Unique Overlap in the Prerequisites for Thrombin Inhibition and Oral Bioavailability Resulting in Potent Oral Antithrombotics

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Despite intense research over the last 10 years, aided by the availability of X-ray structures of enzyme−inhibitor complexes, only very few truly orally active thrombin inhibitors have been found. We conducted a comprehensive study starting with peptide transition state analogues (TSA). Both hydrophobic nonpeptide analogues as well as hydrophilic peptidic analogues were synthesized. The bioavailability in rats and dogs could be drastically altered depending on the overall charge distribution in the molecule. Compound **27,** a tripeptide TSA inhibitor of thrombin, showed an oral bioavailability of 32% in rats and 71% in dogs, elimination half-lives being 58 and 108 min, respectively. The thrombin inhibition constant of compound **27** was 1.1 nM, and in an in vivo arterial flow model, the ED_{50} was 5.4 nmol/kg·min, comparable to known non-TSA inhibitors. A molecular design was found that combines antithrombotic efficiency with oral bioavailability at low dosages.

Introduction

An interesting area of drug discovery research deals with enzymes and receptors geared to recognize peptides or proteins. To find low molecular weight orally applicable ligands for these targets, high throughput screening of compound collections is frequently used. Alternatively, the peptides and proteins themselves form the basis for peptidomimetic approaches in which molecular modification leads to nonpeptide structures. Screening has been especially successful in finding small molecules for G-protein-coupled peptide receptors recognizing, for instance, angiotensin, substance P, cholecystokinin, vasopressin, or corticotropin-releasing factor.[1−5] In the area of protease inhibitors, however, screening has proven less effective, the most important exception being human immunodeficiency virus (HIV) protease. Researchers have relied on peptidomimetic approaches in areas such as elastase, renin, angiotensin-converting enzyme (ACE), and coagulation enzymes.[6−8] The latter class consists of serine proteases such as thrombin, factor Xa, and factor VIIa/TF. Over the past years, thrombin inhibitors, together with platelet receptor antagonists, have been the focal point of attention in pharmaceutical research aimed at finding new orally active antithrombotic agents.[9] Because thrombin plays a pivotal role in the blood coagulation cascade and various X-ray data on thrombin−ligand complexes became available in the early 1990s, a tremendous amount of research toward thrombin inhibitors has been performed.[10] However, thrombin turns out to be a very difficult molecular target for finding orally active antithrombotics. Thrombin expects a peptide in the catalytic cleft with a highly basic arginine moiety in its specificity pocket (P_1 pocket) and, being a serine protease, expects a carbonyl function at the cleavage site. The requirements for the P_2 and P_3 binding pockets are less stringent allowing for turn-inducing hydrophobic moieties of a certain size.[11] After a decade of intense research in many laboratories, only a few orally bioavailable thrombin inhibitors are known. Low antithrombotic potency and side effects in animal models further reduce the list as can be viewed in recent reviews.[12] Apparently, the quest for the ideal orally active thrombin inhibitor is still worthwhile to pursue.

Recently, we reported on several series of potent thrombin inhibitors with isosteric replacements for the highly basic S_1 binding moieties present in earlier leads and seen as prohibitive for intestinal absorption. Acylguanidines, aminoisoquinolines, and piperidines replaced guanidines, benzamidines, and primary amines, respectively, showing that the P_1 pocket can also accommodate more drug friendly entities.[13−15] Now, we wish to report on a comprehensive lead optimization study starting with peptide analogues that include transition state mimics and describe the stepwise improvement toward potent orally active antithrombotics. During this optimization process, publications appeared on the perceived utility of this class of thrombin inhibitors. Concerns were expressed that inhibition with transition state analogues (TSA) would result in low

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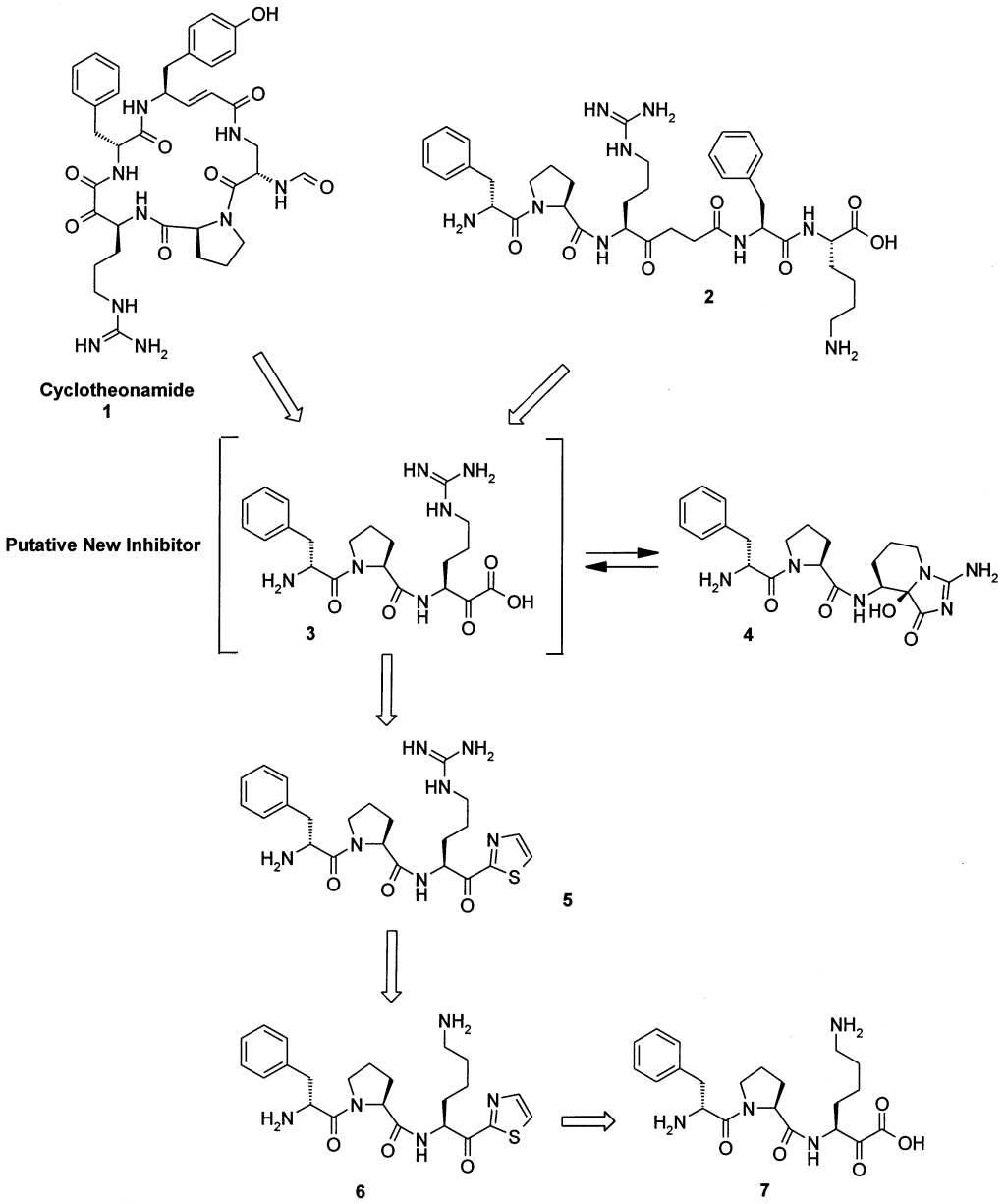
Scheme 1. Design of First Generation Peptidomimetics

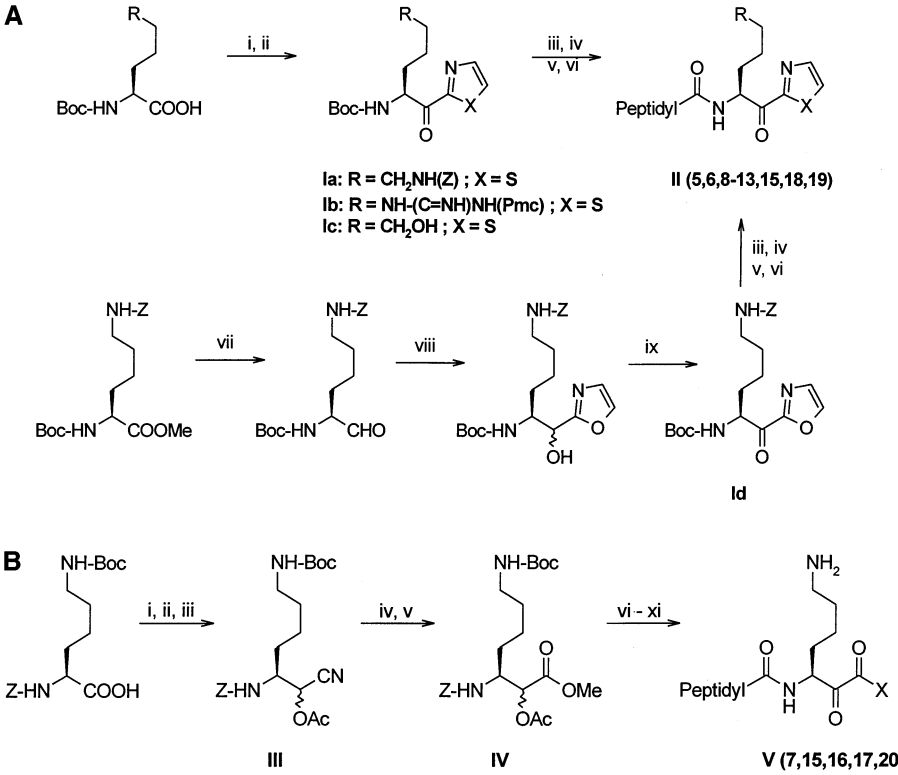


Compound	K_i (nM)
5	**0.3**
6	**146**
7	**1.4**

selectivity over related proteases and, especially, in low k_{on} rates due to slow tight binding kinetics. These points were viewed as being incompatible with the intended use of the inhibitors, i.e., blocking the fast generation of thrombin at the site of a lesion in acute situations.[16,17] Contrary to these concerns, it will be shown that effective antithrombotics, applicable in arterial thrombosis situations, can be generated from this class of transition state mimics.

Starting points for the present series of compounds were peptidic structures **1** and **2** (see Scheme 1) next to earlier work on D-Phe-Pro-Arg aldehydes[18] and boronic acids[19] and work on other serine protease inhibitors.[6] Compound **1** is a natural product called cyclotheonamide with a reported K_i (final) of 1.0 nM, and **2** is a transition state mimic that came out of our earlier research showing a K_i of 28 nM.[20,21] Although very potent in vitro, no oral bioavailability has ever been reported or found for **1** and **2**. The first design cycle started with a hybrid molecule **3** as a putative new inhibitor, and we evolved this into potent orally bioavailable thrombin inhibitors.

Scheme 2. General Strategy for the Synthesis of Thrombin Inhibitors Containing an Active Ketone Species[a]



[a] Panel A describes keto-heterocyclic peptidomimetics and panel B describes the diketo series. Series A: (i) TBTU, O,N-dimethylhydroxylamine·HCl, Et_3N, CH_2Cl_2. (ii) (1) *n*-BuLi/ether, 2-bromothiazole, −78 °C; (2) THF, −78 °C. (iii) 50% TFA/CH_2Cl_2. (iv) DCCI, HOBt, DMF, Et_3N. (v) TFA/thioanisole = 10/1 v/v%. (vi) Purification preparative HPLC. (vii) DiBAL-H, CH_2Cl_2, −78 °C. (viii) 2-(Trimethylsilyl)oxazole. (ix) 80 °C; Dess−Martin. Series B: (i) TBTU, CH_2Cl_2/CH_3OH, Et_3N. (ii) DiBAL-H, CH_2Cl_2, −78 °C. (iii) NaCN, $TEBAC_{cat}$, CH_2Cl_2/water. (iv) 3 M HCl_{gas} in diethyl ether/CH_3OH, 4 °C. (v) Boc_2O, DMF, Et_3N. (vi) H_2, 10% Pd/C, DMF-1 N HCl. (vii) DCCI, HOBt, DMF, Et_3N. (viii) Dioxane/water, pH 10. (ix) Dess−Martin. (x) TFA/CH_2Cl_2. (xi) Purification preparative HPLC.

Chemistry

Chemistry generally involved the stereoselective synthesis of peptide structures with an activated ketone at the C terminus allowing for a transition state type of interaction with thrombin's cleavage site. Essential in these syntheses is the preparation of the amino acid monomers with either a heterocycle or an additional ketone attached to the C terminus of these intermediates. Scheme 2A shows the conversion of an amino acid like lysine to a keto-(2-thiazolyl) derivative using Dondoni type alkylations:[22] first the N-methoxymethylamide[23] was prepared followed by reaction with 2-lithiothiazole at −78 °C. Next, the α amino protective group was removed with trifluoroacetic acid (TFA) in dichloromethane followed by standard peptide coupling reactions to provide fully protected end molecules. After the remaining protective groups were removed and preparative high-performance liquid chromatography (HPLC), the final products were obtained. The oxazole derivates were obtained via the aldehyde, prepared from Boc-Lys(Cbz)-OMe (Scheme 2A). Reaction of the aldehyde with 2-trimethylsilyloxazole and subsequent oxidation of the resulting alcohol yielded the keto-(2-oxazolyl) derivative. This ketone was applied in peptide chain elongation in the same way as the keto-(2-thiazolyl).

Scheme 2B shows a Pinner type reaction[24] sequence to allow for the introduction of additional ketones in the end products. Thus, Z-Lys(Boc)-OMe was first transformed to an aldehyde using diisobutylaluminumhydride (DiBAL-H) followed by a cyanohydrin formation using a phase transfer catalyst; the hydroxyl group, finally, was acetylated to allow for high yield work up procedures. The crucial step was the two step Pinner hydrolysis (III to IV) followed by an in situ Boc protection step. After the required peptide coupling steps, the tripeptides were oxidized using Dess−Martin periodinane reagent.[25] In our hands, this was one of the few reagents that was capable of oxidizing secondary hydroxyl groups while retaining the stereochemistry α to the oxidation site. Finally, deprotection and preparative HPLC purification yielded the products in this series. In accordance with Brady et al.,[16] it was found that the *S* configuration of the lysine moiety remained intact during the synthesis and in solution at low pH. At pH levels above 7.5, time-dependent epimerization could be detected. Biological assays were carried out using strict protocols and time limitations in order to minimize epimerization.

Results and Discussion

In an effort to combine structural features of the thrombin inhibitors **1** and **2**, tripeptide **3** became our initial target (Scheme 1). However, **3** was extremely difficult to synthesize and turned out to be unstable due to the presence of both the reactive keto-carboxylate moiety and the guanidine group. During synthesis, the compound cyclizes spontaneously resulting in the previously unknown heterocyclic compound **4**. The open and closed form appeared to be in a pH- and temperature-controlled creatine−creatinine equilibrium with each other. To avoid this side reaction, we stabilized the

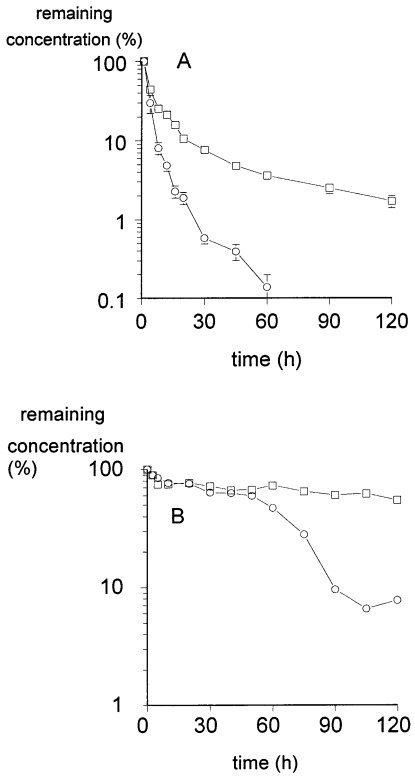


Figure 1. (A) Pharmacokinetics after intravenous administration to rats. Line graph showing dissappearance patterns of the anti-IIa activity of compound **5** (○) and compound **6** (□) after administration of 10 μmol/kg IV in rats. Data are expressed in percent remaining concentration as compared to $T = 1'$ and represent means of three separate experiments ± SEM. (B) Rate of disappearance in rat liver perfusion experiment. The disappearance patterns of the antithrombin activity of the same compounds (**5** (○) and **6** (□)) during liver perfusion are shown. Data are expressed in percent remaining concentration as compared to preperfusion level and represent means of two separate experiments.

molecule by replacing the reactive keto-carboxylate moiety by a keto-thiazole. Indeed, **5** was chemically stable and still an active thrombin inhibitor with a K_i of less than 1 nM. The *N*-methyl analogue of **5** was reported by Costanzo et al. showing a K_i of 3.6 nM for thrombin and no trypsin selectivity.[26,27] Because it was the new lead compound for further optimization studies, analogue **5** was subjected to a more elaborate pharmacological analysis. This resulted in the findings that the K_i ratios of related coagulation proteases over thrombin were generally high except for trypsin. Furthermore, enzyme kinetics revealed no slow binding characteristics of **5**. Unfortunately, in pharmacokinetic studies, less than 5% oral bioavailability was observed and the half-life was only about 5 min (see Figure 1). It was anticipated that efficient hepatic clearance had occurred as has been observed frequently with thrombin inhibitors bearing basic guanidine or benzamidine entities. In addition, haemodynamic side effects have been described for thrombin inhibitors comprising these highly basic groups.[28]

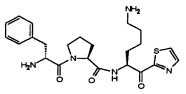
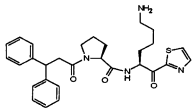
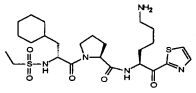
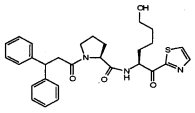
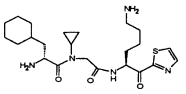
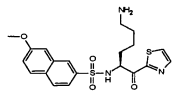
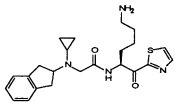
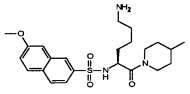
In the next round of optimization, we replaced the guanidine group in **5** with less basic or even neutral moieties. An additional advantage of these modifications is the increase in lipophilicity. A typical example is **6**, which utilizes a lysine instead of an arginine side chain. Although **6** did not show an improvement in oral bioavailability, the half-life of 46 min in rats was an improvement as compared to arginine derivative **5** (see Figure 1). Liver perfusion experiments revealed that **5** was cleared much faster than the corresponding lysine analogue **6**, which remained in circulation for several hours. However, compound **6** exhibited a K_i of 146 nM, warranting further optimization.

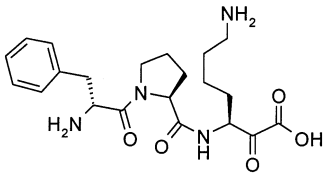
First, we resorted to keto-carboxylate derivative **7**, which turned out to be more active than analogue **6** (K_i of 1.4 nM) and also more stable than its arginine counterpart **3**. It is acknowledged that at the time our patents[29,30] were published describing these lysine derivatives, a number of publications appeared on related heterocycle-activated ketone derivatives and α-keto acids[16,17,31] and derivatives of the D-Phe-Pro-Arg motif.[26,27,32−34] Some of these papers describe the substitution of a lysine for an arginine group. More publications appeared in recent years on this subject focusing on potent thrombin inhibition and selectivity but with limited data on pharmacokinetic behavior.[35−37] To further improve oral bioavailability, two approaches were initiated starting from **6** and **7**. In the first approach, we aimed at improvement of passive diffusion, while in the second approach we incorporated structural features of compounds that are known to be absorbed by active or paracellular transport. The first approach involved the transformation of a tripeptide into a nonpeptide while the second route still allowed for peptidic characteristics.

Following the first approach, we took into account the Lipinski rules for passive diffusion.[38] Reducing the number of H bond donors and acceptors, minimizing the number of rotatable bonds, and lowering the molecular weight, etc. could be achieved. However, we could not obtain a potent thrombin inhibitor with improved Caco 2 permeability (see Table 1 for a representative set of compounds and results). The left panel of Table 1 shows lysine-ketothiazole derivatives with peptide character and therefore rather poor Lipinski scores but with nanomolar potency. The right panel depicts some key compounds such as **13** and **14** that may be described as nonpeptides with permeabilities of 16 and 81 nm/sec, respectively, but without any appreciable potency left.

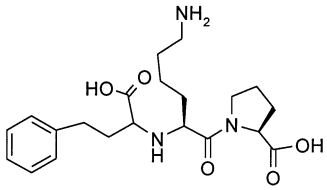
In the second approach, we compared the structural properties of our lead compounds with hydrophilic drugs absorbed at least in part through active transport. To this end, we scrutinized the structural properties of β-lactam antibiotics and ACE inhibitors (e.g., lisinopril). For these drugs, it has been reasoned that the presence of amine and carboxylate groups at specific distances as well as their resemblance to di- and tripeptides render them good substrates for peptide carriers in the intestine.[39,40] In addition, cyclic structures such as a pyrrolidine appear to be required and provide some constraint in these molecules. As shown in Figure 2, the resemblance between lead compound **7** and lisinopril is striking. To create an even better structural overlap, it was decided to introduce a carboxylate group in lead compounds **6** and **7**. In this approach, it was not considered to be necessary to lower the pK_a of the S_1 amine any further. A carboxylate scan was employed to introduce this acidic group at all synthetically accessible positions (see Table 2 for key compounds). Com-

Table 1. Results of the Nonpeptide Approach with In Vitro Thrombin Inhibitory Potency and Transport Across a Monolayer of Caco II Cells

Structure	Example	K_i (nM)	P_{app} (nm/sec)	Structure	Example	K_i (nM)	P_{app} (nm/sec)
	**6**	146	3		**11**	204	0
	**8**	2	5		**12**	>1000	33
	**9**	13	5		**13**	>1000	16
	**10**	>1000	25		**14**	>1000	81



7



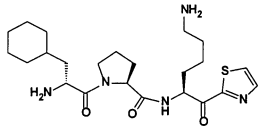
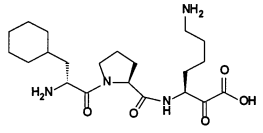
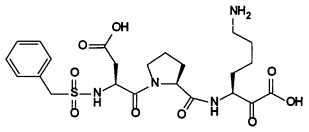
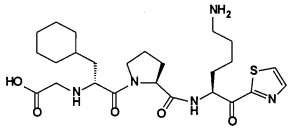
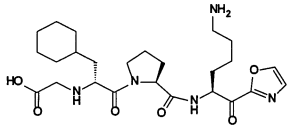
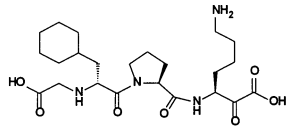
Lisinopril

Figure 2. Structural resemblance between compound **7** and lisinopril.

pound **18** was our first thrombin inhibitor that displayed significant oral bioavailability in rats at oral dosages ranging from 5 to 20 mg/kg. Further optimization of this breakthrough compound on potency and bioavailablility led to the diketo-carboxylate analogue **20**. Introduction of the *N*-methyl carboxylate moiety led to an increase in oral bioavailability in both the keto-(2-thiazolyl) and the diketo-carboxylate series: compare **15** with **18**, respectively, <5 and 23%, and compare **16** with **20**, respectively, 9 and 39% in rats. On the other hand, as exemplified by **17** and **19**, introduction of a carboxylate group in the molecule did not always result in enhanced oral bioavailability.

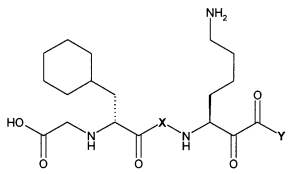
Additional pharmacokinetic studies with nephrectomized rats using the improved lead **20** showed virtually no plasma disappearance indicating that the clearance was almost exclusively renal. Liver perfusion experiments (3 h, 5 mg/kg iv) with **20** revealed a clearance of 0.025 L/hr, which is very low by all standards. Table 3

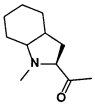
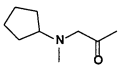
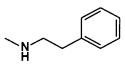
Table 2. Carboxylate Scan of Compound **15** to Arrive at the Second Generation Thrombin Inhibitors[a]

Structure	Example	K_i (nM)	$t_{1/2}$ (min)	% F
	**15**	3.5	17	< 5
	**16**	0.14	16	9
	**17**	15	--	--
	**18**	2.6	11	23
	**19**	0.42	28	< 5 (tox)
	**20**	0.29	19	39

[a] Data are inhibition constants toward thrombin and pharmacokinetics as established in male Wistar rats.

Table 3. Results of Further Optimizations of Compound **20**[a]



Example	X	Y	K_i (nM)	$t_{1/2}$ rat (min)	% F rat	$t_{1/2}$ dog (min)	% F dog
20		OH	0.29	19	39	78	24
21		OH	0.5	21	15	42	1
22		OH	0.29	5	6	--	--
23		OH	0.33	22	37	32	68
24		OH	376	--	--	--	--
25		OH	0.82	8	25	--	--
26		OH	0.21	18	2	--	--
27			1.1	58	32	108	71
28			0.84	17	23	--	--

[a] Two series are shown in this table: one targetting the proline residue and one the diketo moiety. $T_{1/2}$ values were obtained after IV administration. %*F* values were obtained by dividing the AUC after PO administration by the AUC after IV administration normalized for the dose.

shows the elimination half-life of **20** in rat and dog after intravenous administration ($t_{1/2}$ rat = 19 min, $t_{1/2}$ dog = 78 min).

To further guide the lead optimization process, several crystal structures of enzyme−inhibitor complexes were obtained. Key compounds such as **18** and **20** could be crystallized with thrombin showing numerous interactions between the active site of thrombin and the inhibitor structure (see Figure 3 for X-ray on **20**). The extended binding mode observed with these compounds is in accordance with previously published crystallographic data. Some of the lysine and the *N*-methylcarboxylate interactions with thrombin, revealing for instance alternative P_1 interactions through water molecules and backbone amide bonds, were less known.

As the crystal structure did not provide a special clue for further structural modifications, we dwelled on replacement of the proline moiety by known mimics (e.g., compounds **21**−**26**) and studied other keto transition state mimics including the keto carboxylate isopropyl ester **27** and keto-phenylethyl amide **28**. Although the inhibitory activity of most analogues is similar, their oral bioavailability differs markedly. Apparently, the requirements for intestinal absorption could be met with **20** and some closely related analogues. Only the S_1' site of this class of inhibitors may be modified considerably without losing oral bioavailability, e.g., 2-thiazole, carboxylate, isopropyl, and phenylethylamine. In particular, the ester derivative of **20** (i.e., compound **27**) showed oral bioavailabilities of 32 and 71% in rats and dogs, respectively (see Figure 4).

In Caco-2 experiments, we could not measure any degree of membrane transport with representatives of this series. In addition, we performed competition

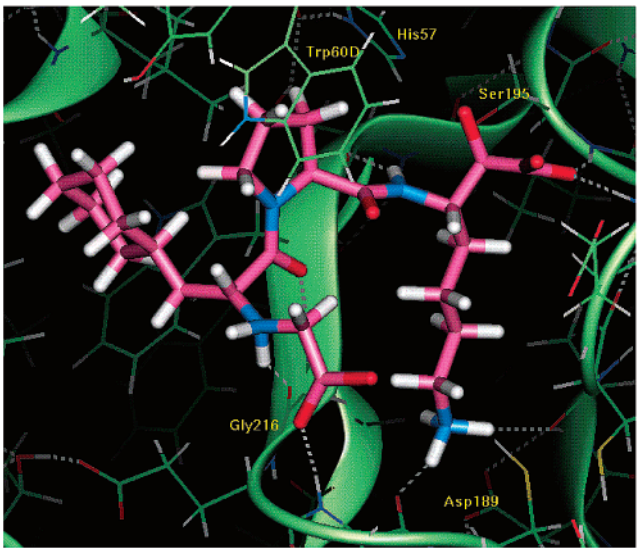


Figure 3. X-ray crystal structure of compound **20** bound to human α-thrombin at 1.59 Å.

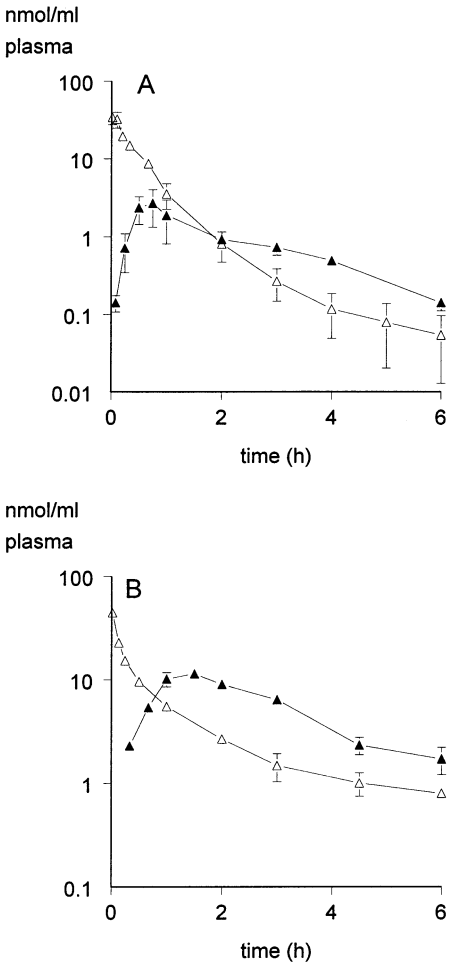


Figure 4. Pharmacokinetics of compound **27** in rats (A) and dogs (B). Line graphs showing disappearance patterns of the anti-IIa activity of compound **27** after i.v. (△) or oral (▲) administration of 10 μmol/kg. Data are expressed in nmol/mL and represent means of two (dogs) or three (rats) separate experiments ± SEM.

experiments with dipeptides and cephalexin but could not find any evidence of active transport in Caco-2 cell layers. Furthermore, we did not find evidence for paracellular transport that sometimes allows polar

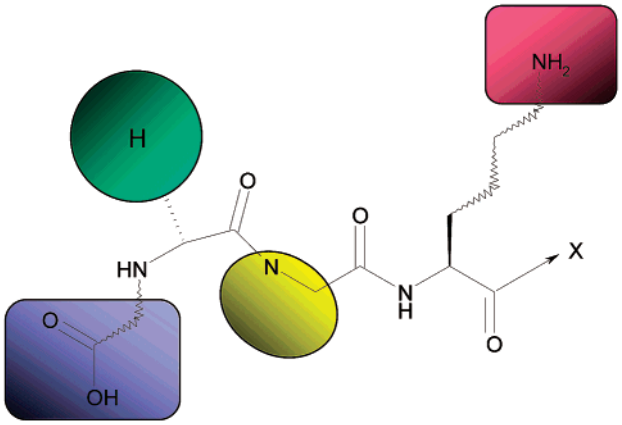


Figure 5. Pharmacophore model for intestinal absorption of polar thrombin inhibitors.

Table 4. Summary of Inhibitory Constants toward Thrombin and Antithrombotic Potencies in the Aortic Flow Model in Rats of Selected Thrombin Inhibitors and Reference Compounds[a]

example/ref compd	K_i (nM)	ED_{50} (mean (95% CI)) (nmol/kg·min)
18	2.6	19 (13; 25)
20	0.29	7.5 (4.7; 11)
27	1.1	5.4 (4.0; 6.6)
argatroban	19[49]	27 (19; 36)
melagatran	2.0[50]	5.2 (4.2; 6.5)

[a] K_I values in nanomolar; ED_{50} values in nmol/kg·min; SD was 25% on average; and data represent means of $n = 4-6$.

compounds to enter the body. Studies aimed at establishing Caco-2 cell transport of ACE inhibitors showed low proton-linked transport of lisinopril and only limited competition with known peptide carrier ligands. Apparently, although clearly absorbed after oral administration in humans, lisinopril is a poor substrate for the peptide carrier present in Caco-2 cells.[41] It remains unclear by which mechanism these hydrophilic compounds are absorbed. Perhaps intramolecular ion-pair formation of such molecules occurs resulting in less hydrophilic and more compact molecules. Despite the fact that we do not fully understand the underlying mechanism, a "pharmacophore" for absorption of these thrombin inhibitors can be envisaged (Figure 5). It is a very restricted window of opportunity best described as carboxy-Pro-Lys with some flexibility at the hydrophobic S_3 moiety and the transition state S_1' moiety.

Finally, we were anxious to compare the in vivo antithrombotic properties with known thrombin inhibitors (see Table 4). Compounds **18**, **20**, and **27** display antithrombotic activities in an aortic flow model in the rat in the same range as melagatran and argatroban, generally viewed as the most advanced drug candidates in this area. The aortic flow model is thought to be predictive for thrombus formation in fast-flowing vessels, i.e., for arterial thrombosis-related diseases.

In vitro data on selectivity profiles of key compounds in the above-described series have been collected. In a typical selectivity screen, activities of a number of human-derived serine proteases were measured. Generally, it consisted of fXa, trypsin, fVIIa/TF, plasmin, tPA, activated protein C, and protrombinase. Most compounds are selective (50−1000) toward the coagulation factors but show no or only limited selectivity (<1−10)

toward the digestive enzyme trypsin. No correlation could be found between oral absorption and trypsin activity.

Although the enzyme kinetics will not be reported in great detail in this paper, it must be stated that some of the compounds, more specifically those with a diketo moiety (Table 3), showed slow binding characteristics. For instance, **20** had a K_i of 0.29 nM and a K_i^* of 0.03 nM. The slow binding effects were obvious only in the first 10−20 min of the kinetic experiments. The results as depicted in Table 4 lead to the conclusion that transition state mimic inhibitors are efficient in arterial models. The slope of the dose response curve in the aortic flow model of a nontransition state mimic like melagatran is not different from compounds such as **20** and **27**. Presumed differences in k_{on} rates appear hardly relevant for the ultimate in vivo effect. The concern that activated ketones in TSAs all show slow tight binding inhibition that may impair successful blocking of thrombin in acute situations appears unwarranted for this series.

In several papers,[16,17] it was mentioned that it is extremely difficult to find the balance between lipophilicity, needed for oral absorption, and antithrombotic potential, measured for instance in an APTT experiment. Failures have been reported in which the lipophilicity was increased to get the desired oral absorption but at the expense of antithrombotic efficacy. Massive protein binding and/or large volumes of distribution may occur, which, for this specific class of compounds that exert their action on circulating blood enzymes, is very unfavorable. Clearly, the current hydrophilic series distinguishes itself in that oral absorption was obtained without compromising the antithrombotic action of the compounds. In that respect, this series offers an alternative to the main stream passive diffusion approaches and may become more important as we learn more about the fate of the leading drug candidates in the clinical arena and why so many drug candidates fail in clinical trials.

Experimental Section

The abbreviations used are as follows: TBTU, O-(benzotriazol-1-yl)-*N*,*N*,*N*,*N*′-tetramethyluronium tetrafluoroborate; Cbz or Z, benzyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; Boc, *tert*-butyloxycarbonyl; THF, tetrahydrofuran; DCU, dicyclohexylurea; DiPEA, *N*,*N*-diisopropylethylamine; Et_3N, triethylamine; TBAF, tetrabutylammonium fluoride; HOBt, 1-hydroxybenzotriazole; DCCI, dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; MS, mass spectrometry; FAB, fast atom bombartment; ESI, electron spray ionization.

Reagent-grade solvents were used without further purification. Evaporation means removal of solvent by use of a Büchi rotary evaporator at 40−50 °C in vacuo. Silica gel used for chromatography was Kieselgel-60 (230−400 mesh). Thin-layer chromatography (TLC) plates coated with silica gel 60 F_{254} (Merck) were used; detection was by UV (254 nm), by USUI, or by treatment with Cl_2/TMB. NMR spectra (^{1}H NMR and ^{13}C NMR) were recorded on either a Bruker AC 200 (200 MHz), Bruker AM 360 (360 MHz), or a Bruker DRX 400 (400 MHz) spectrometer; δ values in parts per million relative to tetramethylsilane are given. Mass spectra were recorded with a Finnigan MAT 90 mass spectrometer (FAB) or a Perkin-Elmer Sciex API-165 apparatus (ESI). Analytical HPLC was done on a Hewlett Packard 1090M or 1100 liquid chromatograph. Reversed phase preparatory HPLC purification was performed on a Waters Prep 4000, using a Supelcosil LC-18-DB or Delta Pak C_{18} (radial compression) prep columns and various gradients (solvent A: 0.5 M phosphate buffer, pH 2.1; solvent B: water; solvent C: acetonitril:water, 6:4 v/v%). All (protected) dipeptides were prepared by synthesis to conventional methodology: coupling of Boc-protected N-terminal L or D amino acid with either L or D amino acid benzyl ester or L or D amino acid methyl ester, followed by hydrogenation or saponification, respectively. HPLC elution of the main peak usually followed by a small second peak with identical mass, in combination with NMR techniques, warranted the diastereomeric purity of the end products.

Preparation of N^8(D-Phenylalanyl-prolyl)-3,8-diamino-6,7,8,8a-tetrahydro-8a-hydroxyimidazo[1,5a]pyridin-1(5H)-one (4). N$^\alpha$,N$^\delta$,N-Tribenzyloxycarbonyl-L-arginine methylester. The pH of a solution of 40 g (66.7 mmol) of N$^\alpha$,N$^\delta$,N-tri-benzyloxycarbonyl-L-arginine, prepared as described previously,[42] and 22.4 g (69.8 mmol) of TBTU in a mixture of CH_2Cl_2 (1080 mL) and MeOH (120 mL) was adjusted at pH 8 with Et_3N. The reaction mixture was stirred for 1 h at room temperature, after which the solution was successively washed with a 5% $NaHCO_3$ solution and water, dried on Na_2SO_4, and evaporated. The solid residue was crystallized from MeOH to give 35 g of Z-Arg(Z)$_2$-OMe (yield 88.8%). TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.60. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.70 (m, 4H), 3.63 (s, 3H), 3.95 (dt, 2H), 4.37 (dd, 1H), 5.07 (s, 2H), 5.12 (s, 2H), 5.22 (s, 2H), 5.52 (d, 1H), 7.30−7.42 (m, 15H), 9.24 (bs, 1H), 9.42 (bs, 1H).

2-Acetoxy-3-(benzyloxycarbonylamino)-6-(dibenzyloxycarbonylguanidino)hexanenitril. A solution of 180 mL (180 mmol) of diisobutylaluminumhydride was added dropwise at −78 °C to a stirred solution of 30 g (47.4 mmol) of Z-Arg(Z)$_2$-OMe in 700 mL of CH_2Cl_2. The mixture was stirred for 1 h at −78 °C, poured in a solution of citric acid in water, and extracted with CH_2Cl_2. The combined extracts were used in the next step. To the solution of the aldehyde was added a solution of 28 g (0.57 mol) of NaCN and 35 g (0.15 mmol) of triethylbenzylammonium chloride in 700 mL of water and 14 mL of acetic anhydride at 0 °C. The mixture was stirred for 30 min at 0−5 °C. The organic layer was separated, washed with water, dried on Na_2SO_4, and evaporated in vacuo. Purification of the residue by chromatography (CH_2Cl_2:MeOH, 9:1) afforded the title compound as a solid (17 g, 57.0%). TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.76. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.98 and 1.99 (s, 3H), 3.90 (m, 1H), 4.10 (m, 1H), 4.15 (m, 1H), 5.05 and 5.07 (s, 2H), 5.09 and 5.10 (s, 2H), 5.23 and 5.24 (s, 2H), 5.38 and 5.44 (d, 1H), 5.79 (d, 1H), 7.32 (m, 15H), 9.29 (br s, 1H), 9.48 (b s, 1H).

3-(Benzyloxycarbonylamino)-6-(dibenzyloxycarbonylguanidino)-2-hydroxy-hexanoic Acid. At −78 °C, hydrogen chloride gas was passed through a solution of 6 g (9.30 mmol) of 2-acetoxy-3-(benzyloxycarbonylamino)-6-(dibenzyloxycarbonylguanidino) hexanenitril in 140 mL of a mixture of Et_2O:MeOH, 3:1, until a 3 M solution was obtained. The mixture was stirred for 16 h at 5 °C, poured into water, and extracted with CH_2Cl_2. The combined extracts were washed with water and 5% $NaHCO_3$ solution and water and dried on Na_2SO_4. The solvent was removed in vacuo yielding the title compound as a gum (6.1 g, quantitative). TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.48. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.65 (m, 4H), 3.65 and 3.68 (s, 3H), 4.22 and 4.27 (d, 1H), 5.03 and 5.06 (AB, 2H), 5.10 (s, 2H), 5.20 and 5.22 (s, 2H), 7.33 (m, 15H), 9.27 (b s,1H), 9.43 (b s,1H).

3-(Benzyloxycarbonylamino)-6-(dibenzoylcarbonylguanidino)-2-oxo-hexanoic Acid Methylester. To a solution of 1.3 g (2.05 mmol) of 3-(benzyloxycarbonylamino)-6-(dibenzyloxycar-bonylguanidino)-2-hydroxy-hexanoic acid in 130 mL of acetone was added 1 mL of 8 N chromic acid solution in aqueous sulfuric acid at 0 °C. The mixture was stirred for 1 h at 0 °C and then poured into water. The precipitate was filtered off, washed with water, and dried in vacuo to give a white solid (1.15 g, 90.7%). TLC (CH_2Cl_2:EtOAc, 8:2) R_f = 0.8. ^{1}H NMR (360 MHz, $CDCl_3$): δ 1.66 (m, 4H), 3.76 (s, 2H), 3.78 (s, 3H), 4.9 (m, 1H), 5.04 (s, 2H), 5.10 (s, 2H), 5.22 (s, 2H), 5.83 (d, 1H), 7.32 (m, 15H), 9.26 (b s, 1H), 9.42 (b s, 1H).

3,8-Diamino-6,7,8,8a-tetrahydro-8a-hydroxyimidazo-[1,5a]pyridin-1(5H)-one. Hydrogen gas was passed through a mixture of 1.5 g (2.37 mmol) of 3-(benzyloxycarbonyl-amino)-6-(dibenzoylcarbonylguanidino)-2-oxo-hexanoic acid methyl-ester, 150 mg of Pd/C 10%, and 2.44 mL of 1 N HCl in DMF (60 mL). Hydrogen was passed through until the reaction was complete. The catalyst was filtered off, and the filtrate was evaporated in vacuo to give a white foam (0.8 g, quantitative). TLC (*n*-BuOH:pyridine:HOAc:water, 10:1:1:2) R_f = 0.2. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.65 (m, 1H), 1.83 (m, 1H), 1.85 (m, 1H), 3.32 (d t, 1H), 3.63 (d d, 1H), 3.78 (d d, 1H).

N^8(D-Phenylalanyl-prolyl)-3,8-diamino-6,7,8,8a-tetrahydro-8a-hydroxyimidazo[1,5a]pyridin-1(5H)-one (4). A solution of Boc-D-Phe-Pro-OH (190 mg, 0.52 mmol), HOBt (108 mg, 0.80 mmol), and DCCI (121 mg, 0.58 mmol) in DMF (10 mL) was stirred for 15 min at 0 °C. 3,8-Diamino-6,7,8,8a-tetrahydro-8a-hydroxyimidazo[1,5a]pyridin-1(5H)-one (300 mg, 0.47 mmol) was added, and the mixture was stirred for 16 h at room temperature. The reaction mixture was evaporated in vacuo to a small volume and diluted with *n*-BuOH. The solution was washed with water, 5% $NaHCO_3$ solution, and brine and evaporated in vacuo. The residue was dissolved in 12.5 mL of 90% TFA and 0.36 mL of anisole, and the reaction mixture was stirred for 2.5 h at room temperature. The volatiles were removed, and the residue was dissolved in *t*-BuOH:water, 1:1. Dowex-2X-8 (acetate form) was added in portions until the pH of the solution was raised to 5−6. The ion exchange resin was filtered off, and the filtrate was lyophilized to give 500 mg of crude **4**. An amount of 190 mg of the residue was purified by RP-18 preparatory HPLC. The fractions containing the desired product were collected, desalted, and lyophilized to yield **4** (42 mg). TLC (*n*-BuOH:pyridine:HOAc:H_2O, 6:1:1:2) R_f = 0.7. ^{1}H NMR (360 MHz, D_2O): δ 1.60 (m, 1H), 1.65 (m, 1H), 1.85 (m, 3H), 2.10 (m, 2H), 2.76 (m, 1H), 3.15 (m, 1H), 3.24 (m, 1H), 3.25 (m, 1H), 3.51 (m, 1H), 3.77 (dd, 1H), 3.92 (dd, 1H), 4.36 (dd, 1H), 4.54 (dd, 1H). ^{13}C NMR (360 MHz, D_2O): δ 190.6, 176.0, 170.7, 167.7, 87.4, 63.5, 56.1, 53.5, 50.8, 40.9, 39.5, 32.4, 27.8, 26.9, 26.4. R_t (LC): 22.13 min, 20% A/80% B to 20% A/40% B/40% C in 40 min; purity 94.6% (LC). MS *m/z* (FAB$^+$): 429 [M + H], 451 [M + Na], 411 [M − H_2O]; (FAB$^-$): 427 [M − H], 409 [M − H − H_2O].

General Procedure for the Preparation of Peptidyl-Lys-(2-thiazolyl) Derivatives (II, Scheme 2A). Preparation of Boc-Lys(Cbz)-(2-thiazolyl) (Ia, Scheme 2A). Boc-Lys(Cbz)-(N-Me)OMe. Boc-Lys(Cbz)-OH·DCHA (10 g, 17.8 mmol) was suspended in CH_2Cl_2 (200 mL). The suspension was washed with cold 0.1 N HCl solution twice. TBTU (6.0 g, 18.7 mmol) and O,N-dimethyl-hydroxylamine hydrochloric acid (1.82 g, 18.7 mmol) were added to the resulting organic phase, and the pH was adjusted to pH 8 by adding Et_3N. The reaction mixture was stirred for 1 h at room temperature. The mixture was washed successively with cold 2 N HCl solution, water, 5% $NaHCO_3$ solution, and water. The organic layer was dried over Na_2SO_4, filtered, and evaporated. Purification of the residue by chromatography (CH_2Cl_2:MeOH, 1:1) afforded Boc-Lys(Cbz)-NMeOMe as a clear oil (7.2 g, 95.5%). TLC (CH_2Cl_2:MeOH, 95:5) R_f = 0.55. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.38−1.76 (m, 6H), 1.42 (s, 9H), 3.13−3.25 (m, 2H), 3.20 (s, 3H), 3.76 (s, 3H), 4.58−4.74 (m, 1H), 4.84−4.98 (m, 1H), 5.09 (s, 2H), 5.19−5.29 (d, 1H), 7.28−7.40 (m, 5H).

Boc-Lys(Cbz)-(2-thiazolyl) (Ia). To a cold (−78 °C), stirred solution of *n*-butyllithium (63.9 mmol) in diethyl ether (58 mL) was added dropwise a solution of 2-bromothiazole (10.5 g, 63.9 mmol) in diethyl ether (30 mL). The solution was stirred at −78 °C for 30 min, after which a solution of Boc-Lys(Cbz)-NMeOMe (8.2 g, 19.4 mmol) in dry THF (75 mL) was added slowly. The mixture was stirred at −78 °C for 1 h, and then, 5% $NaHCO_3$ solution was added. The mixture was allowed to warm to room temperature, and the layers were separated. The aqueous layer was extracted with diethyl ether. The combined organic layers were washed with water, dried over Na_2SO_4, filtered, and evaporated. Purification of the residue by chromatography (EtOAc:heptane, 3:1) afforded Boc-Lys(Cbz)-(2-thiazolyl) as a clear oil (8.6 g, 99.0%). TLC (EtOAc:heptane, 3:1) R_f = 0.77. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.38−1.76 (m, 6H), 1.42 (s, 9H), 3.13−3.25 (m, 2H), 4.83−4.97 (m, 1H), 5.08 (s, 2H), 5.37−5.48 (m, 2H), 7.27−7.40 (m, 5H), 7.70 (d, 1H), 8.03 (d, 1H).

Boc-D-Phe-Pro-Lys-(2-thiazolyl). Boc-Lys(Cbz)-(2-thiazolyl) (500 mg, 1.12 mmol) was dissolved in 50% TFA/CH_2Cl_2 (5 mL) and stirred for 1 h at room temperature. The crude H-Lys(Cbz)-(2-thiazolyl).TFA was isolated in quantitative yield after removal of the solvent by evaporation and used immediately in the next step. TLC (EtOAc:pyridine:HOAc:H_2O, 63:20:6:11) R_f = 0.25.

To a cold (0 °C) solution of Boc-D-Phe-Pro-OH (350 mg, 1 mmol) in DMF (5 mL) were successively added HOBt (145 mg, 1.1 mmol), DCCI (220 mg, 1.1 mmol), and H-Lys(Cbz)-(2-thiazolyl)·TFA (445 mg, 1.1 mmol). The pH was adjusted to pH 8.5 by adding Et_3N. The reaction mixture was stirred at 0 °C for 1 h and then kept overnight at room temperature. The mixture was cooled to −20 °C, and DCU was removed by filtration. The filtrate was evaporated to dryness. The residue was dissolved in EtOAc and washed successively with 5% $NaHCO_3$ solution, 5% $KHSO_4$ solution, and brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by chromatography (CH_2Cl_2:MeOH, 95:5) afforded Boc-D-Phe-Pro-Lys-(2-thiazolyl) (272 mg, 40.9%). TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.80. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80−2.50 (m, 10H), 1.41 (s, 9H), 2.70−2.78 (d, 2H), 3.00−3.60 (m, 5H), 4.27−4.35 (m, 1H), 4.51−4.60 (m, 1H), 5.13 (s, 2H), 5.55−5.67 (m, 2H), 5.59−6.04 (m, 1H), 7.08−7.50 (m, 10H), 7.70 (d, 1H), 8.03 (d, 1H).

H-D-Phe-Pro-Lys-(2-thiazolyl) (6). Boc-D-Phe-Pro-Lys-(2-thiazolyl) (260 mg, 0.38 mmol) was treated with TFA:thioanisole, 10:1 v/v (2.75 mL), for 4 h at room temperature. The reaction mixture was concentrated in vacuo, and the residue was dissolved in water. The aqueous phase was washed extensively with diethyl ether. The water layer, containing H-D-Phe-Pro-Lys-(2-thiazolyl), was charged onto a preparative HPLC Supelcosil LC-18-DB column using a gradient elution system of 20% A/80% B to 20% A/30% B/50% C over 40 min, at a flow rate of 20 mL/min. (A: 0.5 M sodium phosphate buffer, pH 2.1; B: water; C: acetonitril/water; 3/2 v/v). Fractions containing **6** were desalted using a 0.1 M HCl solution and lyophilized. Yield, 117.5 mg of H-D-Phe-Pro-Lys-(2-thiazolyl) (**6**, 68.3%). ^{1}H NMR (360 MHz, D_2O): δ 1.58−1.70 (m, 3H), 1.74−1.94 (m, 5H), 2.11−2.22 (m, 2H), 2.78−2.86 (dt, 1H), 3.06 (t, 2H), 3.17−3.35 (m, 2H), 3.50−3.59 (m, 1H), 4.48 (m, 1H), 4.60 (m, 1H), 5.53 (m, 1H), 7.30−7.52 (m, 5H), 8.14 (d, 1H), 8.18 (d, 1H). R_t (LC): 25.67 min, 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 97.1% (LC). MS *m/z* (FAB$^+$): 458 [M + H].

In an analogues way to the synthesis of **6**, compounds **8**−**11**, **13**, and **15** were prepared from the corresponding dipeptides.

EthylSO$_2$-D-Cha-Pro-Lys-(2-thiazolyl) (8). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8−2.12 (m, 23H), 1.09 (t, 3H), 2.7−2.81 (m, 2H), 2.82−2.93 (m, 2H), 3.42 (m, 1H), 3.59 (dt, 1H), 4.10 (dt, 1H), 4.38 (dd, 1H), 5.26−5.37 (m, 1H), 8.19 (d, 1H), 8.28 (d, 1H). R_t (LC): 25.66 min 20% A/60%B/20% C to 100% C in 40 min; purity 99.3% (LC). MS *m/z* (ESI$^+$): 556 [M + H], 578 [M + Na], 538 [MH − H_2O].

H-D-Cha-(N-Cyclopropyl)-Gly-Lys-(2-thiazolyl) (9). ^{1}H NMR (400 MHz, D_2O): δ 0.72−1.81 (m, 22H), 1.97−2.06 (m, 1H), 2.79−2.85 (m, 1H), 2.92 (t, 2H), 4.13−4.25 (m, 2H), 4.79 (dd, 1H), 5.41 (dd, 1H), 8.01 (d, 1H), 8.06 (d, 1H). R_t (LC): 30.48 min 20% A/80%B to 20% A/20% B/60% C in 40 min; purity 99.9% (LC). MS *m/z* (FAB$^+$): 464.1 [M + H].

Indane-(N-Cyclopropyl)-Gly-Lys-(2-thiazolyl) (10). ^{1}H NMR (400 MHz, CD_3OD): δ 0.93 (broad m, 2H), 1.10 (broad, 2H), 1.58 (m, 2H), 1.66−1.88 (m, 3H), 2.08−2.17 (m, 1H), 2.94 (m, 2H), 3.05 (broad, 1H), 3.27−3.35 (m, 2H), 3.40−3.49 (m, 2H), 4.08 (broad, 2H), 4.49 (m, 1H), 5.58 (dd, 1H), 7.20−7.30 (m, 4H), 8.08 (d, 1H), 8.13 (d, 1H). R_t (LC): 31.71 min 20% A/80%B to 20% A/20% B/60% C in 40 min; purity 99.7% (LC). MS *m/z* (FAB$^+$): 427 [M + H], 853 [2M + H]; (FAB$^-$): 426 [M$^-$], 426 [M + Cl].

3,3-Diphenylpropionyl-Pro-Lys-(2-thiazolyl) (11). ^{1}H NMR (400 MHz, D_2O): δ 1.47−2.32 (m, 10H), 2.72−2.89 (m, 1H), 2.90−3.00 (t, 1H), 3.02−3.18 (m, 1H), 3.29−3.40 (m, 1H), 3.40−3.57 (m, 1H), 3.60−3.70 (m, 1H), 4.31 (dd, 1H), 4.44−4.55 (m, 1H), 5.37−5.47 (m, 1H), 7.21−7.45 (m, 10H), 8.07 (d, 1H), 8.11 (d, 1H). R_t (LC): 32.89 min 20% A/60%B/20% C to 20% A/80% C in 40 min; purity 97.9% (LC). MS *m/z* (FAB$^+$): 519 [M + H], 611 [M + glycerol + H], 1037 [2M + H]; (FAB$^-$): 517 [M − H].

7-Methoxy-2-naphthylsulfonyl-Lys-(2-thiazolyl) (13). ^{1}H NMR (400 MHz, CD_3OD): δ 1.55−1.91 (m, 6H), 2.85−2.97 (m, 2H), 3.88 (s, 3H), 5.20−5.25 (m, 1H), 7.09 (m, 1H), 7.22 (m, 1H), 7.55 (m, 1H), 7.73−7.82 (m, 3H), 8.00 (d, 1H), 8.11 (d, 1H). R_t (LC): 34.06 min 20% A/60%B/20% C to 20% A/80% C in 40 min; purity 88.5% (LC). MS *m/z* (FAB$^+$): 434 [M + H], 526 [M + glycerol + H], 618 [M + 2glycerol + H].

H-D-Cha-Pro-Lys-(2-thiazolyl) (15). ^{1}H NMR (360 MHz, DMSO-d_6): δ 0.80−2.13 (m, 24H), 2.77 (t, 2H), 3.68−3.89 (m, 1H), 4.14 (m, 1H), 4.41 (dd, 1H), 5.28 (m, 1H), 8.18 (d, 1H), 8.27 (d, 1H). R_t (LC): 30.59 min 20% A/80% B to 20% A/20% C/60% C in 40 min; purity 97.8% (LC). MS *m/z* (FAB$^+$): 464 [M + H]; (FAB$^-$): 463 [M$^-$], 498 [M + Cl].

Preparation of HOOC-CH_2-D-Cha-Pro-Lys-(2-thiazolyl) (18). Preparation of the Dipeptide N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-Pro-OH. H-D-Cha-OMe·HCl. To cold (−20 °C) and dry MeOH (195 mL) was added dropwise thionyl chloride (28 mL, 38.6 mmol). H-D-Cha-OH·HCl (40 g, 19.3 mmol) was added, and the reaction mixture was heated under reflux for 5 h. The mixture was concentrated in vacuo and coevaporated with MeOH (three times). The residue was crystallized from MeOH/Et_2O yielding H-D-Cha-OMe·HCl as a crystalline powder. Yield, 40.9 g (96%). TLC (*n*-BuOH:HOAc:H_2O, 10:1:3) R_f = 0.66. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.85−2.00 (m, 13H), 3.84 (s, 3H), 4.07 (dd, 1H).

N-(*tert*-Butyloxycarbonylmethyl)-D-Cha-OMe. *tert*-Butyl-bromo acetate (36 g, 0.185 mol) was added to a stirred solution of H-D-Cha-OMe·HCl (40.9 g, 0.185 mol) in 400 mL of acetonitrile. The pH of the mixture was adjusted to 8.5 with DiPEA. The mixture was stirred for 16 h at room temperature and evaporated in vacuo. The residue was dissolved in CH_2Cl_2, and the solution was washed with water, dried over Na_2SO_4, and evaporated in vacuo. Purification of the residue by chromatography (heptane:EtOAc, 9:1) gave 64 g of N-(*tert*-butyloxycarbonylmethyl)-D-Cha-OMe (115% yield). TLC (EtOAc:heptane, 1:1) R_f = 0.25. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.84−1.96 (m, 22H), 3.16−3.40 (m, 3H), 3.72 (s, 3H).

N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-OMe. The pH of a solution of N-(*tert*-butyloxycarbonylmethyl)-D-Cha-OMe (64 g, 0.185 mol) and di-*tert*-butyl dicarbonate (40.3 g, 0.185 mol) in 500 mL of DMF was adjusted to 8.5 with DiPEA. The mixture was stirred for 16 h at room temperature. The solvent was removed in vacuo. CH_2Cl_2 and water were added to the residue. The organic layer was separated and washed with cold 1 N HCl solution, water, 5% $NaHCO_3$ solution, and water. The organic layer was dried over Na_2SO_4, and the filtrate was evaporated to afford an amorphous solid of N-(*tert*-butyloxycarbonylmethyl)-N-Boc-D-Cha-OMe. Yield, 59.6 g (70%). TLC (EtOAc:heptane, 1:1) R_f = 0.50. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.78−1.90 (m, 31H), 3.55−3.99 (m, 5H), 4.64−5.03 (m, 1H).

N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-OH. A solution of N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-OMe (59.6 g, 0.15 mol) in 900 mL of dioxane/water = 9/1 (v/v) was treated with sufficient 6 N NaOH solution to keep the pH at 12 for 6 h at room temperature. After it was acidified, the mixture was poured into water and was extracted with CH_2Cl_2. The organic layer was washed with water and was dried over Na_2SO_4. The filtrate was evaporated and yielded 54 g of N-(*tert*-butyloxycarbonylmethyl)-N-Boc-D-Cha-OH (93%). TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.6. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.82−2.05 (m, 31H), 3.48−4.02 (m, 2H), 4.27−4.50 (m, 1H).

N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-Pro-OBzl. To a cold (0 °C) solution of N-(*tert*-butyloxycarbonylmethyl)-N-Boc-D-Cha-OH (13.5 g, 35 mmol) in DMF (150 mL) were successively added HOBt (7.09 g, 52.5 mmol), DCCI (7.61 g, 36,8 mmol), H-Pro-OBzl.HCl (9.31 g, 38.5 mmol), and Et_3N (6 mL, 43.4 mmol). The mixture was stirred at 0 °C for 1 h and then kept at room temperature overnight. The mixture was cooled to −20 °C, and DCU was removed by filtration. The filtrate was evaporated to dryness. The residue was dissolved in EtOAc and washed successively with 5% $NaHCO_3$ solution, water, 3% citric acid solution, and brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by chromatography (heptane:EtOAc, 3:1) afforded N-(*tert*-butyloxycarbonylmethyl)-N-Boc-D-Cha-Pro-OBzl (15 g, 75%). TLC (EtOAc:heptane, 1:1) R_f = 0.7. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80−2.35 (m, 35H), 3.28−4.03 (m, 4H), 4.45−5.25 (m, 4H), 7.30−7.41 (m, 5H).

N-(*tert*-Butyloxycarbonylmethyl)-N-Boc-D-Cha-Pro-OH. An amount of 10% palladium on charcoal (750 mg) was added to a solution of N-(*tert*-butyloxy-carbonylmethyl)-N-Boc-D-Cha-Pro-OBzl (15 g, 26.2 mmol) in MeOH (150 mL). The mixture was hydrogenated at atmospheric pressure at room temperature for 1 h. The palladium catalyst was removed by filtration, and the solvent was removed by evaporation at reduced pressure yielding 11.2 g of N-(*tert*-butyloxycarbonylmethyl)-N-Boc-D-Cha-Pro-OH (89%). TLC (EtOAc:pyridine:HOAc:H_2O, 213:20:6:11) R_f = 0.65. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.77−2.39 (m, 35H), 3.30−4.04 (m, 4H), 4.48−5.18 (m, 2H).

In an analogues way to the synthesis of **6**, compound **18** was prepared from the corresponding dipeptide.

HOOC-CH_2-D-Cha-Pro-Lys-(2-thiazolyl) (18). ^{1}H NMR (400 MHz, D_2O): δ 0.59−2.31 (m, 23H), 2.79−2.90 (m, 2H), 3.37−3.65 (m, 4H), 4.11−4.40 (m, 2H), 5.23−5.36 (m, 1H), 7.49−8.00 (m, 2H). R_t (LC) = 32.67 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 97.1% (LC). MS *m/z* (ESI$^+$): 522.1 [M + H].

Preparation of Peptidyl-Arg-(2-thiazolyl) Derivative. Preparation of Boc-Arg(Pmc)-(2-thiazolyl) (Ib, Scheme 2A). In an analogues way to the synthesis of Boc-Lys(Cbz)-(2-thiazolyl), Ib was prepared starting from Boc-Arg(PMc)-OH. TLC (CH_2Cl_2:MeOH, 9:1) R_f = 0.82. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (s, 6H), 1.40 (s, 9H), 1.60−1.73 (bm, 4H), 1.80 (t, 2H), 2.09 (s, 3H), 2.53 (s, 3H), 2.54 (s, 3H), 2.51 (t, 2H), 3.22 (m, 1H), 3.38 (b, 1H), 5.40 (bt, 1H), 5.64 (d, 1H), 6.23 (bs, 1H), 7.71 (d, 1H), 8.03 (d, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 191.9, 164.2, 164.1, 156.2, 153.5, 145.3, 135.5, 134.8, 133.5, 127.0, 124.0, 117.9, 80.3, 73.6, 40.6, 32.9, 28.3, 26.8, 25.4, 21.4, 18.5, 17.4, 12.1. MS *m/z* (ESI$^+$): 608.4 [M + H], 552.2 [M − *t*Bu + H], 508 [M − Boc + H], 1215.8 [2M + H]; (ESI$^-$): 606.4 [M − H].

In an analogous way to the synthesis of **6**, compound **5** was prepared starting from Boc-D-Phe-Pro-OH.

H-D-Phe-Pro-Arg-(2-thiazolyl) (5). ^{1}H NMR (400 MHz, D_2O): δ 1.60−2.30 (complex, 10H), 2.80−2.90 (dt, 2H), 3.20−3.40 (m, 3H), 3.55−3.65 (m, 1H), 4.52 (m, 1H), 4.65 (m, 1H), 5.48 (m, 1H), 7.28−7.57 (m, 5H), 8.18 (d, 1H), 8.23 (d, 1H). R_t (LC) = 13.04 min 20% A/60% B/20% C to 20% A/80% C in 40 min; purity 94.0% (LC). MS *m/z* (FAB$^+$): 486 [M + H].

Preparation of Peptidyl-(ε-hydroxy)-Nle-(2-thiazolyl) Derivative. Preparation of Boc-(ε-hydroxy)-Nle-(2-thiazolyl) (Ic, Scheme 2A). In an analogues way to the synthesis of Boc-Lys(Cbz)-(2-thiazolyl), Ic was prepared starting from Boc-(ε-hydroxy)-Nle-OH. TLC (EtOAc:heptane, 4:1) R_f = 0.50. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.40−2.16 (m, 6H), 1.42 (s, 9H), 3.60−3.69 (t, 2H), 5.36−5.52 (b, 2H), 7.72 (d, 1H), 8.05 (d, 1H). MS *m/z* (FAB$^+$): 315.0 [M + H], 258.9 [M − *t*Bu + H], 214.9 [M − Boc + H]; (FAB$^-$): 313.9 [M − H].

In an analogues way to the synthesis of **6**, compound **12** was prepared from the corresponding dipeptide.

3,3-Diphenylpropionyl-Pro-(ε-hydroxy)-Nle-(2-thiazolyl) (12). ^{1}H NMR (400 MHz, CD_3OD): δ 1.45−2.19 (m, 10H), 2.83−3.27 (m, 2H), 3.32−3.61 (m, 2H), 3.53 (t, 2H), 4.32−4.40 (m, 1H), 4.53−4.60 (m, 1H), 5.47−5.56 (m, 1H), 7.13−7.29 (m, 10 H), 8.00 (d, 1H), 8.07 (d, 1H). R_t (LC): 39.43 min 20% A/60%B/20% C to 20% A/80% C in 40 min; purity 100% (LC).

MS *m/z* (FAB$^+$): 520 [M + H], 612 [M + glycerol + H], 1039 [2M + H]; MS (FAB$^-$): 519 [M$^-$].

Preparation of Peptidyl-Lys-(2-oxazolyl) Derivative. Preparation of Boc-Lys(Cbz)-(2-oxazolyl) (Id, Scheme 2A). Boc-Lys(Cbz)ψ[CHOH]-(2-oxazolyl). To a solution of 975 mg (2.47 mmol) of Boc-Lys(Cbz)-OMe in 25 mL of CH_2Cl_2 at −78 °C under a nitrogen atmosphere was added 6 mL of a 1 M DiBAL-H solution in hexane. After 15 min, the reaction was completed, and the mixture was poured into 150 mL of 2% citric acid solution and filtered. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was coevaporated with toluene to give 920 mg of Boc-Lys(Cbz)-H. This aldehyde (890 mg, 2.44 mmol) was dissolved in 1.4 mL of toluene and 900 mg (6.37 mmol) of 2-(trimethylsilyl)oxazole, prepared according to Edwards et al,[43] was added, and heated at 80 °C. After 60 h, the reaction mixture was concentrated, the residue was dissolved in 5 mL of THF, treated with 3 mL of a 3 M TBAF in THF solution, and stirred at room temperature for 2 h. The mixture was concentrated, dissolved in EtOAc, washed with 3% $NaHCO_3$ solution and brine, dried over $MgSO_4$, and evaporated. Purification of the residue by chromatography (gradient of EtOAc:CH_2Cl_2, 2:1 to ethyl acetate) afforded an oil that was rechromatographed (gradient of EtOAc:heptane, 1:1 to EtOAc:heptane, 1:3) to give the title compound (220 mg, 18%). TLC (EtOAc) $R_f = 0.7$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.82−1.71 (m, 15H), 3.07−3.24 (m, 2H), 4.0 (br. s, 1H), 4.77−5.40 (m, 6H), 7.03 (s, 0.75H) and 7.06 (s, 0.25H), 7.23−7.38 (m, 5H), 7.61 (s, 1H).

Boc-Lys(Cbz)-(2-oxazolyl) (Id). To a solution of 0.22 g (0.51 mmol) of Boc-Lys(Cbz)Ψ[CHOH]-(2-oxazolyl) in 10 mL of CH_2Cl_2 was added 0.22 g (0.52 mmol) of periodinane (Dess−Martin reagent). After it was stirred at room temperature for 1.5 h, 10 mL of aqueous 5% $Na_2S_2O_4$ solution was added and the mixture was stirred for 15 min at room temperature. The organic layer was separated, washed with water, 5% $NaHCO_3$ solution, and brine, dried over $MgSO_4$, and concentrated. Purification of the residue by chromatography (heptane:EtOAc, 1:1) yielded 162 mg of the title compound (79% yield). TLC (EtOAc:heptane, 3:1) $R_f = 0.5$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.33−2.12 (m, 15H), 3.13−3.26 (m, 2H), 4.84−5.47 (m, 6H), 7.29−7.42 (m, 6H), 7.84 (d, 1H).

In an analogues way to the synthesis of **6**, compound **19** was prepared from the corresponding dipeptide.

HOOC-CH$_2$-D-Cha-Pro-Lys-(2-oxazolyl) (19). ^{1}H NMR (400 MHz, D_2O): δ 0.83−2.56 (m, 23H), 3.04−3.17 (2H, m), 3.62−3.91 (m, 4H), 4.32−4.65 (m, 2H), 5.31−5.46 (m, 1H), 7.29−8.28 (m, 2H). R_t (LC): 28.46 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 99.3% (LC). MS *m/z* (FAB$^+$): 506 [M + H], 524 [M + H_2O + H], 598 [M + glycerol + H], 1011 [2M + H], 1029 [2M + H_2O + H]; (FAB$^-$): 504 [M − H], 540 [M + Cl].

Preparation of 7-Methoxynaphthylsulfonyl-Lys-Piperidylamide. (*S*)-1-[6-Amino-2-[[(7-methoxy-2-naphthalene)sulfonyl]amino-1-oxohexyl]-4-methyl-piperidine (14). The procedure described for compound **6** was used. A 300 mg amount of 7-methoxy-2-naphthylsulfonyl-Lys(Boc)-(4-methylpiperidyl) afforded, after chromatography (CH_2Cl_2:MeOH, 98:2) and exchange on Dowex-Cl$^-$, 118 mg of the title compound (44% yield). ^{1}H NMR (400 MHz, CD_3OD): δ −0.24−0.99 (2× d, 2× m, 5H), 1.14−2.33 (m, 10H), 2.66−2.96 (m, 3H), 3.56−4.28 (m, 3H), 3.94 (2× s, 3H), 7.31 (m, 1H), 7.41 (broad, 1H), 7.61−7.67 (m, 1H), 7.88 (dd, 2H), 8.30 (s, 1H). R_t (LC): 31.17 min 20% A/60%B/20% C to 20% A/80% C in 40 min; purity 98.7% (LC). MS *m/z* (FAB$^+$): 448 [M + H], 895 [2M + H]; (FAB$^-$): 446 [M − H], 482 [M + Cl], 518 [M + 2HCl − H], 929 [2M + Cl].

General Procedure for the Preparation of Peptidyl-Lysψ[COCO]-X Derivatives (V, Scheme 2B). Preparation of Cbz-Lys(Boc)ϵ[CHOHCO]-OMe (IV, Scheme 2B). Cbz-Lys(Boc)-OMe. A solution of 28 g (74 mmol) of Cbz-Lys(Boc)-OH and 23.6 g (74 mmol) of TBTU in 500 mL of CH_2Cl_2:MeOH, 9:1, was adjusted to pH 8 by addition of Et_3N and stirred for 2 h at room temperature. The mixture was washed successively with cold 1 N HCl solution, water, 5% $NaHCO_3$ solution, and water and dried over Na_2SO_4. The filtrate was evaporated, and the residue was chromatographed (heptane:EtOAc, 4:1). The fractions containing Cbz-Lys(Boc)-OMe were pooled and evaporated to afford 29.1 g of the title compound (99%). TLC (EtOAc:heptane, 3:1) $R_f = 0.85$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.42 (s, 9 H), 3.10 (br q, 2 H), 3.74 (s, 3 H), 4.38 (br dd, 1 H), 4.57 (br s, 1 H), 5.11 (s, 2 H), 5.38 (br d, 1H), 7.36 (s, 5 H).

Cbz-Lys(Boc)ϵ[cyanoacetate] (III, Scheme 2B). To a cold (−78 °C) solution of 29.1 g (74 mmol) of Cbz-Lys(Boc)-OMe in 800 mL of dry CH_2Cl_2 was added dropwise 222 mL of 1 M solution of DiBAL-H in hexane keeping the reaction temperature below −70° C. The resulting solution was stirred at −78° C for 1 h, and 600 mL of an 5% citric acid solution was added to the reaction mixture. The two layer mixture was stirred at room temperature for 10 min, the layers were separated, and the aqueous layer was extracted twice with CH_2Cl_2. The combined CH_2Cl_2 layers were washed with water, dried over Na_2SO_4, and filtered. The filtrate was stirred under a nitrogen atmosphere and cooled on an icewater bath. A solution of 36.3 g of NaCN (740 mmol) and 4.2 g of triethylbenzylammonium chloride (18.4 mmol) in 600 mL of water was added. Under vigorous stirring, acetic anhydride was added portionwise (2 × 9 mL) over a period of 30 min. The organic layer was separated, and the aqueous layer was extracted twice with CH_2Cl_2. The combined CH_2Cl_2 layers were washed with water, dried over Na_2SO_4, filtered, and evaporated in vacuo. Purification of the residue by chromatography (EtOAc:heptane, 1:1) afforded 26.3 g of Cbz-Lys (Boc)Ψ[cyanoacetate] (82%). TLC (CH_2Cl_2:EtOAc, 7:3) $R_f = 0.6$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.42 (s, 9 H), 2.09 and 2.12 (2 × s, 3 H, diastereomers), 3.10 (complex, 2 H), 4.03 (complex, 1 H), 4.59 (br s, 1 H), 5.11 (s, 2 H), 5.20 (complex, 1 H), 5.45 (br dd, 1H), 7.36 (s, 5 H).

Cbz-Lys(Boc)ϵ[CHOHCO]-OMe (IV, Scheme 2B). A solution of 26.3 g (62 mmol) of Cbz-Lys(Boc)Ψ[cyanoacetate] in 600 mL of Et_2O:MeOH, 3:1, was cooled to −20° C under a nitrogen atmosphere, and 66 g of gaseous hydrogen chloride was introduced keeping the temperature below −5° C. The reaction mixture was kept at 4° C overnight. Water (100 mL) was added dropwise to the reaction mixture keeping the temperature below 5° C. After it was stirred for 16 h at room temperature, the organic layer was separated and washed with water. The aqueous layer was saturated with NaCl and extracted with sec-BuOH:CH_2Cl_2, 3:2. The organic phase was washed with brine, dried over Na_2SO_4, filtered, and evaporated in vacuo to give 25.4 g of the crude amine. This was treated with di-*tert*-butyl dicarbonate (16 g, 73 mmol) in 400 mL of DMF in the presence of Et_3N (pH 8) at room temperature overnight. The solvent was removed by evaporation at reduced pressure. The residue was dissolved in EtOAc, washed with water and brine successively, dried over Na_2SO_4, filtered, and evaporated in vacuo. Purification of the crude product by chromatography (heptane:EtOAc, 6:4) gave Cbz-Lys(Boc)Ψ-[CHOHCO]-OMe (15.8 g, 60%). TLC (EtOAc:pyridine:HOAc:H_2O, 63:20:6:11) $R_f = 0.75$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.42 (s, 9 H), 3.10 (complex, 3 H), 3.74 and 3.80 (2 × s, 3 H, diastereomers), 4.08 (complex, 1 H), 4.18 and 4.33 (2 × dd, 1 H, diastereomers), 4.56 (br s, 1 H), 4.97 (br d, 1 H), 5.05 and 5.11 (2 × s, 2 H, diastereomers), 7.35 (m, 5H).

Boc-D-Phe-Pro-Lys(Boc)ψ[CHOHCO]-OMe. A solution of Cbz-Lys(Boc)Ψ[CHOHCO]-OMe (5.52 g, 13 mmol), 0.55 g of 10% palladium on charcoal, and 6.5 mL of 1 N HCl solution in 50 mL of DMF was hydrogenated for 2 h at room temperature. The catalyst was filtered off, and the filtrate was evaporated to dryness in vacuo. The crude amine was directly used in the coupling with Boc-D-Phe-Pro-OH.

A solution of Boc-D-Phe-Pro-OH (362 mg, 1 mmol) in 3 mL of DMF was treated with HOBt (175 mg, 1.3 mmol) and DCCI (265 mg, 1.3 mmol) at 0 °C for 1 h. At this temperature, a solution of the crude H-Lys(Boc)Ψ[CHOHCO]-OMe (290 mg, 1 mmol) in 2 mL of DMF was slowly added to the reaction mixture and the pH was set to 8 using Et_3N. The reaction was subsequently stirred for 16 h at room temperature. Next, the

mixture was cooled to −20 °C and DCU was removed by filtration after which the filtrate was concentrated in vacuo. The residue was dissolved in EtOAc and washed with 2% citric acid solution, water, 5% $NaHCO_3$ solution, and brine. The organic layers were dried over Na_2SO_4 and concentrated. Purification of the residue by chromatography (CH_2Cl_2:MeOH, 95:5) afforded 511 mg of Boc-D-Phe-Pro-Lys(Boc)Ψ[CHOHCO]-OMe (81%). TLC (EtOAc:pyridine:HOAc:H_2O, 88:31:18:7) R_f = 0.90. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.4−1.5 (2 × s, 18 H), 2.65 (complex, 1H), 3.00 (d, 2H), 3.56 (complex, 1H), 3.78 and 3.80 (2 × s, 3H), 4.12 (complex, 1H), 4.30 (complex, 1H), 4.53 (b dd, 1H), 7.26 (m, 5H). MS *m/z* (FAB^+): 635.2 [M + H], 535.2 [M − Boc + H], 435.1 [M − 2Boc + H].

Boc-D-Phe-Pro-Lys(Boc)ψ[CHOHCO]-OH. Boc-D-Phe-Pro-Lys(Boc)Ψ[CHOHCO]-OMe (510 mg, 0.80 mmol) was dissolved in 20 mL of dioxane:H_2O, 7:3, and treated with 0.8 mL of 2 M NaOH solution portionwise over 30 min at room temperature, keeping the pH at 10−10.5. The reaction mixture was diluted with water, 2 M HCl solution was added until pH 2.0, and the water layer was extracted with CH_2Cl_2 (3×). The combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to obtain 554 mg of Boc-D-Phe-Pro-Lys(Boc)Ψ[CHOHCO]-OH (100% yield). TLC (EtOAc:pyridine:HOAc:H_2O, 63:20:6:11) R_f = 0.30. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.41 (s, 9H), 1.44 (s, 9H), 2.56 (complex, 1H), 2.98 (b d, 2H), 3.09 (b s, 2H), 4.52 (b q, 1H), 7.24 (m, 5H). MS *m/z* (FAB^-): 619 [M − H]; (FAB^+): 621.2 [M + H], 521.2 [M − Boc + H], 421.2 [M − 2Boc + H].

Boc-D-Phe-Pro-Lys(Boc)ψ[COCO]-OH. To a solution of Boc-D-Phe-Pro-Lys(Boc)Ψ[CHOHCO]-OH (554 mg, 0.80 mmol) in 40 mL of CH_2Cl_2 was added Dess−Martin periodinane (339 mg, 0.80 mmol). After it was stirred for 1 h at room temperature, 35 mL of 2% $Na_2S_2O_4$ solution was added and the mixture was stirred for 30 min at ambient temperature. The organic layer was separated, washed with water, dried over Na_2SO_4, filtered, and concentrated to give 700 mg of Boc-D-Phe-Pro-Lys(Boc)Ψ[COCO]-OH (quantitative yield). TLC (EtOAc:pyridine:HOAc:H_2O, 94:20:11:6) R_f = 0.15. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.41 (s, 9H), 1.48 (s, 9H), 2.51 (complex, 1H), 3.0 (complex, 2H), 3.11 (complex, 2H), 3.60 (b s, 1H), 4.30−4.50 (complex, 2H), 4.80 (complex, 1H), 7.25 (m, 5H). MS *m/z* (FAB^+): 616.9 [M + H].

H-D-Phe-Pro-Lysψ[COCO]-OH (7). Boc-D-Phe-Pro-Lys(Boc)Ψ[COCO]-OH (700 mg, 0.80 mmol) was treated with 7 mL of 90% TFA/CH_2Cl_2 for 4 h at room temperature. The reaction mixture was concentrated in vacuo, and the residue was dissolved in water and directly charged on a preparative HPLC DeltaPak RP-C_{18} column using a gradient elution system of 20% A/80% B to 20% A/50% B/30% C in 45 min at a flow rate of 80 mL/min (A: 0.5 M phosphate buffer pH 2.1; B: water; C: acetonitrile/water = 6:4). The fractions containing **7** were isolated, desalted, and lyophilized (71 mg, 21.2%). ^{1}H NMR (400 MHz, D_2O): δ 1.10−1.60 (complex, 6H), 1.67 (m, 2H), 1.92 (complex, 1H), 2.51 (m, 1H), 2.82 (m, 2H), 3.00 (dd, 1H), 3.09 (dd, 1H), 3.36 (m, 1H), 4.04 (dd, 1H), 4.19 and 4.25 (2 x dd, 1H), 4.39 (dd, 1H), 7.15 (m, 2H), 7.26 (m, 3H). R_t (LC): 16.22 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 92.4% (LC). MS *m/z* (FAB^+): 419.1 [M + H], 437.3 [M + H_2O + H], 511.1 [M + glycerol]; (FAB^-): 417.0 [M − H], 509.1 [M + glycerol].

In an analogues way to the synthesis of **7**, compounds **16**, **17**, and **20**−**28** were prepared from the corresponding dipeptides.

H-D-Cha-Pro-Lysψ[COCO]-OH (16). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80−2.13 (m, 24H), 2.77 (t, 2H), 3.70−3.98 (m, 1H), 4.14 (m, 1H), 4.41 (m, 1H), 4.85 (m, 1H). R_t (LC): 22.14 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 97.4% (LC). MS *m/z* (FAB^+): 425 [M + H], 517 [M + glycerol]; (FAB^-): 423 [M − H].

$BnSO_2$-Asp-Pro-Lysψ[COCO]-OH (17). ^{1}H NMR (400 MHz, D_2O): δ 1.12−1.86 (complex, 9H), 2.02−2.15 (m, 1H), 2.41 (m, 1H), 2.66 (m, 1H), 2.84 (t, 2H), 3.14 (m, 1H), 3.36 (m, 1H), 3.99 (dd, 1H), 4.16 (m, 2H), 4.27−4.42 (dd, 2H), 7.27−7.38 (m, 5H). R_t (LC): 21.91 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 94.5% (LC). MS *m/z* (ESI^+): 541.2 [M + H], 559.2 [M + H_2O + H]; (ESI^-): 539.2 [M − H].

HOOC-CH_2-D-Cha-Pro-Lysψ[COCO]-OH (20). ^{1}H NMR (400 MHz, D_2O): δ 0.66−2.13 (m, 23H), 2.51−2.77 (2H, m), 3.16−3.55 (m, 4H), 3.82−4.72 (m, 3H). R_t (LC): 23.11 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 99.1% (LC). MS *m/z* (FAB^+): 483 [M + H], 501 [M + H_2O + H], 575 [M + glycerol + H]; (FAB^-): 481 [M − H], 499 [M + H_2O − H], 573 [M + glycerol − H].

HOOC-CH_2-D-Cha-Azt-Lysψ[COCO]-OH (21). ^{1}H NMR (400 MHz, D_2O): δ 0.93 (m, 2H), 1.00−1.80 (complex, 16H), 2.22 (complex, 1H), 2.62 (complex, 1H), 2.90 (b s, 2H), 3.65 (m, 2H), 4.09 (m, 1H), 4.18 (m, 1H), 4.30 (m, 1H), 4.81 and 4.88 (2 × dd, 1H), 4.93 (dd, 1H). R_t (LC): 21.09 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 99.3% (LC). MS *m/z* (FAB^+): 469.2 [M + H], 487.3 [M + H_2O + H], 561 [M + glycerol]; (FAB^-): 467.1 [M − H], 485 [M + H_2O − H].

HOOC-CH_2-D-Cha-Pec-Lysψ[COCO]-OH (22). ^{1}H NMR (400 MHz, D_2O): δ 0.82−2.20 (complex, 26H), 2.82 (m, 2H), 3.28 (tt, 1H), 3.34−3.55 (complex, 2H), 3,60 (b d, 1H), 4.06 (dd, 1H), 4.51 (m, 1H), 4.78 (dd, 1H), 4.83 and 4.90 (2 × dd, 1H). R_t (LC): 27.25 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 89.2% (LC). MS *m/z* (ESI^+): 497.2 [M + H], 515.4 [M + H_2O + H], 479.4 [M − H_2O + H]; (ESI^-): 495.2 [M − H].

HOOC-CH_2-D-Cha-DehydroPro-Lysψ[COCO]-OH (23). ^{1}H NMR (400 MHz, D_2O): δ 0.94 (m, 2H), 1.15 (m, 4H), 1.20−1.80 (complex, 22H), 1.92 (m, 1H), 2.91 (m, 2H), 3.38−3.62 (complex, 2H), 4.11 (dd, 1H), 4.27−4.39 (complex, 2H), 4.49 and 4.53 (2 × m, 1H), 4.93 (dd, 1H), 5.10 and 5.15 (2 × m, 1H), 5.78 and 5.83 (2 × m, 1H), 6.04 (m, 1H). R_t (LC): 21.58 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 95.9% (LC). MS *m/z* (ESI^+): 481 [M + H], 499 [M + H_2O + H].

HOOC-CH_2-D-Cha-3,3-Dmp-Lysψ[COCO]-OH (24). ^{1}H NMR (400 MHz, D_2O): δ 0.86 (s, 3H), 0.96 (s, 3H), 0.80−1.89 (complex, 21H), 2.83 (m, 2H), 3.47−3.76 (m, 2H), 3.47−3.65 (dd, 2H), 3.89 (s, 1H), 4.15 (dd, 1H), 4.28 (m, 1H). R_t (LC): 29.32 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 97.7% (LC). MS *m/z* (FAB^+): 511.4 [M + H], 529.4 [M + H_2O + H], 603.5 [M + glycerol]; (FAB^-): 509.4 [M − H], 601.5 [M + glycerol].

HOOC-CH_2-D-Cha-Ohi-Lysψ[COCO]-OH (25). ^{1}H NMR (400 MHz, D_2O): δ 0.95 (m, 2H), 1.02−2.02 (complex, 29H), 2.12 and 2.19 (2 × dt, 1H), 2.40 (m, 1H), 2.92 (m, 2H), 3.56−3.70 (m, 2H), 3.76 (m, 1H), 4.17 (dd, 1H), 4.23 (t, 1H), 4.33 and 4.41 (2 × dd, 1H), 4.89 (dd, 1H) (doubling of signals indicates the presence of both Ohi regioisomers). R_t (LC): 33.03 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 92.3% (LC). MS *m/z* (ESI^+): 537 [M + H], 555 [M + H_2O + H].

HOOC-CH_2-D-Cha-(N-cyclopentyl)-Gly-Lysψ[COCO]-OH (26). ^{1}H NMR (400 MHz, D_2O): δ 0.86 (m, 2H), 1.20−1.71 (complex, 6H), 1.78 (complex, 2H), 2.83 (m, 2H), 3.47−3.73 (complex, 2H), 3.90 (dd, 1H), 4.08 (complex, 1H), 4.55 (complex, 1H), 4.85 (m, 1H). R_t (LC): 29.77 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 92.6% (LC). MS *m/z* (FAB^+): 511.2 [M + H], 529.2 [M + H_2O + H], 603.3 [M + glycerol + H]; (FAB^-): 509.1 [M − H], 601.1 [M + glycerol − H].

HOOC-CH_2-D-Cha-Pro-Lysψ[COCO]-OiP (27). ^{1}H NMR (400 MHz, D_2O): δ 0.92 (m, 2H), 1.10 (m, 2H), 1.80 (d, 3H), 1.21 (d, 3H), 1.21−1.82 (complex, 12H), 1.96 (m, 1H), 2.20 (m, 1H), 2.89 (m, 2H), 3.40−3.61 (m, 2H), 3.69 (m, 1H), 4.21 (dd, 1H), 4.31−4.37 (complex, 2H), 4.90 (m, 1H). R_t (LC): 30.68 min 20% A/80% B to 20% A/20% B/60% C in 40 min; purity 96.6% (LC). MS *m/z* (ESI^+): 525.4 [M + H], 543.4 [M + H_2O + H], 507.6 [M − H_2O + H]; (ESI^-): 523.4 [M − H], 541.2 [M + H_2O − H].

HOOC-CH_2-D-Cha-Pro-Lysψ[COCO]-NH-$(CH_2)_2$Ph (28). ^{1}H NMR (400 MHz, D_2O): δ 1.08 (m, 2H), 1.20−2.00 (complex, 11H), 2.09 (m, 2H), 2.31 (m, 1H), 2.93 (m, 2H), 3.03 (m, 2H), 3.44−3.87 (complex, 6H), 4.15 (dd, 1H), 4.43−4.56 (m, 2H), 5.14 (dd, 1H), 7.33−7.49 (complex, 5H). R_t (LC): 38.61 min

20% A/80% B to 20% A/20% B/60% C in 40 min; purity 97.8% (LC). MS *m/z* (ESI^+): 586.0 [M + H], 605.0 [M + H_2O + H], 568.9 [M − H_2O + H].

Determination of Inhibition Constants. The thrombin assay conditions are described in Jetten et al. Human thrombin was used, and inhibition constants were calculated from plots using nine different inhibitor concentrations and always at least in duplicate.[19] Assays for factor Xa, factor VIIa/TF, trypsin, protein C, tPA, plasmin, etc. were performed according to published methods. Values for all serine proteases are the average of the least two determinations, where the variation in the assay is ±10%.

Determination of CACO-2 Transport. The CACO-2 cell permeability studies were performed as described in Walter et al.[44] with the following modifications: Cell passages 25−40 were used; seeding density 6.3×10^4 cells/cm^2 on collagen-coated filters 3.0 μm pore size; three 1 h incubation intervals; and sample analysis by antithrombin activity or specific HPLC method.

Determination of Hepatic Clearance in Perfusion Experiments. The liver of a rat was excised, and circular perfusion was performed during 120 min with 160 mL of a 2.5% bovine serum albumin solution in Krebs−Henseleit buffer, pH 7.4, at 37 °C, containing test compound in an initial concentration of 5 μM. At indicated time points, samples of buffer were taken and concentration of the compound was measured using the chromogenic substrate assay described for the determination of the antithrombin activity. The concentration of the test compound during this 120 min period was compared to the concentration in the buffer at the start of the experiment.

Determination of IV/PO Kinetics in Rats and Dogs. Animals and Anaesthetics. Male Wistar Hsd/Cpb WU rats (body weight, 200−500 g) were obtained from Harlan (Horst, The Netherlands) and anaesthetized intraperitoneally with sodium pentobarbital (Nembutal, 60 mg/kg; Sanofi, Toulouse, France) or methohexital sodium (40 g/L stock solution; 200 μL/100 g body wt Brietal, Eli Lilly). Body temperature was maintained at 37.5 ± 1 °C. Female beagle dogs (6−11 kg) were obtained from Marshall Europe, Lyon, France. All procedures were approved by the Ethics Committee of Animal Welfare in accordance with Dutch guidelines.

Determination of IV/PO Kinetics in Rats and Dogs. One day prior to administration of the serine protease inhibitors, rats (body weight 275−325 g) were anaesthetized by injection of methohexital sodium. The right jugular vein was cannulated with a PE-50 cannula (Clay Adams) filled with saline and led subcutaneously to the neck to be exteriorized. After a recovery period of at least 16 h, the tested compound was administered intravenously via the cannula after which the cannula was rinsed with saline. Depending on the way of administration (intravenously or a orally by gavage) and on the expected half-life of the tested compound, at least six time points were selected for blood sampling. Both ways of administration were carried out in separate animals. Blood was collected from the applied cannula in 0.05 volume 0.2 M Na_2-EDTA·$2H_2O$ and centrifuged at 125 000 N/kg for 2 min after which the plasma was stored below −20 °C until used for determination of the plasma concentration. In case the kinetic study was performed in dogs, both ways of administration were carried out in the same dog with a wash-out period of at least 2 weeks. Blood was collected from the jugular vein by means of a siliconized multisample needle and artificial evacuated blood collection tubes containing 5.8 mg of K_2EDTA for 3 mL blood samples (Terumo, Leuven, Belgium).

The residence time and percent bioavailability of the direct thrombin inhibitors were measured by determination of the anti-IIa activity in plasma. In view of its selectivity of the examined protease inhibitors, inhibition of thrombin linearly relates with the concentration of the measured protease inhibitor. Plasma anti-IIa activity was measured amidolytically with S-2238 (Chromogenix, Cromogenics Ltd., Molndal, Sweden) based on the method of Teien and Lie.[45] All samples were initially diluted with 3 volumes of tris-NaCl buffer, pH 7.4 (composition [mmol/L]: Tris, 50; NaCl, 100), and further with equally prediluted pooled rat (dog) plasma to obtain samples with an antithrombin content within the calibration curve (= range of 10−90% of factor IIa inactivation). After 100 μL of diluted sample with 50 μL of 0.5 U/mL human thrombin (Kordia Laboratory Supplies, Leiden, The Netherlands) was incubated during 2 min, 100 μL S-2238 (1.56 mg/mL) was added and anti-IIa activities were read from a calibration curve, by measuring the difference in optical density at 405 nm after 2 and 22 min (ΔOD_{405}), thus quantifying the residual activity of factor IIa. The calibration curve was obtained from activity measurements for the test compound itself, spiked in prediluted pooled rat (dog) plasma. Anti-IIa activities were expressed as nmol/mL.

The pharmacokinetic parameters were analyzed from the plasma concentration vs time curve using the computerized iterative procedure of MW/Pharm (Medi/ware, Groningen, The Netherlands), based on the Simplex method. Subsequently, the elimination half-lives were calculated using the model of relative error independent of the concentration, the area under the curve (AUC) was determined with the trapezium rule. Assuming linear kinetics, the percent bioavailability was calculated by dividing the AUC obtained after po administration by the mean expected normalized AUC after iv administration of that dose (× 100%).

Determination of Antithrombotic Potency in an Aorta Flow Model in Rats. Thrombus formation on a silk thread positioned in the aorta of a rat was induced as previously described.[46] Rats (350−500 g body wt) were weighed. A catheter (19 cm PT-46, Portex, Portland Plastics, Hythe, U.K.) containing a silk thread (size 4/0, uncoated USP, Pfrimmer, Erlangen, Germany) with two knots, tied at the end of the thread and protruding 3 mm, was inserted into the left common carotid artery. Subsequently, the catheter with thread was led via the carotid artery and the aorta into the left iliac artery. The silk thread was then fixed in position with a small bulldog clamp over the knots, which were just visible through the intact vessel wall. The protruding seal was then cut off the catheter, which was retracted, leaving the silk thread in place. Finally, the carotid artery was clamped, the iliac clamp was removed, and the abdominal cavity was covered provisionally. After 10 min of blood circulation, the silk thread covered with thrombus was carefully removed and gently rinsed. Thrombus weight and length were determined. Details of this well-established procedure have been published elswhere.[45,46]

X-ray Crystallography. Crystallization was essentially performed using the hirugen [53-65]-thrombin exchange procedure at room temperature with the sitting drop vapor diffusion technique. X-ray diffraction data were collected at 100 K as described previously.[47] Molecular replacement was carried out with the aid of the EPMR program and 1.59 Å resolution could be obtained after refinement.[48] The coordinates have been deposited at the Protein Data Bank.

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