a review, see Powers and Kam, [201]). Table 8 illustrates the potency and selectivity profiles of a number of isocoumarins against trypsin-like serine proteases.

The proposed mechanism of action of these agents is shown in figure 11. The isocoumaryl carbon is attacked by the active site serine, forming an acyl-enyzme intermediate **(I)**. This intermediate is then believed to be converted to a highly reactive quinonimine methide intermediate **(II)**, which may undergo further reactions with the enzyme through one of two different pathways.

It has been proposed that either an additional covalent bond could be formed between enzyme and inhibitor, due to alkylation of His 57, to form an irreversible complex **(III)**. Or, alternatively, the quinonimine methide intermediate may react with a solvent molecule to give a second acyl-enzyme-like structure which subsequently deacylates [202].

Table 8. Inhibition constants for the inactivation of a number of serine proteases by substituted isocoumarins [201].



(I) X = guanidino, Y = H, R = Me

(II) X = guanidino, Y = Cl, R = Et

(III) X = guanidino, Y = Cl, R = CH_2CH_2Ph

(IV) X = H, Y = Cl, R = $CH_2CH_2SC(NH)NH_2$

(V) X = H, Y = Cl, R = $CH_2CH_2SC(NH)NH_2$

Inhibitor	Serine protease			
	Bovine thrombin	Bovine factor Xa	Porcine kallikrein	Bovine trypsin
	$K_{obs}/[I]$ ($M^{-1} \cdot s^{-1}$)			
I	290,000	3,100	45,000	310,000
II	55,000	27,000	200,000	110,000
III	30,000	96,000	200,000	110,000
IV	1,400	220	19,000	46,000
V	4,700	5,600	32,000	32,000

Several X-ray crystallographic studies of the complex of substituted isocoumarins with PPE [203–205] have provided evidence in support of both postulated pathways, whilst Chow et al. [206] have performed similar studies on bovine trypsin.

The structural and electronic properties of different substituents on the isocoumarin scaffold have been shown to influence the rate of acylation by Ser 195 as well as the reactivity of the quinonimine methide intermediate and thus the stability of the enzyme-inhibitor complex [203–205]. Substituted isocoumarins have been explored as potential anticoagulants [207–208], with limited success. For example, **94** was shown to be effective in vivo. They have also been shown to have activity against a number of the complement components [209]. However, the authors point out that such agents have inherently low stability in biological buffers and plasma and thus may have only limited applicability as potential therapeutics, perhaps only in situations were a short duration of action is required. More recently, isocoumarin derivatives appear to have found a valuable niche as molecular probes for the study of systemic proteolysis, for example the study of enzymes in CTL cells [210–212]. Taking the concept of using synthetic inhibitors, containing biological reporter groups, as probes for the selective disclosure of proteases in systems, this group have utilized biotinylated isocoumarins to detect serine proteases in activated

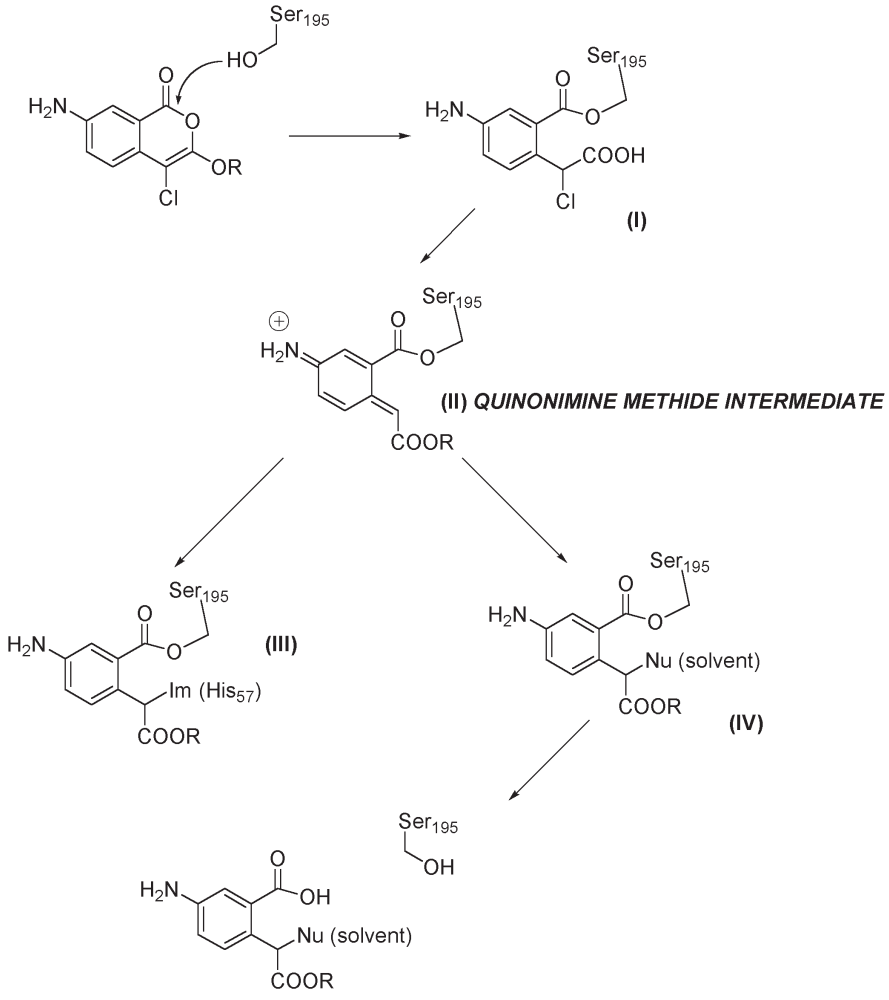


Figure 11. Proposed mechanism of action of isocoumarins [203–205].

CTLs [212–214]. Substrate turnover studies showed the presence of four distinct granzyme-like proteolytic activities in these cells. Molecular biology has shown the presence of at least 10 of these species. The use of biotinylated isocoumarins has also detected the apparent presence of 10 different species using similar technology to the type developed by our group in Belfast (see [65]). It remains to be seen if these molecular biology and proteomics results correlate with each other.

β-lactam-based inhibitors

Of all the heterocyclic serine protease inhibitors which acylate the active site serine residue, the neutral cephalosporins appear to be the most promising as potential clinical agents. *β*-Lactam antibiotics are among the most widely prescribed group of enzyme inhibitors in clinical practice. These agents act by preventing cell wall synthesis via inactivation of transpeptidases such as the penicillin binding proteins (PBPs) and related enzymes. The *β*-lactam structural moiety and the presence of a free carboxylic acid are believed to mimic the configuration of the (D)Ala-(D)Ala substrate of these enzymes [215].

One of the most prevalent problems in antibiotic therapy has been the development of bacterial strains resistant to these drugs. This is generally mediated by cleavage of the *β*-lactam ring component, by a group of serine hydrolases known as *β*-lactamases (for a review, see [216]), leading biological and medicinal chemists to strive for new inhibitors of these enzymes.

An early observation that a benzyl ester of the *β*-lactamase inhibitor clavulinic acid **(95)** was a weak inhibitor of serine proteases [217] led Doherty's group at Merck to develop cephalosporin esters as potential inhibitors of HNE [218]. Intensive investigations into the SAR of such derivatives, based on the *β*-lactam ring system, against HNE led to the conclusion that cephalosporin-based compounds with 7*α* substitution, such as **(96)**, are more potent than their 7*β*-substituted counterparts, with the enzyme preferring small 7*α* substituents, consistent with the known subsite specificity of HNE for small aliphatic side chains at P_1. *α* substitution was presumed to reflect

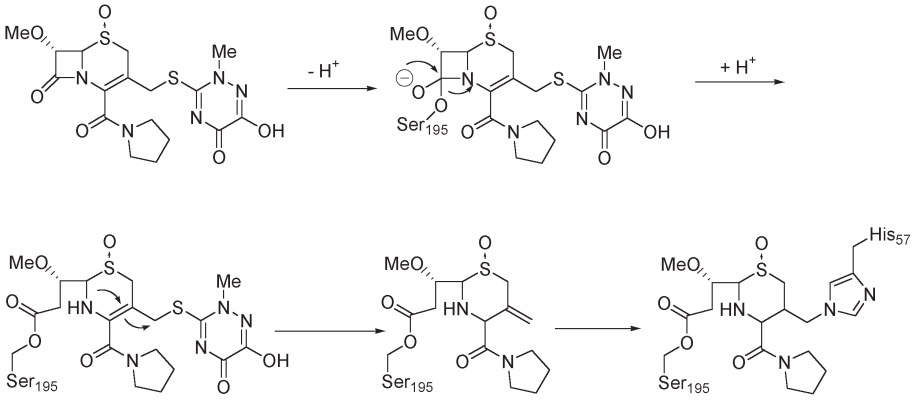


Figure 12. Proposed mechanism of inhibition of HNE by 7-methoxy-substituted cephems [222].

the optimal stereochemistry observed in all mammalian enzymes.

SAR studies showed that a sulphone, as opposed to sulphide or sulphoxide, substituent in the cephalosporin core structure is required for the most potent inhibition. As observed with other heterocyclic serine protease inhibitors, structural modification profoundly affects the mode of inhibition of these agents, leading them to behave as alternative substrates or irreversible inhibitors. 7α-chloro substitution leads to irreversible inhibition of PPE. Crystallographic analysis of the resultant enzyme-inhibitor complex shows that the 3-alkoxy-alkyl group is displaced from the cephalosporin ring system by the imidazole of the catalytic His 57 residue [219].

Numerous attempts have been made to elucidate the mechanism of the reaction of different inhibitors with HNE and PPE. Knight et al. [220] provided evidence for the formation of a noncovalent (Michaelis) complex between enzyme and inhibitor, since k_{app} values, obtained for the inhibition of HNE by two cephalosporin derivatives, L-658 758 **(97)** and L-659 286 **(98)**, were saturable. In addition they suggested that original postulates for the mechanism of inhibition did not fully explain the kinetics of inhibition by such compounds. Subsequently, the action of the cephalosporins against elastases has been the subject of a series of eloquent experiments by the group at Merck. Sophisticated chemical and kinetic studies, combined with more recently developed sensitive techniques in protein chemistry, such as electrospray mass spectroscopy and two-dimensional (2D) NMR (the former having been utilized by an independent group for similar work [221]), have been used in an attempt to clarify the inhibitory mechanism in great detail [222–226]. Such is the complexity of the mechanism of inhibition by these compounds that this is still an area of active investigation.

The initial crystallographic work by Navia et al. led to the belief that the reaction (with the 7α-chloro-substituted cephalosporin) with PPE proceeded via a 'double-hit' mechanism: initial acylation of Ser 195, after attack of the Oγ atom, on the lactam amide bond, followed by a second reaction, nucleophilic attack of His 57 imidazole on the 3-substituent of the ring, resulting in the irreversible formation of an alkyl bond. This mechanism is not, however, absolutely required for formation of a stable acyl-enzyme complex. Figures 12 and 13 show the proposed mechanisms for the inhibition of HNE by a 7α-methoxy-substituted derivative and PPE by the 7α-chloro-substituted compound, respectively.

Since the original discovery, a vast number of analogues of monocyclic and bicyclic β-lactams [222, 224, 227, 228], derivatives of cephalosporins [229–234], penems

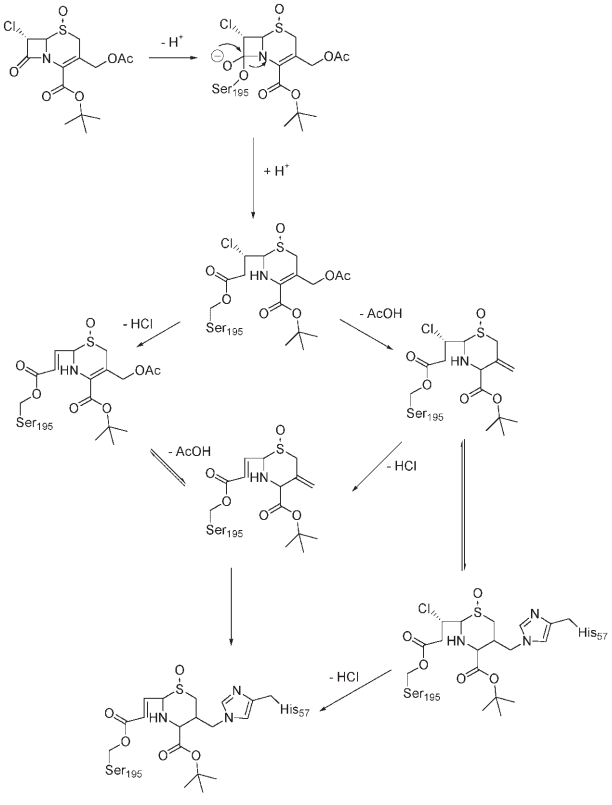


Figure 13. Potential mechanisms of HNE inhibition by 7-chloro-substituted cephems [219].

[235] and penicillins [236, 237] have been synthesized and evaluated as inhibitors of HNE.

As a result of this considerable success with HNE, a number of researchers have attempted to extend the search to other serine proteases. Monocyclic β-lactams have been reported as inhibitors of prostate-specific antigen (PSA). PSA has been shown to have a number of interesting biological activities, including the cleavage of insulin-like growth factor binding protein-3 (IGFBP-3) [238, 239]. A lead inhibitor **(99)** (K_i = 8.98 μM) was shown to form a stable acyl-enzyme with Ser 195 at the active site. Elaboration of this initial compound led to a more potent inhibitor **(100)** (K_i = 0.22 μM) [240].

More recently, monocyclic β-lactams have been reported as mechanism-based inhibitors of the serine protease from human cytomegalovirus (HCMV), acylating the active site serine in the expected manner [241]. One compound in particular, with favourable solubility and selectivity characteristics for HMCV protease over mammalian serine proteases **(101)**, was identified. Combinatorial synthetic approaches have recently been undertaken to prepare libraries of these monocyclic β-lactams as potential serine protease inhibitors [242]. Whilst this work was particularly slanted towards the discovery of new HNE inhibitors, the potential for the resultant large numbers of compounds generated in screens for serendipitous discovery systems is enormous, thus leading to β-lactams with activity against other serine proteases.

Han's group have developed monocyclic β-lactams as inhibitors of other serine proteases, describing the synthesis and biological evaluation of β-lactam analogues designed to act against the trypsin-like serine proteases, in particular, thrombin [243, 244]. Azetidin-2-one derivatives with 3-guanidinopropyl substitution **(102–104)** were evaluated against thrombin, trypsin and plasmin. Variation of the 4-position of the lactam ring, combined with different substituents on the lactam nitrogen, was examined. Substitution at the 4-position was found to be important for good inhibition, with polar substituents enhancing selectivity for thrombin. Intraperitoneal administration of **104** resulted in significant increase in clotting time ex vivo when administered to rats, thus indicating that these inhibitors may have useful activity as antithrombotic agents.

Metal-potentiated compounds

Katz et al. have recently reported an entirely new concept in the development of highly selective trypsin-like serine protease inhibitors. Based on the use of compounds with the ability to form a ternary complex with enzyme and a 'bridging' Zn(II) atom, the mediation of Zn(II) in the enzyme-inhibitor interaction enhances potency by anything from 1000–100,000-fold [245]. Structural studies confirm spatial coordination between a Zn(II) atom and the Ser195 Oγ atom and His 57 imidazole Nε2 atoms in the catalytic triad [246]. Originally based on the nonspecific trypsin-like serine protease inhibitor, bis(5-amidino-2-benzimidazolyl)methane [BABIM; **105**], additional functionalities incorporated into this molecule facilitate binding to the specificity sites of individual enzymes, thus enhancing selectivity **(106–108)**. For example, BABIM **(105)** K_i(tryptase) is 2.5 μM; however, in the presence of Zn^{2+}, the K_i value is some 500-fold lower (50 nM). More recently, kinetic studies aimed at elucidating the mechanism of inhibition of tryptase by metal-potentiated inhibitors illustrate a slow, tight-binding mechanism with extremely slow dissociation rates, with half-lives in the order of hours [247].

Conclusions

It is clear from the wealth of knowledge gained over the years that the serine proteases play a critical role in the development and maintenance of many chronic debilitating illnesses as a result, significant effort has been expended towards develop new compounds with the ability to modify these enzymes in biological systems. The key aim of researchers has and must continue to be the development of agents with the ability to inhibit serine proteases selectively. The advent of genomics and the growth of emerging technologies, such as proteomics and combinatorial chemistry, is slowly providing drug development scientists with the means to identify potential toxicity and selectivity problems early in the discovery process and will surely steer serine (and other) protease inhibitor development towards new and exciting end points.

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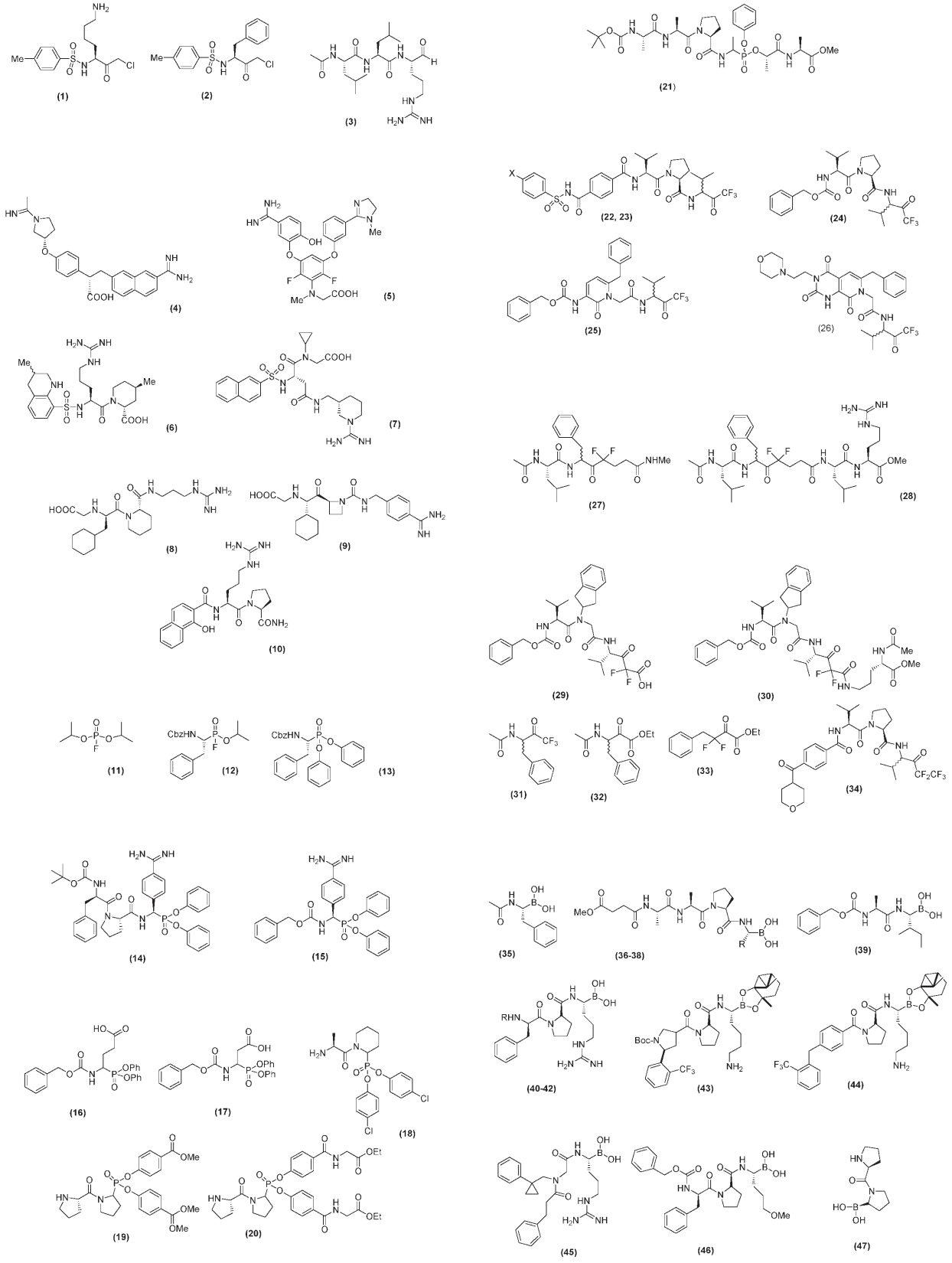
Scheme 1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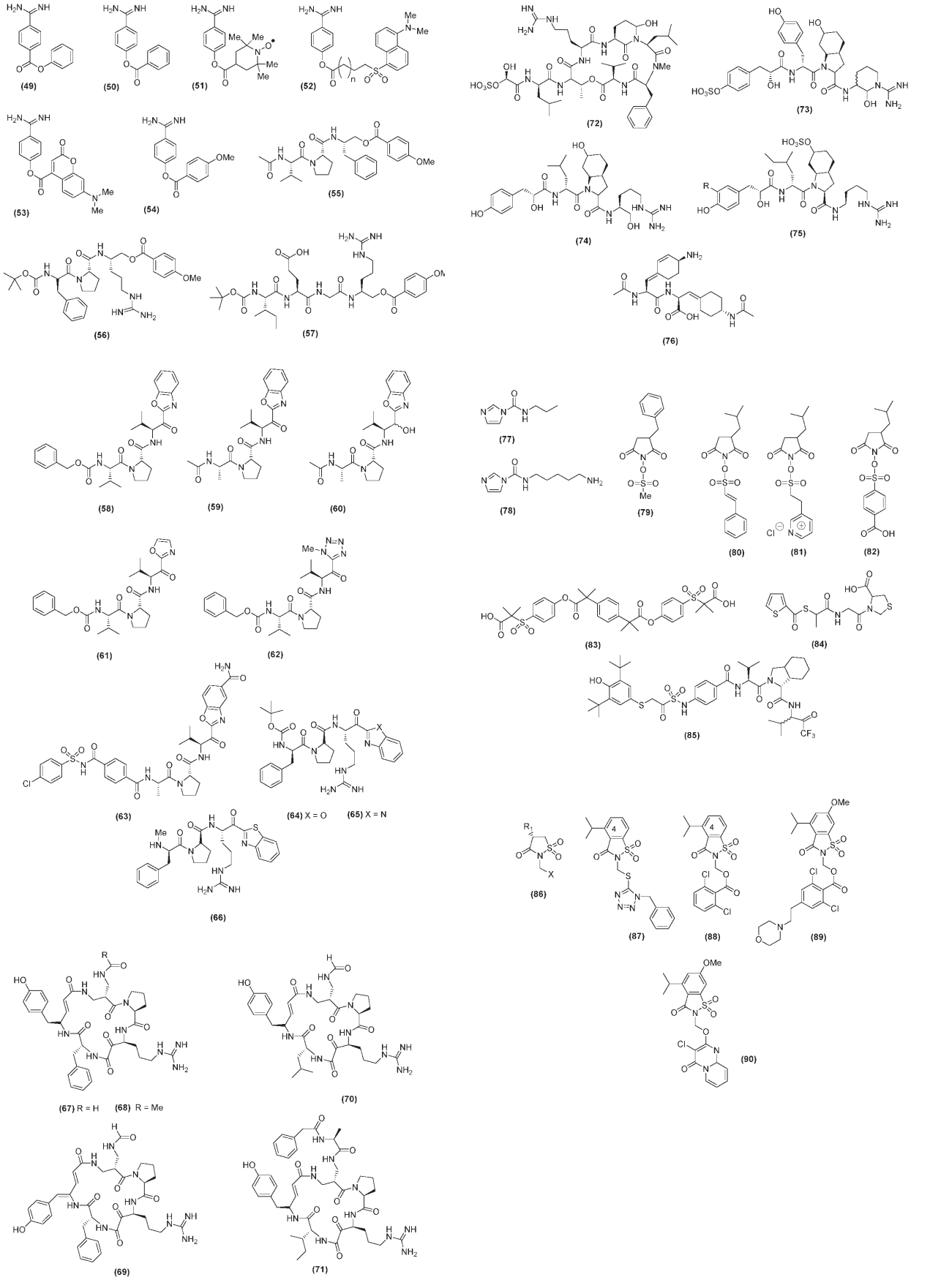
Scheme 1 (continued)



Scheme 1 (continued)