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Review

Inhibitors of proteases and amide hydrolases that employ an α-ketoheterocycle as a key enabling functionality

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Abstract—This article reviews the scientific literature on the application of α-ketoheterocycles to the discovery of potent enzyme inhibitors. The α-ketoheterocycle functionality provides a moderately electrophilic ketone carbonyl with 'tunable' reactivity, as well as a structural template for introducing new interactions in the enzyme active-site cleft. This type of moiety has served an important role in the design of active-site-directed inhibitors of diverse serine and cysteine proteases, and of fatty acid amide hydrolase (FAAH). Potent inhibitors have been identified for, inter alia, elastase, thrombin, factor Xa, tryptase, chymase, cathepsin K, cathepsin S, and FAAH. For example, **6e** is an orally active inhibitor of human neutrophil elastase that entered human clinical studies, **52h** is an orally bioavailable inhibitor of human chymase, and **82m** is a FAAH inhibitor with in vivo endocannabinoid-enhancing activity.

Contents

Keywords: Protease; Hydrolase; Peptidase; Inhibitor; Structure-based drug design; Ketoheterocycle; Transition-state analogue.
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1. Introduction

Proteases (or proteinases), one of the largest classes of enzymes in living organisms, are responsible for catalyzing the hydrolytic cleavage of amide bonds in proteins and peptides.[1] Because of their broad distribution and important biochemical functions, proteases play crucial roles in normal physiology, and in pathophysiology. Thus, inhibitors of various proteases have eminent therapeutic relevance.[1] Indeed, several protease inhibitors have entered advanced clinical studies, and some have reached the market.[1]

There are four main categories of proteases, according to their mechanism of action and the key catalytic moiety in the enzyme active site: serine (or threonine[2]), cysteine, aspartic, and metallo.[1] To accomplish amide-bond hydrolysis ('proteolysis'), a peptide substrate is bound within the enzyme active-site cleft, usually as an extended β-strand.[1c] The substrate is further recognized on the basis of its specific sequence characteristics, particularly the pattern of amino acid side chains. Commonly, these recognition domains are represented by the terminology of Schechter and Berger, which designates the amino acid residues that surround the scissile bond (/) of the substrate peptide or protein as $H_2N\ldots\sim$P3–P2–P1/P1′–P2′–P3′$\sim\ldots CO_2H$, and the corresponding enzyme subsites as ∼S3–S2–S1/S1′–S2′–S3′∼.[3] For the serine protease family, there is a subclassification according to the amino acid residue (P1) that is favored within the S1 subsite (known as the 'specificity pocket'): trypsin-like for a basic residue (Arg, Lys), chymotrypsin-like for an aromatic residue (Phe, Tyr, Trp), elastase-like for an aliphatic residue (Val, Leu, Ile), and caspase-like for an acidic residue (Asp). When considering the design of active-site-directed protease inhibitors, it is prudent to take such inherent enzyme properties into account.

A foremost approach for identifying novel enzyme inhibitor ligands is protein structure-based design. From this perspective, a protein structure is essential, whether from X-ray diffraction, NMR spectroscopy, or homology modeling. The structural features of the active-site cleft are analyzed to determine possible domains and groups for developing key interactions. This task can benefit from the use of computer-assisted drug design methods. Thus, one can pinpoint promising functional groups and surfaces within the enzyme active site. For example, there may be β-sheet hydrogen bonding or hydrophobic interactions to exploit for molecular recognition. Then, one needs to decide on the type of inhibitor ligand to use.[1b] Would it be desirable to have a ligand that is (1) freely reversible, (2) irreversibly reactive by covalent bond formation (e.g., a chloromethylketone group), or (3) somewhat reactive, but reversible (e.g., a trifluoromethylketone group)? Relative to drug discovery, the therapeutic utility of irreversible inhibitors has been drawn into question because of their chemical reactivity and, thus, potential for toxicity. However, there is a subtype of irreversible inhibitor, the mechanism-based or 'suicide-substrate' inhibitor, which becomes reactive only when it is activated by the targeted enzyme itself.[4] For a drug candidate, an inhibitor that is somewhat reactive and reversible may be more applicable since it will not form a stable covalent bond with the target enzyme, or with off-target enzymes. Rather, the reactive moiety in this instance will form a reversible covalent bond that usually will involve a residue of the catalytic machinery. Such inhibitors often contain a *moderately reactive* electrophile, such as an aldehyde, an activated ketone carbonyl, or a nitrile.[1a,5,6] Some examples of effective ketone groups are: α-fluoroalkyl ketones, α-keto amides, and α-ketoheterocycles.

The primary purpose of this review is to survey the scientific literature concerning applications of the α-ketoheterocycle functionality to the design of active-site-directed enzyme inhibitors. In particular, we will be addressing observations in the areas of serine proteases, cysteine proteases, and amide hydrolases. This functionality, in a suitable format, has proven to be advantageous for two main reasons. It has a moderately electrophilic ketone carbonyl that is 'tunable' to form readily reversible covalent bonds with active-site groups and it offers a molecular template for attaching substituents to introduce new binding interactions.

2. Serine protease inhibitors

Serine proteases represent a vast family of enzymes that employ an active-site serine to hydrolyze a scissile amide bond.[1a] The active site is usually characterized by a Ser-His-Asp catalytic triad (Ser-195, His-57, Asp-102).[1a,7] Mechanistically, hydrolysis of a peptide amide bond involves addition of the hydroxyl of Ser-195 to the carbon atom of the amide carbonyl to form a tetrahedral intermediate, which is an oxido-orthoamide.[1a,7] The 'oxyanion' occupies a specific site in the enzyme known as the 'oxyanion hole', which is rigid and well-conserved across the serine protease class.[1a,7] In this recognition pocket, the interactions with a substrate involve multiple hydrogen-bond-donor groups on the enzyme. In addition, there are often multiple hydrogen-bonding interactions between the amide backbone of the substrate and the amide backbone of the protease. Relative to drug discovery, the most extensively studied serine protease targets have probably been α-thrombin (factor IIa), human neutrophil elastase, and factor Xa.[7,8] α-Ketoheterocycle-based inhibitors have made a significant contribution to this field.

2.1. Elastase inhibitors

The proteolytic enzyme elastase (EC 3.4.21.37), which degrades connective tissue and extracellular matrix, is involved in inflammatory disorders, especially with respect to the lungs.[9] Human neutrophil elastase (HNE) is released from neutrophils and controlled by endogenous proteinase inhibitors, such as α_1-proteinase inhibitor, under physiological conditions. However, when there is an imbalance between HNE and its endogenous inhibitors in certain pathological conditions, the administration of synthetic inhibitors (i.e., drugs) could be therapeutically useful.[9] HNE has the standard serine

protease catalytic triad and favors a substrate with a branched aliphatic amino acid side chain in the S1 specificity pocket.

Several elastase inhibitors have advanced to Phase 2 clinical trials,[9c–f] but development of them has been abandoned.[9d,e] It is unfortunate that two orally active agents, peptidyl trifluoromethylketone ZD-8321[10] and peptidyl α-ketoheterocycle ONO-6818, have been terminated. Sivelestat (ONO-5046), a parenteral drug from Ono Pharmaceutical, is approved for marketing in Japan,[9d] but its development in the USA (in partnership with Lilly) was ended in 2003.[11]

For the purpose of inhibiting HNE, Edwards et al. pioneered the application of α-ketoheterocycles in drug design.[9a,12] A key premise of this structural approach is that certain types of heterocycles would be sufficiently electron-withdrawing to activate a ketone carbonyl for nucleophilic addition of the Oγ of Ser-195 in the HNE active site. The electronic properties of the heterocycle could be manipulated to regulate the degree of carbonyl electrophilicity and suitable substitution might gain other useful interactions within the active-site cleft. Secondarily, a suitably positioned nitrogen atom in the heterocycle could hydrogen bond with Nε of the proximate His-57 to reinforce the 'mechanism-based' inhibition. Such tunability could be helpful for modulating physicochemical, ADMET (absorption, distribution, metabolism, excretion, and toxicology), and biological properties to improve the druggability of inhibitor molecules. These principles have been demonstrated in the early studies with HNE, and also with other serine proteases, as we will discuss later.

A seminal paper by Edwards et al. reported the first examples of peptide-based α-ketoheterocycles as serine protease inhibitors.[12a] They found that tripeptide α-ketobenzoxazoles Cbz-Val-Pro-Val-(2-benzoxazole) (**1**) and Ac-Ala-Pro-Val-(2-benzoxazole) (**2**) are potent inhibitors of HNE, with K_i values of 3 and 73 nM, respectively. A series of analogues of **1** with alterations of the α-ketobenzoxazole was quite informative.[12a] Whereas the trifluoromethylketone (K_i = 1.6 nM) and α-ketooxazoline (K_i = 0.6 nM) cognates had nearly the same potency as **1**, the methylketone was much less potent (K_i = 8000 nM) and the C-terminal aldehyde was somewhat less potent (K_i = 41 nM). The alcohol that directly corresponds to ketone **1** had sharply attenuated HNE inhibition (K_i = 21,000 nM), consistent with the importance of the electrophilic ketone. A 1.9-Å X-ray crystal structure of **2** complexed with porcine pancreatic elastase (PPE) depicted hemiketal formation with Ser-195 and suggested a hydrogen-bonding interaction between the benzoxazole nitrogen and the Nε of His-57 (Fig. 1).[12a] These results support the fundamental concepts of the structure-based drug design approach that was used.

Follow-up reports by Edwards and coworkers offered details on the medicinal chemistry for this class of HNE inhibitors.[12b,c] General exploration with the heterocycle unit proved interesting, as the degree of HNE

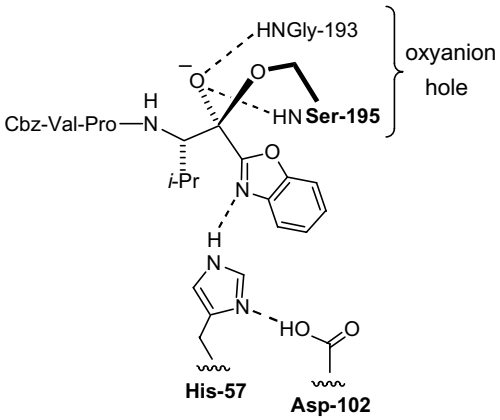


Figure 1. Diagram representing the interactions of **1** in the catalytic region of the active site of PPE (or HNE).

inhibition was considerably sensitive to structure (Table 1). Benzoxazole **1** was ~1000 times more potent than benzofuran **3a**, reflecting the importance of an sp^2 donor nitrogen. The oxazole set (**1** and **3d**) was better that the corresponding thiazole set (**3b** and **3c**) by a factor of 8–10. The imidazole groups (**3f–h**) and the π-deficient pyridine group (**3i**) provided very weak inhibitors. The degree of enzyme inhibition here did not correlate well with the electron-withdrawing properties of the different heterocycles.[12b,13] The more potent derivatives (K_i < 1000 nM) were deemed to form a hemiketal with the γO of Ser-195 and a hydrogen bond with the Nε of His-57.

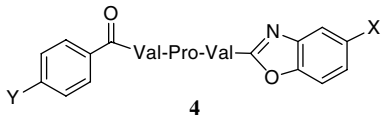
In a series of Cbz-Val-Pro-Val compounds containing substituted benzoxazoles, the level of HNE inhibition resided in a narrow range (K_i = 0.4–4 nM) despite a wide variation in the substituents (e.g., OMe, Cl, CN, CO_2Me, CO_2H, $CONH_2$, and CH_2OH). Since these substituents spanned from electron-donating (OMe) to electron-withdrawing (e.g., CN), modulation of the electronic properties of the heterocycle was apparently not a crucial factor in this case. Two of the most potent HNE inhibitors possessed a carbomethoxy group (K_i = 0.4–0.5 nM).[12c] More extensive studies were pursued with ArC(O)-Val-Pro-Val-(2-benzoxazole) analogues (e.g., **4**; Table 2).[12c] Analogues with X = carbomethoxy exhibited optimal potency, and the introduction of a sizable arylsulfonamido-

Table 1. In vitro inhibition of HNE by tripeptide α-ketoheterocycles Cbz-Val-Pro-Val-Het (**1**; **3**)[a]

Compound	Het	K_i (nM)
1	2-Benzoxazole	3
3a	2-Benzofuran	3400
3b	2-Benzothiazole	25
3c	2-Thiazole	270
3d	2-Oxazole	28
3e	2-Oxazoline	0.6
3f	2-(1-Me-imidazole)	80,000
3g	2-(1-Me-benzimidazole)	12,000
3h	2-Benzimidazole	5600
3i	2-Pyridine	22,000

[a] Data were taken from Ref. 12b.

Table 2. In vitro inhibition of HNE by tripeptide α-keto-2-benzoxazoles (**4**)[a]



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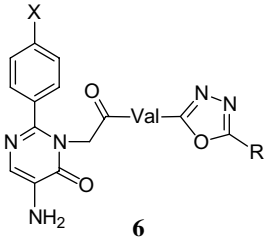
Compound	X	Y	K_i (nM)
4a	H	CO_2Me	2.3
4b	H	CO_2-*t*-Bu	0.34
4c	H	CO_2H	11
4d	H	SO_2NH_2	1.3
4e	CO_2Me	SO_2NH_2	0.35
4f	H	b	0.2
4g	CO_2Me	b	0.06
4h	H	c	0.33
4i	CO_2Me	c	0.1
4j	OMe	c	0.66
4k	OH	c	0.81
4l	$CONH_2$	c	0.2

[a] Data were taken from Ref. 12c.
[b] 4-Chlorophenylsulfonylaminocarbonylamino.
[c] 4-Chlorophenylsulfonylaminocarbonyl.

Table 3. Biological data for pyrimidinone α-keto-1,3,4-oxadiazoles (**6**)[a]



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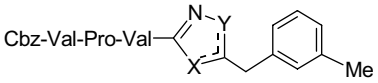
Compound	R	X	K_i (nM)	ED_{50}[b] (mg/kg)
6a[c]	CH_2(3-Me-C_6H_4)	F	0.64	13
6b[c]	CMe_2(3-Me-C_6H_4)	F	1.4	—
6c[c]	CMe_2Ph	F	0.52	10
6d[c]	*t*-Bu	F	6.4	6.5
6e[c]	*t*-Bu	H	3.6	6.7
6f[d]	*t*-Bu	H	12	5.1

[a] Data were taken from Ref. 15.
[b] Dosed orally to hamsters.
[c] (*S*) enantiomer.
[d] This compound (ONO-6818) is the racemate corresponding to **6e**.

carbonyl substituent at the N-terminus resulted in consistent subnanomolar potency, especially as represented by **4g** (K_i = 0.06 nM) and **4i** (K_i = 0.1 nM). In a hamster acute lung injury model, certain compounds (administered intratracheally by aerosol), such as **4l**, were effective protectants. The type of substitution on the benzoxazole ring had a substantial effect on the observed in vivo activity.[14]

Ohmoto et al.[15] and Wieczorek et al.[16] described tripeptidyl oxadiazoles, such as **5**, along with several related compounds. 1,3,4-Oxadiazole **5a** exhibited very potent HNE inhibitory activity (K_i = 0.025 nM),[15,16] in conjunction with slow-tight binding kinetics.[16] The corresponding 1,2,4-oxadiazole, **5b**, was about 20 times less potent (K_i = 0.49 nM) than **5a**, but it remained in the subnanomolar range.[16] In fact, many derivatives in this series were potent HNE inhibitors (K_i < 5 nM).[16] Compound **5a** was greater than 1000-fold selective over chymotrypsin, cathepsin G, and trypsin.[16]



5 **a**: X = O, Y = N
b: X = N, Y = O

Although **5a** did not possess the desired oral activity in a hamster acute lung injury model (30 mg/kg),[15] better compounds were obtained by chemically modifying the tripeptide portion of **5**, especially by introducing the previously exploited[17] 5-amino-2-phenylpyrimidin-6-one subunit.[15,16] Consequently, Ohmoto et al. generated a series of highly potent, orally active, nonpeptide inhibitors (**6**; Table 3),[15] from which **6f**, the racemate corresponding to **6e**, was selected for in vivo studies. In an X-ray study, a co-crystal derived from **6f** and PPE (1.9 Å) was found to contain (*S*)-enantiomer **6e**, as would be expected, and the **6e**·PPE complex displayed many of the predicted intermolecular interactions (Fig. 2).[15,18] Additional pyrimidinone analogues were presented in a full paper.[19]

Use of the racemate, **6f**, emanated from the fact that stereochemical equilibration (e.g., of **6e**) occurred on incubation in whole blood from different species. For example, the racemization half-life for the *R* and *S* enantiomers of **6f** in hamster whole blood at 37 °C was just ca. 10 min.[19] (More detailed information on this stereomutation issue is contained in the following subsection.) In the hamster lung injury model, oral administration of a suspension of **6f** (ONO-6818) in 0.5% aqueous carboxymethyl cellulose gave an ED_{50} of 1.4 mg/kg. The pharmacokinetic parameters for **6f** in fasted rats (3 mg/kg, po) were impressive, with $t_{1/2}$ = 4.5 h,

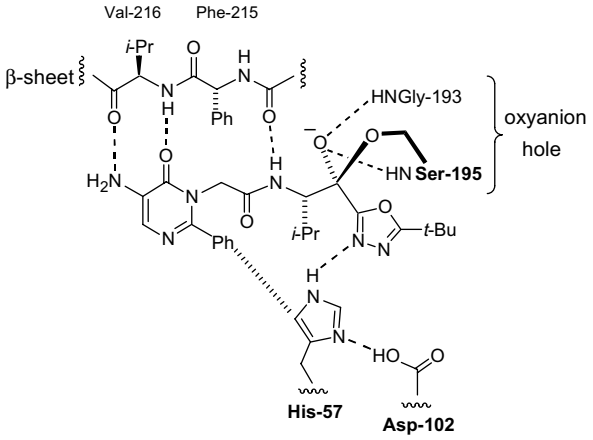
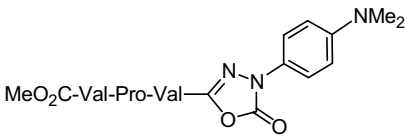


Figure 2. Diagram representing the interactions of **6e** in the active site of PPE. Intermolecular hydrogen bonding (dashed lines) with β-sheet residues Phe-215 and Val-216, and an aromatic π-stacking interaction (hashed line) with His-57, are shown.

C_{max} = 257 ng/mL, t_{max} = 0.6 h, and F = 50%.[18] On the basis of the results with **6f**, this compound (as a racemate) was advanced into human clinical study.[20]

Further work on tripeptidic analogues bearing a 1,3,4-oxadiazolin-2-one group led to other orally active HNE inhibitors, such as **7** (K_i = 6.4 nM; ED_{50} = 7.5 mg/kg).[21] Although the intrinsic potency against HNE was weaker in this chemical series, relative to **5**, for example, it is remarkable that reasonably good oral activity could be obtained with such derivatives, especially given their tripeptide nature and high molecular weight (>500 Da).



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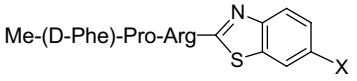
Additional α-ketoheterocycle-based inhibitors of HNE are the subject of patents or patent applications assigned to Cortech, Inc.,[22] Ono Pharmaceutical Co., Ltd,[23] and Kyowa Hakko Kogyo KK.[24]

2.2. Inhibitors of thrombin and other coagulation proteases

Inhibitors of serine proteases in the blood coagulation cascade, such as α-thrombin (EC 3.4.21.5), factor VIIa (EC 3.4.21.21), factor Xa (EC 3.4.21.6), and factor XIa (EC 3.4.21.27), have potential utility as anticoagulant and antithrombotic agents. α-*Thrombin* is a central enzyme in this cascade, and thus plays a key role in regulating thrombosis and hemostasis.[25] This trypsin-class enzyme cleaves fibrinogen to generate fibrin, which polymerizes into a hemostatic plug. It also activates thrombin receptors (PAR-1; PAR-4) on human platelets and other cell types.[26] The action of thrombin on platelets induces their aggregation into thrombi. For nearly two decades, researchers have been avidly pursuing thrombin inhibitors as drug candidates.[27]

Numerous direct thrombin inhibitors have been taken into the clinic and some parenteral drugs reached the market.[27–29] Although the oral direct thrombin inhibitor ximelagatran, a double prodrug of melagatran, advanced to late-stage clinical development and was approved for therapeutic use in certain global markets,[28,30] the drug was withdrawn from medical practice shortly after its introduction because of an adverse risk-benefit profile relative to liver toxicity.[31] Currently, there are no orally administered thrombin inhibitors on the market, although a few compounds are in advanced stages of clinical development.[28,32]

The first α-ketoheterocycle-based thrombin inhibitors were reported by our research group in 1996.[33] Initially, the D-Phe-Pro-Arg recognition motif for thrombin's S3–S2–S1 domain[34] was utilized with an intent to extend interactions into the S1′ area by appending suitable C-terminal heterocyclic units.[33] This objective arose from our observation that cyclotheonamide A, a macrocyclic pentapeptide that inhibits thrombin, capitalized on S1′ binding interactions.[35] Investigation of a series of compounds of general structure Me-(D-Phe)-Pro-Arg-Het (**8**; **9**) yielded potent thrombin inhibitors (Table 4).[33] Among the heterocycle (Het) groups that delivered high potency (K_i < 10 nM) were 2-benzothiazole (**8a**; RWJ-50353), 2-thiazole (**8b**), 2-benzoxazole (**8c**), and 2-(1-methylbenzimidazole) (**8e**). The relative inhibition of thrombin versus trypsin varied, although **8a** and **8e** showed moderate selectivity (15- and 35-fold, respectively). 2-Benzothiazole **8a**, with a subnanomolar K_i value of 0.2 nM, was clearly superior to the other derivatives. The importance of the ring sulfur atom was demonstrated by the loss in potency for 2-benzoxazole **8c** (30-fold) and the importance of the benzene ring was revealed by the loss in potency for 2-thiazole **8b** (10-fold) and 2-(4,5,6,7-tetrahydrobenzothiazole) **8g** (15-fold). The ring nitrogen atom played a critical role as demonstrated by the dramatic loss in potency for 2-benzothiophene **8d** (12,000-fold). The imidazole (**8f**) and pyridine (**8h**) groups gave weak inhibitors, although their attenuation was not as severe as in the case of analogous inhibitors of HLE (vide supra).[12b] Evidently, the 2-benzothiazole group was optimal, and substitution of it with small electron-donating groups, as in **9a** (K_i = 0.14 nM) and **9b** (K_i = 0.15 nM), had little effect on potency. On the other hand, there was a minor reduction of potency with electron-withdrawing groups, as in **9c** (K_i = 1.3 nM) and **9d** (K_i = 2.0 nM; RWJ-51438).[33b] The reference C-terminal aldehyde Me-(D-Phe)-Pro-Arg-CHO (efegatran) was ~90-fold less potent (K_i = 18 nM) than **8a**. The C-terminal alcohols (mixture of two diastereomers) corresponding to **8a** had greatly reduced thrombin inhibition (K_i values of 5300 and 1600 nM; 26,000- and 8000-fold weaker than **8a**), consistent with the electrophilic ketone concept.[33b]



8a: X = H **9c**: X = CO_2Me
9a: X = OMe **9d**: X = CO_2H
9b: X = F

Table 4. Inhibition of human α-thrombin (thr) and bovine trypsin (try) by tripeptide α-ketoheterocycles Me-(D-Phe)-Pro-Arg-Het (**8**)[a]

Compound	Het	thr K_i (nM)	try K_i (nM)
8a	2-Benzothiazole	0.2	3.1
8b	2-Thiazole	2.1	1.2
8c	2-Benzoxazole	6.2	13
8d	2-Benzothiophene	2400	1300
8e	2-(1-Me-Benzimidazole)	8.1	290
8f	2-(1-Me-Imidazole)	200	5800
8g	2-(H_4-Benzothiazole)[b]	3.4	4.4
8h	2-Pyridine	85	73
8i	2-(4-CO_2Et-Thiazole)	4.5	5.4

[a] Data were taken from Ref. 33b.
[b] 4,5,6,7-Tetrahydro derivative.

X-ray crystal structures of the ternary complexes **8a**–thrombin–hirugen (2.1 Å)[33a,36] and **9d**–thrombin–hirugen (1.7 Å)[33b,37] displayed hemiketal formation with the γO of Ser-195 and interactions in the S1′ region of the enzyme, encompassing a hydrogen bond between the benzothiazole ring nitrogen and the Nε of His-57 and an aromatic π interaction with Trp-60D of thrombin's 60A–60I insertion loop (Figs. 3 and 4). Interestingly, the Lys-60F side chain was displaced by the sizable benzothiazole ring of **8a** into an anomalous *gauche*-conformation. In contradistinction, the side chain of Lys-60F formed a salt-bridge with the benzothiazole-carboxylate of **9d**, and adopted a normal extended, *anti*-conformation (Fig. 5). Since **8a** has a 10-fold greater affinity for thrombin than **9d**, there is no benefit from an increase in binding energy through this salt-bridge, perhaps because of structural perturbations across the enzyme active site with **9d** (Fig. 6). The increased affinity and better selectivity of such α-keto-benzothiazole inhibitors may be attributable in part to the aromatic stacking interaction with Trp-60D, which resembles the S1′ interaction observed in the X-ray crystal structure of the cyclotheonamide A–thrombin–hirugen complex.[35] Additionally, energy-contour calculations with the computer program GRID indicated favorable affinity between the sulfur atom of the benzothiazole and a hydrophobic patch on the surface of thrombin.[33b]

In the previous subsection, we mentioned that the *R* and *S* enantiomers of **6f** underwent rapid racemization in hamster whole blood at 37 °C,[17] due to lability of the proton on the stereogenic center adjacent to the activated ketone. This property of peptide-based α-ketoheterocycles appears to be more general, such that stereomutation may take place during synthetic operations, biochemical assays, or in vivo studies.[33b] Indeed, the Arg α-ketoheterocycle subunit was sensitive to such stereomutation, especially under alkaline conditions (even mildly so).[33b] The epimerization of α-keto carbethoxy thiazole **8i** (L-Arg/D-Arg = 98:2) was assessed in various buffers at pH 7.4, 7.85, and 8.4. Whereas the L-Arg content of **8i** decreased just slightly at pH 7.85 and 7.4 after 35 min at 37 °C (from 98% to ~90%), it decreased to 73% at pH 8.4. However, **8i** did not evince epimerization in distilled water (pH 6.5) after standing for 3 h at 23 °C, although it changed to a 73:27 L-Arg/D-Arg mixture after 16 days. With benzothiazole **9d** (L-Arg/D-Arg = 98:2) in a pH 7.4 buffer at 37 °C, the L-Arg content decreased to 70% after 0.5 h, 60% after 1 h, and 50% (equilibrium mixture) after 6.5 h. Exposure of **8a** to rabbit or rat plasma at 37 °C (pH 7.4), which is representative of physiological conditions in vivo, resulted in epimerization to a 53:47 mixture of L-Arg/D-Arg diastereomers after just 60 min.

Selectivity profiles were determined for **8a** and **9d**.[33b] Although their selectivity versus trypsin was unremarkable, **8a** and **9d** were very selective versus other serine proteases, such as factor Xa (2400- and 600-fold), plasmin (12,000- and 390-fold), tissue plasminogen activator and urokinase plasminogen activator (u-PA) (23,000- and 8500-fold). Compounds **8a** and **9d** exhibited potent

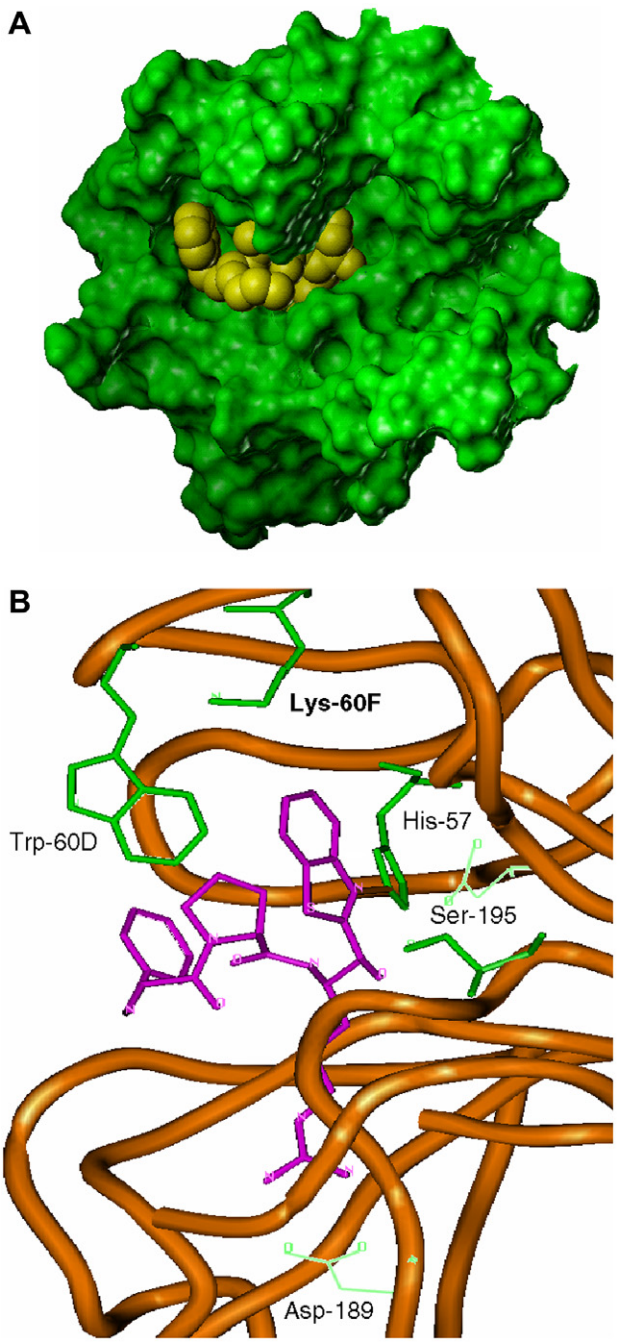


Figure 3. X-ray crystal structure of **8a**·human α-thrombin (2.1 Å). (A) View of the complex with thrombin shown as a Connolly electron-density surface (green) and **8a** shown as a space-filled model (yellow). The S3–S1 domain is occupied by D-Phe-Pro-Arg and the S1′ region is occupied by the benzothiazole group. (B) View of the active-site environment with thrombin shown as a ribbon diagram (orange) with certain side chains (green) installed and **8a** shown as a stick model of its nonhydrogen atoms (magenta). The Ser-195 Oγ is suitably disposed to form a hemiketal structure with **8a**; the benzothiazole nitrogen resides within favorable hydrogen-bonding distance relative to the His-57 Nε; the benzothiazole participates in an aromatic stacking interaction with Trp-60D; the side chain of Lys-60F is folded into a gauche conformation.

in vitro antithrombotic activity as measured by inhibition of gel-filtered human platelet aggregation induced by α-thrombin (IC_{50} = 30–40 nM).[33b,38] Since these ad-

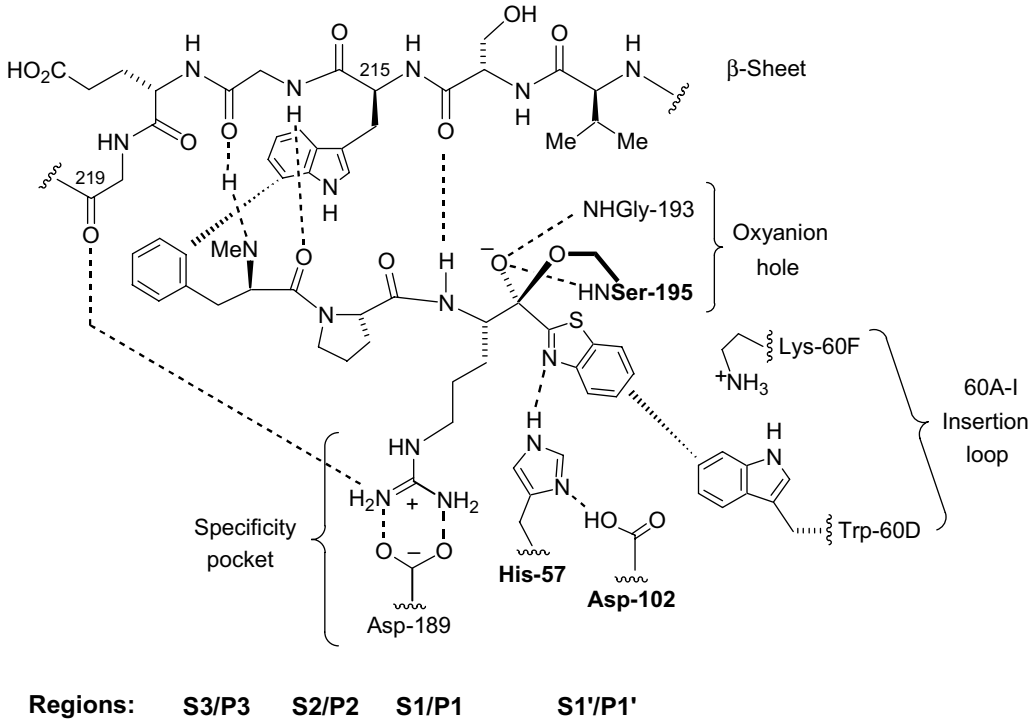


Figure 4. Diagram representing the interactions of **8a** in the active site of α-thrombin (intermolecular hydrogen bonding, dashed lines; hydrophobic interactions, hashed lines).

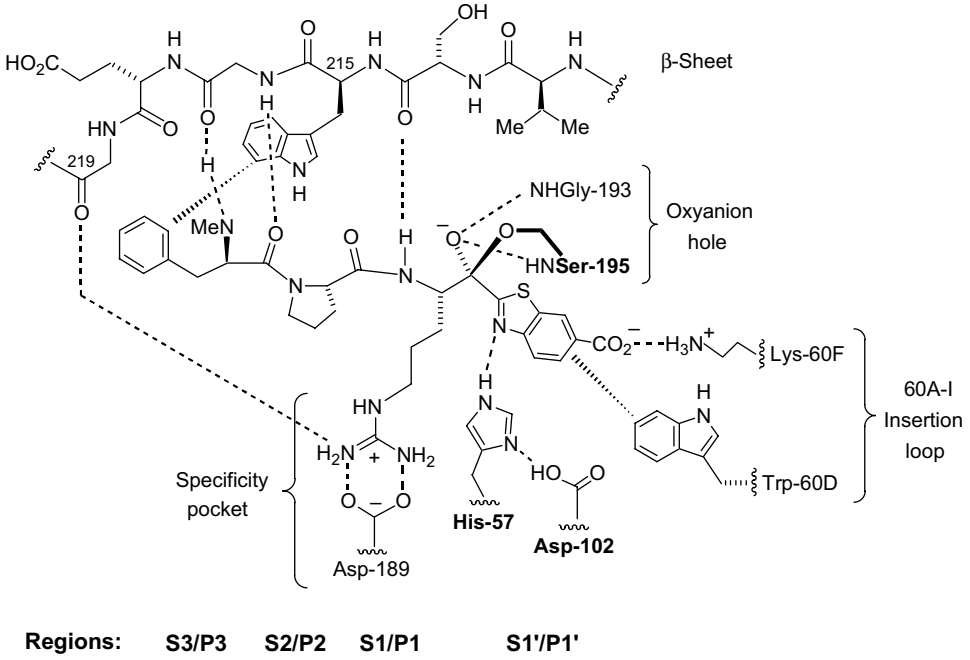


Figure 5. Diagram representing the interactions of **9d** in the active site of α-thrombin (intermolecular hydrogen bonding, dashed lines; hydrophobic interactions, hashed lines).

vanced leads did not possess useful oral bioavailability, they were investigated in vivo by parenteral administration, and thus were found to be potent anticoagulant/antithrombotic agents. In the canine arteriovenous shunt (e.g., Fig. 7) and rabbit deep vein thrombosis models, on intravenous administration, ED_{50} values in the range of 0.1–0.5 mg/kg were realized.[33b,38] Whereas **8a** caused hypotension and ECG side effects in guinea pigs, this problem was largely resolved with benzothiazole carboxylate **9d**.

Costanzo et al. explored numerous analogues of **8a** that were structurally modified on the 'tripeptide' backbone or side chains.[33b] Attempted truncation of the backbone

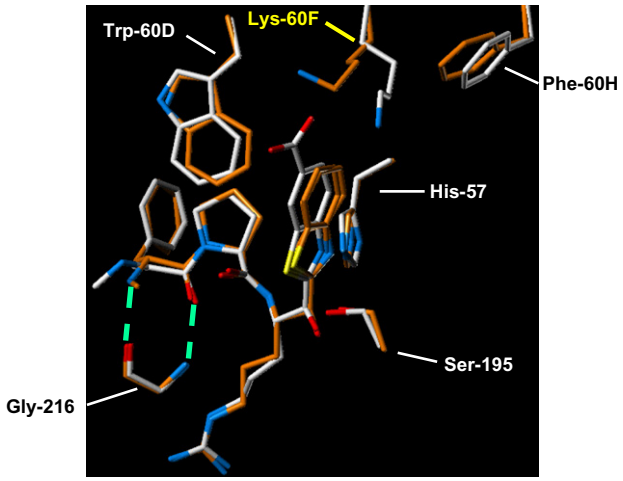


Figure 6. Comparison of the thrombin complexes involving **8a** (orange) and **9d** (white).

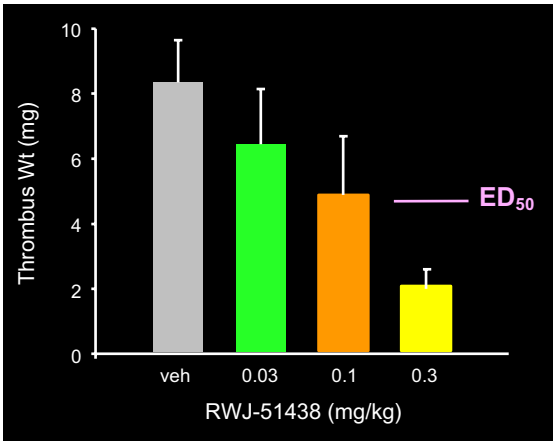
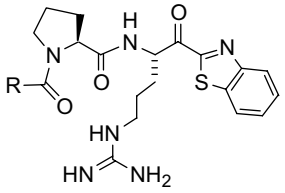
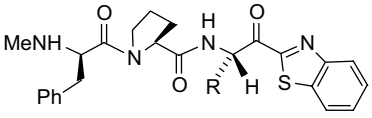


Figure 7. Dose–response for **9d** in inhibiting thrombus accumulation on a thrombogenic fiber in the canine arteriovenous shunt model with intravenous administration.

by eliminating the D-Phe, as with **10**, dramatically diminished potency (IC_{50} = 14,000 nM). By contrast, D-diphenylalanine analogue **11** exhibited remarkable potency and slow-tight binding behavior, with a K_i value of 0.00065 nM. Thus, the analogue with two phenyl groups and no terminal NHMe group, **12**, manifested substantial potency (K_i = 1.1 nM). The opposite, D-Arg diastereomer of **8a** was at least 100-fold less potent (K_i = 17 nM), and replacement of the Arg side chain in **8a** with a less basic 4-aminobutyl group resulted in a 200-fold loss in potency (K_i = 38 nM). Some of the more interesting compounds in this study, from a potency standpoint, were **13**–**17**, in which there are P1 or P3 alterations. An important point surfaced in that an effective thrombin-recognition motif from one type of active series will not necessarily translate into an α-ketoheterocycle series. For example, although **18** is the 2-benzothiazole analogue of super-potent boronic acid thrombin inhibitor **19**,[39] it only possessed rather weak potency (K_i = 400 nM).[33b]



10 R = Me
11 R = D-$Ph_2CHCH(NHMe)$-
12 R = Ph_2CHCH_2



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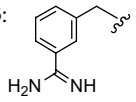
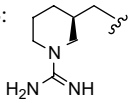
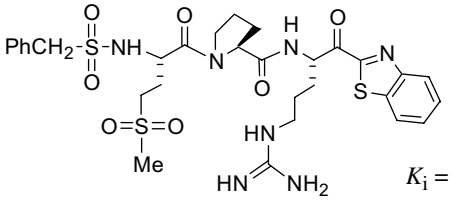


K_i = 1.0 nM

14:



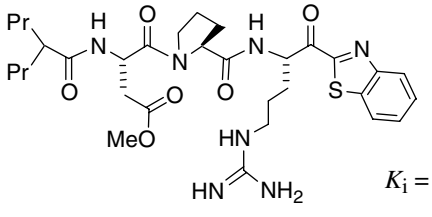
K_i = 1.6 nM



16

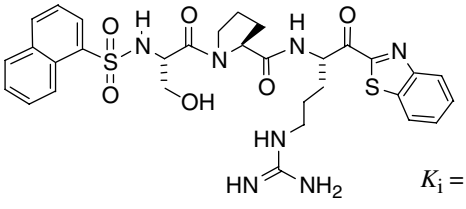
K_i = 1.5 nM

Akiyama et al.[40] reported on related tripeptide α-ketothiazole thrombin inhibitors, and Tamura et al.[41] reported on some related α-ketobenzoxazole and α-ketobenzimidazole derivatives.



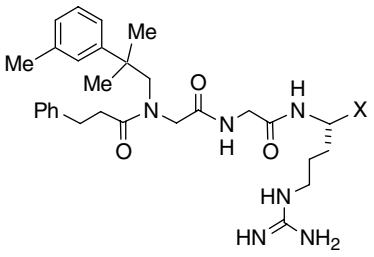
15

K_i = 1.4 nM



17

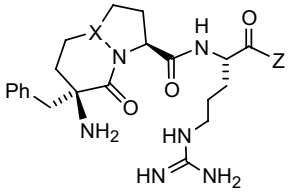
K_i = 0.8 nM



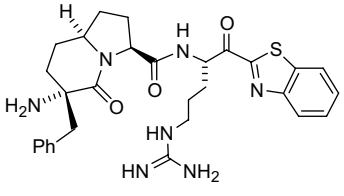
18 X = C(O)-2-benzothiazole
19 X = $B(OH)_2$

Thrombin inhibitors that have a novel P3–P1′ β-strand mimetic, in the context of a bicyclic template for the D-Phe-Pro segment, were described by Boatman et al.[42] Considering the diazabicyclo[3.2.0]nonane class, 2-thiazole **20** had a K_i value of 2.4 nM and 2-benzothiazole **21** had a K_i value of 0.65 nM. In the azabicyclo[3.2.0]nonane class, 2-benzothiazole **22** had a K_i value of 0.85 nM, similar to that for **21**; however, the diastereomer of **22** with the amino and benzyl groups exchanged (**23**) had a less potent K_i value of 10 nM. There was reasonable parallelism to the potency of related α-keto amide derivatives, such as **24** (K_i = 0.071 nM). The oral bioavailability of **21** (MOL144) was ~25% in rats and nonhuman primates, whereas the oral bioavailability of **22** (MOL174) was only ~2%.[42] Compounds **21** and **22** were very effective in blocking platelet deposition in a baboon arteriovenous shunt model on intravenous administration.[42]

X-ray crystal structures of **20** and **23** complexed with thrombin were determined (~2.1 Å).[43] As mentioned above for **8a**, a hemiketal formed with Ser-195 Oγ and interactions occurred in the S1′ region of thrombin that entailed a hydrogen bond between the heterocycle ring nitrogen and His-57 Nε and an stacking interaction between the heterocycle and Trp-60D.



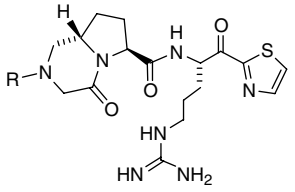
20 X = N; Z = 2-thiazole
21 X = N; Z = 2-benzothiazole
22 X = α-CH; Z = 2-benzothiazole
24 X = N; X = $C(O)NH(CH_2)_2Ph$



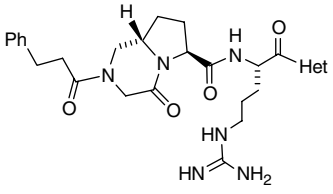
23

Related bicyclic thrombin inhibitors **25** were described by St-Denis et al., in a collection of 27 derivatives with R usually being a hydrophobic acyl substituent.[44] A subset of 3-arylpropanoyl compounds, such as **25a** (K_i = 0.6 nM), exhibited subnanomolar K_i values (0.1–0.9 nM). Compounds **25b** and **25c** were also potent inhibitors (K_i = 5.0 and 1.0 nM, respectively). An X-ray co-crystal structure of **25a**· thrombin showed the phenylpropanoyl group occupying the S3 pocket, a hemiketal formed between Ser-195 Oγ, and the expected interactions within the S1′ region. Plummer et al. examined some analogues of **25a** in which the heterocycle was varied (**26**).[45] The IC_{50} values changed from 5 nM for 2-thiazole **25a** to <1 nM for **26a**, to 51 nM for **26b**, to 2400 nM for **26c**, and to 340 nM for **26d**. Clearly, the 2-benzothiazole group provided the most potent thrombin inhibitor, consistent with structure–activity results mentioned above relative to the Me-(D-Phe)-Pro-Arg motif.[33] Further research efforts identified **27** as a potent thrombin inhibitor (IC_{50} < 1 nM) with high selectivity over trypsin (IC_{50} = 590 nM).[45] Other modifications of the P1 position led to the 1-amidinyl-4-piperidine side chain, as in potent thrombin inhibitor **28** (IC_{50} = 1.0 nM), which also possessed excellent selectivity over trypsin (IC_{50} = 11,900 nM).[46]

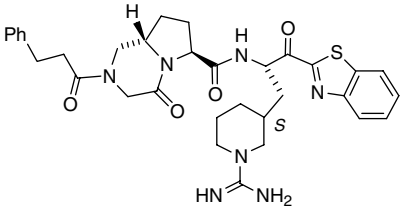
A detailed X-ray crystallographic study of **25a** and **27** complexed with thrombin and trypsin was performed to gain an appreciation for the structural factors that are responsible for the much better selectivity of **27**.[47] Narasimhan et al.[47] proposed that the remarkable thrombin selectivity imparted by the Arg-mimetic unit in **27** was caused by differential interaction of this ligand's side chain with the enzyme residue at position 192, Gln-192 in thrombin and Glu-192 in trypsin.



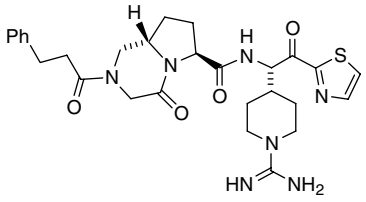
25 **a**: R = $Ph(CH_2)_2C(O)$
b: R = $PhO(CH_2)_2C(O)$
c: R = D-Trp



26 **a**: Het = 2-benzothiazole
b: Het = 1-Me(2-benzimidazole)
c: Het = 1-Me(2-imidazole)
d: Het = 2-pyridine



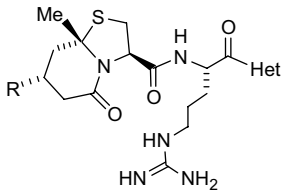
27



28

Continued evolution of the bicyclic piperazinone class resulted in potent, selective thrombin inhibitors, **29**, with an amino-containing side chain in the S1 pocket (Table 5).[48] The benzothiazole derivative (**29a**; $K_i = 0.7$ nM) was more potent than the thiazole derivative (**29b**; $K_i = 3.2$ nM) as usual; the 5-(1-methyltetrazole) derivative (**29d**; $K_i = 5.0$ nM) was nearly equipotent with **29b**. Since the arginine compound that corresponds to **29b** had a comparable K_i value of 0.2 nM, the *N*-sulfonyl bicyclic lactam motif is furnishing very effective molecular recognition. Also, it is noteworthy that the conformationally constrained 4-aminocyclohexyl group can work quite well as a P1 probe in this series (**29**), even though it is less basic. In the aforementioned Me-D-Phe-Pro-Arg series,[33b] use of this side chain in place of the Arg side chain caused a 500-fold decrease in potency, again indicating that potency trends are not necessarily reflected across different series (also, viz. **18** and **19**). Despite the presence of the amino side chain, the compounds in this bicyclic lactam class did not possess useful oral bioavailability. In vivo antithrombotic activity was verified for several potent thrombin inhibitors on intravenous administration in a rat arterial thrombosis model.[48] Compound **29b** inhibited the aggregation of washed human platelets induced by α-thrombin with an IC_{50} of 23 nM, and prolonged the clotting time of human plasma to twice the value of a control (vehicle) in coagulation assays at a concentration of 3 μM.[49]

Thia-bicyclic lactams **30** were also explored.[50] In this case, the 2-benzothiazole group was not much better than the 2-thiazole group. For example, **30a** and **30b** had K_i values of 18 and 16 nM, respectively, and **30c** and **30d** had K_i values of 18 and 10 nM, respectively.

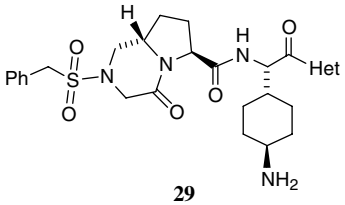


30 **a**: R = CH_2Ph; Het = 2-benzothiazole
b: R = CH_2Ph; Het = 2-thiazole
c: R = 2-CF_3-(quinolin-6-yl)CH_2; Het = 2-benzothiazole
d: R = 2-CF_3-(quinolin-6-yl)CH_2; Het = 2-thiazole

The 1-amidinyl-3-piperidine side chain, which yielded favorable results with **27**, was applied to thrombin inhibitors with a piperazine-1,4-dione template, as in **31** (Table 6).[51] Derivatives with R = benzyl, **31a** and **31d**, showed reasonable potency, and optimization of the β-benzyl series by addition of chloro substituents led to potent inhibitor **31f**, with a K_i value of 1.2 nM and selectivity over trypsin of ~500-fold.[52] Compound **31f** was not orally bioavailable in dogs, but was effective in vivo on intravenous administration in a rat arterial thrombosis model.

An X-ray structure of **31d**·thrombin revealed that the ligand adopts an anomalous binding mode (Fig. 8).[51b] Although the guanidine was installed in the S1 pocket, the Ser-195 Oγ did not interact with the carbonyl of the α-ketothiazole. Rather, the ketothiazole group pointed toward the solvent with its carbonyl involved in a hydrogen bond with Gly-219 Nα. The benzyl group occupied the S3 pocket and the phenylpropyl group extended through S2 into the S1′ region, apparently with its phenyl making a hydrophobic interaction with His-

Table 5. Inhibition of α-thrombin (thr) and trypsin (try) by bicyclic piperazinone α-ketoheterocycles (**29**)[a]

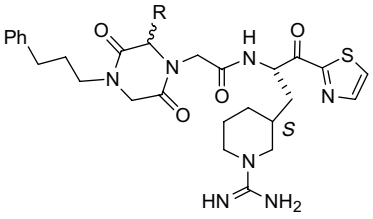


29

Compound	Het	thr K_i (nM)	try K_i (nM)
29a	2-Benzothiazole	0.72	1400
29b	2-Thiazole	3.2	8000
29c	2-(4-Me-Thiazole-5-CO_2H)	80	65,000
29d	5-(1-Me-Tetrazole)	5	35,000
29e	5-(1-CO_2Me-Tetrazole)	2600	>400,000

[a] Data were taken from Ref. 48. Human enzymes were used.

Table 6. Inhibition of α-thrombin (thr) and trypsin (try) by piperazinedione α-ketothiazoles (**31**)[a]



31

Compound	R	thr K_i (nM)	try IC_{50} (nM)
31a	α-Benzyl	24	80% at 100 μM
31b	α-Methyl	65	24,000
31c	H	500	91,000
31d	β-Benzyl	55	9100
31e	β-Methyl	880[b]	340
31f	β-(2-Cl-$C_6H_4CH_2$)	1.2	9700

[a] Data were taken from Ref. 51. Human enzymes were used.
[b] This is an IC_{50} value.

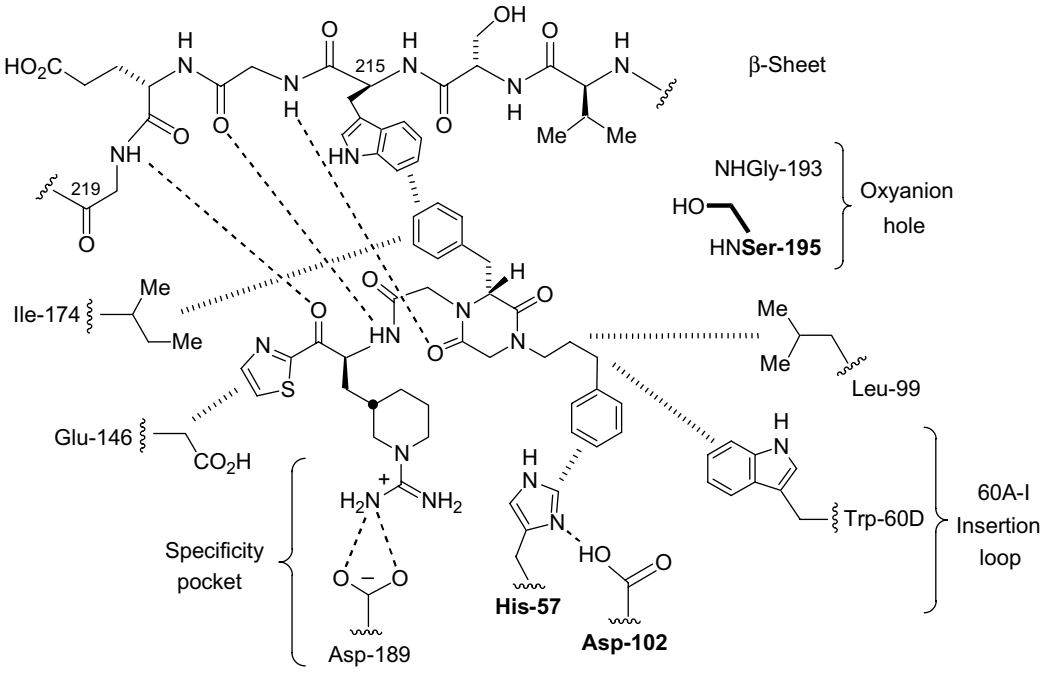
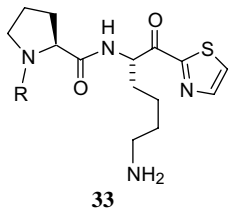


Figure 8. Diagram representing the intermolecular interactions in the X-ray structure of **31d**·human α-thrombin (intermolecular hydrogen bonding, dashed lines; hydrophobic interactions, hashed lines).

57. The NH and one of the piperazine carbonyls of **31d** formed hydrogen bonds with the β-sheet Gly-216 C=O and Gly-216 Nα, respectively. Changing the S3-resident phenylpropyl group of **31d** to related moieties had a limited effect on thrombin inhibition: for example, phenylpropyl, IC_{50} = 170 nM; Ph, IC_{50} = 1200 nM; and 4-Cl-phenylpropyl, IC_{50} = 110 nM.[51b] Interestingly, the X-ray structure of thrombin complexed with the arginine analogue of **31d**, a weaker inhibitor (IC_{50} = 3400 nM), revealed a conventional α-ketoheterocycle binding mode, with the Arg side chain in S1, the ketothiazole group in S1′, and hemiketal formation with Ser-195.[51b] However, the benzyl group was directed toward the solvent, as opposed to being positioned in S2, and the piperazinedione did not have its carbonyl hydrogen bonded to Gly-216 Nα.

Adang et al.[53] examined some piperidin-4-yl α-keto thiazoles and found that **32** is a moderately potent thrombin inhibitor (IC_{50} = 120 nM). Despite this modest result, Adang et al.[54] went on to study compounds with a 4-aminobutyl side chain as an Arg replacement (**33**; Table 7), in pursuit of orally bioavailable inhibitors. Although placement of the 4-aminobutyl group in the P1 position diminished potency for ligands with D-Phe in the P3 position,[33b,54] this adverse effect could be counteracted by using a D-Cha residue in P3 (**33c**; K_i = 3.5 nM). Moreover, the oxazole analogue of **33d** had a subnanomolar K_i value (Table 7), although its oral bioavailability in rats was <5%. By contrast, the oral bioavailability of **33c** was a fairly workable 23%. This effort led to thrombin inhibitor **34** (K_i = 1.1 nM), which proved to be orally bioavailable in rats (32%) and dogs (71%), and efficacious in a rat arterial flow model. In general, this chemical series did not exhibit meaningful selectivity over trypsin.

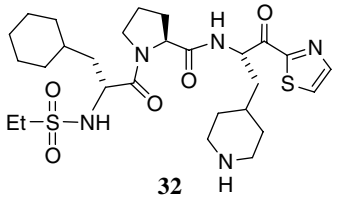
Table 7. Inhibition of human α-thrombin by 4-aminobutyl α-keto-thiazoles (**33**)[a]



33

Compound	R[b]	K_i (nM)
33a	D-Phe	150
33b	$Ph_2CHCH_2C(O)$	200
33c	D-Cha	3.5
33d[c]	HO_2CCH_2-(D-Cha)	2.6
33e	$EtSO_2$-(D-Cha)	2.0

[a] Data were taken from Ref. 54.
[b] Cha stands for cyclohexylalanine.
[c] The 2-oxazole analogue of **33d** had a potent K_i value of 0.42 nM.



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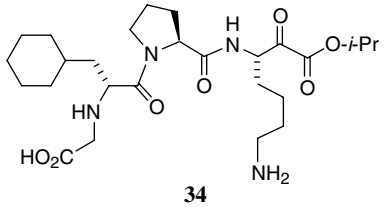


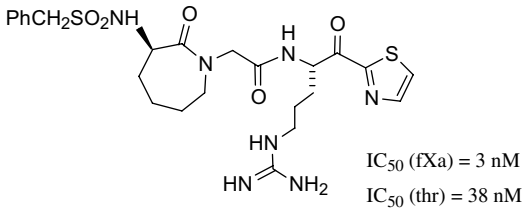
34

Another trypsin-like serine protease in the blood coagulation cascade is *factor Xa* (fXa), a pivotal enzyme located at the confluence of the intrinsic and extrinsic coagulation pathways.[55,56] A supramolecular prothombinase complex is formed between fXa, factor Va, prothrombin, surface-bound phospholipids, and Ca^{2+} en route to the generation of α-thrombin from prothrombin.[56] Inhibitors of fXa can be very effective as anticoagulant and antithrombotic agents.[57] Several small-molecule fXa inhibitors, such as rivaroxaban (BAY 59-7939) and apixaban (BMS-562427), are currently in late-stage clinical trials.[57c]

In 2001, Zhu et al. reported on a series of fXa inhibitors with a (D-Arg)-Gly-Arg-Het sequence (**35**; Table 8).[58] The design concept involved an Arg at P1 to occupy the S1 pocket (Asp-189), an α-ketoheterocycle to interact with Ser-195 and His-57, and a D-Arg at P3 to bind within the S4 'cation hole' (Glu-97).[59] Prototype **35a** inhibited fXa with an IC_{50} value of 8 nM, with good selectivity over several other serine proteases; also, it was effective in blocking thrombosis in a rabbit stasis model.[58] Analogues **35b–f** are additional examples of potent fXa inhibitors. *N*-Benzylsulfonyl analogue **35e**, an exceedingly potent fXa inhibitor with a K_i value of 0.014 nM, displayed a slow-tight binding mechanism.[60] Besides its potent inhibition of fXa (IC_{50} = 0.5 nM), **35e** inhibited factor XIIa (IC_{50} = 50 nM) and plasma kallikrein (IC_{50} = 14 nM).[61] This compound was effective in rabbit venous and arteriovenous-shunt thrombosis models with intravenous infusion.[61]

Ketothiazole **35b** showed better enzyme selectivity than ketobenzothiazole **35c** with respect to kallikrein (IC_{50} values of 120 vs 29 nM), and the selectivity of ketobenzoxazole **35d** was less attractive still (IC_{50} values for tissue plasminogen activator (t-PA), plasmin, and kallikrein were 29, 36, and 13 nM, respectively). In general, the various (D-Arg)-Gly-Arg-Het compounds inhibited trypsin to the same extent as fXa. It was feasible to replace the basic D-Arg residue in **35e** with hydrophobic amino acids, such as D-Phe (IC_{50} = 2 nM), D-Trp (IC_{50} = 2 nM), and D-Cha (IC_{50} = 0.6 nM), although the latter also inhibited thrombin with an IC_{50} value of 40 nM.[58] Additionally, the P1 Arg side chain in **35e** could be replaced by less basic groups, such as piperidin-4-ylmethyl (IC_{50} = 28 nM), 4-pyridylmethyl (IC_{50} = 44 nM), and Lys (IC_{50} = 1 nM). Substitution of Arg by Trp was surprisingly effective (IC_{50} = 39 nM), while affording excellent trypsin selectivity (IC_{50} > 180,000 nM). Some simplified analogues with one stereocenter, such as $PhSO_2$-Gly-(Me-Gly)-Arg-(2-thiazole) (IC_{50} = 6 nM), were potent fXa inhibitors as well.[58]

Given that the tripeptide-based fXa inhibitors tended to have very short half-lives in vivo ($t_{1/2}$ < 1 h in rats), peptide-mimetics were explored.[58,62] Compounds **36** and **37** turned out to be potent dual inhibitors of fXa and thrombin.[58,62a] In an attempt to attain selectivity for fXa, and to simplify the structure, two series of piperazinones, **38** and **39**, were devised (Tables 9 and 10).[58,62] Incorporation of a sulfonamide group into **38** led to single-digit nanomolar potency and good selectivity for inhibition of fXa versus thrombin, such as with **38d**, which was efficacious as an anticoagulant in a rabbit venous thrombosis model. A wide variety of arylsulfonamide analogues was found to have potent fXa inhibition with good selectivity over thrombin.[58,62a] However, certain compounds in this series lacked selectivity over trypsin and did not show attractive pharmacokinetic properties. Series **39** also yielded numerous compounds with potent inhibition of fXa and good selectivity over thrombin.[58,62b] Interestingly, a broad range of R substituents was acceptable and their stereochemistry was not necessarily a critical factor (e.g., cf. **39c** and **39d**).



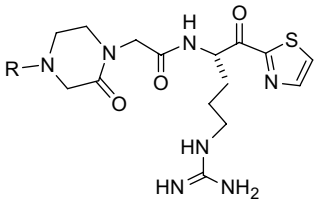
36

Table 8. Inhibition of human factor Xa by tripeptide α-ketoheterocycles X-(D-Arg)-Gly-Arg-Het (**35**)[a]

Compound	X	Het	IC_{50} (nM)
35a	H	2-Thiazole	8
35b	$PhCH_2CH_2SO_2$	2-Thiazole	1
35c	$PhCH_2CH_2SO_2$	2-Benzothiazole	2
35d	$PhCH_2CH_2SO_2$	2-Benzoxazole	2
35e	$PhCH_2SO_2$	2-Thiazole	0.6[b]
35f	$MeSO_2$	2-Thiazole	2

[a] Data were taken from Ref. 58.
[b] K_i value of 0.014 nM (Ref. 60).

Table 9. Inhibition of factor Xa (fXa) and α-thrombin (thr) by piperazinone α-ketothiazoles (**38**)[a]

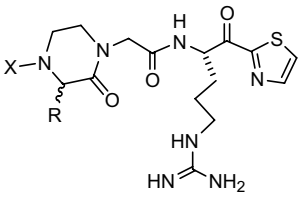


38

Compound	R	fXa IC_{50} (nM)	thr IC_{50} (nM)
38a	$PhCH_2$	770	14,000
38b	PhC(O)	320	7000
38c	$PhSO_2$	8	420
38d	$(4\text{-Cl-}C_6H_4)SO_2$	2	1000
38e	$PhCH_2C(O)$	17	11,000
38f	$PhCH_2SO_2$	4	390

[a] Data were taken from Refs. 58 and 62a. Human enzymes were used

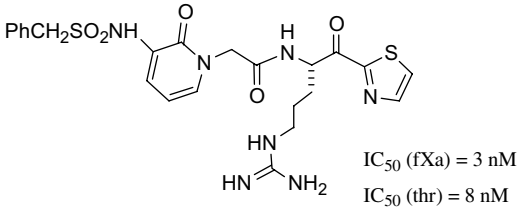
Table 10. Inhibition of factor Xa (fXa) and α-thrombin (thr) by piperazinone α-ketothiazoles (**39**)[a]



39

Compound	R[b]	X	fXa IC_{50} (nM)	thr IC_{50} (nM)
39a	α-$H_2NC(NH)NH(CH_2)_3$–	H	21	200,000
39b	β-$H_2NC(NH)NH(CH_2)_3$–	H	11	150,000
39c	α-$H_2NC(NH)NH(CH_2)_3$–	$PhCH_2SO_2$	13	3000
39d	β-$H_2NC(NH)NH(CH_2)_3$–	$PhCH_2SO_2$	3	12,000
39e	α-$MeOC(O)CH_2$–	$PhCH_2SO_2$	8	5000
39f	β-$MeOC(O)CH_2$–	$PhCH_2SO_2$	85	1000
39g	α-$PhCH_2$–	$PhCH_2SO_2$	0.9	510
39h	β-$PhCH_2$–	$PhCH_2SO_2$	59	2000

[a] Data were taken from Refs. 58 and 62b. Human enzymes were used.
[b] The α orientation has the 3(*R*) configuration; the β orientation has the 3(*S*) configuration.



37

As part of the extrinsic blood coagulation pathway, the trypsin-like serine protease *factor VIIa* (fVIIa) combines with exposed tissue factor (TF) to form a complex (fVIIa/TF), which activates the zymogens factor IX and factor X to their respective serine proteases.[56,57a,63] The resultant factors IXa and Xa are help to convert prothrombin to α-thrombin, which then induces blood clotting. Selective inhibition of the fVIIa/TF complex may provide useful anticoagulation with a decreased risk of bleeding side effects.[64]

A tripeptide α-ketothiazole library of general structure $PhCH_2SO_2$-(D-Phe)-X-Arg-(2-thiazole), where X = an L-amino acid, was prepared to identify inhibitors of the fVIIa/TF complex (**40**; Table 11).[65] The most potent compound was Phe-derivative **40c**, which had a fVIIa/TF IC_{50} value of 42 nM. This compound also strongly inhibited fXa (IC_{50} = 27 nM), but not α-thrombin (IC_{50} = 4000 nM). The 2-fluorophenylalanine analogue, **40d**, was nearly as potent as **40c** and very selective over thrombin, but not fXa. Serine derivative **40f** inhibited all three enzymes in the same realm. An X-ray crystal structure of **40e** bound to the active site of fVIIa/TF was determined.[65] The Arg side chain of the ligand occupied the S1 pocket, the α-ketothiazole interacted with Ser-195 and His-57 in the standard

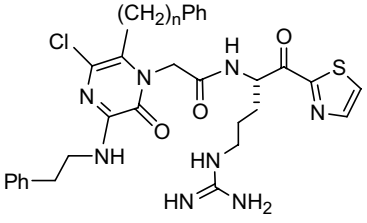
Table 11. Inhibition of factor VIIa/tissue factor (fVIIa), thrombin (thr), and factor Xa (fXa) by tripeptide α-ketothiazoles $PhCH_2SO_2$-(D-Phe)-X-Arg-(2-thiazole) (**40**)[a]

Compound	X	fVIIa IC_{50} (nM)	thr IC_{50} (nM)	fXa IC_{50} (nM)
40a	Ile	300	19,000	380
40b	Asn	1000	60,000	2100
40c	Phe	42	4000	27
40d	2-F-Phe	90	>30,000	210
40e	3-Py-Ala	200	100,000	290
40f	Ser	110	40	240

[a] Data were taken from Ref. 65. Human enzymes were used.

way, and there were two hydrogen bonds involving Gly-216 and Gly-219.

South et al. prepared two pyrazinone α-ketothiazoles, **41**, as potential inhibitors of fVIIa/TF.[66] These compounds inhibited fVIIa/TF with moderate potency, but were not very selective over fXa, thrombin, or trypsin. For example, **41b** had IC_{50} values of 290 nM (fVIIa), 2900 nM (fXa), 110 nM (thrombin), and 470 nM (trypsin).



41 **a**: n = 0
b: n = 1

Factor XIa (fXIa) is a trypsin-like serine protease that is critical to the amplification phase of the blood coagulation cascade. In the intrinsic coagulation pathway,

thrombin mediates the activation of the zymogen factor XI to generate fXIa, which proceeds to activate factors IX and X as part of an amplification process for hemostasis.[67] Selective inhibitors of fXIa might attenuate thrombosis without completely blocking normal hemostasis.[67e]

Deng et al. prepared a series of dipeptide α-ketothiazoles with the structure RC(O)-Val-Arg-(2-thiazole) as potential inhibitors of the fXIa (**42**; Table 12).[68] The most potent inhibitor was **42e** (IC_{50} = 120 nM). They obtained X-ray crystal structures for three different fXIa–ligand complexes, involving **42a**, **42c**, or **42e**,[69] and there was considerable similarity of inhibitor interactions across these structures. The Arg side chain occupied the S1 pocket, the α-ketothiazole bound in the standard manner with Ser-195 and His-57, the Val side chain occupied the S2 region, and the N-terminal carbonyl formed a hydrogen bond with Gly-216 Nα. For **42c** and **42e**, the N-terminal moiety occupied the S4 region, but only **42e** seemed to benefit potency-wise from the extra binding interactions.

Further work with tripeptide α-ketothiazoles RNHC(O)-X-Val-Arg-(2-thiazole), **43**, led to more potent fXIa inhibitors, some of which had very good selectivity (Table 13).[70] Tyrosine analogue **43e** achieved single-digit nanomolar potency and was highly selective over fXa and thrombin, but it potently inhibited kallikrein (IC_{50} = 10 nM) and trypsin (IC_{50} = 12 nM). In in vitro testing with human plasma, **43e** and **43f** were very potent anticoagulants. Additionally, **43e** was active in vivo in a rat model of venous thrombosis with intravenous administration. An X-ray structure of fXIa·**43f** showed the Arg side chain in S1, the α-ketothiazole bound in the standard manner with Ser-195 and His-57, the Val side chain in S2, hydrogen bonding of the Val and urea carbonyls with Lys-192 Nε, and the citrulline side chain in S4.[69]

Table 12. Inhibition of human factor XIa (fXIa) by dipeptide α-ketothiazoles RC(O)-Val-Arg-(2-thiazole) (**42**)[a]

Compound	R	fXIa IC_{50} (nM)
42a	NH_2	500
42b	(3,4-di-Cl-C_6H_3)CH_2NH	480
42c	$Me_2CHCH_2CH_2$	850
42d	3-Cl-$C_6H_4C(O)$	250
42e	(*R*)-3-Cl-$C_6H_4CH(OH)$	120

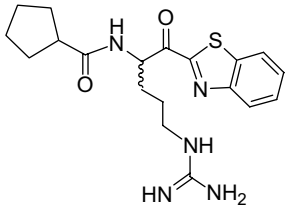
[a] Data were taken from Ref. 68.

2.3. Inhibitors of other mammalian serine proteases

Peptidyl α-ketoheterocycle derivatives have been used to develop inhibitors for additional serine proteases from mammals. In this section, we will address several therapeutically important enzymes, such as tryptase (EC 3.4.21.59), chymase (EC 3.4.21.39), and dipeptidyl peptidase IV (EC 3.4.14.5; DPP-IV).

Tryptases are trypsin-like serine proteases that are produced in mast cells and basophils within the immune system. [71] This heparin-stabilized, homotetrameric enzyme is stored in the granules of mast cells and released on activation by allergic stimuli. Thus, tryptase appears to be very relevant to mast cell-dependent inflammatory conditions, such as allergic asthma, inflammatory bowel disorder, and psoriasis. β-Tryptase is the prototypical family member and major variety present in mast cells.

The first α-ketoheterocycle-based tryptase inhibitors were reported by our research group in 2003,[72] as an extension of our observations with analogues of thrombin inhibitors. While attempting to reduce the molecular size of thrombin inhibitors such as Me-(D-Phe)-Pro-Arg-(2-benzothiazole), **8a**, we synthesized truncated derivative **44** (as a racemate).[73] Although **44** was a very weak thrombin inhibitor (K_i = 12,300 nM), it retained fairly potent inhibition of trypsin (K_i = 30 nM). An X-ray crystal structure of this compound (as the 2*S* enantiomer) complexed with bovine β-trypsin (1.8 Å) depicted the ketone carbonyl covalently linked to Ser-195 Oγ, to form a hemiketal, the benzothiazole nitrogen hydrogen bonded to His-57 Nε, the amide carbonyl hydrogen bonded to Gln-192 Nδ, the amide NH hydrogen bonded to the Ser-214 C=O, and the guanidine within S1.[73]



44

Table 13. Inhibition of factor XIa (fXIa), factor Xa (fXa), and α-thrombin (thr) by tripeptide α-ketothiazoles RNHC(O)-X-Val-Arg-(2-thiazole) (**43**)[a]

Compound	R	X[b]	fXIa IC_{50} (nM)	fXa IC_{50} (nM)	thr IC_{50} (nM)
43a	(3,4-di-Cl-C_6H_3)CH_2	Phe	93	14,000	6700
43b	(3,4-di-Cl-C_6H_3)CH_2	Leu	63	>20,000	2600
43c	(2,4-di-Cl-C_6H_3)CH_2	Leu	45	13,000	390
43d	(*R*)-(4-Br-C_6H_4)CH(Me)–	Cha	10	2700	980
43e	(*R*)-(4-Br-C_6H_4)CH(Me)–	Tyr	6	1600	2000
43f	(*R*)-(4-Br-C_6H_4)CH(Me)–	Cit	30	1900	1100

[a] Data were taken from Ref. 70. Human enzymes were used.
[b] Cha, cyclohexylalanine; Cit, citrulline.

Since trypsin and tryptase have analogous, shallow active-site clefts, we tested **44** for inhibition of human β-tryptase and obtained a respectable K_i value of 88 nM.[72] We also found that thrombin inhibitor **8a** is a potent tryptase inhibitor, as well (K_i = 6.5 nM).[72] Thus, **44** was modified by converting the cyclopentanoyl group into an *N*-acyl amino acid unit, hoping to pick up another hydrogen bond with the active-site β-sheet, such as through Gly-216. A series of dipeptide compounds, represented by **45**, was explored (Table 14). *N*-Ac-Pro analogue **45a** looked very promising with its tryptase K_i value of 19 nM. The *N*-Ac-(D-Pro) diastereomer, **45b**, was much diminished in potency (K_i = 14,000 nM). The *N*-benzoyl analogue, **45c**, was not as potent (K_i = 250 nM), but the *N*-methanesulfonyl (Ms) analogue, **45d**, had decent potency (K_i = 33 nM). Leu derivative **45f** was just 2-fold less potent than **45a**, but Gly derivative **45e** was considerably weaker (K_i = 470 nM). Hydroxyproline analogue **45g** (RWJ-56423; K_i = 10 nM) became a candidate for advanced biological evaluation.[72] While **45g** was not selective over trypsin (K_i = 8.1 nM), it was 30-fold selective versus kallikrein, 200-fold selective versus u-PA, and >500-fold selective versus thrombin, factor Xa, plasmin, and t-PA.

An X-ray co-crystal structure of **45g**·trypsin (1.9 Å) was determined in lieu of a tryptase co-crystal (Fig. 9).[72] The ketone carbonyl formed a hemiketal with Ser-195 Oγ, the benzothiazole nitrogen was hydrogen bonded to His-57 Nε, and the guanidine was in S1. There were hydrogen bonds as follows: P1 amide carbonyl to Gln-192 Nδ, P1 amide NH to the Ser-214 C═O, and *N*-acetyl to Gly-216 Nα. Subsequently, we solved the structure of **45g**·tryptase and found that the interactions in the active site are essentially the same.[74] It is notable that the 2-thiazole analogue of **45g**[72] (not shown) was nearly 20-fold less potent in inhibiting tryptase (K_i = 180 nM), even though no key interactions due to the fused benzene ring in **45g** are evident in the X-ray structure. This decrease in potency with the 2-thiazole derivative is consistent with observations for inhibitor series **3** (Table 1), **8** (Table 4), and **29** (Table 5), but it is not universal (viz. series **35**, Table 8). The latter inconsistency may deter one from ascribing the better potency of 2-benzothiazole vis-à-vis 2-thiazole strictly to the relative π electron-withdrawing properties.[13a] Depending on the specific situation, a different weighting may pertain to activation of the electrophilic ketone versus enhancement of the donor ability of the ring nitrogen.[13a] Perhaps, there are also subtle hydrophobic interactions at play in the S1′ region, such as with the Cys-42/Cys-58 disulfide bond in tryptase.[33b,72] In any case, for a particular enzyme of interest, the 2-benzothiazole may turn out to be an optimal group for α-ketoheterocycle-based inhibitors. Thus, it should be considered as a component in any preliminary study set.

Table 14. Inhibition of human β-tryptase (trpt) and bovine trypsin (trps) by dipeptide α-ketobenzothiazoles R-X-Arg-(2-benzothiazole) (**45**)[a]

Compound	R	X[b]	trpt K_i (nM)	trps K_i (nM)
45a	Ac	Pro	19	4.0
45b	Ac	D-Pro	14,000	960
45c	Bz	Pro	250	160
45d	Ms	Pro	33	38
45e	Ac	Gly	470	260
45f	Ac	Leu	41	7
45g	Ac	(4*R*-OH)-Pro	10	8.1
45h	Ac	(4*R*-OH)-(D-Pro)	380	170

[a] Data were taken from Ref. 72.

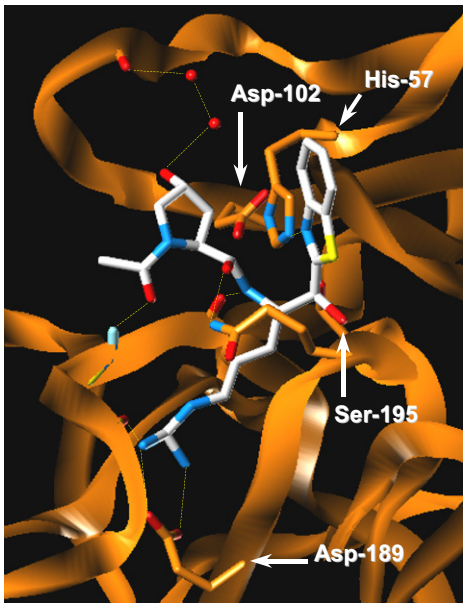
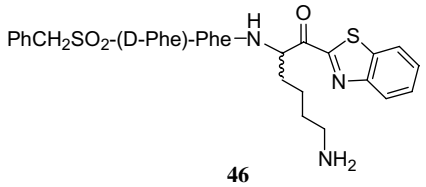


Figure 9. X-ray crystal structure of **45g**·bovine β-trypsin (1.9 Å). View of **45g** (white stick model with the heteroatoms in the standard coloring scheme) bound in the active site of trypsin (orange ribbon bearing specific amino acid side chains). Hydrogen bonds are depicted by yellow broken lines; the two red spheres are water molecules.

Compound **45g** can easily epimerize, especially under mildly basic conditions, because of its labile proton α to the keto group.[72] Stability studies on closely related **8a** in rat and rabbit plasma showed that the Arg α-proton is completely equilibrated in 2 h under physiological conditions (pH 7.4, 37 °C).[33b] Consequently, a mixture of the two diastereomers (L-Arg/D-Arg = 1.2:1; RWJ-58643), from equilibration of **45g** prior to HPLC purification and salt formation, was used for in vivo studies.[72] On aerosol administration to conscious, allergen-sensitized sheep, RWJ-58643 exhibited marked antiasthmatic activity, strongly blunting the early response, abolishing the late response, and essentially eliminating airway hyperreactivity.[72] Single diastereomer **45g** advanced into Phase 2 human clinical trials.

McGrath et al. used tripeptide library screening and structure-based design, including molecular fragment analysis, to derive tryptase inhibitors containing a key α-ketoheterocycle.[75] They reported potent tryptase inhibitors **46** (K_i = 23 nM), **47** (K_i = 3 nM), and **48** (K_i = 25 nM), the latter two lacking a P2 residue but adding an extension on the P1 residue to capture interactions in the S1′–S2′ region. Compounds **47** and **48**, with reduced peptide character, had about 50-fold selectivity over trypsin,[75] and **47** was >1000-fold selective

over thrombin, factor Xa, and factor VIIa.[76] Additional studies on α-keto-(1,2,4-oxadiazole) derivatives of this type were pursued, resulting in many potent inhibitors of tryptase.[76–78] For example, **49** had a tryptase K_i value of 1.5 nM and 275-fold selectivity versus trypsin.[76]



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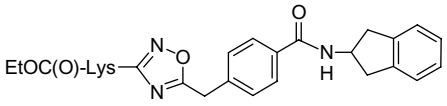

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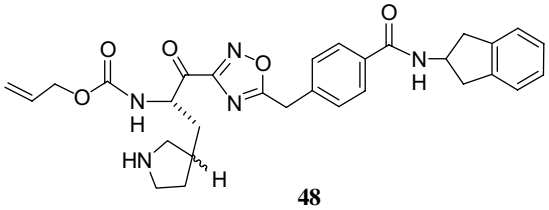

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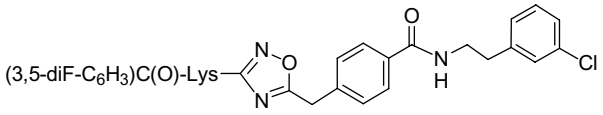
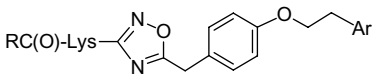


49

Exploration of ether-linked benzyl carbamates with a 1,2,4-oxadiazole core, such as **50**, led to interesting ethyl carbamate analogue **51** (K_i = 5 nM), which was 35-fold selective for tryptase versus trypsin, 80-fold selective versus plasmin, and >1000-fold selective versus kallikrein, u-PA, and thrombin.[77] Although this compound showed just low oral bioavailability in rats (F = 8%), it was 68% bioavailable on intraperitoneal administration in mice. Thus, **51** was assessed in a mast cell-dependent model of asthma in mice and found to inhibit ovalbumin-induced airway hyperreactivity and inflammation in a dose-dependent manner.[77] Although further analogue studies around **50** (e.g., with R highly varied) afforded potent tryptase inhibitors, compounds with improved oral bioavailability were not identified.[78]



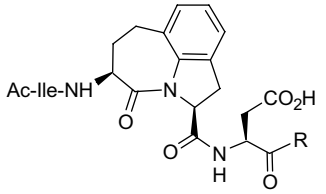
50 R = $PhCH_2O$

51 R = EtO; Ar = Ph

An X-ray structure of **48**·tryptase (2.5 Å) showed hemiketal formation with the ketone and hydrogen bonding between the 1,2,4-oxadiazole nitrogen (N_4) and His-57. In addition to the standard hydrogen bonding interaction between the P1 NH and Ser-214 C=O, there was a new interaction in S1′ between the benzamide NH and the Cys-58 C=O.[75] The P1 extension also had hydrophobic contacts with the side chains of Val-35, Phe-41, Ala-60, and Lys-60D in the S1′–S2′ region. Similar protein–ligand interactions were seen in the X-ray structure of **47**·tryptase (2.3 Å).[75,76] Thus, this inhibitor series nicely illustrates the use of an α-ketoheterocycle as a scaffold for displaying additional molecular subunits that garner new protein–ligand interactions.

Chymase is a chymotrypsin-like serine protease that is stored in and released from mast cells, within the immune system.[71e,79] Inhibitors of this enzyme have therapeutic potential in the treatment of immune-mediated inflammatory disorders, such as asthma, and cardiovascular disorders, such as atherosclerosis.[79b,80] Akahoshi et al. described a series of potent, nonpeptide α-ketoheterocycle inhibitors of human chymase (**52**; Table 15),[81,82] which capitalize on the 5-amino-2-phenylpyrimidin-6-one subunit.[15–17] Variation of heterocycles with R = 4-fluorophenyl indicated that 2-benzothiazole (**52b**) and 2-benzoxazole (**52c**) groups were reasonably effective, although the K_i values did not drop below 100 nM. Nevertheless, these two α-ketoheterocycles were approximately 2-fold more potent as chymase inhibitors than the corresponding trifluoromethylketone.[82] An improvement in potency was realized by substitution of the benzoxazole at the 5-position, such as with methoxy (**52e**; K_i = 73 nM) and carbomethoxy (**52f**; K_i = 23 nM). And adjusting the R group to Ph (**52g**) or 3-methoxyphenyl (**52h**) attained single-digit nanomolar levels. Analogue **52h** (Y-40079) was about 200-fold selective over chymotrypsin, 80-fold selective over cathepsin G, and inactive against elastase and thrombin. Notably, **52h** had oral bioavailability in rats of about 20%, with a lengthy residence time in plasma.

Granzyme B (EC 3.4.21.79) is a caspase-like serine protease that is released by cytotoxic T lymphocytes, which are major effector cells in the immune system.[83] This enzyme is involved in activating apoptosis and has been implicated in autoimmune diseases, such as rheumatoid arthritis.[83] Given granzyme B's preference for an Asp at P1, Willoughby et al. investigated tricyclic proline-mimetics with C-terminal electrophilic traps, such as aldehydes and α-ketoheterocycles, as in **53**.[84] Ketoheterocycles **53a** and **53b** were effective inhibitors of granzyme B, with K_i values of 85 and 7 nM, respectively, and the corresponding aldehyde, **53c**, had a K_i value of 8 nM. These three compounds also inhibited caspase 3.

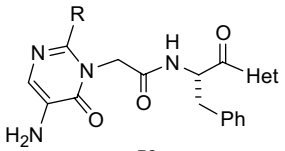


53 **a**: R = 2-benzothiazole

b: R = 2-(5-Ph-1,3,4-oxadiazole)

c: R = H

Table 15. Inhibition of human chymase (chma) and bovine chymotrypsin (chmo) by aminopyrimidinone α-ketoheterocycles (**52**)[a]



52

Compound	Het	R	chma K_i (nM)	chmo K_i (nM)
52a	2-Thiazole	4-F-C_6H_4	3800	3500
52b	2-Benzothiazole	4-F-C_6H_4	180	170
52c	2-Benzoxazole	4-F-C_6H_4	120	230
52d	2-Oxazoline	4-F-C_6H_4	590	9300
52e	2-(5-MeO-Benzoxazole)	4-F-C_6H_4	73	48
52f	2-(5-CO_2Me-Benzoxazole)	4-F-C_6H_4	23	53
52g	2-(5-CO_2Me-Benzoxazole)	Ph	5.6	47
52h	2-(5-CO_2Me-Benzoxazole)	3-MeO-C_6H_4	4.8	940

[a] Data were taken from Ref. 81.

C3 convertase (EC 3.4.21.43),[85] a trypsin-like enzyme in the immune system, has been probed with peptidyl α-ketoheterocycles.[86] In contrast to the inhibition of C3 convertase by the cyclic dodecapeptide compstatin (IC_{50} = 3.4 μM), tripeptide α-ketoheterocycles Ac-Leu-Ala-Arg-Het, with Het = 2-benzothiazole or 2-thiazole (**54a** or **54b**), derived from the C3 peptide cleavage sequence, were not effective.[86] These two compounds maximally inhibited thrombin and trypsin at a high concentration of 100 μM.

The *prolyl endopeptidase* (PEP) family of serine proteases (EC 3.4.21.26) is able to cleave peptides, such as substance P and vasopressin, at internal proline residues.[87] Tsutsumi and coworkers reported on a series of dipeptide α-ketoheterocycles, acyl-Pro-Pro-Het (**55**; Table 16), some of which were potent inhibitors of porcine PEP.[88] In general, the structure–activity relationship for the more potent compounds showed little sensitivity relative to the type of heterocycle used in that the compounds with a donor sp^2 nitrogen atom in the ketoheterocycle adjacent to the keto group resided in a narrow potency range, with IC_{50} values of 4–9 nM (viz. **55a–c**; **55e–j**).[88b] In the absence of an adjacent sp^2 ring nitrogen (e.g., **55d**), there was a 10- to 500-fold decrease in potency. Reduction of the keto group in **55a** to an alcohol or installation of D-Pro at P1 in **55a** lowered potency by >1000-fold. In ex vivo studies in rats with oral administration at 10 mg/kg, **55a**, **55b**, **55f**, and **55h** inhibited kidney PEP activity, and **55a**, **55b**, and **55h** inhibited brain PEP activity, with reasonably long durations of action.[89]

Table 16. Inhibition of porcine kidney prolyl endopeptidase (PEP) by dipeptide α-ketoheterocycles RC(O)-Pro-Pro-Het (**55**)[a]

Compound	Het	R	PEP IC_{50} (nM)
55a	2-Thiazole	$Ph(CH_2)_3$	5.0
55b	2-Thiazole	$PhCH_2O$	8.5
55c	4-Thiazole	$Ph(CH_2)_3$	13
55d	5-Thiazole	$Ph(CH_2)_3$	1100
55e	2-Thiazoline	$Ph(CH_2)_3$	3.8
55f	2-Imidazole	$Ph(CH_2)_3$	9.0
55g	2-Benzothiazole	$Ph(CH_2)_3$	4.0
55h	2-Benzothiazole	$PhCH_2O$	5.6
55i	2-Benzoxazole	$Ph(CH_2)_3$	5.6
55j	2-Pyridine	$Ph(CH_2)_3$	6.9

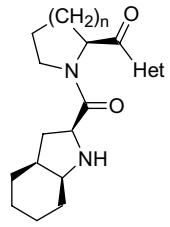
[a] Data were taken from Ref. 88b.

Dipeptidyl peptidase IV is a serine protease related to PEP in that it is also specific for cleaving peptides at a Pro-X site.[90] Some noteworthy substrates degraded by this cell-surface enzyme are glucagon-like peptide-1 (GLP-1), neuropeptide Y, and substance P.[90,91] Inhibitors of DPP-IV have potential therapeutic utility for the treatment of diabetes and the management of pain,[90,92] and various compounds have advanced to late-stage clinical trials, such as sitagliptin (MK-431) and the 2-cyanopyrrolidines saxagliptin (BMS-477118) and vildagliptin (LAF 237).[90b–d] Sitagliptin is marketed by Merck and Co. in several countries for the treatment of type 2 diabetes.[93]

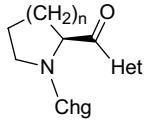
Ferraris et al. investigated dipeptide α-ketoheterocycles **56** and **57**, from which they identified some DPP-IV inhibitors with IC_{50} values in the range of 30–45 nM (Table 17).[94] Some of these compounds were fairly unstable in solution because of cyclization between the amino and keto groups. An ex vivo study was conducted in rats with the diastereomer of **56c** that contains the (2*R*)-azetidinecarboxylic acid subunit. With oral dosing at 50 mg/kg, there was enzyme inhibitory potency over a 6-h period.

Proteins are targeted for degradation by the proteasome, and the 26S proteasome has a catalytic core known as the *20S proteasome*. Proteolysis occurs in the 20S proteasome with the aid of a key active-site threonine residue.[95] Since the proteasome fulfills a generalized proteolytic function, it has different catalytic subunits that recognize basic, acidic, and hydrophobic amino acid side chains in the P1 position. Bortezomib (PS-341), a 20S proteasome inhibitor that is marketed for the treatment of multiple myeloma, was designed to act at the chymotrypsin-like cleavage site.[95a,b]

Table 17. Inhibition of dipeptidyl peptidase IV (DPP-IV) by dipeptide α-ketoheterocycles (**56** and **57**)[a]



56



57

Compound	Het	n[b]	DPP-IV IC_{50} (nM)
56a	2-Thiazole	1	44
56b	2-Benzothiazole	1	30
56c	2-Benzothiazole	0	80
56d	2-Pyridine	1	2000
56e	2-Thiophene	1	50,000
57a	2-Benzothiazole	1	50
57b	2-Benzothiazole	0	42
57c	2-Thiazole	1	42
57d	2-Thiazole	0	30

[a] Data were taken from Ref. 93. Presumably recombinant human enzyme was used. Chg, (*S*)-cyclohexylglycine.
[b] With $n = 0$, contains a (2*S*)-azetidinecarboxylic acid subunit.

Rydzewski et al. developed a series of α-keto-1,3,4-oxadiazoles that yielded some remarkably potent inhibitors of the 20S proteasome (**58**; Table 18).[96] Generally, the compounds of interest targeted the chymotrypsin-like proteolytic activity, rather than the trypsin-like or post-glutamyl peptide-like proteolytic activity. Analogues **58b–d** exemplify the selective, subnanomolar inhibitors. Reasonably potent growth inhibition with PC3 cells, a human prostate cancer cell line, was observed for **58b** (IC_{50} = 200 nM).

2.4. Inhibitors of viral, bacterial, and protozoal serine proteases

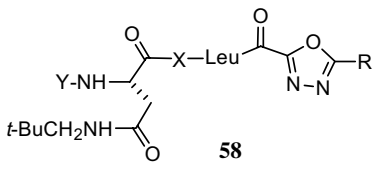
Viruses contain proteolytic enzymes that mediate the maturation of their essential polyprotein during replication.[97] Inhibitors of such proteases can serve as antiviral agents, as amply illustrated by several marketed drugs for the treatment of AIDS (acquired immunodeficiency syndrome), which function by inhibiting a key aspartyl protease in the human immunodeficiency virus (HIV). The fundamental principles of drug design are applicable to the discovery of novel inhibitors of viral proteases.[97] However, it is important to appreciate that viruses can develop drug resistance in response to pharmacotherapy by adapting to protease inhibitors through site-specific mutations in the protease.

Human cytomegalovirus (*HCMV*) *protease* (EC 3.4.21.-), a member of the serine protease family with little structural homology to mammalian chymotrypsin or bacterial subtilisin, is essential for capsid formation during viral replication.[97b] The active site of HCMV contains a Ser-His-His catalytic triad (Ser-132, His-63, His-157) that basically overlays the Ser-His-Asp catalytic triad of α-chymotrypsin. HCMV, one of nine human herpes viruses, is an important pathogen for which there is a serious unmet medical need.[97b] Thus, active-site-directed inhibitors of HCMV protease might provide useful therapeutic agents.

In 1997, Ogilvie et al.[98] reported on peptidomimetic inhibitors of HCMV protease, just after its X-ray structure had been disclosed.[99] While many inhibitors of interest had a trifluoromethylketone group at the C-terminus (e.g., **59d**), some were also α-ketoheterocycles (e.g., **59a–c**).[98] Although compounds **59a–c** had just moderate-to-weak IC_{50} values of 0.6, 1.1, and 11 μM for inhibition of recombinant HCMV N_o protease, they were comparable in potency to **59d** (IC_{50} = 1.1 μM). However, benzoxazole **59a** was reasonably potent as an inhibitor of porcine pancreatic elastase (PPE), with an IC_{50} value of 0.08 μM. Interestingly, **59a** was nearly 100 times more potent against PPE than analogous benzothiazole **59b** (IC_{50} = 9 μM), indicating a clear-cut distinction between similar C-terminal heterocycles, at least with the PPE enzyme.

The α-ketoheterocycle Val-Phe-(D-Ser)-(2-benzothiazole) was found to be a very weak inhibitor of HCMV protease and chymotrypsin.[100]

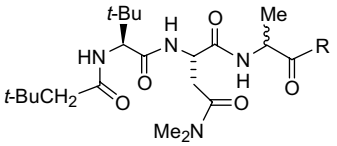
Table 18. Inhibition of the human 20S proteasome activities by α-keto-2-(1,3,4-oxadiazoles) (**58**)[a]



58

Compound	R	X	Y	ChT-L K_i (nM)	Tryp-L K_i (nM)	PGPH K_i (nM)
58a	Ph	Ala	Mesyl	5.7		
58b	Ph	Ala	*p*-Tosyl	0.72	>300,000	>900,000
58c	Ph	MeSer	*p*-Tosyl	0.72	>900,000	>900,000
58d	4-Me_2N-C_6H_4	Ala	*p*-Tosyl	0.51	290,000	>300,000

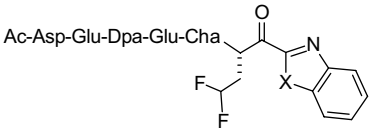
Abbreviations: ChT-L, chymotrypsin-like proteasome activity; Tryp-L, trypsin-like proteasome activity; PGPH, post-glutamyl peptide hydrolase-like proteasome activity; MeSer, *O*-methylserine.
[a] Data were taken from Ref. 96.



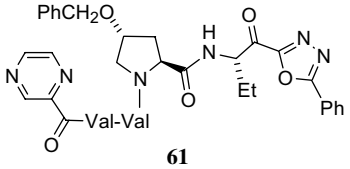
59 **a**: R = 2-benzoxazole
b: R = 2-benzothiazole
c: R = 2-(oxazolyl[4,5-*b*]pyridine)
d: R = CF_3

Human hepatitis C virus (*HCV*) *protease* constitutes one domain of the chymotrypsin-like HCV NS3 protein (NS, nonstructural) and is responsible for processing the HCV polyprotein.[97a,101] The active site of HCV NS3 protease contains a catalytic triad comprised of Ser-139, His-57, and Asp-81.[102] This serine protease is a compelling target for the development of anti-HCV drugs, and several compounds [SCH 503034, telaprevir (VX-950), and ciluprevir (BILN-2061)] have advanced into human clinical studies.[103]

Among the wide range of peptidomimetic inhibitors reported,[103a,c] there are two publications that address α-ketoheterocycles.[104] Hexapeptides **60a** and **60b** (Dpa, diphenylalanine; Cha, cyclohexylalanine), despite their large size, were relatively modest inhibitors of HCV NS34A protease, with IC_{50} values of 150 and 600 nM, respectively.[104a] In sharp contrast, potent inhibition was observed with the corresponding C-terminal aldehyde (IC_{50} = 0.5 nM), indicating a deficiency of the α-ketoheterocycle approach in this case. Ketooxadiazole **61** was a relatively weak inhibitor (K_i = 35 μM), as was its aldehyde analogue (K_i = 16 μM).[104b]



60 **a**: X = S
b: X = O



61

In tropical and subtropical areas around the world, there are approximately 100,000,000 cases per year of dengue fever, which is caused by dengue virus.[105a] The *dengue virus NS3 protease* (EC 3.4.21.91), a trypsin-like serine protease with a catalytic triad defined by Ser-135, His-51, and Asp-75, is essential for viral replication.[105] Yin et al. evaluated tetrapeptide α-ketoheterocycles Bz-Nle-Lys-Arg-Arg-Het (Het = 2-benzoxazole or 2-thiazole), which turned out to be very weak inhibitors (K_i values of 83 or 43 μM).[105b] In contrast, the corresponding aldehyde was about 10-fold more potent (K_i = 5.8 μM), and the boronic acid analogue [C(O)-Het replaced by $B(OH)_2$] was substantially more potent (K_i = 43 nM).

β-*Lactamases* (EC 3.5.2.6), which are grouped into classes A, B, C, and D according to the homology of their primary amino acid sequences, are responsible for the development of bacterial resistance to β-lactam antibiotics, such as drugs of the penicillin and cephalosporin types.[106] In general, β-lactamases are serine proteases, with the exception of the class B enzymes, which are metallo β-lactamases.[106] Kumar et al. employed α-ketoheterocycles $PhCH_2C(O)$-Gly-Het (**62**) in pursuit of inhibitors of class C β-lactamases (cephalosporinases).[107] Five derivatives tested for inhibition of the P99 β-lactamase from *Enterobacter cloacae* were found to have very weak potency. For example, the best inhibitor of β-lactamase, $PhCH_2C(O)$-Gly-2-(thiazole-4-CO_2H), had a K_i value of 110 μM. Perhaps, additional molecular recognition elements would improve the binding characteristics and, thus, the degree of β-lactamase inhibition.

A *prolyl aminopeptidase* (EC 3.4.11.5; PAP) from *Serratia marcescens*, a Gram-negative, pathogenic bacterium in the Enterobacteriaceae family, was probed by Inoue et al. with some simple, P1–P1′ α-ketoheterocycles.[108] They investigated Pro-Het, Ala-Het, and sarcosine-Het [Het = 2-(5-*t*-butyl-1,3,4-oxadiazole] and found that the proline derivative, **63**, is a moderate PAP inhibitor (K_i = 500 nM). An X-ray crystal structure of **63** complexed with PAP showed that the ligand occupies the active site in the expected region. From the figures presented, it appears that the catalytic Ser-113 Oγ may interact with the ketone carbon atom of **63** to some extent.

Chagas' disease is a chronic illness caused by the protozoan parasite *Trypanosoma cruzi*, which contains a *prolyl endopeptidase* (EC 3.4.21.26; also called prolyl oligopeptidase) designated as POP Tc80.[109] Joyeau et al. identified Cbz-Leu-Gly-Pro-(2-benzothiazole) as a moderate inhibitor of POP Tc80 (IC_{50} = 200 nM).[109b] Bal et al. explored a series of Pro-Pro isoxazoles and isoxazolines, and identified several very potent inhibitors of POP Tc80, as well as human PEP (Table 19).[110] For example, isoxazole **64b** and isoxazoline **64e** exhibited K_i values of 0.28 and 0.21 nM, respectively. The related 2-thiazole derivative, **55b**, mentioned above with respect to inhibition of porcine PEP,[88] was also a subnanomolar inhibitor of POP Tc80.[109a,110] Compound **64c** was studied for in vitro antitrypanosomal activity against *T. cruzi* and two other organisms. The ED_{50} values (mg/mL) were 6.7 for *T. cruzi*, 2.2 for *T. b. rhodesiense*, and 13.5 for *Leishmania donovani*.[110] An investigation of host cell invasion indicated that **63c** did not interfere with attachment to the surface of mammalian host cells, but rather prevented the infection of such cells (i.e., entry blockade).[109] It is noteworthy that series **64** represents the sole reported example of using an α-keto-3-isoxazolyl moiety for inhibitor design, and the outcome is rather impressive.

3. Cysteine and metalloprotease inhibitors

Cysteine proteases represent a large family of enzymes that employ an active-site cysteine to hydrolyze amide

Table 19. Inhibition of POP Tc80 and human prolyl endopeptidase (PEP) by α-ketoheterocycles $PhCH_2OC(O)$-Pro-Pro-Het (**64**)[a]

Compound	Het	POP Tc80 K_i (nM)	PEP K_i (nM)
64a	3-Isoxazole	72	36
64b	3-(5-$PhCH_2OCH_2$-Isoxazole)	0.28	4
64c	3-(5-Ph-Isoxazole)	1.9	15
64d[b]	3-(5-$PhCH_2OCH_2$-Isoxazoline)	1.2	6
64e[c]	3-(5-Ph-Isoxazoline)	0.21	10
64f[c]	3-(5-CN-Isoxazoline)	7.5	38
55b	2-Thiazole	0.26	16

[a] Data were taken from Ref. 110.
[b] Single diastereomer.
[c] Mixture of two diastereomers.

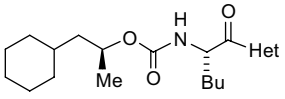
bonds in proteins or peptides.[1a,111] The active site has many mechanistic features in common with that of serine proteases, although a key cysteine thiol is utilized as the nucleophile during proteolysis. The transition state for cysteine proteases similarly involves formation of a tetrahedral intermediate. However, in this case the sulfur atom of Cys adds to the amide carbonyl carbon to give an oxido-orthothioamide, which interacts with an oxyanion hole.[1a,111] Again, there are multiple hydrogen-bonding interactions between the amide backbone of the substrate and the amide backbone of the enzyme. The catalytic site of papain-like cysteine proteases is highly conserved and defined by a Cys-His-Asn catalytic triad (Cys-25, His-159, Asn-175).[111b,112] The nucleophilic Cys γS is apparently ionized prior to substrate binding, unlike the Ser γO in serine proteases.[111b] Since papain-like cysteine proteases have diverse roles in physiology and pathology, there is opportunity for inhibitors to serve as drugs for diseases such as osteoporosis, arthritis, asthma, atherosclerosis, cancer, and parasitic infection.[111b] From a drug discovery standpoint, efforts have been directed to inhibitors of cathepsins B, K, L, and S.[111b,113] Compounds that target cathepsins S and K have entered clinical development, and the cathepsin K inhibitor balicatib (AAE581) has advanced into Phase 2 studies.[113b]

3.1. Inhibitors of cathepsins K and S

The papain-like cysteine protease *cathepsin K* (EC 3.4.22.38; cat K) is a major effector of osteoclastic bone resorption.[111b,113b,114] Consequently, this enzyme is an attractive target for the treatment of osteoporosis and other bone disorders associated with bone degradation. A range of active-site-directed cat K inhibitors, including irreversible agents and compounds that can form a reversible covalent bond with Cys-25 (i.e., a hemithioketal), have been explored.[114] Potent inhibitors of cat K, such as relacatib (SB-462795), which entered human clinical trials, have shown antiresorptive activity in normal and ovariectomized monkeys.[114]

Tavares et al. worked on a series of peptide α-ketoheterocycles, **65**, that contains moderate-to-weak cat K inhibitors (Table 20).[115] The best compound reported was carbethoxythiazole **65c** (IC_{50} = 230 nM). Since the corresponding C-terminal aldehyde was much more potent (IC_{50} = 2.7 nM), the α-ketoheterocycle approach did not deliver much impact in this situation.

Table 20. Inhibition of recombinant human cathepsin K (cat K) by peptide α-ketoheterocycles (**65**)[a]



65

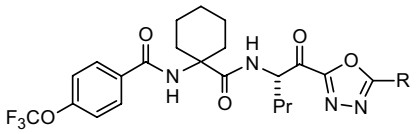
Compound	Het	cat K IC_{50} (nM)
65a	2-Thiazole	3100
65b	2-(4-Ph-Thiazole)	7100
65c	2-(4-CO_2Et-Thiazole)	230
65d	3-(5-Me-1,2,4-Oxadiazole)	8100
65e	2-(5-Ph-1,3,4-Oxadiazole)	4000

[a] Data were taken from Ref. 115.

However, much more promising results were obtained by Palmer et al. with a different recognition motif and an α-keto-1,3,4-oxadiazole subunit (**66**; Table 21).[116] Compounds such as **66d**–**f** attained single-digit nanomolar potency, and **66f** had >100-fold selectivity versus cathepsins B, L, and S. These researchers probed the furanyl-1,3,4-oxadiazole theme further by altering the P2 residue (**67**; Table 22).[116] Clearly, the P2 group is required for potent inhibition of cat K (viz. **67a**–**c**), but it could be reduced in size relative to **66f**. In fact, **67d** afforded spectacular potency against all four cathepsins under study. The analogue of **66f** with a dimethylamino group instead of a trifluoromethoxy group was not only a potent cat K inhibitor (K_i = 0.95 nM) with good selectivity over cathepsins B, L, and S, but also inhibited bone resorption in an in vitro protocol (IC_{50} = 130 nM).[116]

Another interesting series of cat K inhibitors involved dipeptide α-ketobenzoxazoles **68** (Table 23).[117] While the compounds shown potently inhibited cat K, they also had undesirable off-target inhibition. For example, **68b** was a single-digit nanomolar inhibitor of cat K, and potently inhibited cat S (K_i = 6.6 nM) and cat L (K_i = 20 nM). In X-ray structures of the co-crystals **68a**·cat K and **68b**·cat K, the peptide scaffold mapped onto positions S3 to S1′ in the enzyme active site.[117] The piperidine amide of these inhibitors fit into the S3 pocket, primarily defined by the side chains of Asp-61 and Tyr-67. The interactions between the P2 group and the S2 domain contributed ample binding affinity, especially from hydrophobic contacts and β-sheet-type hydrogen bonds (P2 amide NH with Gly-66 C=O; the

Table 21. Inhibition of cathepsin K (cat K) and cathepsins B, L, and S by dipeptide α-keto-2-(1,3,4-oxadiazoles) (**66**)[a]

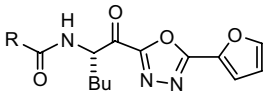


66

Compound	R	cat K K_i (nM)	cat B K_i (nM)	cat L K_i (nM)	cat S K_i (nM)
66a	Ph	29	73,000	19,000	45,000
66b	4-MeO-C_6H_4	22	57,000	37,000	20,000
66c	3-CF_3-C_6H_4	54	1100	1400	3300
66d	Et	7.9	98	300	470
66e	MeO	3.3	93	160	320
66f	2-Furanyl	1.7	220	1000	350

[a] Data were taken from Ref. 116. Recombinant human enzymes were used, except for cat B, which was derived from human liver.

Table 22. Inhibition of cathepsin K (cat K) and cathepsins B, L, and S by α-keto-2-(1,3,4-oxadiazoles) (**67**)[a]

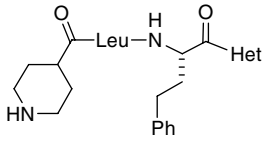


67

Compound	R	cat K K_i (nM)	cat B K_i (nM)	cat L K_i (nM)	cat S K_i (nM)
67a	$PhCH_2CH_2$	9600	25,000	100,000	26,000
67b	$PhCH_2O$	3000	34,000	28,000	2000
67c	*i*-$PrCH_2O$	500	16,000	14,000	380
67d	$PhCH_2OC(O)$-Leu	<0.25	2	1	<0.25

[a] Data were taken from Ref. 116. Recombinant human enzymes were used, except for cat B, which was derived from human liver.

Table 23. Inhibition of cathepsin K (cat K) and cathepsins B, L, and S by dipeptide α-keto-2-benzoxazoles (**68**)[a]



68

Compound	Het	cat K K_i (nM)	cat B K_i (nM)	cat L K_i (nM)	cat S K_i (nM)
68a	2-Benzoxazole	9.5	2100	30	
68b	2-(Benzo[*c*]benzoxazole)	2.9	640	20	6.6
68c	2-(5-*t*-Bu-Benzoxazole)	8.5	7000	70	30
68d	2-(Benzoxazole-5-SO_2NH_2)	40	90	220	40

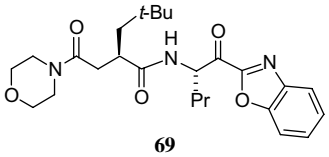
[a] Data were taken from Ref. 117. Recombinant human enzymes were used, except for cat B, which was derived from human liver.

P2 carbonyl with Gly-66 Nα; the P1 amide NH with Asn-158 C═O). Interestingly, the homophenylalanine side chain did not make any notable binding interactions with the enzyme. Cys-25 formed a covalent bond with the ketone carbonyl of the inhibitors via its sulfur atom and the benzoxazole ring occupied the S1′ pocket with its oxazole portion juxtaposed to the catalytic His-159. From the 1.9-Å structure of **68b**·cat K, it is evident that the benzoxazole oxygen formed a hydrogen bond with Asn-158 C═O (as well as the P1 amide NH) and to His-159 Nε (O–N distance = 3.1 Å). This benzoxazole binding mode differs from that seen with comparable serine protease inhibitors, which had the benzoxazole *nitrogen* hydrogen bonded to His-57 Nε (vide supra).

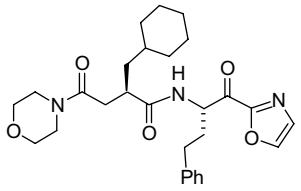
Cathepsin S (EC 3.4.22.27; cat S), a cysteine proteinase of the papain superfamily, plays a critical role in the generation of a major histocompatibility complex (MHC) class II restricted T-cell response by antigen-presenting cells.[118] Therefore, selective inhibition of this enzyme may be useful in modulating class II restricted T-cell responses in immune-related disorders, such as

rheumatoid arthritis, multiple sclerosis, and extrinsic asthma.[113b,118] A diversity of active-site-directed cat K inhibitors have been explored.[118]

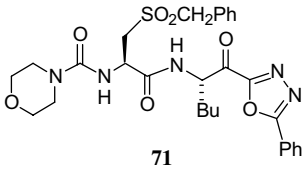
Although there are no scientific publications in print that describe α-ketoheterocycle-based inhibitors of cat S, numerous compounds are the subject of patents or patent applications.[118d,119] Some noteworthy examples will be presented here. Compounds **69**–**72** had K_i values below 100 nM,[119a–d] **73** had a K_i value below 10 nM,[119e] and **74** had an IC_{50} value of 6.6 nM with >100-fold selectivity versus cathepsins B, K, and L.[119f] It was indicated that **75** exhibits dose-dependent anti-inflammatory activity in a murine ovalbumin-challenge model (<30 mg/kg, orally).[119g]



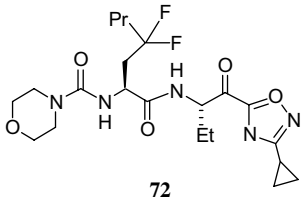
69



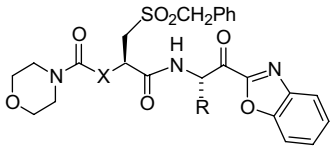
70



71

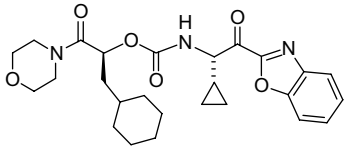


72



73 X = O; R = Et

75 X = NH; R = Pr



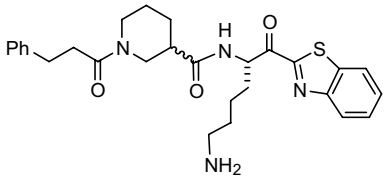
74

Collaborative research between Sanofi–Aventis and Celera Genomics (formerly Axys Pharmaceuticals, Inc.) identified compounds with an α-keto-2-benzoxazole moiety that exhibit robust cat S inhibition.[120] To obtain a development candidate, the initial cat S inhibitor leads were optimized to improve potency, selectivity, and pharmacokinetics. An α-ketobenzoxazole derivative was stated to possess anti-inflammatory activity in an in vivo model on oral administration.[120]

3.2. Inhibitors of miscellaneous cysteine proteases

The *calpains* are calcium-dependent cysteine proteases that participate in calcium-regulated cellular processes, such as signal transduction, apoptosis, cell proliferation, and cell differentiation.[121] Thus, calpains can play an important role in various human diseases. Tao et al. reported on a series of peptidyl α-ketoheterocycles as modest inhibitors of calpain I.[122] At a concentration of 10 μM, Cbz-Leu-Leu-Phe-(2-thiazole) and Boc-Leu-Leu-(2-imidazole) displayed 54% and 77% calpain I inhibition, respectively.

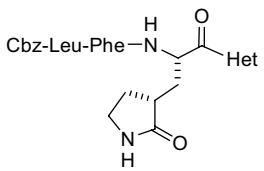
The cysteine proteases *Arg*- and *Lys-gingipains* (Rgp and Kgp; EC 3.4.22.37 and EC 3.4.22.47) are secreted by the bacterium *Porphyromonas gingivalis*, the major pathogen in periodontal disease.[123] Compound **76** (A71561) was found to potently inhibit Kgp, with a K_i value of 0.9 nM, while showing no inhibition of Rgp's at 100 μM.[123a] This Kgp inhibitor was just 30-fold selective over trypsin, but 120,000-fold selective over cat B. Biodata in this paper suggest the therapeutic potential of Kgp inhibitors for controlling *P. gingivalis* infections.



76

Human rhinovirus 3C protease (3CP; EC 3.4.22.28) is a cysteine protease that is a crucial component in the viral replication cycle. Dragovich et al. identified some

Table 24. Inhibition of human rhinovirus 3C protease (3CP) by tripeptide α-ketoheterocycles (**77**)[a]

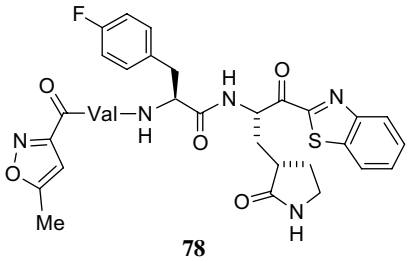


77

Compound	Het	3CP K_i (nM)	Anti-3CP EC_{50} (μM)
77a	2-Benzothiazole	65	3.2
77b	2-Thiazole	700	7.9
77c	2-Pyridine	170	4.0
77d	2-Benzothiophene	4700	>10

[a] Data were taken from Ref. 124. The 3CP was from human rhinovirus serotype-14.

α-ketoheterocycles with a tripeptide motif as 3CP inhibitors (**77**; Table 24).[124] Benzothiazole derivative **77a** was reasonably potent against 3CP (K_i = 65 nM) and exhibited antiviral activity (EC_{50} = 3.2 μM) as well. The frank erosion of potency with benzothiophene **77d** and the reduced-ketone (i.e., alcohol) analogue of **77a** is consistent with standard α-ketoheterocycle interactions in the 3CP active site. In this case, there would be a hemithioketal formed with the active-site Cys and a hydrogen bond formed with the active-site His. In fact, the paper mentions an X-ray crystal structure of **77a**·3CP wherein these interactions were confirmed (without giving any specific details).[124] These findings led to analogue **78**, which manifested excellent 3CP inhibition (K_i = 4.5 nM) and enhanced antiviral activity (EC_{50} = 0.34 μM). This level of antiviral activity was comparable to that of related peptide aldehydes.



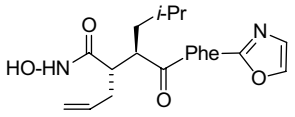
78

3.3. Inhibitors of metalloproteases

The metalloproteases constitute a wide variety of zinc(II)-dependent enzymes, which are classified by the nature of the most prominent active-site functional group: metallocarboxypeptidases (EC 3.4.17) and metallo-endopeptidases (e.g., EC 3.4.24).[125] Because of the obligatory metal center in the active site, a preponderance of metalloprotease inhibitors possess a distinct zinc-chelating functionality, such as a hydroxamate, sulfhydryl, or carboxylate group.[1b,e,126] An archetypal example of a successful drug class from this enzyme category is inhibitors of angiotensin-converting enzyme (ACE; EC 3.4.15.1).[1b,e] Matrix metalloproteases (MMPs; EC 3.4.24.-) are another important enzyme category, of which there are 26 different isozymes in the human genome.[1e,126] Because most small-molecule MMP inhibitors bind within the prime-side pocket of the enzyme's active site, the attainment of good selectivity has been problematic.[1e] Given the lack of a crucial Ser or Cys residue in the active site of MMPs, one might not be readily disposed to employ a reactive α-ketoheterocycle for inhibitor design. Nevertheless, there are a few reports on this approach in the scientific literature.[127–129]

Histone deacetylases (HDACs; EC 3.5.1.-) remove acetyl groups from ε-*N*-acetyl-Lys residues of histones to down-regulate gene expression.[130] Vasudevan et al. studied several α-ketoheterocycles as potential HDAC inhibitors.[127] A survey of (4-Ph-C_6H_4)O(CH_2)$_6$C(O)R derivatives determined that the following R groups, inter alia, are not favorable for obtaining HDAC inhibition (IC_{50} > 50 μM): 2-thiazole, 2-benzoxazole, 2-pyridine, 2-imidazole, and 2-(1-Me-imidazole). On the other hand, the 2-oxazole, 2-oxazoline, and 5-tetrazole groups yielded moderately potent HDAC inhibitors, with IC_{50} values in the range of 1–2 μM. A comparable result was obtained with R = CF_3 (IC_{50} = 2.9 μM); however, R = C(O)NHMe was much better (IC_{50} = 110 nM). Building upon the 2-oxazole platform, these researchers developed some reasonably potent HDAC inhibitors (**79**; Table 25).[127] Compounds **79**, **79d**, and **79f** had double-digit nanomolar IC_{50} values, and **79f** exhibited significant cellular antiproliferative activity (IC_{50} = 2.3 μM) that appeared to be histone-relevant.

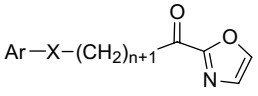
A series of heteroarylketones bearing a terminal hydroxamic acid group gave rise to numerous, potent *matrix metalloprotease* inhibitors.[128] For example, **80** inhibited MMP-1, MMP-2, MMP-3, and MMP-7 with IC_{50} values of 2.7, 7.2, 1.8, and 4.2 nM. However, from the structure–activity relationship (SAR) of this series, the bioactivity is not really dependent on a particular structural feature of the heterocycle, as illustrated by the high potency for a phenylketone analogue.



80

Methionine aminopeptidase (MetAP; EC 3.4.11.18) enzymes perform a critical aspect of protein maturation by eliminating the N-terminal Met residue from the polypeptide chain of newly biosynthesized proteins.[131] Douangamath et al. found that the α-ketoheterocycles Met-(2-thiazole) and Met-(2-pyridine) weakly inhibit *Staphylococcus aureus* MetAP I (IC_{50} = 19 and 16 μM; possibly racemates).[129] X-ray crystal structures of MetAP I complexed with Met-(2-thiazole) and Met-(2-pyridine) displayed a *gem*-diol (hydrated) form of the ketone group, which could mimic the tetrahedral intermediate for peptide amide-bond cleavage. This struc-

Table 25. Inhibition of histone deacetylase (HDAC) by α-keto-2-oxazoles (**79**)[a]



79

Compound	*n*	X	Ar[b]	HDAC IC_{50} (nM)
79a	5	NH	2-Np	430
79b	5	O	3-Ph-C_6H_4	620
79c	5	NHC(O)	3-Ph-C_6H_4	60
79d	4	NHC(O)	3-Ph-C_6H_4	310
79e	5	NHC(O)	2-Indole	90
79f	5	NHC(O)	AnThz	30

[a] Data were taken from Ref. 127

[b] 2-Np, 2-naphthyl; AnThz, 2-[4-(4-methoxyphenyl)thiazole].

tural feature, in conjunction with the nitrogen of the heterocycle and the Nε2 of His-178, established a favorable metal-binding environment.

4. Hydrolase and transferase inhibitors

Many classes of proteins hydrolyze the carbon–nitrogen bond in carboxamide substrates (EC 3.5.1.-).[132] In addition to the protease/peptidase enzymes already discussed in this review, there are amidases (amide hydrolases), which cleave the amide bond in diverse *N*-acyl amines.[132] A common class of amidases operates on aliphatic carboxamides (EC 3.5.1.4). In general, enzymes in the 'amidase signature family' are present in non-mammalian species, and are especially prevalent in bacteria and fungi.[132,133] An exception to this picture is conferred by *fatty acid amide hydrolase* (FAAH; EC 3.5.1.4), a mammalian enzyme that degrades fatty acid amides.[133]

Oleamide (9*Z*-octadecenamide) and anandamide (*N*-arachidonylethanolamide) are members of a large family of fatty amides that serve as endogenous signaling molecules.[133,134] Anandamide behaves as an endocannabinoid in that it binds to and activates cannabinoid CB1 and CB2 receptors; additionally, it activates the vanilloid receptor (VR1 or TRPV1), an ion channel involved in pain transmission. These bioactive fatty amides are hydrolyzed to the corresponding fatty acids by the membrane-bound enzyme FAAH,[133,135] a serine amide hydrolase with a Ser-Ser-Lys catalytic triad (Ser-241, Ser-217, Lys-142) in its active site.[133,136] Since FAAH appears to be a pivotal regulator of the levels of anandamide in vivo, selective FAAH inhibitors may have potential as therapeutic agents.

Boger and coworkers used an α-ketoheterocycle approach to devise exceptionally potent inhibitors of FAAH (**81**; Table 26).[137] Derivatives based on oleyl (C_{17}–CO) and arachidonyl (C_{19}–CO) groups were highly effective against the rat enzyme, as were those based on smaller hydrocarbon substituents. Inhibitory potency varied dramatically for different heterocycles, with the best heterocycles being oxazolopyridines. For example, oleyl oxazolopyridine **81i** had a K_i value of 2.3 nM, which was 35-fold better than its oleyl trifluoromethyl congener (K_i = 82 nM). Surprisingly, the 2-thiazole (**81a**) and 2-benzothiazole (**81b**) groups were quite ineffective. Several subnanomolar inhibitors, such as **81l**–**n**, were identified (K_i = 0.2–0.7 nM). Surprisingly, with the α-keto-2-oxazolo[4,5-*b*]pyridine in place, the R group could be contracted to butyl and still yield decent potency (**81p**; K_i = 50 nM). Compound **81n** was remarkably potent against recombinant human FAAH, with a K_i value of 0.094 nM. Reduction of the carbonyl group in **81i** or **81l** to an alcohol caused a large drop in potency of 800- or 2000-fold, respectively, consistent with the α-ketoheterocycle concept. While the electrophilic carbonyl is an essential component in the design of these FAAH inhibitors, other aspects of molecular recognition connected with the heterocycle are also very important. One such factor would be the basic pyridine nitrogen atom of the oxazolopyridines.

Table 26. Inhibition of fatty acid amide hydrolase (FAAH) by α-ketoheterocycles RC(O)-Het (**81**)[a]

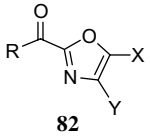
Compound	Het	R[b]	FAAH K_i (nM)[c]
81a	2-Thiazole	Oleyl	>100,000
81b	2-Benzothiazole	Oleyl	>100,000
81c	2-Oxazole	Oleyl	17
81d	2-Benzoxazole	Oleyl	370
81e	3-Pyridazine	Oleyl	130
81f	5-(2-Me-Tetrazole)	Oleyl	65
81g	5-(1-Me-Tetrazole)	Oleyl	3700
81h	2-Oxazolo[5,4-*c*]pyridine	Oleyl	3.7
81i	2-Oxazolo[4,5-*b*]pyridine	Oleyl	2.3
81j	2-Pyridazine	Arachidonyl	47
81k	2-Oxazolo[4,5-*b*]pyridine	Arachidonyl	1.0
81l	2-Oxazolo[4,5-*b*]pyridine	Undecyl	0.57
81m	2-Oxazolo[4,5-*b*]pyridine	Heptyl	0.69
81n	2-Oxazolo[4,5-*b*]pyridine	$Ph(CH_2)_5$	0.20
81o	2-Oxazolo[4,5-*b*]pyridine	$Ph(CH_2)_3$	6.9
81p	2-Oxazolo[4,5-*b*]pyridine	Butyl	50

[a] Data were taken from Ref. 137a. The enzyme from rats was used.
[b] Oleyl, (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_7$; arachidonyl, (*Z*,*Z*,*Z*,*Z*)-$Me(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2$.
[c] Inhibition of recombinant human FAAH (compound K_i): **81d**, 73 nM; **81i**, 13 nM; **81n**, 0.1 nM.

FAAH inhibitors **81i** and **81l** were found to be selective over triacylglycerol hydrolase (TGH) by 30- and 100-fold, respectively, but **81n** was not selective.[138] The hexamethylene homologue of **81n** and **81o** was 10-fold selective for FAAH (IC_{50} = 0.28 nM)[137a] versus TGH, while being very selective (>10,000-fold) over the mammalian hydrolases lipoprotein lipase (LPL), monoacylglycerol lipase (MAGL), and carboxylesterase 1 (CE-1).[139] The moderate FAAH inhibitor **81d** (K_i = 370 nM) manifested a central nervous system (CNS) behavioral effect in rats (50 mg/kg, intraperitoneally) that was analogous to oleamide, possibly due to an increase in endogenous cannabinoid levels.[140] However, potent FAAH inhibitor **81i**, on administration to mice at 50 mg/kg intraperitoneally, failed to enhance the analgesic pharmacological activity imparted by anandamide.[139]

Boger et al. continued efforts on the α-ketooxazole theme (**82**; Table 27), leading to some potent FAAH inhibitors.[141] For example, derivatives of **82** with a 2-pyridine (**82d**, **82h**, **82m**) or 2-oxazole (**82f**, **82o**) on the 5-position of the α-ketooxazole subunit had K_i values in the range of 2–20 nM.[141a] Success was also realized with α-ketooxadiazoles (**83**; Table 28).[141b] In fact, several compounds demonstrated subnanomolar potency, such as **83e** (K_i = 0.29 nM). Monte Carlo simulations for **82d** and **82m**, covalently bound to FAAH via the γO of Ser-241, furnished some structural features for these complexes.[141a,142] There was a hydrogen-bond network between the enzyme, the pyridine nitrogen atom, and the oxazole oxygen atom, such that the oxazole oxygen interacted with Ser-217 and the pyridine nitrogen interacted with Lys-142 and the hydroxyl of Thr-236.

Table 27. Inhibition of fatty acid amide hydrolase (FAAH) by α-keto-2-oxazoles (**82**)[a]

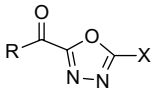


82

Compound	R[b]	X	Y	FAAH K_i (nM)[c]
82a	Oleyl	H	Ph	490
82b	Oleyl	Ph	H	320
82c	Oleyl	H	2-Pyridine	31
82d	Oleyl	2-Pyridine	H	18
82e	Oleyl	3-Pyridazine	H	14
82f	Oleyl	2-Oxazole	H	12
82g	Oleyl	2-Furan	H	54
82h	Undecyl	2-Pyridine	H	2.2
82i	Heptyl	2-Pyridine	H	49
82j	Butyl	2-Pyridine	H	3000
82k	$Ph(CH_2)_3$	2-Pyridine	H	120
82l	$Ph(CH_2)_5$	2-Pyridine	H	11
82m	$Ph(CH_2)_6$	2-Pyridine	H	4.7
82n	$Ph(CH_2)_6$	3-Pyridazine	H	5.6
82o	$Ph(CH_2)_6$	2-Oxazole	H	4.6

[a] Data were taken from Ref. 141a. The recombinant rat enzyme was used.
[b] Oleyl, (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_7$.
[c] Inhibition of recombinant human FAAH (compound, K_i): **82d**, 10 nM; **82m**, 9.0 nM.

Table 28. Inhibition of fatty acid amide hydrolase (FAAH) by α-keto-1,3,4-oxadiazoles (**83**)[a]



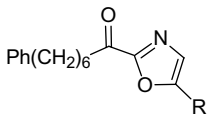
83

Compound	R[b]	X	FAAH K_i (nM)
83a	Oleyl	H	90
83b	Oleyl	Ph	16
83c	Oleyl	2-Pyridine	3.0
83d	$Ph(CH_2)_6$	Ph	2.0
83e	$Ph(CH_2)_6$	2-Pyridine	0.29
83f	$Ph(CH_2)_6$	2-Furan	0.56
83g	$Ph(CH_2)_8$	2-Pyridine	0.83

[a] Data were taken from Ref. 141b. The recombinant rat enzyme was used.
[b] Oleyl, (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_7$.

2-[5-(2-Pyridyl)oxazole] **82m** was 300-fold selective for FAAH (IC_{50} = 2.1 nM) versus TGH, while being very selective (>10,000-fold) over MAGL, CE-1, and LPL.[139,141a] This compound also potently inhibited a second mammalian FAAH (FAAH-2).[134b] Pyridyl-oxadiazole analogues **83c** and **83g** were 5000- and 600-fold selective for FAAH versus TGH.[141b] Potent FAAH inhibitor **82m** (OL-135) administered to mice intravenously potentiated the analgesic activity of anandamide with an ED_{50} value of 1.9 mg/kg.[139] This compound also produced CB1-dependent analgesia and elevated endocannabinoid levels in mice (10 mg/kg, intraperitoneally), with a protracted duration of 240 min.[139] Indeed, there was marked antinociception in the mouse tail immersion and hot-plate tests for thermal pain sensation.[139,143]

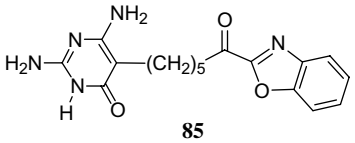
Boger and coworkers studied the effect of oxazole ring substituents on FAAH inhibition.[144] Systematic alteration of the R group on the oxazole 5-position, as in **84a–d**, defined a fundamental relationship between the K_i values and Hammett σ_p constants. The spectrum of R groups elicited a 200-fold variation in K_i values, ranging from Me (σ_p = −0.15; K_i = 80 nM) to CN (σ_p = 0.7; K_i = 0.4 nM). Compounds bearing the strongest electron-withdrawing substituents, such as **84c** and **84d**, exhibited subnanomolar potency. It was proposed that the observed correlation reflects the effect of the R substituent on the reactivity of the electrophilic carbonyl group. Further work elaborated on pyridyl-oxazole **82m** (K_i = 4.7 nM), with the 2-pyridine ring being variously substituted.[144b] Thus, the 4-methyl (**84e**) and 4-methoxy analogues had K_i values of 0.6 and 0.8 nM, respectively, whereas the 3-carbomethoxy analogue had a K_i value of 130 nM.[144b] Notably, potent FAAH inhibitor **84e** was 2000-fold selective for FAAH over TGH. An intensive structure–activity campaign was executed with respect to modifying the 6-phenylhexyl segment of **82m** and **84**.[145] Thus, structure **82** (Table 27) with R = 2-(4-biphenyl)ethyl, X = H, and Y = 2-pyridine was found to have optimal, subnanomolar potency (K_i = 0.75 nM).[145a] Analogously, structure **84** with R = CN and 2-(4-biphenyl)ethyl in place of the 6-phenylhexyl group had superpotent K_i values of 0.20 and 0.26 nM for rat FAAH and recombinant human FAAH, respectively.



84 **a**: R = Me [−0.15; 80 nM]
b: R = F [0.15; 30 nM]
c: R = CF_3 [0.55; 0.8 nM]
d: R = CN [0.7; 0.4 nM]
e: R = 2(4-Me-pyridine)

Glycinamide ribonucleotide transformylase (GAR TF; EC 2.1.2.2) and *aminoimidazole carboxamide transformylase* (AICAR TF; EC 2.1.2.3) are folate-dependent enzymes that transfer a formyl group.[146] GAR TF catalyzes the third step of de novo purine biosynthesis, the conversion of glycinamide ribonucleotide to *N*-formyl glycinamide ribonucleotide, which is accompanied by conversion of 10-formyltetrahydrofolate to tetrahydrofolate. AICAR TF catalyzes a later step in the pathway involving formyl group transfer from 10-formyltetrahydrofolate to the exocyclic amino group of aminoimidazole carboxamide to generate 5-formylaminoimidazole-4-carboxamide ribonucleotide. Boger and coworkers explored α-ketoheterocycle derivatives as potential inhibitors of these formyl

transferases.[147] In general, the compounds examined from a series containing a relatively simplified pharmacophore showed weak inhibitory action, at best.[147a] For example, **85** was a weak inhibitor of *Escherichia coli* GAR TF (K_i = 15 μM), and was virtually inactive against recombinant human GAR TF and AICAR TF (K_i > 100 μM). In contrast, a more structurally elaborate α-ketobenzoxazole, **86**, which has an L-glutamate appendage like native tetrahydrofolate, inhibited recombinant human GAR TF with a K_i value of 0.6 μM.[147b] Since the alcohol analogue from reduction of the ketone in **86** was just 3-fold less potent (K_i = 1.8 μM), the electrophilic carbonyl is not likely to play a mechanistic role in this case.



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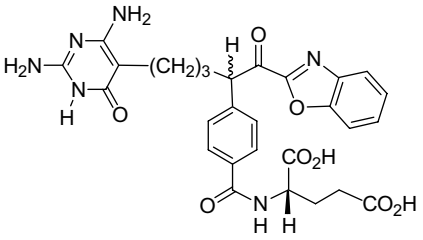


86

5. Synthetic chemistry

Some common synthetic procedures have become standard tools for supplying α-ketoheterocycles of interest as potential enzyme inhibitors. In this section, the presentation is not intended to be comprehensive; rather, we endeavor to illustrate the main methods that researchers have utilized.

5.1. Imidate method

The crux of this synthetic method is the conversion of an aldehyde cyanohydrin into the corresponding hydroxyl imidate with HCl (Pinner reaction), formation of a heterocycle with a suitable bifunctional amine, elaboration of the recognition motif, and oxidation of the alcohol to a ketone. This method is exemplified by the synthesis of α-ketobenzoxazole **2** (Fig. 10).[12a] The product was purified as a free base and isolated as a 9:1 diastereomeric mixture, because of some epimerization at the stereocenter α to the ketone.

In an example from our thrombin inhibitor studies, protected tripeptide aldehyde **87** was converted to imidate **88** en route to α-ketobenzothiazole **8a** (Fig. 11).[33b] This product was purified and isolated by reverse-phase HPLC, with some trifluoroacetic acid in the eluant, which formed a salt (49:1 diastereomeric mixture). Use of an acid-addition salt helps to prevent epimerization at the stereocenter α to the ketone.

A variant of this synthetic approach involves use of a thioimidate, which can be obtained by methylation of a thioamide. Tamura et al. resorted to this method to prepare targets such as **89**, when they encountered difficulties with the imidate route for 2-benzoxazole synthesis (Fig. 12).[41]

The imidate chemistry is applicable to the preparation of α-ketooxazolines from 1,2-amino alcohols[12b,81] and α-ketothiazolines from 1,2-amino thiols.[88b]

5.2. Weinreb amide method

This generally applicable method entails the addition of a metalated heterocycle to an *N*-methyl-*N*-methoxycarboxamide (Weinreb amide) to generate an α-ketoheterocycle. The ketone formed, even though it is desired in the final product, is reduced to the alcohol to avoid stereomutation of the stereocenter α to the heterocycle. The

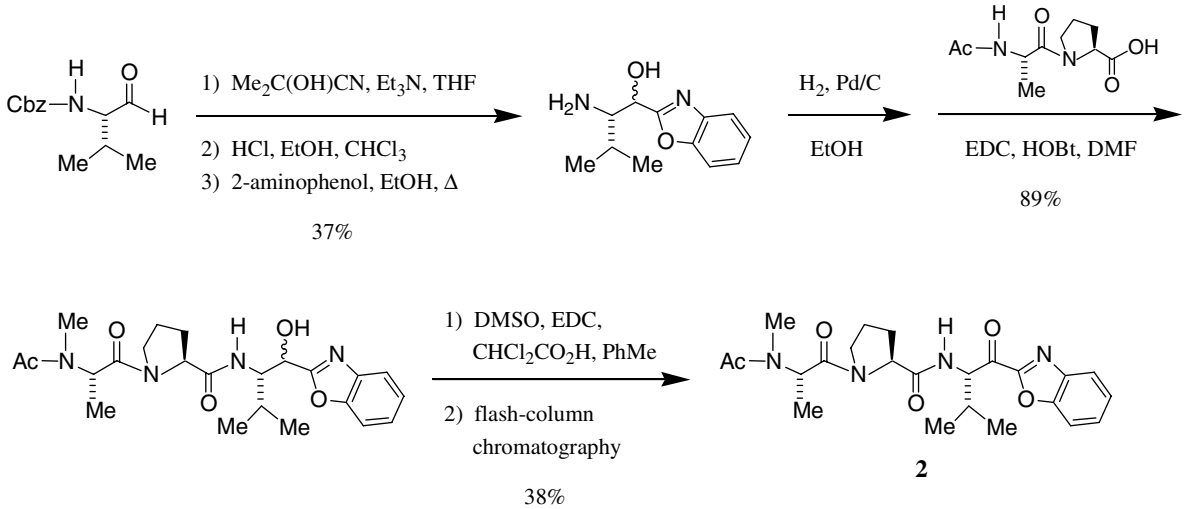


Figure 10. Synthesis of **2** via the imidate route [EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole].

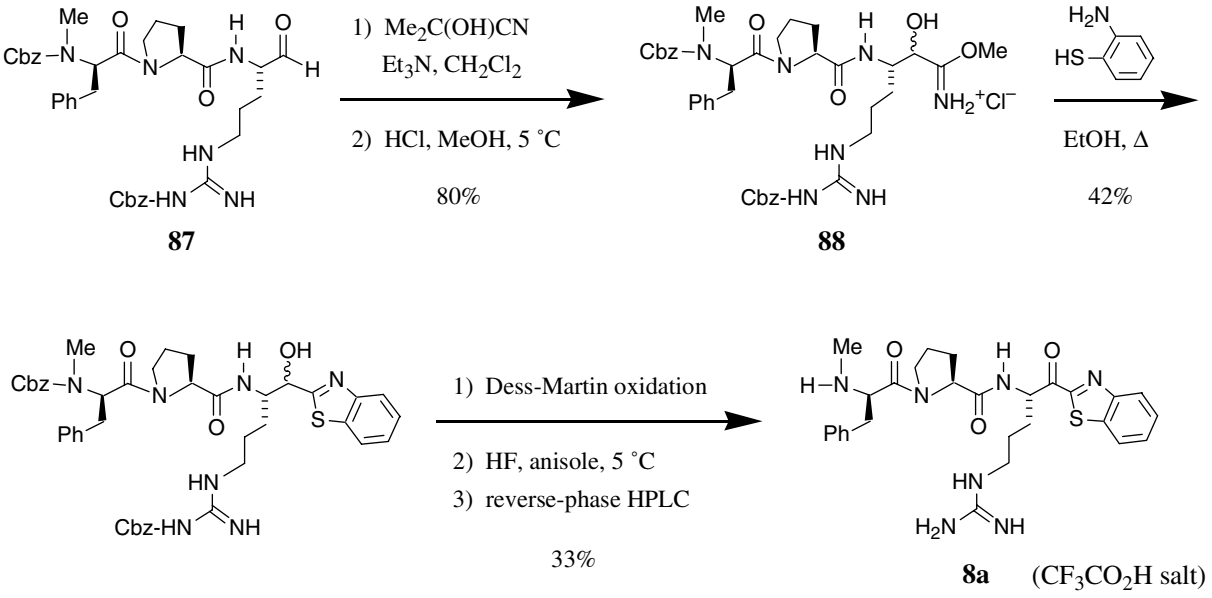


Figure 11. Synthesis of **8a** via the imidate route. The Dess–Martin oxidation employs the periodinane 1,1,1-tris(acetyloxy)-3*H*-1,2-benziodoxol-3-one in dichloromethane (Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4155).

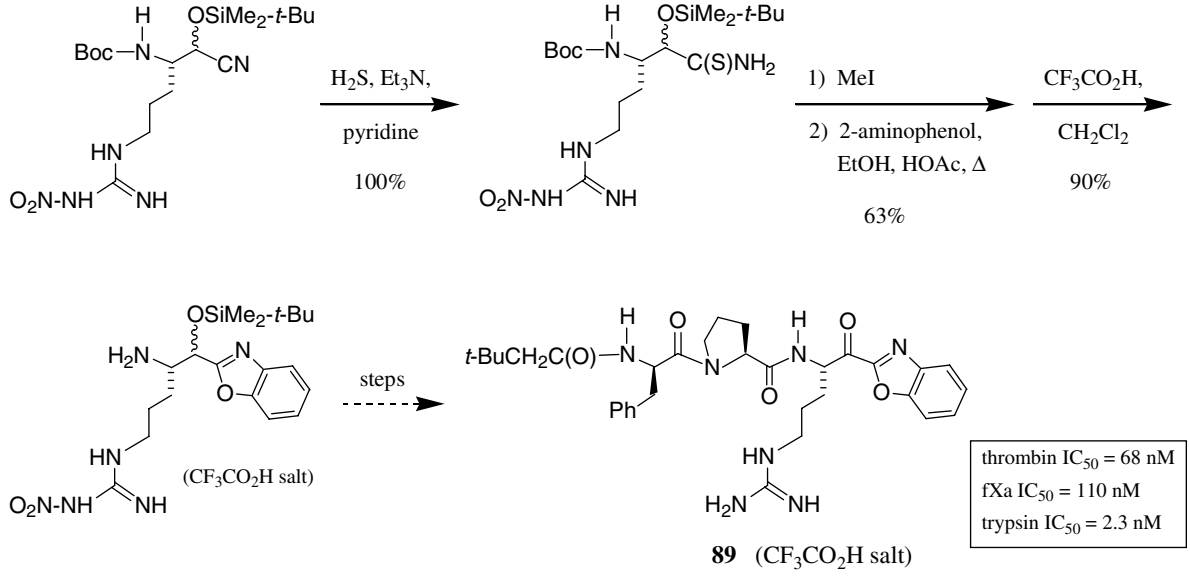


Figure 12. Synthesis of α-ketobenzothiazole **89** via an intermediate thioimidate.

recognition motif is attached and the alcohol is oxidized to the ketone in the penultimate step. This method is illustrated for the synthesis of α-ketobenzothiazole **8a** (Fig. 13).[33b]

5.3. Ester-addition chemistry

By analogy to the Weinreb amide route, one can utilize an ester for addition of metalated heterocycles. Since this process often leads to overaddition, which adversely impacts the yield, it is not generally recommended for high-value substrates. For example, Kumar et al. reacted 2-lithio-[1-($Me_3SiCH_2CH_2OCH_2$)-imidazole] with $PhCH_2C(O)$-Gly-OMe in THF at −78 °C to give the α-ketoimidazole in 42% yield, which was carried on to $PhCH_2C(O)$-Gly-2-(imidazole).[107] Also, Leung et al. reacted RMgBr or RLi with various 5-aryl-substituted 1,3,4-oxadiazole-2-carboxylates to obtain α-keto-1,3,4-oxadiazoles, such as **83d** (Table 28).[141b]

The addition of organometallic reagents to an ester can be more efficient under certain circumstances, such as when steric bulk is present. For example, ester **90**, with steric hindrance at the α stereocenter, was condensed with 2-lithiobenzothiazole to form the intermediate α-ketobenzothiazole, which was re-

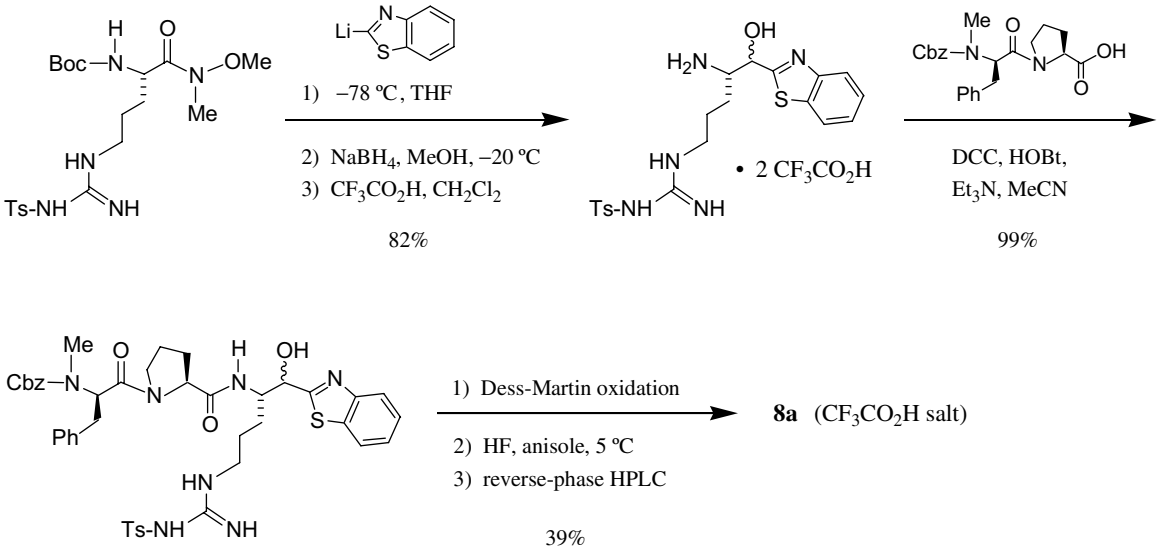


Figure 13. Synthesis of **8a** via the Weinreb amide route [DCC, *N*,*N*′-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole]. See caption of Figure 11 regarding the Dess–Martin oxidation.

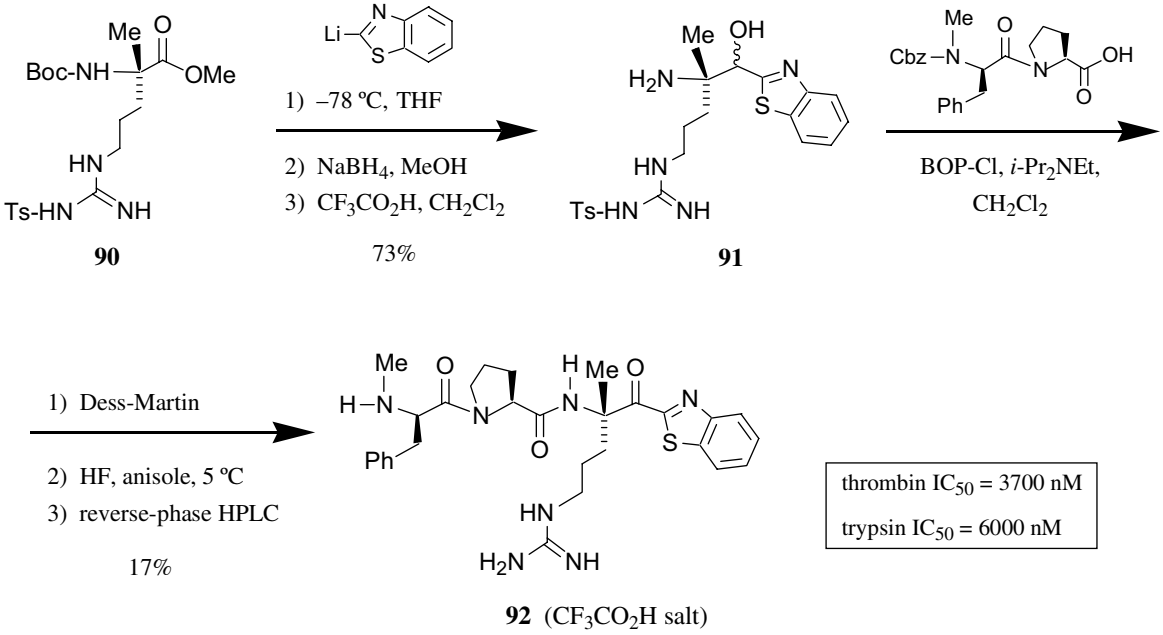


Figure 14. Synthesis of **92**, the α-methylarginine analogue of **8a**, via lithio-heterocycle addition to an ester [BOP, bis(2-oxo-3-oxazolidinyl)phosphonic]. See caption of Figure 11 regarding the Dess–Martin oxidation.

duced and deprotected to supply **91** in 73% overall yield.[33b,148] This chemistry led to target **92** (14:1 diastereomeric mixture), the α-methylarginine analogue of **8a** (Fig. 14).[33b]

Liebeskind and coworkers have developed a procedure for preparing N-protected peptidyl ketones with high enantiomeric purity from peptidyl thiol esters by coupling with aryl and heteroaryl boronic acids.[149] This mild chemistry could conceivably be used to obtain α-ketoheterocycle-based enzyme inhibitors.

The coupling of organometallic reagents with acid chlorides has been used in certain systems.[127,141a]

5.4. Aldehyde-addition chemistry

Lithio-heterocycles, and related metallo species, can be added to aldehydes to give intermediate α-hydroxy heterocycles en route to targeted enzyme inhibitors. Some examples of this method are contained in the synthesis of α-ketotetrazole thrombin inhibitor **29d** (Fig. 15);[48] the conversion of Boc-prolinal into Pro-Pro-Het inhibitors of PEP (viz. **55**;

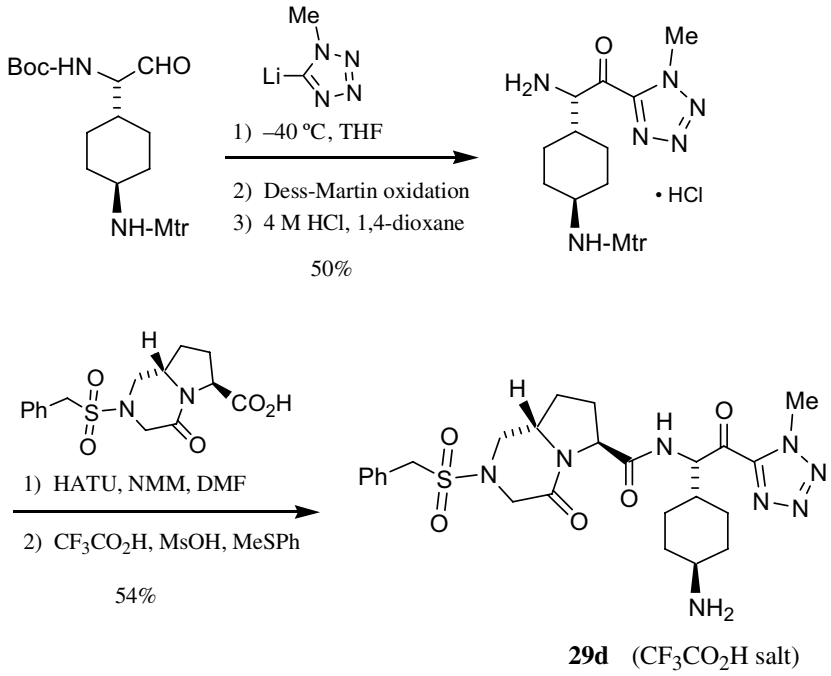


Figure 15. Synthesis of **29d** via aldehyde-addition chemistry [HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Ms, mesyl; Mtr, (4-methoxy-2,3,6-trimethylphenyl)sulfonyl; NMM, *N*-methylmorpholine]. See caption of Figure 11 regarding the Dess–Martin oxidation.

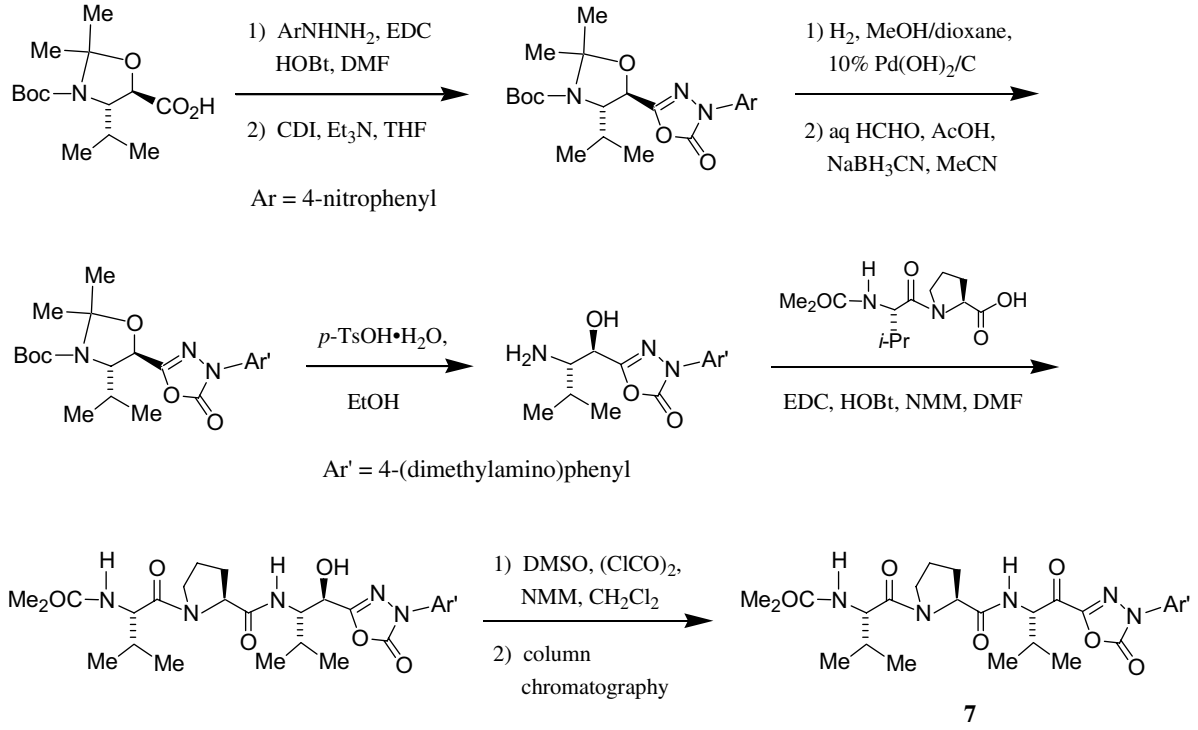
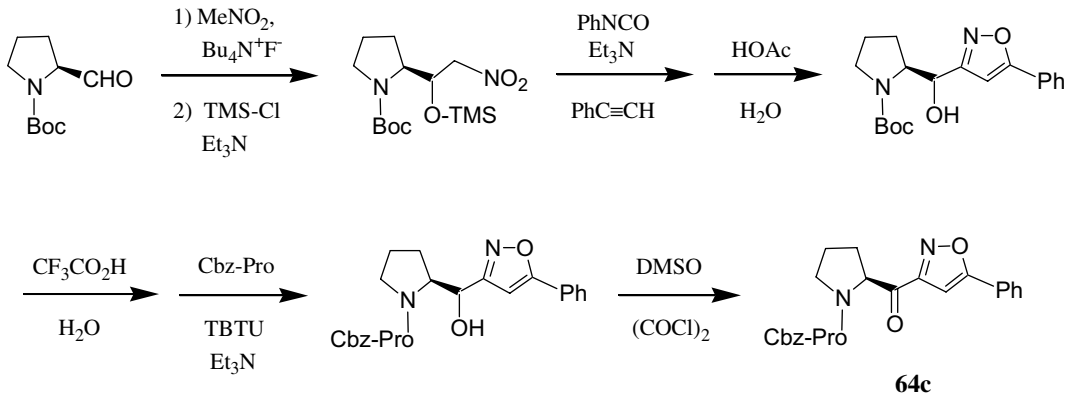


Figure 16. Synthesis of **7** [CDI, 1,1′-carbonyldiimidazole; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine].

Table 16);[88b] and the synthesis of certain FAAH inhibitors.[145a]

The chemistry developed by Dondoni and coworkers with silyl-substituted heterocycles can also be useful,[150] especially for α-keto-2-oxazoles since 2-lithio-oxazoles can be thermally unstable to ring-opening.[12b] Edwards and coworkers capitalized on this reaction with 2-(trimethylsilyl)oxazole to transform a tripeptide aldehyde into elastase inhibitor **3e** (Table 1).[12b]



64c

Figure 17. Synthesis of **64c** [TBTU, *N*,*N*, *N*′,*N*′-tetramethyluronium tetrafluoroborate; TMS, trimethylsilyl].

5.5. Other chemistry

Various α-keto-1,3,4-oxazolidin-2-one derivatives, such as orally active HNE inhibitor **7**, were prepared by using hydrazide cyclization chemistry (Fig. 16).[21]

Bal et al. relied on de novo heterocycle assembly via 1,3-dipolar cycloaddition chemistry to obtain α-keto-3-isoxazole and α-keto-3-isoxazoline inhibitors of POP Tc80 (e.g., Fig. 17).[110]

6. Concluding remarks

It is abundantly clear that α-ketoheterocycle derivatives can furnish potent inhibitor molecules for a wide variety of protease enzymes, as well as for the amide hydrolase FAAH. However, this approach to inhibitor design is not applicable universally. With some enzyme targets, α-ketoheterocycles have provided inferior inhibitors compared with other electrophiles, such as the aldehyde, trifluoromethylketone, α-keto amide, or boronic acid groups. On the other hand, there are comparative cases where α-ketoheterocycles have yielded better inhibitors (except, perhaps, relative to the boronic acid group). Consequently, in working with a new enzyme target of interest, it would be advisable to implement the following game plan: explore several different types of electrophilic groups *and* explore different types of α-ketoheterocycles. With respect to α-ketoheterocycles, one ought to adhere to some crucial design conditions. (1) The heterocycle should be one that attracts electron density sufficiently to activate the ketone carbonyl for nucleophile addition. This can be readily achieved by a collection of π-rich heterocycles that possess an sp^2 hybridized nitrogen next to the position of carbonyl attachment. (2) The electrophilic ketone must be positioned properly within the enzyme active site to take full advantage of the catalytic residue that it is expected to trap. (3) A secondary interaction with the catalytic machinery should be installed. Fortunately, the above-mentioned sp^2 nitrogen can serve a dual purpose and accomplish this objective. (4) Enzyme–ligand interactions within the S4–S3–S2–S1 regions, or a subset thereof, should be incorporated, such as from a substrate-based recognition motif. Having additional interactions in the S1′–S2′ area can also be valuable, such as from appending suitable substituents to the heterocycle.

So, what might the future hold for enzyme inhibitors that rely on α-ketoheterocycles? Whereas much research has been directed to finding inhibitors of diverse proteases, there has been much less attention devoted to amide hydrolases (amidases). Indeed, the successful results with inhibitors of FAAH bode well for further applications in this arena. At the risk of going out on a limb, we suggest that α-ketoheterocycle-based enzyme inhibitors could play an important role in the field of esterases, many of which utilize serine-dependent catalysis. Some limited work with esterases has already been touched on amidst the studies with FAAH inhibitors.[137–139,141a,b,144,145a]

There has been debate about whether transition state analogue-based inhibitors, which form weak covalent bonds with the enzyme, would be suitable as marketed drugs.[27d,f] There may be issues as to their drug delivery and immunogenic potential. Such molecules can be problematic in terms of supplying quality clinical candidates because of undesirable pharmacokinetics. Aspects of this problem, as it pertains to inhibitors of proteases, may relate to the need for peptide-like structures and high molecular weights (>500 Da). The case of thrombin inhibitors can be instructive in that some issues apply regardless of the type of inhibitor. Currently, there are no orally administered, direct thrombin inhibitors, either 'covalent' or 'noncovalent', on the global market. Although the noncovalent inhibitor ximelagatran was introduced in 2006, it had to be withdrawn shortly thereafter because of toxicity issues.[31] Since ximelagatran is actually a double prodrug of melagatran, special structural manipulation was required to obtain the desired oral drug-like properties. Other noncovalent thrombin inhibitors that entered the clinic have not progressed to the stage of marketing approvals.[27a–e] Thus, neither covalent nor noncovalent thrombin inhibitors have delivered on the ultimate goal of a marketed oral drug. For thrombin inhibitors at least, it would seem that the verdict is still out as to the relative advantages or disadvantages of these two inhibitor types in medical practice.

Considering orally delivered drugs, it has been difficult for pharmaceutical companies to develop marketable protease inhibitors. Nevertheless, there have been some notable successes, among which are inhibitors of ACE,[1,151] DPP-IV,[93] HIV protease,[1,152] renin,[153] and β-lactamases.[106a,b,154] Considering parenteral-only drugs, there are also marketed inhibitors of elastase,[9d] thrombin,[27–29] and the 20S proteasome.[95a,b] Orally active compounds that inhibit other proteases, such as fXa,[57c] HCV NS3·4A protease,[103] cat K,[113,114] and cat S,[155] have advanced into human clinical trials. While there are no marketed α-ketoheterocycle-based enzyme inhibitors at present, it is encouraging that several orally bioavailable compounds have surfaced,[15,21,42,54,81,82,89,94] including the clinical agent **6f** (ONO-6818).[15] With this backdrop, it may not be too farfetched to envision the possible emergence of an α-ketoheterocycle-based drug in the future.

Acknowledgments

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