Secondary Structure Peptide Mimetics: Design, Synthesis, and Evaluation of *β*-Strand Mimetic Thrombin Inhibitors

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Constrained dipeptide mimetic templates were designed to mimic the secondary structure of peptides in a *β*-strand conformation. Two templates corresponding to the D-Phe-Pro portion of the thrombin inhibitor D-Phe-Pro-ArgCH_2Cl were synthesized and converted into nine α-ketoamide and α-ketoheterocycle inhibitors of thrombin. Additionally, a template corresponding to L-Phe-Pro was synthesized and converted to a thrombin inhibitor. The in vitro inhibition of thrombin by these compounds was determined, and those corresponding to the D-Phe-Pro were found to be more potent inhibitors than the L-Phe-Pro mimetic. The α-ketoamides were found to be more potent than the α-ketoheterocycles but had much slower on rates. By comparison of a series of α-ketoamide analogues, it is apparent that the there is a preference for binding of bulky hydrophobic substituents in the P′ portion of the thrombin active site. Three of the inhibitors (MOL098, MOL144, and MOL174) were screened against a series of coagulation and anticoagulation enzymes and found to be selective for inhibition of the coagulation enzymes. Two of the inhibitors were tested in in vitro models of intestinal absorption and found to have low absorption potential. The compounds were then tested in vivo in both rats and primates, and one of them (MOL144) was approximately 25% absorbed in both species. This study has delineated the synthesis of constrained dipeptide *β*-strand mimetics and validated the potential for compounds of this type as potent thrombin inhibitors and possible drug leads.

Introduction

The utilization of 20 amino acid side chains, displayed on a limited array of topological templates (i.e., common secondary structure motifs such as reverse turns, *β*-strands, and α-helices) provides the elements required to delineate ligand−receptor and enzyme substrate/inhibitor interactions for a multitude of processes. We have been engaged in the development of mimics of these secondary structure motifs[1,2] and in particular the utilization of combinatorial libraries of these "privileged templates" to probe biological structure−function relationships and to develop orally available pharmaceutical agents.

The trypsin-like serine proteases form a large and diverse family of enzymes which includes the enzymes in the blood coagulation cascade. Sequencing of these proteases has shown the presence of a homologous trypsin-like core with insertions that can modify specificity and which are generally responsible for interactions with other macromolecular components.[3] With regard to the recognition of proteolytic substrates and proteinaceous inhibitors by their cognate enzymes, inspection of numerous X-ray crystal structures has highlighted the fact that an extended strand motif is uniformly adopted by the inhibitor/substrate in the active site.[4] Inhibition of certain enzymes in this family may be beneficial in ameliorating associated disease states. One potential target is the serine protease thrombin which is key among the enzymes in the blood coagulation cascade in thrombus formation.

Thrombin exhibits remarkable specificity in the removal of fibrinopeptides A and B of fibrinogen, through the selective cleavage of two Arg−Gly bonds of the 181 Arg/Lys-Xaa sequences in fibrinogen.[5] Fibrinogen is subsequently cross-linked by factor XIIIa, which is also activated by thrombin, giving an insoluble fibrin clot. Thrombin also activates platelets by binding to a specific PAR-1 receptor on the cell surface and cleaving between residues Arg41 and Ser42 to produce a tethered ligand which activates the receptor.[6] Activation of platelets results in shape change, platelet aggregation, and release of secretory granules, all of which strengthen the blood clot. Thrombin also proteolytically activates factors V and VIII, thereby amplifying its own production.

PPACK (D-Phe-Pro-ArgCH_2Cl), a compound which had earlier reached phase II clinical evaluation, has been shown to be a potent inhibitor of thrombin,[7] and many thrombin inhibitors based on this tripeptide sequence have been synthesized.[8,9] The X-ray crystal structure of PPACK−thrombin has been determined and reveals that the peptide binds in an extended strand conformation similar to that of thrombin's natural substrate, fibrinogen.[10] The templates **1** and **2** (Chart 1) were designed as mimics for an extended *β*-strand secondary structure. We have synthesized these templates with substituents chosen to match those of the D-Phe-Pro portion of PPACK and converted these to a number of potent and selective inhibitors of thrombin.

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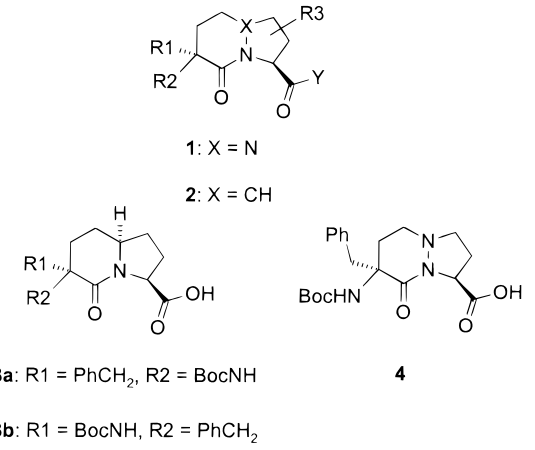
Chart 1. Bicyclic β-Strand Mimetic Templates



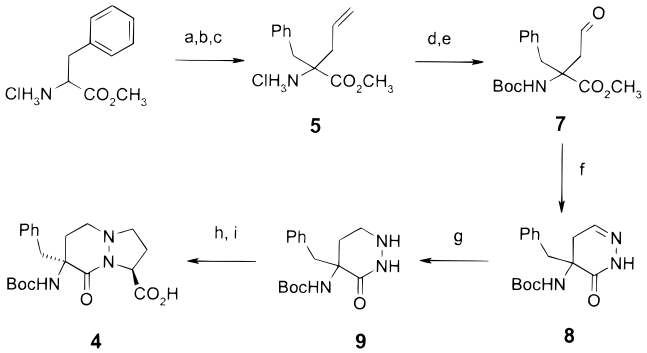
Results and Discussion

Design and Modeling. The bicyclic structures **1** and **2** were chosen as structures that would rigidify the peptide backbone of the peptidomimetic inhibitors and place the encompassed functional groups in approximately the orientation of an idealized peptide. This strategy has been used extensively in the production of potential β-turn mimetics[11] but has not been used for the production of β-strand mimetics. The β-turn mimetics of this type generally encompass the *i+1* and *i+2* residues of the turn and position the *i* and *i+3* residues for formation of a hydrogen bond between the respective amide NH and carbonyl. However, bicyclic structures such as **1** and **2** also orient the atoms encompassed by the template in positions that are approximately the same as those in an extended strand. We chose to exploit this similarity.

Monte Carlo conformational searches were carried out on the β-sheet templates **1** and **2** with R1 = CH_3CONH, R2 = CH_3, R3 = H, and Y = $NHCH_3$ in a simulated water environment (GBSA solvation[12]) using MM2/MMOD force fields.[13] Force-field parameters for the angles and bond lengths were adopted from Amber* force field, and the N(sp^2)−N(sp^3) distance (1.41 Å) was from X-ray crystal data.[14,15] No dihedral force constants were assigned for the C−N−N−C. Low-energy conformations were compared with dialanine peptides with ideal parallel and antiparallel β-sheet conformations, (ϕ,ψ)=(−119,113) and (−139,135), respectively. Boltzmann population p_i for conformer i at room remperature (T = 300 K) was estimated from the energy difference of conformer i from the global minimum energy, assuming the energy barriers between the conformers are negligible, i.e., $p_i = [\exp(-\Delta E/RT)]/[\sum_{i-1}^{\infty} \exp(-\Delta E/RT)]$. The summations in the denominator were truncated at conformer j with ΔE_j = 50 kJ/mol. Boltzmann weighted average rmsd (root mean-square deviation) values at the seven superimposable heavy atom positions were 0.4 and 0.5 Å for **1** and 0.5 Å and 0.5 Å for **2** against parallel and antiparallel β-strands, respectively. More importantly, the templates **1** and **2** (R1 = CH_3, R2 = NH_2, R3 = H, and Y = NH_2) superimposed very well against BPTI, which is believed to represent a canonical loop proteolysis substrate mimic, at the P2−P3 sites and against PPACK with a Boltzmann average rmsd value of 0.2 and 0.4 Å, respectively.

Chemistry. For the production of potential inhibitors of thrombin, we chose to synthesize the templates **3** and **4** (Chart 1) with substituents matching those of PPACK. Syntheses of 2-oxo-3-(*N*-Boc-amino)-1-azabicyclo[4.3.0]-nonane-9-carboxylic acids (**3**) have been published previously.[16,17] The synthesis of racemic 3-(*N*-Bocamino)-3-benzyl-2-oxo-1,6-diazabicyclo[4.3.0]nonane-9-carboxylate (**4**) is detailed in Scheme 1.[18]

Scheme 1[a]

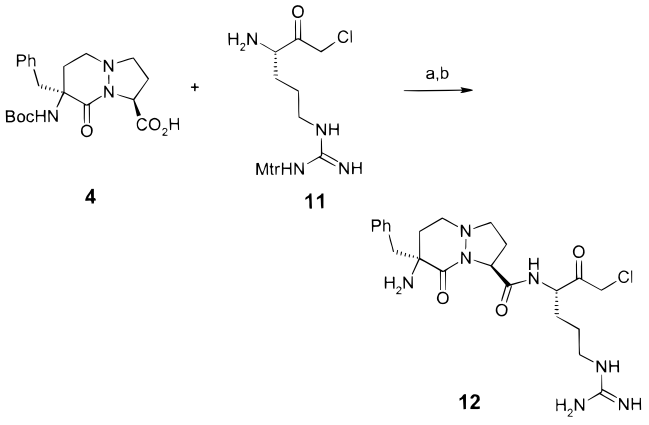


[a] Reagents and conditions: (a) PhCHO, Et_3N, CH_2Cl_2, rt; (b) LDA, −78 °C, then allyl bromide, −78 °C → rt; (c) 1 M HCl (aq), MeOH, rt, 1 h; (d) Boc_2O, $NaHCO_3$, THF/H_2O, rt, 3 days; (e) O_3, CH_2Cl_2/MeOH; (f) hydrazine, THF, reflux, 3 days; (g) Pt_2O (cat.), H_2, MeOH, rt, 48 h; (h) HCHO, ethyl acrylate, reflux; (i) LiOH (2 equiv, 1 M aq), THF, rt, 1.5 h.

The benzaldimine of phenylalanine methyl ester was formed and alkylated with allyl bromide, and the imine was hydrolyzed to give (±)-α-allylphenylalanine methyl ester, **5**. Protection of **5** as the *N*-Boc derivative and ozonolysis of the olefin followed by reductive workup gave the aldehyde ester **7** needed for formation of the heterocyclic core of the peptide mimetic. Treatment of the aldehyde ester with hydrazine at room temperature gave mixtures of cyclic hydrazone **8** and isomeric acyclic hydrazones. TLC and NMR evidence indicated rapid formation of the acyclic hydrazone and slow formation of the heterocycle. It was found that heating the reaction mixture for extended periods gave the desired cyclic hydrazone **8** in good yield. The slow rate of this cyclization is probably due to a combination of slow reaction of the hindered ester and equilibration of the acyclic hydrazone isomers. Reduction by sodium cyanoborohydride mediated by *p*-toluenesulfonic acid[19] and basic hydrolysis of the cyanoborane intermediate was inefficient and capricious. However, hydrogenation of **8** over Adam's catalyst was more reproducible and cleanly yielded the tetrahydropyridazinone **9**. The 1,3-dipolar cycloaddition of the iminium ion, formed by treatment of **9** with formaldehyde, in the presence of a large excess of ethyl acrylate gave a mixture of diastereomeric and regioisomeric products from which the desired bicyclic template could be isolated in 27% yield. Hydrolysis of the ester gave the Boc-protected amino acid template **4**.

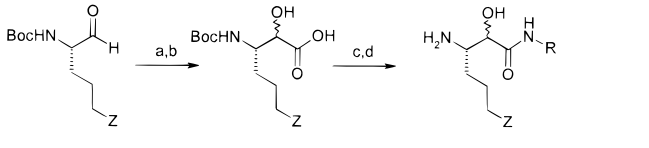
An inhibitor of thrombin, **12** (MOL098), was produced by coupling the template with HArg(Mtr)CH_2Cl (**11**) followed by TFA deprotection (Scheme 2). Although the thrombin inhibitory activity of **12** was good, rivaling that of PPACK, the possibility of toxicity due to nonspecific alkylation of the highly reactive chloromethyl ketone led us to pursue alternative inhibitory strategies. We chose to synthesize a number of arginine derivatives with activated electrophilic carbonyl groups in order to

Scheme 2[a]



[a] Reagents and conditions: (a) *i*-BuOCOCl, NMM, THF, −50 °C, 10 min then **11**; (b) TFA, H_2O, $PhSCH_3$, rt.

Scheme 3[a]



13a, b **14a, b**

a: Z=CH_2NHCbz
b: Z=NHC(=NBoc)NHMtr

16: R=cyclohexyl; Z=CH_2NHCbz
17a: R=benzyl; Z=NHC(=NH)NHMtr
17b: R=phenethyl; Z=NHC(=NH)NHMtr
17c: R=*p*-chlorophenethyl; Z=NHC(=NH)NHMtr
17d: R=*p*-methoxyphenethyl; Z=NHC(=NH)NHMtr

[a] Reagents and conditions: (a) $(MeS)_3CH$, *n*-BuLi, THF, −78 °C; (b) $HgCl_2$, HgO, MeOH, rt; (c) RNH_2, EDC, HOBt, *i*-Pr_2NEt, THF, rt; (d) *p*-TsOH, THF, rt or TFA, CH_2Cl_2, rt.

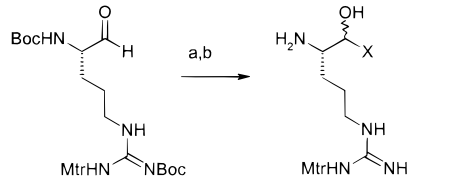
take advantage of the well-characterized covalent interaction of such groups with Ser195 of thrombin. The α-ketoester functionality had been shown to function well in this capacity in other active site inhibitors of elastase[20] and thrombin.[21]

BocArg(Mtr)-OH and BocLys(Cbz)-OH were homologated to the hydroxy acids **14a**,[22]**b**[23] via the aminoaldehyde derivatives **13** (Scheme 3). The hydroxy acids were coupled with several amines to produce the α-hydroxy amides **16** and **17a**−**d**. The Boc groups were selectively removed under acidic conditions to give the hydroxyamines used for coupling with the *β*-strand template.

Recently, α-ketoheterocycles have been employed as activated carbonyl compounds that function as covalent active site inhibitors of the serine proteases elastase[24] and prolyl endopeptidase.[25] After we had initiated our investigations of inhibitors of this type, a study reporting highly potent α-ketoheterocycle inhibitors of thrombin based on the D-Phe-Pro-ArgX motif was published.[26] Addition of 2-lithiothiazole and 2-lithiobenzothiazole to aldehyde **13b** was inefficient when stoichiometric amounts of the lithium reagents were used in THF but proceeded smoothly with a large excess of the reagent in diethyl ether. Selective removal of both Boc protecting groups using TFA in methylene chloride gave the amino alcohols **18** suitable for coupling with amino acid derivatives.

Coupling of the amino alcohols **16**, **17a**−**d**, and **18a**,**b** with the *β*-strand templates **3a** and **4** using EDC and

Scheme 4[a]

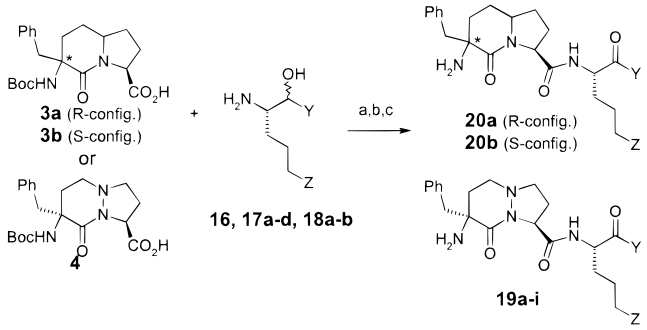


13b

18a: X=2-thiazolyl
18b: X=2-benzothiazolyl

[a] Reagents and conditions: (a) benzothiazole, *n*-BuLi, Et_2O, −78 °C; (b) TFA, CH_2Cl_2 (1:4), rt, 0.5 h.

Scheme 5[a]



[a] Reagents and conditions: (a) EDC, HOBt, *i*-Pr_2NEt, THF, rt; (b) Dess−Martin periodinane, CH_2Cl_2, rt; (c) TFA, H_2O, PhSMe (19:1:1), rt, HPLC separation.

Table 1

compd	corporate code	X	Y	Z
18a	MOL106	N	CONHcyclohexyl	CH_2NH_2
18b		N	$CONHCH_2Ph$	$NHC(=NH)NH_2$
18c	MOL126	N	$CONHCH_2CH_2Ph$	$NHC(=NH)NH_2$
18d	MOL150	N	$CONHCH_2CH_2Php$-Cl	$NHC(=NH)NH_2$
18e		N	$CONHCH_2CH_2Php$-OMe	$NHC(=NH)NH_2$
18f	MOL127	N	2-thiazolyl	$NHC(=NH)NH_2$
18g	MOL144	N	2-benzothiazolyl	$NHC(=NH)NH_2$
18h	MOL174	CH	2-benzothiazolyl	$NHC(=NH)NH_2$
18i	MOL168[a]	CH	2-benzothiazolyl	$NHC(=NH)NH_2$

[a] Synthesized from the diastereomer of the template shown in Scheme 5, i.e., template **3b**.

HOBt gave the corresponding amides as mixtures of four diastereomers (Scheme 5). Oxidation of the secondary alcohols with Dess−Martin periodinane gave the ketones which were deprotected with TFA to give the thrombin inhibitors as mixtures of two diastereomers. The diastereomers were separated by HPLC to give the inhibitors **19a**−**h** listed in Table 1. In an analogous manner, the diastereomer of the template **3b** was coupled, oxidized, and deprotected to produce the inhibitor **19i**. This inhibitor was produced in order to ascertain the importance of the D-configuration for potent thrombin inhibition as precedented in the peptide analogues.

Biology. The results of in vitro assays of the inhibitors against thrombin are summarized in Table 2. It is clear that the templates mimic the peptide conformation in the D-Phe-Pro-Arg thrombin inhibitors well as **12** showed thrombin inhibitory activity nearly identical to that of PPACK. The α-ketoamide inhibitors (**19a**,**c**,**d**) are slow tight-binding inhibitors of thrombin, whereas the α-ketoheterocycle inhibitors have a much higher on rate. This is readily apparent during enzyme assays of these compounds. Although all compounds were equilibrated with the enzyme for 30 min prior to assay, the α-ketoheterocycle inhibitors gave similar results when

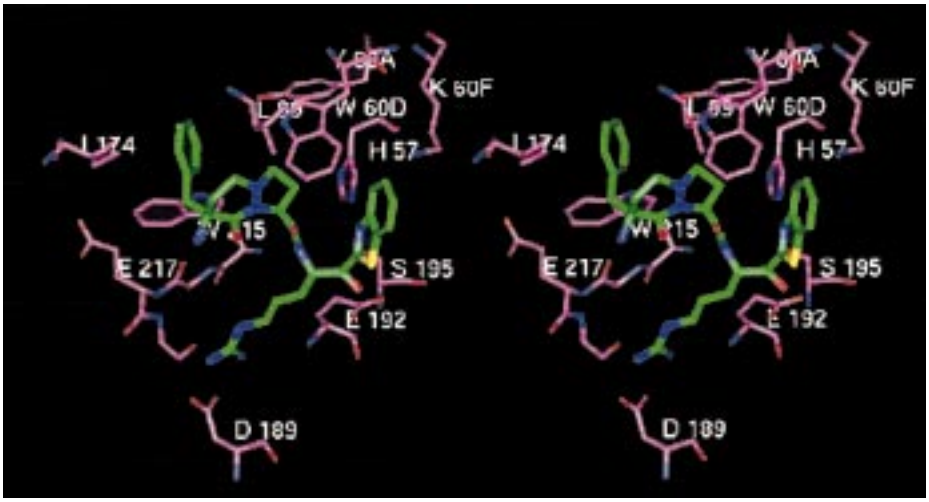


Figure 1. Modeled **18g** bound to human α-thrombin active site based on the X-ray crystal structures of **18f**,**i**.

Table 2. Inhibition of Thrombin and Trypsin and Thrombin On Rate

compd	corporate code	thrombin K_i (nM)	thrombin K_{on} (1/M·s)	trypsin K_i (nM)
PPACK		1.5[a]	ND	ND
12	MOL098	1.2[a]	2.4×10^6	ND
18a	MOL106	2000	ND	ND
18b		0.073	8.8×10^5	0.073
18c	MOL126	0.071	6.4×10^5	0.027
18d	MOL150	0.31	3.8×10^5	ND
18e		0.45	2.5×10^5	ND
18f	MOL127	2.4	$>10^7$	ND
18g	MOL144	0.65	$>10^7$	0.64
18h	MOL174	0.85	$>10^7$	0.23
18i	MOL168	10	$>10^7$	ND

[a] IC_{50} (nM).

no preequilibration was used suggesting a much higher on rate. The actual on rates were measured,[27] and as can be seen in Table 2, the ketoheterocycles are much higher. The reasons for these kinetic differences are not yet known; however, the α-ketoamides have a high propensity for hydrate, hemiacetal, or hemiaminal (with the guanidine side chain) formation in protic media. The solution kinetics of the α-ketoamides may have some effect on the kinetics of interactions with proteolytic enzymes.[28] Although the inhibitory potency of the α-ketoamides is high, the slow on rate was expected to be a significant factor in in vivo efficacy, and indeed the in vivo results were poorer (see discussion below). **19i** suffered a greater than 10-fold loss in potency as compared to **19h** verifying that the configuration corresponding to D-phenylalanine at the P3 site is optimal for binding of the template to thrombin. X-ray crystallographic data shows that the hydrophobic phenyl ring of **19i** is in a solvated region of thrombin which probably contributes significantly to the decrease in binding and lower K_i.[29]

Although a large number of inhibitors of thrombin have been synthesized and their thrombin complexes studied by X-ray crystallography, little is known about interactions of inhibitors with the P′ residues of thrombin. From the ketoamide inhibitors investigated in this study, a few inferences can be made regarding P′ selectivity. **19a** is a lysine version of the inhibitors with a bulky cyclohexyl moiety near the scissile bond. From previous studies[30] it can be surmised that a change from arginine to lysine results in a ca. 100-fold loss in potency; however, **19a** suffers a 6400−2800-fold loss in potency as compared to the other ketoamides in this study. The additional loss in inhibitory potency may be attributed to poorer interactions of the shorter nonaromatic cyclohexane group with the P′ residues of thrombin. X-ray crystallographic studies[27] indicate that bulky S′ groups can be accommodated by thrombin; however, it appears that such groups require flexibility in order to attain a conformation that will increase interactions with P′ residues. The ketoamide inhibitors that incorporate benzyl and phenethyl groups in the S′ site have much better inhibitory potency than **19a**. The length of the spacer seems to have little effect on the P′ interactions as **19b** (Y = CONHbenzyl) and **19c** (Y = CONHphenethyl) have similar thrombin K_i values. Substitution on the phenyl ring is detrimental to thrombin inhibition as both **19d**,**e** suffered losses in potency as compared to **19c**.

X-ray crystallography has shown that the α-ketoheterocycles bind with the heterocycle in a position similar to that of the cyclohexyl in **19a**. However, the interaction of the aromatic S′ residue is much stronger than that of the cyclohexyl moiety. We have used the X-ray crystal structures of thrombin-bound **19f**,**i** to model the interactions of **19g** bound to human α-thrombin (Figure 1). The major P′ contributions to this increase in thrombin binding appear to be a favorable stacking interaction between the aromatic heterocycle and Trp60D and a hydrogen bond to His57 of thrombin. **19g** binds in the active site and mimics the extended strand conformation of PPACK bound to thrombin very well. The electrophilic carbonyl is bound to Ser195 in the active site, and the guanidyl side chain extends through the specificity pocket and interacts with Asp189. The bicyclic core of the mimetic is embedded in the apolar S2 region of the active site, and the five-membered ring closely mimics the proline of PPACK. The phenyl ring of **19g** (D-Phe-like portion) is positioned within the aryl D-53 binding site and interacts with Leu99, Ile174, and Trp215.

12 and **19g**,**h** were tested more extensively in in vitro assays for inhibition of coagulation and anticoagulation enzymes (Table 3). Comparison of the data for PPACK with those of the constrained β-strand indicates that the template imparts greater selectivity for thrombin than the more flexible peptide. The selectivity of the α-keto-

Table 3. Inhibition and Selectivity of Coagulation vs Anticoagulation Enzymes

enzyme[a]	PPACK[b]	**12** (MOL098)[b]	**19g** (MOL144)[c]	**19h** (MOL174)[c]
thrombin	1.5 (1)	1.2 (1)	0.65 (1)	0.085 (1)
factor Xa	165 (110)	385 (320)	270 (415)	19.3 (230)
factor VIIa	200 (133)	140 (117)	270 (415)	200 (2300)
protein C	281 (187)	528 (440)	3320 (5110)	1250 (15000)
plasmin	699 (466)	978 (815)	415 (640)	251 (3000)
urokinase	508 (339)	927 (772)	600 (920)	335 (4000)
t-PA	106 (70)	632 (527)	495 (760)	93 (1100)

[a] Inhibitor concentration (selectivity = K_i other/K_i thrombin). [b] IC_{50} (nM). [c] K_i (nM).

heterocycle inhibitors is even greater showing particularly good selectivity for thrombin over the anticoagulation enzymes (protein C, plasmin, urokinase, and t-PA). This trend is especially evident in **19h** where the selectivity is greater than 1000-fold for thrombin versus all of the anticoagulation enzymes tested.

19g,**d**,**h** were evaluated in an in vivo arterio-venous shunt thrombosis model in baboons.[31] This model allows for evaluation in real time of ^{111}In platelet deposition on a dacron thrombogenic surface. Subsequently, fibrinogen deposition can also be evaluated. Administration of the agent was performed locally, immediately upstream of the shunt via continuous iv infusion. Both **19g**,**h** at 1 μg/min were very effective in blocking platelet deposition, whereas **19d** despite having a more potent equilibrium K_i than **19g** was not as effective presumably due to the lower K_{on}.

On the basis of these considerations, **19g**,**h** were evaluated for their bioavailability both in vitro and in vivo. The potential for oral absorption was estimated using IAM chromatography.[32] The log(k'_{IAM}/MW) values calculated for **19g**,**h** were 0.031 and −0.709, respectively. It is difficult to extrapolate these values to prediction of oral absorption since a structurally similar set of compounds has not been evaluated using this system.[33] However, comparison of **19g**,**h** indicates that, of these two, **19h** has a slightly higher potential for oral bioavailability. Evaluation of transepithelial permeability of **19g**,**h** in the Caco-2 cell assay[34] gave calculated P_{app} values of 0.30×10^{-6} cm/s for **19g** and 0.25×10^{-6} cm/s for **19h**.[35] On the basis of these data, the absorption potential of both compounds would be considered low.

The bioavailability of **19g**,**h** was evaluated in rats and nonhuman primates. The compounds were administered as solutions to conscious, catheterized animals via intravenous and oral routes. Serial plasma samples were analyzed for thrombin inhibitory activity and pharmacokinetic parameters calculated by noncompartmental methods. The bioavailability of **19g** approximated 25% in both rat and primate, while **19h** bioavailability approximated 2% in both species. Although **19g**,**h** display similar characteristics in the in vitro assays, **19g** was consistently at least 10-fold more bioavailable in vivo.

In summary, this work has delineated the use of constrained templates that mimic the β-strand secondary structure of proteins. These templates have been used for the production of a series of potent inhibitors of thrombin. Further utilization of this approach for the inhibition of proteases as well as other enzymes which recognize β-strand motifs will be reported in due course.

Experimental Section

Precoated analytical TLC plates were purchased from EM Science (silica gel 60 F_{254}) and were visualized with UV light or by treatment with phosphomolybdic acid (PMA). Flash chromatography was carried out on EM Science silica gel 60 with the solvent indicated. Preparative HPLC separations were accomplished on a Waters Symmetry C18 column operated at room temperature and eluted at a 1.5 mL/min flow rate, using a linear gradient of 100% H_2O containing 0.1% TFA−90%H_2O/MeCN containing 0.1% TFA over 30 min, with diode array detection. ^{1}H NMR data were obtained using a Varian Unity 500 spectrometer. Mass spectral data were obtained on a Fisons VG Quattro mass spectrometer operated in the mode indicated. Solvents and reagents were purchased from Aldrich Chemical Co. and used as received. THF was distilled from sodium benzophenone prior to use.

Synthesis of α-Allylphenylalanine Methyl Ester Hydrochloride (5). To a mixture of L-phenylalanine methyl ester hydrochloride (7.19 g, 33.3 mmol) and benzaldehyde (3.4 mL, 33.5 mmol) stirred in CH_2Cl_2 (150 mL) at room temperature was added triethylamine (7.0 mL, 50 mmol). Anhydrous magnesium sulfate (2 g) was added, and the mixture was stirred for 14 h and then filtered through a 1-in. pad of Celite with CH_2Cl_2. The filtrate was concentrated under reduced pressure to ca. one-half of its initial volume and then diluted with an equal volume of hexanes. The mixture was extracted twice with saturated aqueous $NaHCO_3$, H_2O, and brine and then dried over anhydrous Na_2SO_4 and filtered. Concentration of the filtrate under vacuum yielded 8.32 g of colorless oil. ^{1}H NMR analysis indicated nearly pure (>95%) phenylalanine benzaldimine. The crude product was used without further purification.

To a solution of diisopropylamine (4.3 mL, 33 mmol) stirred in THF (150 mL) at −78 °C was added dropwise a solution of *n*-butyllithium (13 mL of a 2.5 M hexane solution, 33 mmol). The resulting solution was stirred for 20 min; then a solution of phenylalanine benzaldimine (7.97 g, 29.8 mmol) in THF (30 mL) was slowly added. The resulting dark red-orange solution was stirred for 15 min; then allyl bromide (3.1 mL, 36 mmol) was added. The pale yellow solution was stirred for 30 min at −78 °C and then allowed to warm to room temperature and stirred an additional 1 h. Saturated aqueous ammonium chloride was added, and the mixture was poured into ethyl acetate. The organic phase was separated, washed with water and brine, and then dried over anhydrous sodium sulfate and filtered. Concentration of the filtrate under vacuum yielded 8.54 g of racemic α-allylphenylalanine benzaldimine as a viscous yellow oil.

To a solution of α-allylphenylalanine benzaldimine (5.94 g, 19.3 mmol) stirred in methanol (50 mL) was added 5% aqueous hydrochloric acid (10 mL). The solution was stirred at room temperature for 2 h and then concentrated under vacuum to an orange-brown caramel. The product was typically used without purification but could be recrystallized from $CHCl_3$/hexanes to give white crystals (3.56 g, 62% from phenylalanine): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.86 (3 H, br s, NH_3^+), 7.32−7.26 (5H, m, Ar*H*), 6.06 (1 H, dddd, J = 17.5, 10.5, 7.6, 7.3 Hz, C*H*=CH_2), 5.33 (1 H, d, J = 17.5 Hz, CH=CH_2), 5.30 (1 H, d, J = 10.5 Hz, CH=CH_2), 3.70 (3 H, s, COOCH_3), 3.41 (1 H, d, J = 14.1 Hz, ArCH_2), 3.35 (1 H, d, J = 14.1 Hz, ArCH_2), 2.98 (1 H, dd, J = 14.5, 7.3 Hz, CH_2CH=CH_2), 2.88 (1 H, dd, J = 14.5, 7.6 Hz, CH_2CH=CH_2).

Synthesis of *N*-(*tert*-Butoxycarbonyl)-α-allylphenylalanine Methyl Ester (6). To a solution of the crude D,L-α-allylphenylalanine hydrochloride, **5** (565 mg, 2.21 mmol), stirred in a mixture of THF (15 mL) and water (5 mL) was added di-*tert*-butyl dicarbonate followed by solid sodium bicarbonate in small portions. The resulting two-phase mixture was vigorously stirred at room temperature for 3 days and then diluted with ethyl acetate. The organic phase was separated, washed with water and brine, and then dried over anhydrous sodium sulfate and filtered. Concentration of the filtrate under vacuum yielded a colorless oil that was purified by column chromatography (5−10% EtOAc in hexanes gradient elution)

to yield 596 mg (84%) of *N*-(*tert*-butyloxycarbonyl)-α-allylphenylalanine: TLC $R_f = 0.70$ (silica, 20% EtOAc in hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26−7.21 (3 H, m, Ar*H*), 7.05 (2 H, d, $J = 6.1$ Hz, Ar*H*), 5.64 (1 H, dddd, $J = 14.8$, 7.6, 7.2, 7.2 Hz, C*H*=CH_2), 5.33 (1 H, br s, BocN*H*), 5.12−5.08 (2 H, m, CH=CH_2), 3.75 (3 H, s, COOCH_3), 3.61 (1 H, d, $J = 13.5$ Hz, ArCH_2), 3.21 (1 H, dd, $J = 13.7$, 7.2 Hz, CH_2CH=CH_2), 3.11 (1 H, d, $J = 13.5$ Hz, ArCH_2), 2.59 (1 H, dd, $J = 13.7$, 7.6 Hz, CH_2CH=CH_2), 1.47 (9 H, s, COC(CH_3)$_3$).

Synthesis of 2-Benzyl-2-[(*tert*-butoxycarbonyl)amino]-4-oxobutanoate (7). Ozone was bubbled through a solution of 2.10 g (6.57 mmol) of the olefin **6** stirred with solid $NaHCO_3$ at −78 °C in a mixture of CH_2Cl_2 (50 mL) and methanol (15 mL) until the solution was distinctly blue in color. The mixture was stirred an additional 15 min; then dimethyl sulfide was slowly added. The resulting colorless solution was stirred at −78 °C for 10 min and then allowed to warm to room temperature and stirred for 6 h. The mixture was filtered, and the filtrate was concentrated under vacuum to 2.72 g of viscous pale yellow oil which was purified by chromatography (10−20% EtOAc in hexanes gradient) to yield 1.63 g (77%) of pure aldehyde **7** as a viscous colorless oil: TLC $R_f = 0.3$ (silica, 20% EtOAc in hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.69 (1 H, br s, C*H*O), 7.30−7.25 (3 H, m, Ar*H*), 7.02 (2 H, m, Ar*H*), 5.56 (1 H, br s, N*H*), 3.87 (1 H, d, $J = 17.7$ Hz, ArC*H*), 3.75 (3 H, s, OCH_3), 3.63 (1 H, d, $J = 13.2$ Hz, C*H*CHO), 3.08 (1 H, d, $J = 17.7$ Hz, ArC*H*), 2.98 (1 H, d, $J = 13.2$ Hz, C*H*CHO), 1.46 (9 H, s, OC(CH_3)$_3$).

Synthesis of 4-[(*tert*-Butoxycarbonyl)amino]-4-benzyldihydrohydropyridazin-3(2*H*)-one (8). To a solution of the aldehyde **7** (1.62 g, 5.03 mmol) stirred in THF (50 mL) at room temperature was added hydrazine hydrate (0.32 mL, 6.5 mmol). The resulting solution was stirred at room temperature for 10 min and then heated to reflux for 3 days. The solution was allowed to cool to room temperature and then concentrated under vacuum to 1.59 g of colorless foam. The foam was dissolved in ethyl acetate and filtered through a 2-in. plug of silica gel with ethyl acetate. Concentration of the filtrate under vacuum gave 1.16 g (76%) of the cyclic hydrazone **8** as a white foam: TLC $R_f = 0.7$ (50% EtOAc in hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (1 H, br s, N=C*H*), 7.32−7.26 (3 H, m, Ar*H*), 7.17 (1 H, br s, NN*H*), 7.09 (2H, m, Ar*H*), 5.55 (1 H, br s, BocN*H*), 3.45 (1 H, d, $J = 17.7$ Hz, ArC*H*), 3.29 (1 H, d, $J = 13.5$ Hz, CH_2CH), 2.90 (1 H, d, $J = 13.5$ Hz, CH_2CH), 2.88 (1 H, dd, $J = 17.7$, 1.3 Hz, ArC*H*), 1.46 (9 H, s, OC(CH_3)$_3$); MS (CI+, NH_3) *m*/*z* 304.1 (M + H$^+$).

Synthesis of 4-[(*tert*-Butoxycarbonyl)amino]-4-benzyltetrahydropyridazin-3(2*H*)-one (9). The cyclic hydrazone **8** (21.28 g, 70.15 mmol) was dissolved in 200 mL of methanol, and PtO_2 (1.59 g, 7.01 mmol) was added. The flask was flushed twice with H_2 gas; then a balloon filled with H_2 was attached, and the mixture was stirred vigorously overnight. An additional 0.80 g (3.52 mmol) of PtO_2 was added, and stirring under an H_2 atmosphere was continued for another day. The mixture was filtered through Celite with the aid of ethyl acetate; then the filtrate was concentrated under vacuum to a white foam. The foam was purified by flash chromatography (50% ethyl acetate/hexanes eluent) to give 14.37 g (67%) of the hydrazide **9** as a white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41−7.35 (4 H, m, Ar*H*, NHN*H*), 7.18 (2 H, m, Ar*H*), 7.08 (1 H, br s, N*H*NH), 5.32 (1 H, br s, BocN*H*), 3.47 (1 H, d, $J = 12.8$ Hz, ArCH_2), 3.38 (1 H, dd, $J = 12.8$, 5.5 Hz, NCH_2), 2.72 (1 H, d, $J = 12.8$ Hz, ArCH_2), 2.45 (1 H, ddd, $J = 15.1$, 15.0, 5.5 Hz, CH_2CH_2), 2.38−2.30 (2 H, m, CH_2CH_2), 1.44 (9 H, s, OC(CH_3)$_3$); MS (CI+, NH_3) *m*/*z* 306.2 (M + H$^+$).

Synthesis of Ethyl 7-Benzyl-7-[(*tert*-butoxycarbonyl)amino]-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxylate (10). To a solution of the cyclic hydrazide **9** (4.07 g, 13.32 mmol) stirred in ethyl acrylate (200 mL) at 90 °C was added formaldehyde (1.2 mL of a 37% aqueous solution). The mixture was heated to reflux for 4 h and then allowed to cool to room temperature and concentrated under vacuum to a white foam. The products were separated by column chromatography (5−10% acetone/$CHCl_3$) to yield 0.851 g of the highest R_f isomer of the bicyclic ester and 0.552 g (25% overall) from a second chromatography of impure fractions: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27−7.21 (3 H, m, Ar*H*), 7.09 (2 H, d, $J = 6.5$ Hz, Ar*H*), 5.59 (1 H, br s, BocN*H*), 4.52 (1 H, dd, $J = 9.1$, 3.4 Hz, C*H*CO$_2$Et), 4.21 (2 H, m, OCH_2CH$_3$), 3.40 (1 H, d, $J = 12.5$ Hz, ArCH_2), 3.32 (1 H, d, $J = 12.5$ Hz, ArCH_2), 3.10 (2 H, m, NCH_2), 2.79 (1 H, br m, NCH_2), 2.66 (1 H, br m, NCH_2), 2.66 (1 H, br m, CH_2CH_2), 2.54 (1 H, br m, CH_2CH_2), 2.46 (1 H, m, CH_2CH_2), 2.18 (1 H, m, CH_2CH_2), 1.44 (9 H, s, OC(CH_3)$_3$), 1.28 (3 H, t, $J = 7.0$ Hz, OCH$_2$CH_3); MS (CI+, NH_3) 418.4 (M + H$^+$).

Synthesis of 7-Benzyl-7-[(*tert*-butoxycarbonyl)amino]-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxylate (4). To a solution of the ethyl ester **10** (1.383 g, 3.312 mmol) stirred in THF (30 mL) was added aqueous lithium hydroxide (1 M, 6.6 mL). The resulting mixture was stirred at room temperature for 1.5 h; then the reaction was quenched with 5% aqueous citric acid. The mixture was extracted twice with ethyl acetate; then the combined extracts were washed with water and brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to 1.30 g (100%) of white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34−7.29 (3 H, m, Ar*H*), 7.16−7.13 (2 H, m, Ar*H*), 5.44 (1 H, s, BocN*H*), 4.72 (1 H, dd, $J = 9.1$,5.7 Hz, C*H*CO$_2$H), 3.36 (1 H, d, $J = 12.9$ Hz, ArCH_2), 3.09 (1 H, d, $J = 12.9$ Hz, ArCH_2), 3.08, (2 H, m, NCH_2), 2.65 (1 H, dd, $J = 15.4$, 7.4 Hz, NCH_2), 2.55−2.37 (5 H, m), 1.44 (9 H, s, OC(CH_3)$_3$).

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(2-chloroacetyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (12). To a solution of the acid **4** (24 mg, 0.062 mmol) and *N*-methylmorpholine (0.008 mL) stirred in THF (1 mL) at −50 °C was added isobutyl chloroformate. The resulting cloudy mixture was stirred for 10 min; then 0.016 mL (0.14 mmol) of *N*-methylmorpholine was added followed by a solution of HArg(Mtr)CH_2Cl (**11**; 50 mg, 0.068 mmol) in THF (0.5 mL). The mixture was kept at −50 °C for 20 min and then was allowed to warm to room temperature during 1 h. The mixture was diluted with ethyl acetate and extracted with 5% aqueous citric acid, saturated aqueous sodium bicarbonate, and brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to 49 mg of colorless glass. Separation by column chromatography yielded 12 mg of less polar diastereomer and 16 mg of the more polar diastereomer: ^{1}H NMR (more polar diastereomer, 500 MHz, $CDCl_3$) δ 7.93 (1 H, br s, N*H*), 7.39−7.31 (3 H, m, Ar*H*), 7.16 (2 H, d, $J = 6.9$ Hz. Ar*H*), 6.52 (1 H, s, Mtr-Ar*H*), 6.30 (1 H, br s, N*H*), 5.27 (1 H, s), 4.74 (1 H, dd, $J = 9.1$, 6.9 Hz, C*H*CONH), 4.42 (1 H, br d, $J = 6.8$ Hz, CH_2Cl), 4.33 (1 H, d, $J = 6.8$ Hz, CH_2Cl), 3.82 (3 H, s, OCH_3), 3.28 (1 H, d, $J = 13.3$ Hz, ArCH_2), 3.26−3.12 (4 H, m, NCH_2, NHCH_2), 2.98 (1 H, d, $J = 13.3$ Hz, ArCH_2), 2.69 (3 H, s, ArCH_3), 2.60 (3 H, s, ArCH_3), 2.59−2.33 (4 H, m, NCH$_2$CH_2C, NCH$_2$CH_2CH), 2.25−2.10 (3 H, m,), 2.11 (3 H, s, ArCH_3), 1.77 (1 H, br m), 1.70−1.55 (3 H, br m), 1.32 (9 H, s, OC(CH_3)$_3$).

The more polar diastereomer (16 mg, 0.021 mmol) was dissolved in 95% TFA/H_2O (1 mL), and the resulting solution was stirred at room temperature for 48 h and then concentrated under vacuum. The residue was triturated with ether, washed twice with ether and then dried under high vacuum for 14 h. Purification of the product by HPLC yielded 5 mg of pure compound **12**: MS (EI+) *m*/*z* 477.9 (M$^+$).

Preparation of (3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-*N*-benyl-2-hydroxy-6-[[[(*tert*-butoxycarbonyl)imino]{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]amino}methyl]-amino]hexanamide (15a). To a solution of the acid **14b** (65 mg, 0.105 mmol), HOBT (17 mg, 0.126 mmol), and EDC (24 mg, 0.126 mmol) in THF (5 mL) was added benzylamine (14 μL, 0.126 mmol) followed by diisopropylethylamine (28 μL, 0.158 mmol). The reaction mixture was stirred at room temperature overnight and diluted with 5% citric acid. The organic layer was separated and the aqueous phase extracted with EtOAc (3×). The combined extracts were washed with saturated solution of $NaHCO_3$ and brine, dried over Na_2SO_4,

and filtered. After concentration the crude product was purified by chromatography (EtOAc/Hx, 1:1) to give 33 mg (44%) of the amide: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.83 (s, 1 H, $NHSO_2$), 8.34 (t, $J = 5$ Hz, 1 H, N*H*CH_2), 7.33 (m, 1 H, CONH), 7.29 (m, 5 H, aromatic), 6.53 (s, 1 H, *m*-benzene sulfonyl CH), 5.26 (d, $J = 9.5$ Hz, 1 H, N*H**Boc*), 4.85 (d, $J = 5$ Hz, 1 H, OH), 4.45 (m, 2 H, ArCH_2N), 4.19 (dd, $J = 5.5$, 3.0 Hz, 1 H, C*H*OH), 3.99 (m, 1 H, C*H*NH*Boc*), 3.82 (s, 3 H, OCH_3), 3.63 (m, 1 H, one guanidine CH_2CH_2), 3.18 (m, 1 H one guanidine CH_2CH_2), 2.68 and 2.58 (each s, 3 H, *o*-benzene sulfonyl CH_3), 2.12 (s, 3 H, *m*-benzene sulfonyl CH_3), 1.73−1.62 (m, 4 H, guanidine $CH_2CH_2CH_2$), 1.49 (s, 9 H, guanidine *Boc*), 1.38 (s, 9 H, NH*Boc*); MS (ES+) *m*/*z* 706.5 (M + H$^+$).

Preparation of (3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-*N*-phenethyl-2-hydroxy-6-[[[(*tert*-butoxycarbonyl)imino]-{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]amino}-methyl]amino]hexanamide (15b). Amide **15b** was prepared in a fashion similar to **15a** from the acid **14b** and phenethylamine: 26 mg (77%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.84 (s, 1 H, $NHSO_2$), 8.34 (t, $J = 5$ Hz, 1 H, N*H*CH_2), 7.28 (m, 3 H, aromatic), 7.21 (m, 2 H, aromatic), 7.04 (m, 1 H, CONH), 6.55 (s, 1 H, *m*-benzene sulfonyl CH), 5.16 (d, $J = 8.5$ Hz, 1 H, N*H**Boc*), 4.56 (d, $J = 5$ Hz, 1 H, OH), 4.11 (dd, $J = 5.0$, 3.0 Hz, 1 H, C*H*OH), 3.98 (m, 1 H, C*H*NHBoc), 3.84 (s, 3 H, OCH_3), 3.66 (m, 1 H, one guanidine CH_2CH_2), 3.51 (m, 2 H, ArCH_2-CH_2N), 3.17 (m, 1 H, one guanidine CH_2CH_2), 2.81 (t, $J = 7.5$ Hz, 2 H, ArCH_2CH_2N), 2.71 and 2.65 (each s, 3 H, *o*-benzene sulfonyl CH_3), 2.14 (s, 3 H, *m*-benzene sulfonyl CH_3), 1.68−1.52 (m, 4 H, guanidine $CH_2CH_2CH_2$), 1.49 (s, 9 H, guanidine *Boc*), 1.39 (s, 9 H, NHBoc); MS (FAB+) *m*/*z* 720.6 (M + H$^+$) (FAB−) *m*/*z* 718.5 (M−H$^+$).

Preparation of (3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-*N*-(4-chlorophenethyl)-2-hydroxy-6-[[[(*tert*-butoxycarbonyl)imino]{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]amino}methyl]amino]hexanamide (15c). Amide **15c** was prepared in a similar fashion to **15a** from the acid **14b** and 4-chlorophenethylamine: 55 mg (75%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.82 (s, 1 H, $NHSO_2$), 8.34 (t, $J = 5$ Hz, 1 H, N*H*CH_2), 7.25 (m, 2 H, aromatic), 7.12 (m, 3 H, aromatic and CONH), 6.55 (s, 1 H, *m*-benzene sulfonyl CH), 5.20 (d, $J = 9.0$ Hz, 1 H, N*H*Boc), 4.66 (d, $J = 5$ Hz, 1 H, OH), 4.11 (m, 1 H, C*H*OH), 3.99 (m, 1 H, C*H*NHBoc), 3.83 (s, 3 H, OCH_3), 3.64 (m, 1 H, one guanidine CH_2CH_2), 3.47 (m, 2 H, ArCH_2CH_2N), 3.17 (m, 1 H, one guanidine CH_2CH_2), 2.78 (t, $J = 7.5$ Hz, 2 H, ArCH_2CH_2N), 2.70 and 2.64 (each s, 3 H, *o*-benzene sulfonyl CH_3), 2.14 (s, 3 H, *m*-benzene sulfonyl CH_3), 1.68−1.52 (m, 4 H, guanidine $CH_2CH_2CH_2$), 1.49 (s, 9 H, guanidine Boc), 1.39 (s, 9 H, NH*Boc*); MS (ES+) *m*/*z* 754.6 (M + H$^+$).

Preparation of (3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-*N*-(4-methoxyphenethyl)-2-hydroxy-6-[[[(*tert*-butoxycarbonyl)imino]{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-amino}methyl]amino]hexanamide (15d). Amide **15d** was prepared in a fashion similar to **15a** from the acid **14b** and 4-methoxyphenethylamine: 57 mg (78%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.83 (s, 1 H, $NHSO_2$), 8.34 (t, $J = 5$ Hz, 1 H, N*H*CH_2), 7.11 (m, 2 H, aromatic), 7.04 (m, 1 H, CONH), 6.83 (m, 2 H, aromatic), 6.54 (s, 1 H, *m*-benzene sulfonyl CH), 5.22 (d, $J = 9.0$ Hz, 1 H, N*H*Boc), 4.66 (d, $J = 5$ Hz, 1 H, OH), 4.11 (m, 1 H, CH_2OH), 3.96 (m, 1 H, C*H*NHBoc), 3.83 (s, 3 H, benzene sulfonyl OCH_3), 3.78 (s, 3 H, aromatic OCH_3), 3.62 (m, 1 H, one guanidine CH_2CH_2), 3.46 (m, 2 H, ArCH_2CH_2N), 3.19 (m, 1 H, one guanidine CH_2CH_2), 2.74 (t, $J = 7.5$ Hz, 2 H, ArCH_2-CH_2N), 2.70 and 2.64 (each s, 3 H, *o*-benzene sulfonyl CH_3), 2.14 (s, 3 H, *m*-benzene sulfonyl CH_3), 1.70−1.52 (m, 4 H, guanidine $CH_2CH_2CH_2$), 1.49 (s, 9 H, guanidine *Boc*), 1.39 (s, 9 H, NH*Boc*); MS (ES+) *m*/*z* 750.7 (M + H$^+$).

Preparation of (3*S*)-3-Amino-*N*-benzyl-2-hydroxy-6-[(imino{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-amino}methyl)amino]hexanamide (17a). To a solution of **15a** (25 mg, 0.035 mmol) in THF (5 mL) was added of *p*-toluenesulfonic acid monohydrate (24 mg, 0.124 mmol). The reaction mixture was stirred at room temperature overnight to give a baseline spot by TLC. The solution was concentrated in vacuo and the residue washed twice with ether to give a yellowish-white solid: ^{1}H NMR (500 MHz, $CDCl_3$) consistent with the expected product; however, individual peak assignment was difficult due to signal broadening; MS (FAB+) *m*/*z* 506.4 (M + H$^+$).

Preparation of (3*S*)-3-Amino-*N*-phenethyl-2-hydroxy-6-[(imino{[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-amino}methyl)amino]hexanamide (17b). The amine **17b** was prepared in a fashion similar to **17a** from **15b**: MS (ES+) *m*/*z* 520.4 (M + H$^+$).

Preparation of (3*S*)-3-Amino-*N*-(4-chlorophenethyl)-2-hydroxy-6-[(imino{[(4-methoxy-2,3,6-trimethylphenyl)-sulfonyl]amino}methyl)amino]hexanamide (17c). The amine **17c** was prepared in a fashion similar to **17a** from **15c**: MS (ES+) *m*/*z* 554.5 (M + H$^+$).

Preparation of (3*S*)-3-Amino-*N*-(4-methoxyphenethyl)-2-hydroxy-6-[(imino{[(4-methoxy-2,3,6-trimethylphenyl)-sulfonyl]amino}methyl)amino]hexanamide (17d). The amine **17d.** was prepared in a fashion similar to **17a** from **15d**: MS (ES+) *m*/*z* 550.5 (M + H$^+$).

Synthesis of *N*-[4-Amino-5-(1,3-benzothiazol-2-yl)-5-hydroxypentyl]-*N*′-[(4-methoxy-2,3,6-trimethylphenyl)-sulfonyl]guanidine (18b). To a solution of benzothiazole (1.55 mL, 14 mmol) stirred in anhydrous diethyl ether (60 mL) at −78 °C under a dry argon atmosphere was added a solution of *n*-butyllithium (2.5 M in hexane, 5.6 mL, 14 mmol) dropwise over a period of 10 min. The resulting orange solution was stirred for 45 min; then a solution of the arginal **13b** (1.609 g, 2.819 mmol) in diethyl ether (5 mL) was slowly added. The solution was stirred for 1.5 h; then saturated aqueous ammonium chloride solution was added, and the mixture was allowed to warm to room temperature. The mixture was extracted with ethyl acetate (3 × 100 mL), and the combined extracts were extracted with water and brine and then dried over anhydrous sodium sulfate and filtered. Concentration of the filtrate under vacuum yielded a yellow oil that was purified by flash chromatography (30% then 40% ethyl acetate/hexanes eluent) to yield 1.22 g (61%) of the hydroxybenzothiazoles (ca. 2:1 mixture of diastereomers by ^{1}H NMR analysis) as a white foam.

The mixture of hydroxybenzothiazoles (1.003 g, 1.414 mmol) was stirred in CH_2Cl_2 (12 mL) at room temperature, and trifluoroacetic acid (3 mL) was added. The resulting solution was stirred for 1.5 h and then concentrated under reduced pressure to brown gum. The gum was triturated twice with diethyl ether; then the residue was dried under high vacuum to give 0.923 g (89%) of **17b** as a pale yellow foam: MS (EI+) *m*/*z* 506.2 (M + H$^+$).

Synthesis of *N*-[4-Amino-5-(1,3-thiazol-2-yl)-5-oxopentyl]-*N*′-[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]-guanidine (18a). 18a was synthesized in a fashion analogous to that of **18b** from the aldehyde **13b** and 1,3-thiazole in 67% overall yield.

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(1,3-benzothiazol-2-ylcarbonyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19g). The bicyclic acid **4** (151 mg, 0.387 mmol) and HOBt hydrate (71 mg, 0.46 mmol) were dissolved in THF (5 mL), and diisopropylethylamine (0.34 mL, 1.9 mmol) was added followed by EDC (89 mg, 0.46 mmol). After stirring for 10 min a solution of the amine **18b** (273 mg, 0.372 mmol) in THF (1 mL) was added along with a THF (0.5 mL) rinse. The mixture was stirred at room temperature for 15 h and then diluted with ethyl acetate and extracted sequentially with 5% aqueous citric acid, saturated aqueous sodium bicarbonate, water, and brine. The organic solution was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to 297 mg of a yellow glass: ^{1}H NMR a mixture of four diastereomeric amides; MS (ES+) *m*/*z* 877 (M$^+$).

The crude hydroxybenzothiazole (247 mg, 0.282 mmol) was dissolved in CH_2Cl_2 (5 mL), and Dess−Martin periodinane (241 mg, 0.588 mmol) was added. The mixture was stirred at room temperature for 6 h and then diluted with ethyl acetate and stirred vigorously with 10% aqueous sodium thiosulfate for 10 min. The organic solution was separated, extracted with

saturated aqueous sodium bicarbonate, water, and brine, and then dried over anhydrous sodium sulfate and filtered. Concentration of the filtrate under vacuum yielded 252 mg of yellow glass: ^{1}H NMR a mixture of two diastereomeric ketobenzothiazoles.

The ketobenzothiazole (41 mg, 0.047 mmol) was dissolved in 95% aqueous trifluoroacetic (0.95 mL) acid, and thioanisole (0.05 mL) was added. The resulting dark solution was stirred for 30 h at room temperature and then concentrated under vacuum to a dark brown gum. The gum was triturated with diethyl ether and centrifuged. The solution was removed, and the solid remaining was triturated and collected as above two more times. The yellow solid was dried in a vacuum desiccator for 2 h and then purified by HPLC to give 2.5 mg of **19g**: MS (ES+) *m*/*z* 563.5 (M + H$^+$).

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(cyclohexylamino)-2-(oxoacetyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19a). 19a was synthesized in a similar fashion from the acid **4** and the amine **16**.

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-{2-(phenethylamino)-2-oxoacetyl}butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-caoxamide (19b). 19b was synthesized in a similar fashion from the acid **4** and the amine **17a**: MS (ES+) *m*/*z* 536.6 (M + H$^+$).

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-{2-(phenethylamino)-2-oxoacetyl}butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19c). 19c was synthesized in a similar fashion from the acid **4** and the amine **17b**.

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-{2-[(4-chlorophenethyl)amino]-2-oxoacetyl}butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo-[1,2-*a*]pyridazine-1-carboxamide (19d). 19d was synthesized in a similar fashion from the acid **4** and the amine **17c**: MS (ES+) *m*/*z* 611.3 (M + H$^+$); HPLC t_R = 19.8 min.

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-{2-[(4-methoxyphenethyl)amino]-2-oxoacetyl}butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo-[1,2-*a*]pyridazine-1-carboxamide (19e). 19e was synthesized in a similar fashion from the acid **4** and the amine **17d**: MS (ES+) *m*/*z* 607.4 (M + H$^+$); HPLC t_R = 18.2 min.

Synthesis of (1*S*,7*S*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(1,3-thiazol-2-ylcarbonyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19f). 19f was synthesized in a similar fashion from the acid **4** and the amine **18a**: MS (ES+) *m*/*z* 513.5 (M + H$^+$).

Synthesis of (1*S*,7*R*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(1,3-benzothiazol-2-ylcarbonyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19h). 19h was synthesized in a similar fashion from the acid **3a** and the amine **18b**: MS (ES+): *m*/*z* 562.4 (M + H$^+$); HPLC t_R = 19.8 min.

Synthesis of (1*S*,7*R*)-7-Amino-*N*-[(1*S*)-4-{[amino(imino)methyl]amino}-1-(1,3-benzothiazol-2-ylcarbonyl)butyl]-7-benzyl-8-oxohexahydro-1*H*-pyrazolo[1,2-*a*]pyridazine-1-carboxamide (19i). 19i was synthesized in a similar fashion from the acid **3b** and the amine **18b**: MS (ES+) *m*/*z* 562.4 (M + H$^+$).

Inhibition Assays. Both thrombin and trypsin inhibition assays were performed at room temperature in 96-well microplates using either a Bio-Rad microplate reader (model 3550) or a Molecular Devices SpectroMax 250 or Labsystems Fluoroskan Ascent fluorescence plate reader. Solutions of 1 mM of testing compounds in water or 10 mM in DMSO served as the stock solutions for both of the inhibition assays. The hydrolysis of the chromogenic substrate *N*-*p*-tosyl-Gly-Pro-Arg-pNA (Sigma) was monitored at 405 nm, and the fluorogenic substrate *N*-*p*-tosyl-Gly-Pro-Arg-AMC (Sigma) was monitored at 345-nm excitation wavelength and 450-nm emission wavelength. The reaction progress curves were recorded by reading the plates, typically 100 times at 21-s intervals. Initial rates were determined by unweighted nonlinear least-squares fitting to a first-order reaction in either GraFit (Erithacus Software Ltd., London, England) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The determined initial velocities were then nonlinear least-squares fitted against the concentrations of a tested compound using GraFit or Prism to obtain K_i. The general format of these assays were 100 μL of a substrate solution and 100 μL of inhibitor solution were placed in a microplate well; then 50 μL of enzyme solution was added to initiate the reaction.

In thrombin assays, 0.05−0.2 nM human thrombin (Sigma T-6759) and 40 μM *N*-*p*-tosyl-Gly-Pro-Arg-pNA or 20 μM *N*-*p*-tosyl-Gly-Pro-Arg-AMC were used in pH 8.0 Tris buffer (Tris, 50 mM; Tween 20, 0.1%; BSA, 0.1%; NaCl, 0.15 M; $CaCl_2$, 5 mM).

In trypsin assays, 4 nM bovine trypsin (Sigma T-1005) and 40 μM *N*-*p*-tosyl-Gly-Pro-Arg-pNA or 20 μM *N*-*p*-tosyl-Gly-Pro-Arg-AMC were used in 50 mM Tris, pH 8.0, 0.15 M NaCl, and 5 mM $CaCl_2$ buffer containing 0.1% Tween 20 (v/v).

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