Design and Synthesis of Potent and Highly Selective Thrombin Inhibitors

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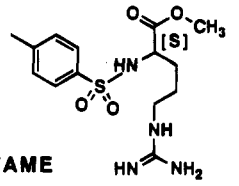
Thrombin, a serine protease, plays a central role in the initiation and propagation of thrombotic events. An extensive search for new thrombin inhibitors was performed, using an unconventional approach. Screening of small basic molecules for binding in the recognition pocket of thrombin led to the discovery of (aminoiminomethyl)piperidine (amidinopiperidine) as a weak, but intrinsically selective, thrombin inhibitor. Elaboration of this molecule provided compounds which inhibit thrombin with K_i's in the range of 20–50 nM and with selectivities of 1000–4000 against trypsin. These inhibitor compounds show a new and unexpected binding mode to thrombin. Modification of the central building block and then of one of the hydrophobic substituents led to the discovery of a new family of thrombin inhibitors which has reverted to the former binding mode to thrombin. This last class of compounds shows inhibitory activities in the picomolar range, low toxicity, and a short plasma half life which favors its use for an intravenous application. From this series of thrombin inhibitors, **19f** (Ro 46-6240) was selected for clinical development as an antithrombotic agent for intravenous administration.

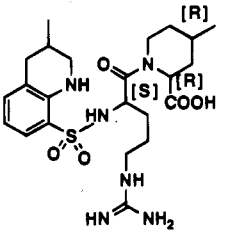
Introduction

Thrombotic vascular disease is a major cause of mortality in the industrialized world. Thrombin, a trypsin-like serine protease and a key enzyme in the blood coagulation cascade plays a major role in the development of the disease state, catalyzing the formation of polymerizable fibrin from fibrinogen as well as stimulating platelet aggregation. The currently used antithrombotics, heparin and coumarins, suffer from well-documented liabilities. Heparin applied parenterally depends on antithrombin III for its antithrombotic activity, interferes with platelet function, and is neutralized by platelet release products. It exhibits low bioavailability as well as dose dependent pharmacokinetics.[1] All these factors might be responsible for the relatively narrow therapeutic range of antithrombotic efficacy and for the bleeding liability. The orally active coumarins act with a delay of several days, need to be monitored due to variable dose response, and are affected by the vitamin K content of the diet.[2] A new antithrombotic with an immediate onset of action is therefore clearly needed, and thrombin is an attractive target for therapeutic intervention.[3] Considerable effort has recently been directed toward the development of inhibitors of thrombin.[4] Hirudin,[5] a polypeptide isolated from the medical leech, is currently in phase III clinical trials[6] as is Hirulog-8,[7] one of a series of peptide inhibitors derived from hirudin. A variety of low molecular weight thrombin inhibitors are also under investigation.[8]

The development of synthetic low molecular weight inhibitors of thrombin can be traced back to Nα-tosylargininemethyl ester, TAME,[9] which is a poor substrate. Independent lines of development led to the identification of the lead inhibitor Argatroban[10] (MD-805) by Okamoto and co-workers[11–13] and of the lead inhibitor NAPAP, Nα-((2-naphthylsulfonyl)glycyl)-DL-*p*-amidinophenylalanylpiperidide, by Stürzebecher and co-workers.[14,15] Both are still clearly related to TAME, with NAPAP having extra a glycine "spacer".



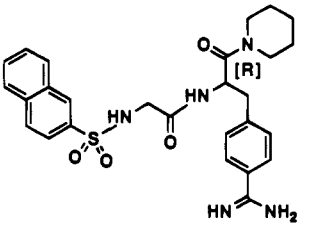
TAME



Argatroban, MD-805

K_i = 0.020 μM

Tr / Th = 210



[R] - N A P A P [15]

K_i = 0.004 μM

Tr / Th = 30

Initial synthetic work in our laboratories on low molecular weight thrombin inhibitors derived from these lead structures led to compounds which either were toxic and/or had severe hemodynamic side effects. It was thus thought worthwhile to investigate an alternative approach to the discovery of inhibitors independent of the known lead structures. A search for small basic compounds showing both affinity for thrombin and some selectivity against trypsin was initiated. The criterion of selectivity was introduced as it was realized that many enzymes of the coagulation system are closely related to thrombin and trypsin and share the same primary substrate specificity, having a recognition pocket with an aspartic acid at the bottom which binds preferentially the basic amino acids arginine and

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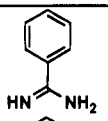
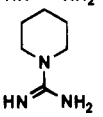
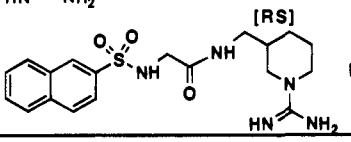
Table 1. In Vitro Activities of Selective Building Blocks

compd.	K_i [μM] Thrombin	K_i [μM] Trypsin	selectivity (Tr/Th)	formula[a]
Benz-amidine 	0.300	0.031	0.1	$C_7H_8N_2$
1  0.5 H_2SO_3	0.150	0.360	2.4	$C_6H_{13}N_3 \cdot 0.5\ H_2SO_3 \cdot 0.12\ CH_4N_2SO_3$[b]
3a  HCl	0.480	75	156	$C_{19}H_{25}N_5O_3S \cdot 1\ HCl \cdot 0.08\ H_2O$[c]

[a] All new compounds analyzed correctly (±0.4%) for C, H, N, except were noted. [b] N: calcd, 24.78; found, 23.90. [c] C: calcd, 51.87; found, 51.34; H: calcd, 5.96; found, 6.43.

lysine. It could well be desirable not to interfere with these related enzymes. Trypsin is the least specific enzyme of this family, and so the ratio of K_i trypsin/K_i thrombin (indicated below as Tr/Th) was chosen to provide a first indication of selectivity.

We now report that this approach led to the discovery of a new arginine mimetic and later to derivatives of D-phenylalanyl-3-[(aminomethyl)amidino]piperidine, which form a new class of low molecular weight, competitive, and reversible thrombin inhibitors.

Initial Considerations. (1) The nature of the primary recognition pocket of thrombin could be inferred from the known crystal structures of inhibited trypsin.[16] Trypsin-like serine proteases all have a highly conserved hydrophobic pocket which has an aspartic acid (189) at the bottom interacting with the basic side chain functional groups of arginine and lysine. Thrombin is arginine specific, and the specificity was thought to arise from the sequence change at position 190, which is serine in trypsin and alanine in thrombin.

(2) Benzamidine was known to bind to trypsin by making the predicted salt bridge to Asp-189.[16] Other similar small molecules were expected to bind in the same way to both trypsin and thrombin.

(3) Small molecules of this type are referred to as "needles". It was assumed that for thrombin inhibitors interacting with the active site region some kind of needle is a prerequisite.

(4) Benzamidine shows selectivity for trypsin. It was expected, by analogy, that it would be possible to find needles showing selectivity for thrombin.

(5) A "needle screening" was implemented, using amidolytic assays selected to best identify thrombin and trypsin inhibitors of low affinity.

(6) The best needles using the double criterion of affinity and selectivity were subject to further elaboration using building blocks expected from previous studies to give enhanced thrombin affinity.

The advantage of this procedure was that a very large amount of information could be collected with a minimum of chemical expenditure. More than 200 simple synthetic building blocks were evaluated and led to the identification of the piperidine **1** (Table 1), which exhibits thrombin selectivity by a factor of about 2 as well as a 2-fold increase in activity compared to benzamidine. Development of this fragment provided the lead compound **3a** with an affinity (K_i) for thrombin of 0.5 μM and a selectivity of 150 against trypsin.

The D-Amino Acid Series of Inhibitors. At this stage of the project the X-ray crystal structure of human thrombin was determined.[15] The major difference between the structure of thrombin and that of the other related serine proteases was seen to be the presence uniquely in thrombin of an insertion loop (the Tyr-Pro-Pro-Trp loop). This creates near to the active site two hydrophobic pockets which we call the P-pocket and the D-pocket (proximal and distal relative to the catalytic center). The lead inhibitors NAPAP and MD-805 bind with the larger aryl group in the D-pocket (aryl binding pocket),[17] the smaller piperidide in the P-pocket (S_2 hydrophobic pocket),[17] and the oxyanion hole empty. This "*inhibitor binding mode*" stands in marked contrast to earlier models based on "substrate-like" binding where the carbonyl group of the basic amino acid was placed in the oxyanion hole (Figure 1).

A computer modeling study of **3a** showed that it was possible to superpose the naphthalene moiety exactly on that of the corresponding group of NAPAP and at the same time place the piperidide in the recognition pocket with the amidino group interacting with Asp189 as expected. This hypothetical binding mode immediately suggested that the P-pocket could be filled with a hydrophobic group if this were attached to the α carbon of the glycyl moiety of the inhibitor so as to give a D-amino acid.

One of the suggested inhibitors, a D-phenylalanine derivative **3b**, was readily synthesized and tested. The measured K_i for thrombin of 0.050 μM with a selectivity of 840 against trypsin was most encouraging. Further analogs (Table 2) established these D-amino acid derivatives as an interesting new series of lead inhibitors.

In order to determine the best overall configuration the tryptophan derivatives **3d**, **3e**, **3f**, and **3g**, respectively, were synthesized. The binding data (Table 2) show that the preferred configuration is *R* at the amino acid, as predicted, and *S* at the piperidine ring.

A number of D-amino acid derivatives were synthesized and found to have K_i's in the low nanomolar range. It became apparent, however, that the structure–activity relationships for this series of compounds were not as expected. For instance, compounds **3h**, **3i**, **3k**, and **3l** show high affinities for thrombin (Table 2),

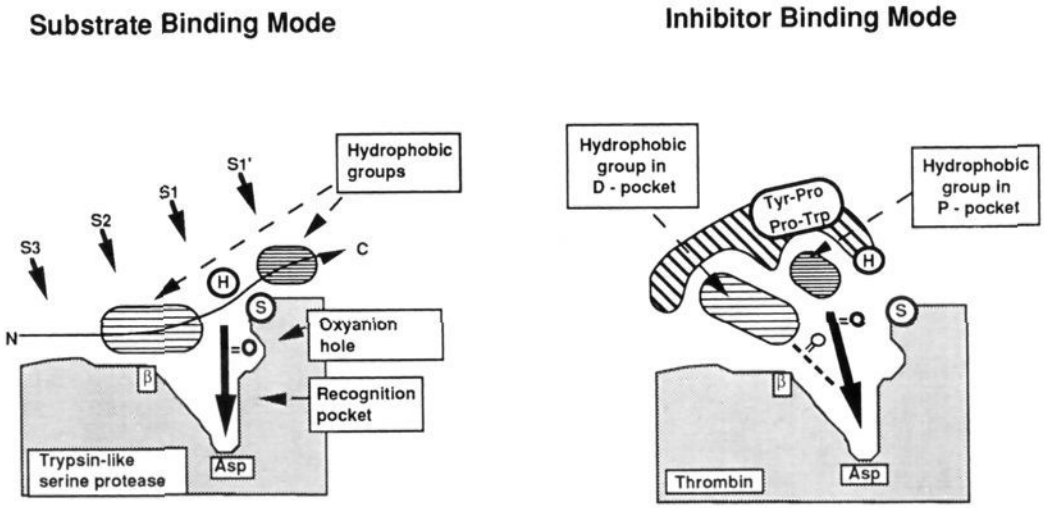
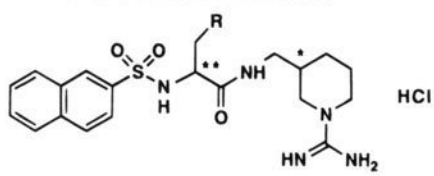


Figure 1. Left, Substrate binding mode: Schematic drawing of the supposed mode of inhibitor binding to a trypsin-like serine protease, based on the known mode of binding of substrates. The notation for substrate binding is that of Schechter and Berger.[44] Subsites on the enzyme are named S1, S2, ..., and S1′, S2′, ..., substituent positions corresponding to these sites are named P1, P2, ..., and P1′, P2′, ... The basic amino acid side chain, drawn as a bold arrow, is placed in the P1 position with its carbonyl binding in the oxyanion hole. The flanking hydrophobic side chains of the inhibitor are put into the P2 and P1′ positions, respectively. The histidine and serine of the catalytic center are shown as H and S, respectively. Peptide substrates bind in the direction N → C as shown and make an antiparallel β interaction with Gly216, indicated by β. Right, Inhibitor binding mode: Schematic drawing of the mode of binding of NAPAP and MD-805 to human thrombin as revealed by the crystal structure analyses of the inhibitor–protein complexes.[15] The Tyr-Pro-Pro-Trp loop, unique to thrombin, helps form two hydrophobic pockets in which the two hydrophobic moieties of the inhibitors bind. The hydrophobic P-pocket, proximal to the catalytic center, corresponds to the S2 subsite for substrates and binds the inhibitor piperidide. The D-pocket, distal to the catalytic center, is also hydrophobic and binds the aryl moieties of these inhibitors (hence also called the "aryl binding site"[17]). The amidinophenylalanine side chain of NAPAP is drawn as a bold arrow; the arginine side chain of MD-805 is drawn as a dotted arrow. Note that the oxyanion hole is empty and the entry of the basic amino acids into the recognition pocket is different for both inhibitors and different from that of the "substrate binding mode", although both preserve the antiparallel β interaction with Gly-216.

Table 2. In Vitro Activities of the D-Amino Acid Series of Thrombin Inhibitors



compd	R	**	*	K_i (μM) thrombin	K_i (μM) trypsin	selectivity (Tr/Th)	formula[a]
3b	phenyl	(*R*)	(*RS*)	0.047	42	840	$C_{26}H_{31}N_5O_3S$·HCl·0.60EtOH·0.11H_2O[b]
3c	phenyl	(*R*)	(*S*)	0.032	26	810	$C_{26}H_{31}N_5O_3S$·HCl·0.36EtOH·0.02H_2O[c]
3d	3-indolyl	(*R*)	(*RS*)	0.018	33	1833	$C_{28}H_{32}N_6O_3S$·HCl·0.42EtOH·0.15H_2O
3e	3-indolyl	(*S*)	(*RS*)	0.660	27	41	$C_{28}H_{32}N_6O_3S$·HCl·0.50EtOH·0.1H_2O[d]
3f	3-indolyl	(*R*)	(*S*)	0.009	17	1889	$C_{28}H_{32}N_6O_3S$·HCl·0.89EtOH·0.13H_2O[e]
3g	3-indolyl	(*R*)	(*R*)	0.130	130	1000	$C_{28}H_{32}N_6O_3S$·HCl·0.59EtOH·0.22H_2O[f]
3h	4-NO_2-phenyl	(*R*)	(*RS*)	0.024	31	1292	$C_{26}H_{30}N_6O_5S$·HCl·0.48EtOH·0.21H_2O
3i	4-NO_2-phenyl	(*R*)	(*S*)	0.010	12	1212	$C_{26}H_{28}N_6O_5S$·HCl·0.41H_2O[g]
3k	4-AcNH-phenyl	(*R*)	(*RS*)	0.038	20	526	$C_{28}H_{34}N_6O_4S$[h]
3l	4-(*p*-tos)-NH-phenyl	(*R*)	(*RS*)	0.063	32	508	$C_{33}H_{38}N_6O_5S_2$[h]

[a] All new compounds analyzed correctly (±0.4%) for C, H, N, except were noted. [b] C: calcd, 58.58; found, 58.15. [c] C: calcd, 58.71; found, 58.23. [d] C: calcd, 58.82; found, 58.37. [e] C: calcd, 58.62; found, 58.07. [f] 0.46% residue, C: calcd, 58.77; found, 58.02. [g] C: calcd, 54.49; found, 53.80; H: calcd, 5.10; found, 5.64. [h] Characterized by ^{1}H-NMR, IR, and MS.

despite the fact that the phenyl moiety carries a substituent in the 4-position. Both the inhibitor binding model and the known structure–activity relationships for NAPAP and Argatroban[11-13] indicate that the P-pocket accepts preferentially a hydrophobic group which may not be extended in this manner without severe loss of affinity. An X-ray structure determination of **3c** bound to human thrombin was undertaken (see methods) and showed that this compound indeed binds in a novel "*second inhibitor binding mode*" (Figures 2 and 3) in which the two hydrophobic groups have exchanged places.

The naphthalene group runs across the front of the P-pocket and into the back of the D-pocket, the side chain of the D-amino acid is in a rather exposed position at the front of the D-pocket, and the sulfone accepts a hydrogen bond from the NH of Gly216. The second inhibitor binding mode explains nicely the observed structure–activity relationships for all of this series of compounds. In particular, it is clear (Figure 3) that substituents such as those in the 4-position of the phenyl group of compounds **3i–l** will lie along the surface of the protein and thus may be both large and hydrophilic.

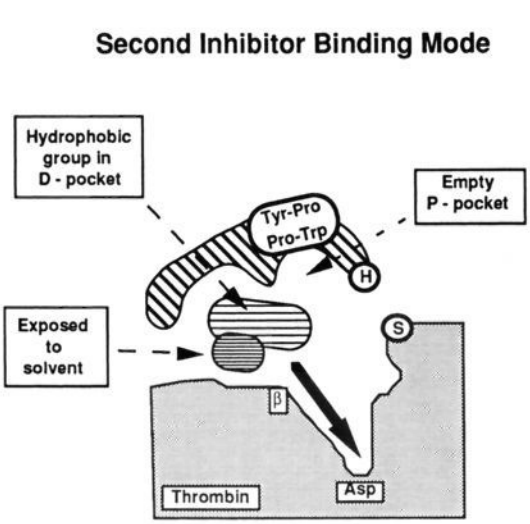


Figure 2. Second inhibitor binding mode: Schematic drawing of a D-phenylalanine derivative binding to human thrombin, notation as in Figure 1. The naphthylsulfonyl group runs from the front of the P-pocket (S2 subsite) to the back of the D-pocket (aryl binding site), whereas the benzyl ring of the phenylalanine lies more on the surface of the protein, exposed to solvent. Surprisingly there is no hydrophobic substituent deep in the P-pocket. Note that the amidinopiperidine substituent, represented as a bold arrow, enters the recognition pocket from a position further to the left than that seen for previous inhibitors.

There is thus enormous potential for synthetic variation of this part of the inhibitor. Many such variants were made, the binding constants (data not shown) falling generally in the range of those in Table 2.[18] Notwithstanding this possibility for synthetic variation without loss of affinity, the presence of an unnatural amino acid as the central building block was found to be disadvantageous from the synthetic point of view.

The L-Aspartate Series of Inhibitors. Although many synthetic routes to unnatural amino acids have been reported, it nevertheless was considered worthwhile to evaluate other central templates which might provide easier synthetic access to a wide range of side chain variants. Aspartic acid was identified as a simple and readily available central unit. A first possibilty was to attach, as before, the 3-(aminomethyl)-1-amidinopiperidine unit (**7**) to the α-carboxyl and the arylsulfonyl group to the amino acid nitrogen, leaving the side-chain β-carboxyl available for derivatization. Compounds of this type were found to be similar in activity as those described above (data not shown).

There also exists, however, a second possibility of attaching the basic group to the β-carboxyl, leaving the α-carboxyl free for derivatization, but at the expense of introducing an extra methylene group in the framework of the inhibitor. It could be shown that compounds of that type are more potent if the configuration of the aspartic acid is *S*, corresponding to the naturally occurring L-aspartic acid (data not shown). Our interest was aroused by a number of these latter compounds (**18a**,**b**, **19d**, Table 3), where the structure–activity relationships had clearly reverted to that expected for the "first inhibitor binding mode".[11–14]

We determined the X-ray crystal structure of **18b** bound to human thrombin and confirmed reversion to the "first binding mode" (data not shown). The cyclic amide binds in the hydrophobic P-pocket, as predicted. Compound **19d** was synthesized and showed a promising inhibitory activity. This compound has a piperidide identical to that of the lead compound MD-805.[19] The X-ray crystal structure of **19d** was determined and is shown in Figure 4. The piperidide lies in the P-pocket, displaced about 1.5 Å from the position of the corresponding moiety of MD-805.[15]

The Picomolar Thrombin Inhibitors. Compound **19d** has high affinity to thrombin and was notable for its low toxicity; however, the piperidide has two stereocenters and is difficult to synthesize. A search for simple piperidide substitutes was thus initiated, supported by computer-modeling experiments. Two simple possibilities are shown in Figure 5. The *N*-methylleucine derivative still retains one stereocenter, but the N-substituted glycine derivative has no stereocenters and is synthetically readily accessible.

L-Aspartic acid derivatives containing N-substituted glycines were found to belong to the group of most potent, low molecular weight, reversible thrombin inhibitors known to us.[8] As shown in Table 4, small hydrophobic substituents at the glycine nitrogen are preferred. Larger hydrophobic groups are tolerated, but the *N*-methyl compound (**19i**) binds poorly. The corresponding ethyl ester compounds (**18e–i**) are 2–3 orders of magnitude less active than the corresponding acids.

We determined the X-ray crystal structure of **19g**, shown in Figure 6. The glycine N-substituent is in the P-pocket and clearly has to be hydrophobic and of small to medium size. The carboxylate group of the N-substituted glycine of the inhibitor may be seen to make three favorable new interactions:

(i) One carboxylate oxygen is now positioned so as to accept a hydrogen bond from the NH of the amide linking the piperidine needle to the aspartic acid template of the inhibitor.

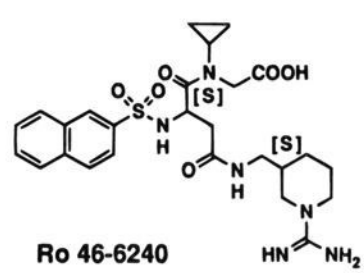
(ii) The side of the carboxylate group packs well against the 4-position of the piperidine ring.

(iii) The other carboxylate oxygen is within hydrogen bonding distance of both the histidine and the serine of the catalytic center.

As the first two of these interactions are intramolecular they may contribute toward making this inhibitor conformation more favorable in solution.

These new interactions lead to binding constants to thrombin approximately 2 orders of magnitude better than that of MD-805.

From this series of new competitive and reversible low molecular weight thrombin inhibitors[20] compound **19f** (Ro 46-6240)[21] was selected, on the basis of initial experiments, as having the most appropriate pharmacological properties (low toxicity and a half-life of 15 min) for clinical investigation.



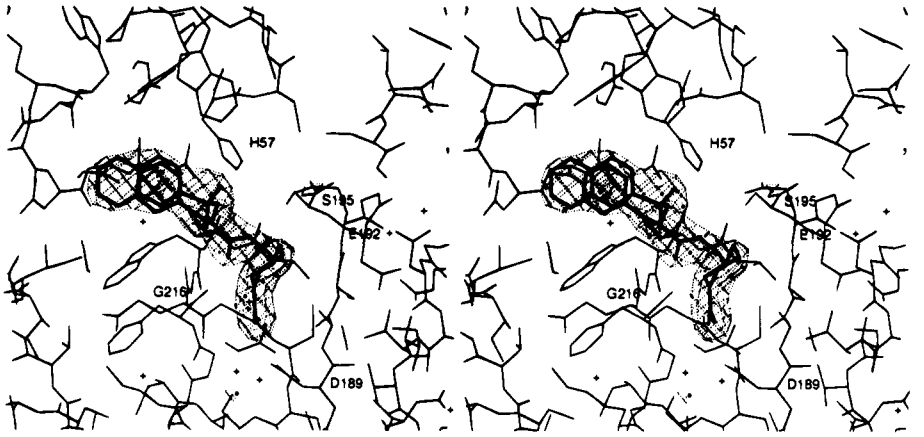
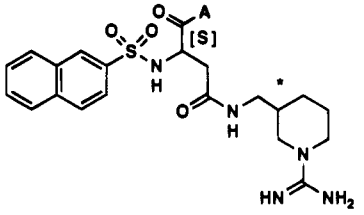
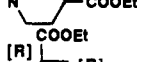
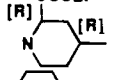
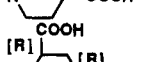
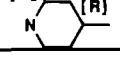


Figure 3. Omit map difference electron density for **3c**. The contour level is 0.08 electrons per Å^3. The residues labeled are H57, S195, the histidine and serine of the catalytic triad; D189, the aspartic acid at the bottom of the recognition pocket; G216, the glycine at the left hand side of the recognition pocket; and E192, the glutamic acid at the right hand side of the recognition pocket. Crosses mark the positions of water molecules. At this contour level there is no residual density in the active site region other than for further water molecules and some disorder of E192. As well as the expected interaction with D189, hydrogen bonds (not shown) are also found between the inhibitor and glycine 218 (two), glycine 216 (NH to the sulfone), and a water deep in the recognition pocket. The electron density for the phenyl group is rather weak.

Table 3. In Vitro Activities of the Aspartic Acid Series of Thrombin Inhibitors (Cyclic Amides)



compd.	A	salt	*	K_i [μM] Thrombin	K_i [μM] Trypsin	selectivity (Tr/Th)	formula[a]
18a		HCl	[RS]	0.019 00	7.50	395	$C_{25}H_{34}N_6O_5S{\cdot}1HCl{\cdot}0.28H_2O$[b]
18b		HCl	[RS]	0.004 8	28.0	5833	$C_{28}H_{40}N_6O_4S{\cdot}1HCl{\cdot}0.22H_2O$[c]
18c		HCl	[RS]	0.093	5.40	58	$C_{29}H_{40}N_6O_6S{\cdot}1HCl{\cdot}0.21EtOH{\cdot}0.22H_2O$
18d		AcOH	[S]	0.003 1	1.10	355	$C_{30}H_{42}N_6O_6S{\cdot}1.0AcOH{\cdot}1.29H_2O$[d]
19c		-	[RS]	1.400	130	93	$C_{27}H_{36}N_6O_6S{\cdot}0.23H_2O$
19d		-	[S]	0.006 7	0.78	116	$C_{28}H_{38}N_6O_6S{\cdot}0.29H_2O$

[a] All new compounds analyzed correctly (±0.4%) for C, H, N, except were noted. [b] C: calcd, 52.95; found, 52.44; N: calcd, 14.82; found, 14.32. [c] C: calcd, 56.70; found, 56.24. [d] H: calcd, 7.01; found, 7.60.

Conclusion

Screening of small, basic molecules for thrombin binding and intrinsic thrombin selectivity led to the identification of 1-amidinopiperidine. This was elaborated in two stages to provide compounds which inhibit thrombin with K_i's in the range of 20–50 nM and with selectivities of 1000–4000 against trypsin. Modification, for synthetic reasons, of the central building block of these compounds, followed by simplification of one of the other moieties, led to the discovery of a new family of thrombin inhibitors. These show inhibitory activities in the picomolar range and many exhibit low toxicity and a favorable pharmacokinetic behavior. The cyclopropyl derivative **19f** (Ro 46-6240) was selected for further development.

Chemistry

All target compounds shown in Tables 1–4 were prepared according to Schemes 1–4.

The novel thrombin inhibitors of the D-amino acid series can be prepared as outlined in Scheme 1. Sulfonylation of the appropriate amino acids with 2-naphthalenesulfonyl chloride using Schotten–Baumann conditions led to the intermediates **2a–h.** Coupling of these N-sulfonylated amino acids with either the racemic (**7a**) or enantiomerically pure (**7b,c**) needle 3-

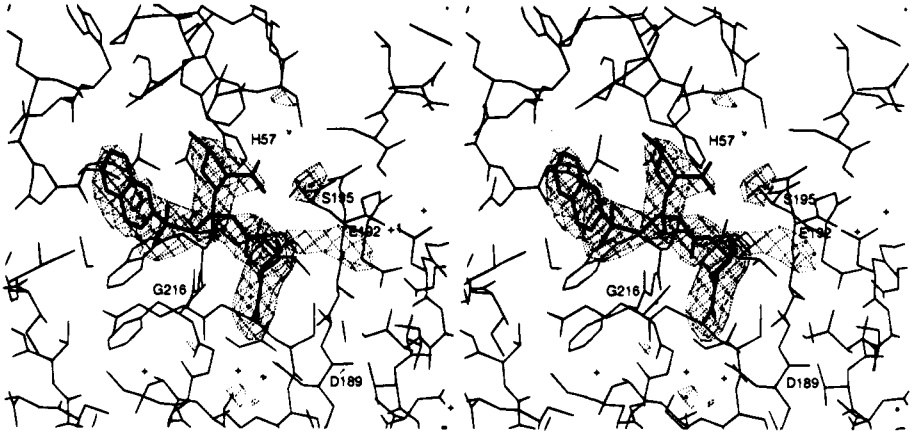


Figure 4. Omit map difference electron density for **19d**. The contour level is 0.1 electrons per Å^3. Residue labels as above. Residual small electron density peaks may be interpreted as further water molecules. The inhibitor refined to an occupancy of 50% (see methods). E192 is disordered and the extra electron density in front of this residue comes from the disordered amino-terminal region of a neighboring molecule in the crystal. In addition to those between inhibitor **3c** (above) and the protein, a second hydrogen bond is found to glycine 216, another to a water in the oxyanion hole, and another to a probable water above the Oγ of serine 195. The inhibitor has also an internal hydrogen bond to the carboxylate.

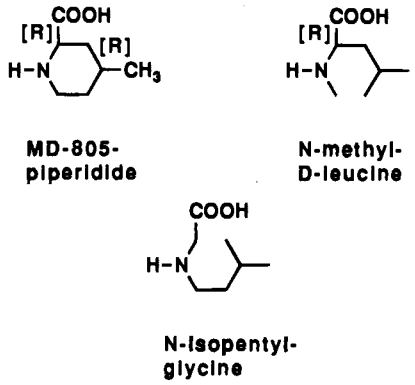


Figure 5.

(aminomethyl)-1-amidinopiperidine led to the target compounds **3a–l**.

The synthesis of the racemic intermediate **7a** is outlined in Scheme 2. *N*-Boc-protected 3-picolylamine **4** was hydrogenated to the piperidine **5a**. Amidination of the piperidine ring following a protocol described by Mosher et al.[22] led to **6a** as the salt (0.5 H_2SO_3). Cleavage of the protective group gave the racemic building block **7a** as the hydrochloride salt.

The preparation of the enantiomerically pure intermediate **7b** is depicted in Scheme 3. Treatment of protected (*S*)-3-(hydroxymethyl)piperidine **8b**[23,24] with methanesulfonyl chloride led to mesylate **9b**. After conversion of **9b** to the azide **10b** catalytic hydrogenation gave the amine **11b**. Protection of the amino function with benzyl chloroformate afforded **12b**. Cleavage of the Boc protecting group and standard amidination as described above led to the guanidine **13b**. Deprotection of the primary amine by catalytic hydrogenation finally yielded enantiomerically pure **7b**.

The enantiomer **7c** was prepared in the same way, starting with the (*R*)-alcohol **8c**.

Scheme 4 illustrates the synthesis of the thrombin inhibitors of the aspartic acid family. Standard coupling methods (BOP) were used to convert the amines H-A and *N*-Boc-protected aspartic acid β-benzyl ester (cyclic amides) or β-*tert*-butyl ester (substituted glycines), respectively, to intermediates **15a–i**. Cleavage of the BOC protecting group with either hydrochloric acid or *p*-toluenesulfonic acid and subsequent sulfonylation under sligthly basic conditions gave intermediates **16a–i**. Removal of the β-ester function by either catalytic hydrogenation (**16a–c**) or with TFA (**16d–i**) led to the free acids **17a–i** which in turn were coupled with **7a–c** (standard BOP coupling conditions) to give **18a–i**. Hydrolysis of the ethyl ester (**18c–i**) provided the target compounds **19c–i**.

Experimental Section

The abbreviations used are as follows: BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide.

Chemistry. 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*. All organic extracts were dried over Na_2SO_4. Normal-phase silica gel used for flash chromatography was Kieselgel-60 (230–400 mesh); reverse-phase silica gel used was Lichroprep RP 18 (40–63 μm); both were supplied by E. Merck, A. G., Darmstadt, Germany. TLC plates coated with silica gel 60 F_{254} (Merck) were used; detection was by UV (254 nm), by I_2 vapor, or by treatment with Cl_2/*o*-toluidine. Ion-exchange resin used was Dowex 1 × 2 (Cl^- form; 200–400 mesh) from Fluka, Switzerland. Melting points were determined with a Büchi 512 apparatus and are uncorrected. Proton NMR spectra (^{1}H-NMR) were recorded on a Bruker AC 250 spectrometer; δ values in ppm relative to tetramethylsilane are given. Optical rotations $[\alpha]^{20}_D$ were determined with a Perkin-Elmer 241 polarimeter, *c* in g/100 mL. Mass spectra (MS) were recorded with an MS 901 AEI apparatus. Results of elemental analysis were within 0.4% of the theoretical values unless otherwise noted.

Preparation of (*RS*)-3-(Aminomethyl)piperidine-1-carboxamidine Dihydrochloride (7a). (Pyridin-3-ylmethyl)-carbamic Acid *tert*-Butyl Ester (4). To a solution of 3-picolylamine (30 g, 0.277 mol) in dioxane (300 mL) was added a solution of di-*tert*-butyl dicarbonate (66.6 g, 0.305 mol) in dioxane (200 mL). After 3 h at room temperature the solvents were evaporated, and the residue was chromatographed with EtOAc to give **4** (55.5 g, 100%) as a colorless oil: ^{1}H-NMR ($CDCl_3$) δ 1.46 (s, 9 H), 4.33 (d, J = 5 Hz, 2 H), 4.96 (br, s, 1 H), 7.27 (dd, J = 5, 8 Hz, 1 H), 7.35 (d, J = 8 Hz, 1 H), 8.52 (s + d, J = 5 Hz, 2 H); MS *m/z* (EI) 209 (M + H), 152 (M − isobutylene). Anal. ($C_{11}H_{16}N_2O_2$·0.17H_2O) C, H, N.

(*RS*)-(Piperidin-3-ylmethyl)carbamic Acid *tert*-Butyl Ester (5). To a solution of **4** (45.5 g, 0.218 mol) in MeOH (220 mL) was added ruthenium on alumina (4.6 g), and the mixture was stirred under a hydrogen atmosphere of 100 bar at 60 °C. The reaction mixture was filtered and the filtrate evaporated

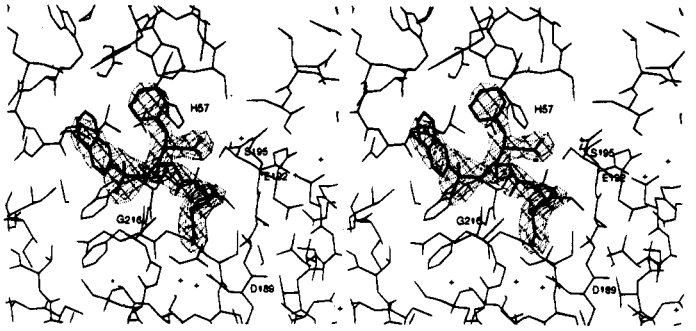
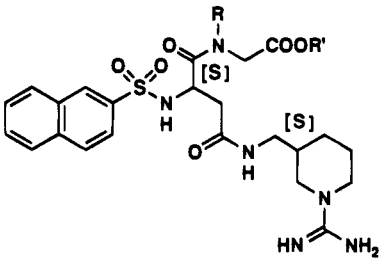


Figure 6. Omit map difference electron density for **19g**. The contour level is 0.18 electrons per Å^3. At this contour level there is no residual density in the active site region. Residue labels as above. The inhibitor makes almost exactly the same hydrogen bonds to the protein and to bound water as does **19d** shown above. An important difference, however, is that the carboxylate has rotated down and back. One oxygen still accepts the internal hydrogen bond and the one from the water in the oxyanion hole, but the other now interacts with both the serine and histidine of the catalytic triad. The carboxylate also now packs well against the 4-position of the amidinopiperidine of the inhibitor, protecting it from the solvent.

Table 4. In Vitro Activities of the Aspartic Acid Series of Thrombin Inhibitors (Substituted Glycines)



compd	R	R′	salt	K_i (μM) thrombin	K_i (μM) trypsin	selectivity (Tr/Th)	formula[a]
18e	cyclohexyl	Et	HCl	0.11	27.0	250	$C_{31}H_{44}N_6O_6S$·HCl·0.12H_2O[b]
18f	cyclopropyl	Et	HCl	0.086	30.0	350	$C_{28}H_{38}N_6O_6S$·HCl·0.11H_2O[c]
18g	benzyl	Et	HCl	0.070	8.60	120	$C_{32}H_{40}N_6O_6S$·HCl·0.61EtOH·0.21H_2O
18h	n-butyl	Et	HCl	0.330	48.0	150	$C_{29}H_{42}N_6O_6S$·HCl·0.33H_2O
18i	methyl	Et	HCl	0.013	23.0	1800	$C_{26}H_{36}N_6O_6S$·HCl·0.37H_2O
19e	cyclohexyl	H		0.000 71	3.80	5400	$C_{29}H_{40}N_6O_6S$·0.10H_2O[d]
19f	cyclopropyl	H		0.000 27	1.90	7143	$C_{26}H_{34}N_6O_6S$·0.72MeOH·0.45H_2O
19g	benzyl	H		0.000 68	0.150	220	$C_{30}H_{36}N_6O_6S$·EtOH·0.16H_2O[e]
19h	*n*-butyl	H		0.000 86	3.10	3600	$C_{27}H_{36}N_6O_6S$·0.07H_2O
19i	methyl	H		0.100	20.0	200	$C_{24}H_{32}N_6O_6S$·0.34H_2O[f]

[a] All new compounds analyzed correctly (±0.4%) for C, H, N, except were noted. [b] C: calcd, 55.97; found, 55.24; N: calcd, 12.63; found, 11.71. [c] C: calcd, 53.97; found, 53.40. [d] 0.78% residue. [e] C: calcd, 58.70; found, 58.24. [f] C: calcd, 54.12; found, 53.42.

to give **5** (46 g, 89%): mp 69–70 °C dec; ^{1}H-NMR ($CDCl_3$) δ 1.0–1.20 (m, 1 H), 1.32–1.84 (m, 5 H), 1.44 (s, 9 H), 2.30 (dd, J = 12, 14 Hz, 1 H), 2.54 (ddd, J = 5, 12, 15 Hz, 1 H), 2.88–3.10 (m, 4 H), 4.54 (br s, NH); MS m/z (FAB) 214 (M^+), 141 (M − OtBu). Anal. ($C_{11}H_{22}N_2O_2$) C, H, N.

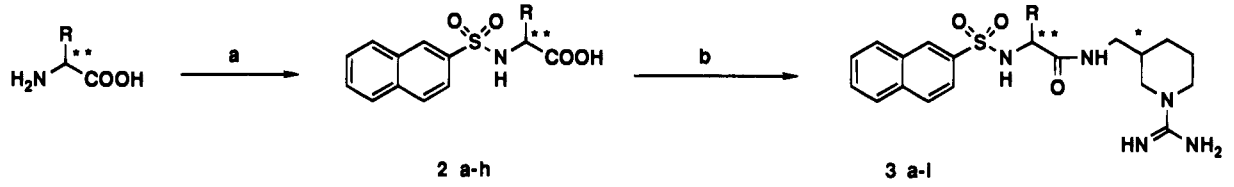
(*RS*)-[[1-(Aminoiminomethyl)piperidin-3-yl]methyl]-carbamic Acid *tert*-Butyl Ester (6a). A mixture of **5** (38.8 g, 0.180 mol) in DMF (900 mL) and NEt_3 (74.5 mL, 0.540 mol) was treated with formamidinesulfinic acid[22] (26.5 g, 0.214 mol) at room temperature for 15 h. The reaction mixture was filtered, the filtrate evaporated, the residue taken up in EtOAc and washed twice with H_2O. The organic layer was dried, evaporated, and azeotroped successively with EtOH, toluene, and dichloroethane. The residue was suspended in ether, filtered, and dried to yield colorless **6a** (44 g, 82%): mp >240 °C dec; ^{1}H-NMR (DMSO-d_6) δ 1.08–1.26 (m, 1 H), 1.38 (s, 9 H), 1.5–1.82 (m, 4 H), 2.60–2.76 (m, 1 H), 2.80–3.00 (m, 3 H), 3.75 (d, J = 12 Hz, 2 H), 6.94 (t, J = 5 Hz, 1 H), 8.10 (br, s, 4 H); MS m/z (ISP) 257 (M + H). Anal. ($C_{12}H_{24}N_4O_2$·0.5H_2SO_3·0.25H_2O) H, S; C: calcd, 48.47; found, 47.01; N: calcd, 18.84; found, 17.92.

(*RS*)-3-(Aminomethyl)piperidine-1-carboxamidine Dihydrochloride (7a). **6a** (41.9 g, 0.141 mol) was dissolved in 1 N hydrochloric acid (423 mL) and stirred for 4 h at 50 °C. The solvents were evaporated, and the residue was azeotroped three times with EtOH, suspended in hot EtOH (250 mL), cooled, and filtered to give colorless **7a** (27.3 g, 85%). A sample was passed through an ion-exchange column (Dowex, Cl^--form): mp >250 °C dec; ^{1}H-NMR (D_2O) δ 1.30–1.50 (m, 1 H), 1.53–1.74 (m, 1 H), 1.79–1.94 (m, 1 H), 1.94–2.16 (m, 2 H), 2.88–3.22 (m, 4 H), 3.70–3.90 (m, 2 H); MS m/z (FAB) 156 (M). Anal. ($C_7H_{16}N_4$·0.02H_2O) C, H, N.

Preparation of (*S*)-3-(Aminomethyl)piperidine-1-carboxamidine Dihydrochloride (7b). (*R*)-[[(Methylsulfonyl)oxy]methyl]piperidine-1-carboxylic Acid *tert*-Butyl Ester (9b). A solution of *tert*-Butyl (*R*)-3-(hydroxymethyl)-1-piperidinecarboxylate **8b**[23,24] (67.8 g, 0.314 mol) and NEt_3 (65.8 mL, 0.472 mol, 1.5 equiv) in CH_2Cl_2 (1200 mL) was cooled to 0 °C and treated under cooling dropwise with a solution of methanesulfonyl chloride (25 mL, 0.321 mol, 1.02 equiv) in CH_2Cl_2 (100 mL) in such a way that the reaction temperature did not exceed 0 °C. The mixture was stirred for 1.5 h at 0 °C, the solvents were evaporated, the residue was redissolved in EtOAc (800 mL), insoluble salts were removed by filtration, the filtrate was evaporated, and the residue was purified by chromatography with EtOAc/hexane (1:1) to yield **9b** (92 g, 98%) as a yellow oil: $[\alpha]^{20}_D$ = −21.6° (c = 0.9, EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.20–1.60 (m, 2 H), 1.46 (s, 9 H), 1.61–1.76 (m, 1 H), 1.84 (m, 1 H), 1.97 (m, 1 H), 2.80 (dd, J = 8, 14 Hz, 1 H), 2.93 (ddd, J = 4, 8, 12 Hz, 1 H), 3.03 (s, 3 H), 3.82 (m, 1 H), 3.95 (m, 1 H), 4.10 (m, 2 H); MS m/z (thermospray) 294 (M + H). Anal. ($C_{12}H_{23}NO_5S$) C, H, N, S.

(*R*)-3-(Azidomethyl)piperidine-1-carboxylic Acid *tert*-Butyl Ester (10b). A mixture of ester **9b** (90 g, 0.306 mol)

Scheme 1. Preparation of the D-Amino Acid Series of Thrombin Inhibitors[a]



2 a-h

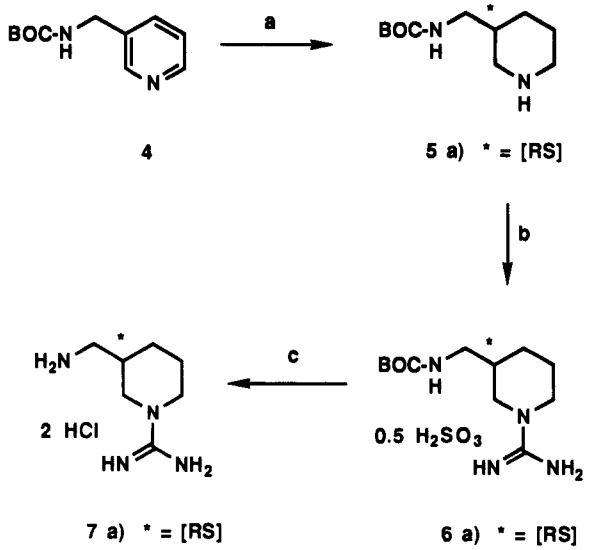
3 a-l

a) R = H
b) R = benzyl, ** = [R]
c) R = 3-indolinylmethyl, ** = [R]
d) R = 3-indolinylmethyl, ** = [S]
e) R = 4-NO_2-benzyl, ** = [R]
f) R = 4-NH_2-benzyl, ** = [R]
g) R = 4-AcNH-benzyl, ** = [R]
h) R = 4-(p-tos)-NH-benzyl, ** = [R]

a) R = H, * = [RS]
b) R = benzyl, ** = [R], * = [RS]
c) R = benzyl, ** = [R], * = [S]
d) R = 3-indolinylmethyl, ** = [R], * = [RS]
e) R = 3-indolinylmethyl, ** = [S], * = [RS]
f) R = 3-indolinylmethyl, ** = [R], * = [S]
g) R = 3-indolinylmethyl, ** = [R], * = [R]
h) R = 4-NO_2-benzyl, ** = [R], * = [RS]
i) R = 4-NO_2-benzyl, ** = [R], * = [S]
k) R = 4-AcNH-benzyl, ** = [R], * = [RS]
l) R = 4-(p-tos)-NH-benzyl, ** = [R], * = [RS]

[a] (a) 2-Naphthyl-SO_2Cl, 1 N NaOH, ether; (b) **7a–c**, BOP, 4-ethylmorpholine, DMF.

Scheme 2. Synthesis of the (*RS*)-3-(Aminomethyl)piperidine-1-carboxamidine Hydrochloride[a]



4

5 a) * = [RS]

6 a) * = [RS]

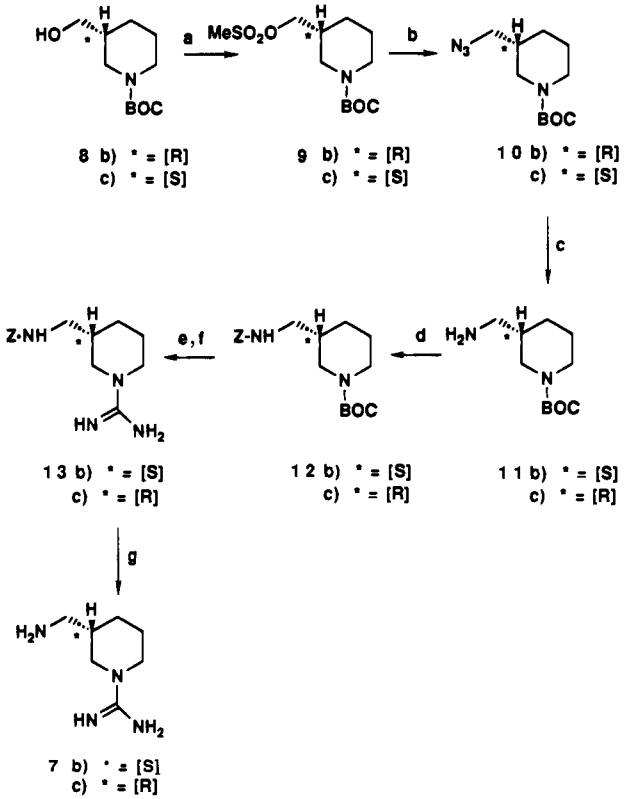
7 a) * = [RS]

[a] (a) H_2, 100 bar, 60 °C, RuO_2, MeOH; (b) formamidinesulfinic acid, NEt_3, DMF; (c) 1 N HCl.

and sodium azide (66 g, 0.920 mol, 3 equiv) in DMF (600 mL) was stirred for 24 h at 50 °C. The reaction mixture was filtered, the solvent evaporated, and the residue partitioned between H_2O and EtOAc. The organic layers were separated, washed twice with H_2O, dried, and evaporated to yield colorless resin **10b** (75 g, 98%): $[\alpha]^{20}_D = -7.5°$ (c = 0.7, EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.26 (m, 1 H), 1.46 (s + m, 10 H), 1.60–1.92 (m, 3 H), 2.67 (br, s, 1 H), 2.86 (m, 1 H), 3.22 (d, J = 6 Hz, 2 H), 3.79–4.06 (m, 2 H); MS m/z (FAB) 185 (M − isobutylene), 156 (M − N_2 − isobutylene). Anal. ($C_{11}H_{20}N_4O_2$) C, H; N: calcd, 23.32; found, 22.45.

(*S*)-3-(Aminomethyl)piperidine-1-carboxylic Acid *tert*-Butyl Ester (11b). A mixture of azide **10b** (60.7 g, 0.252 mol) in EtOH (1500 mL) and Adams catalyst on charcoal (12 g) was hydrogenated at room temperature for 1.5 h under a pressure of 10 bar. The catalyst was filtered off and the filtrate evaporated to yield oily **11b** (53.1 g, 98%): ^{1}H-NMR ($CDCl_3$) δ 1.07–1.25 (m, 1 H), 1.28 (s, 2 H), 1.46 (s + m, 11 H), 1.58–1.73 (m, 1 H), 1.84 (m, 1 H), 2.59 (m, 3 H), 2.89 (m, 1 H), 3.74–4.03 (m, 2 H); MS m/z (FAB) 197 (M − NH_3), 141 (M − NH_3 − isobutylene).

Scheme 3. Synthesis of the (*S*)-3-(Aminomethyl)piperidine-1-carboxamidine Hydrochloride[a]



8 b) * = [R]
c) * = [S]

9 b) * = [R]
c) * = [S]

10 b) * = [R]
c) * = [S]

11 b) * = [S]
c) * = [R]

12 b) * = [S]
c) * = [R]

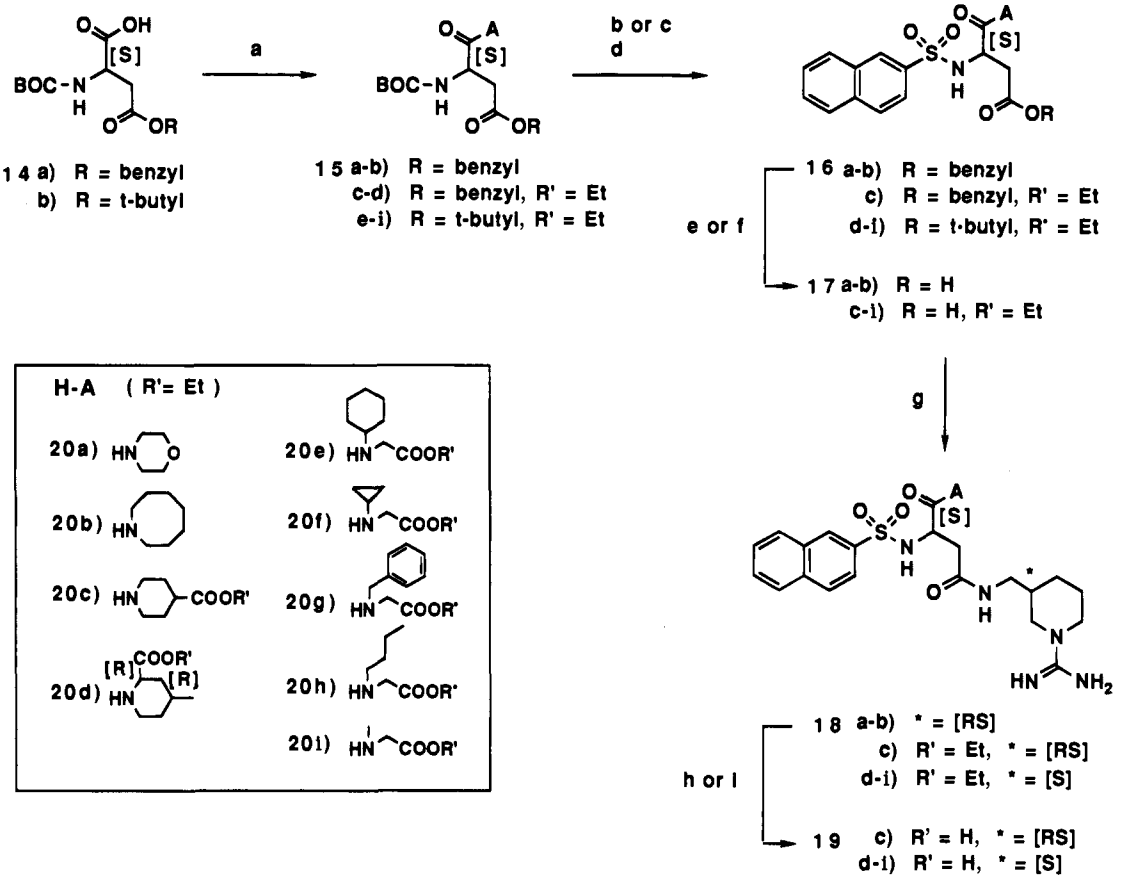
13 b) * = [S]
c) * = [R]

7 b) * = [S]
c) * = [R]

[a] (a) $MeSO_2Cl/NEt_3$, CH_2Cl_2; (b) NaN_3, DMF; (c) H_2, Pd/C, EtOH; (d) Z-Cl, NaOH, $Bu_4N^+HSO_4^-$, hexane–H_2O; (e) HCl, AcOEt; (f) formamidinesulfinic acid, NEt_3, DMF; (g) H_2, Pd/C, EtOH–1 N HCl.

(*S*)-[[(Benzyloxycarbonyl)amino]methyl]piperidine-1-carboxylic Acid *tert*-Butyl Ester (12b). To a solution of amine **11b** (57.5 g, 0.268 mol) in hexane (2400 mL) were added H_2O (600 mL), tetrabutylammonium hydrogen sulfate (21.3 g, 0.063 mol, 0.23 equiv), and 1 N NaOH (600 mL, 0.6 mol). The mixture was cooled to 4 °C and treated dropwise with benzyl chloroformate (53 mL, 0.376 mol, 1.4 equiv) in such a way that the reaction temperature did not exceed 10 °C. The mixture was vigorously stirred at room temperature for 3 h

Scheme 4. Preparation of the L-Aspartic Acid Series of Thrombin Inhibitors[a]



[a] (a) H-A (**20a–i**), BOP, 4-ethylmorpholine, DMF; (b) 2 M HCl in AcOEt; (c) *p*-Tos-OH·H_2O, dioxane; (d) 2-naphthyl-SO_2Cl, $NaHCO_3$, dioxane–water; (e) H_2, Pd/C, EtOH; (f) TFA, CH_2Cl_2; (g) **7a–c**, BOP, 4-ethylmorpholine, DMF; (h) 1 N LiOH, EtOH; (i) 1 N NaOH, EtOH.

(TLC control). Then the organic layer was separated and washed successively with aqueous citric acid (5%), H_2O, bicarbonate, and brine. The organic extracts were dried and evaporated, yielding colorless **12b** (102.1 g, >100%). This raw material was used for the further steps. A sample was purified by chromatography on silica gel with EtOAc–hexane (1:4) for spectral data: ^{1}H-NMR ($CDCl_3$) δ 1.12–1.31 (m, 1 H), 1.45 (s, 9 H), 1.54–1.86 (m, 4 H), 2.77 (dd, J = 9, 13 Hz, 1 H), 2.88–3.26 (br, m, 3 H), 3.60–3.84 (m, 2 H), 4.91 (br, s, 1 H), 5.10 (s, 2 H), 7.35 (s, 5 H); MS m/z (FAB) 247 (M – COOtBu). Anal. ($C_{19}H_{28}N_2O_4$·0.28H_2O) C, H, N.

(*S*)-1-[[(Aminoiminomethyl)piperidin-3-yl]methyl]carbamic Acid Benzyl Ester Hydrochloride (13b). To a solution of **12b** (74.5 g, 0.214 mol) in EtOAc (570 mL) was added a solution of 4 M HCl in EtOAc (40 mL). The mixture was stirred for 4 h at room temperature and then evaporated to dryness. The residue was dissolved in DMF (660 mL), NEt_3 (115 mL, 0.828 mol) and formamidine sulfonic acid (26.9 g, 0.216 mol) were added, and the suspension was stirred at room temperature overnight. The mixture was filtered, the filtrate evaporated, and the residue purified by chromatography (EtOAc/MeAc/AcOH/H_2O, 16:2:1:1). The purified product was redissolved in 1 M hydrochloric acid and the solvent evaporated to yield crystalline, colorless **13b** (42 g, 62%): mp 183–185 °C dec; ^{1}H-NMR (DMSO-d_6) δ 1.16–1.52 (m, 2 H), 1.72 (m, 3 H), 2.80 (dd, J = 10, 14 Hz, 1 H), 2.98 (m, 3 H), 3.76 (m, 2 H), 5.02 (s, 2 H), 7.36 (s, 5 H), 7.45 (s, 4 H, $H_2NC{=}NH_2^+$); MS m/z (FAB) 291 (M + H). Anal. ($C_{15}H_{22}N_4O_2$·1.0HCl·0.02H_2O) C, H, N, Cl.

(*S*)-3-(Aminomethyl)piperidine-1-carboxamidine Dihydrochloride (7b). To a solution of **13b** (42 g, 0.290 mol) in EtOH (850 mL) and 1 N HCl (850 mL) was added palladium on charcoal (4.2 g). The mixture was hydrogenated for 6 h at room temperature. Then the catalyst was filtered off, and the filtrate was evaporated and azeotroped twice with EtOH. The residue was suspended in EtOH (50 mL) filtered, and dried to give crystalline, colorless **7b** (20.6 g, 70%). A sample was purified by chromatography on an ion-exchange column (Dowex, Cl^--form) and recrystallization from MeOH-Et_2O: mp 252–254 °C; $[\alpha]^{20}_D$ = −16.9° (c = 1, H_2O); ^{1}H-NMR (D_2O) δ 1.30–1.50 (m, 1 H), 1.52–1.73 (m, 1 H), 1.78–1.94 (m, 1 H), 1.94–2.16 (m, 2 H), 2.86–3.20 (m, 4 H), 3.78 (m, 2 H); MS m/z (FAB) 156 (M). Anal. ($C_7H_{16}N_4$·2.0HCl·0.05H_2O) C, H, N, Cl.

The other enantiomer, (*R*)-3-(aminomethyl)piperidine-1-carboxamidine dihydrochloride (**7c**), was prepared by the same procedure but starting with *tert*-butyl (*S*)-3-(hydroxymethyl)-1-piperidinecarboxylate.[23,24]

Preparation of (*R*)-*N*-[[(*S*)-1-(Aminoiminomethyl)piperidin-3-yl]methyl]-3-indol-3-yl-2-[(naphth-2-ylsulfonyl)amino]propionamide Hydrochloride (3f). (*R*)-3-Indol-3-yl-2-[(naphth-2-ylsulfonyl)amino]propionic Acid (2c). To a solution of D-tryptophan (20.4 g, 0.1 mol) in 1 N NaOH (100 mL, 0.1 mol) was added within 20 min a solution of 2-naphthalenesulfonyl chloride (45.3 g, 0.2 mol) in ether (400 mL). The reaction mixture was kept at room temperature with gentle cooling and stirred for 1 h. Then additional 1 N sodium hydroxide (100 mL, 0.1 mol) was added three times at 1 h intervals. After the last addition, stirring was continued for another 2 h. The precipitate formed was filtered, washed three times with ether, suspended in water (250 mL), and acidified with concentrated hydrochloric acid (25 mL). After extraction with EtOAc (2 × 250 mL), the organic layers were washed with H_2O and brine, dried, and evaporated to give crude material (42.7 g). 3-fold recrystallization from EtOAc–hexane (1:1) yielded **2c** (21.1 g, 53%): mp 177–178 °C; $[\alpha]^{20}_D$ = +62.8° (c = 0.5, EtOH); ^{1}H-NMR (DMSO-d_6) δ 2.84 (dd, J = 7, 14 Hz, 1 H), 3.06 (dd, J = 6.5, 14 Hz, 1 H), 3.97 (m, 1H), 6.78 (t, J = 7.5 Hz, 1 H), 6.96 (t, J = 7.5 Hz, 1 H), 7.07 (d, J = 2 Hz, 1 H), 7.12–7.30 (m, 2 H), 7.56–7.75 (m, 3 H), 7.92–8.10 (m, 3 H), 8.27 (s, 1 H), 8.39 (d, J = 9 Hz, 1 H), 10.79 (s, 1 H), 12.59 (s, 1 H); MS m/z (FAB) 394 (M). Anal. ($C_{21}H_{16}N_2O_4S$) C, H, N, S.

(*R*)-*N*-[[(*S*)-1-(Aminoiminomethyl)piperidin-3-yl]-methyl]-3-indol-3-yl-2-[(naphth-2-ylsulfonyl)amino]pro-

pionamide Hydrochloride (3f). **2c** (1.2 g, 3.0 mmol) was dissolved in DMF (12 mL) and treated with 4-ethylmorpholine (1.9 mL, 15 mmol), BOP (1.33 g, 3.0 mmol), and **7b** (0.82 g, 3.6 mmol). The dark solution was stirred overnight, the solvents were evaporated, and the residue was chromatographed with an H_2O–acetonitrile gradient on RP-18 silica (1.1 g, 64%). The crude material was taken up in EtOH–H_2O (1:1) and passed through an ion-exchange column (Dowex, Cl^- form) to yield slightly yellow **3d** (0.9 g, 53%): ^{1}H-NMR (DMSO-d_6) δ 0.72–0.94 (m, 1 H), 1.0–1.36 (m, 3 H), 1.36–1.54 (m, 1 H), 2.28–2.90 (m, 5 H), 2.94–3.10 (m, 1 H), 3.36–3.66 (m, 2 H), 4.02 (dd, J = 7, 14 Hz, 1 H), 6.80 (m, 1 H), 6.96 (m, 1 H), 7.10 (d, J = 3 Hz, 1 H), 7.18–7.50 (m, 2 H), 7.37 (s, 4 H, $H_2NC{=}NH_2^+$), 7.56–7.75 (m, 3 H), 7.88–8.04 (m, 4 H), 8.20–8.34 (s + d, J = 7 Hz, 1 H + NH), 10.81 (d, J = 3 Hz, NH); MS m/z (ISP) 533 (M + H). Anal. ($C_{28}H_{32}N_6O_3S$·1.0HCl·0.89EtOH·0.13H_2O) H, N, Cl; C: calcd, 58.62; found, 58.07.

In an analogous way to the synthesis of **2c**, compounds **2a**,**b** and **2d**,**e** were prepared from the corresponding amino acids.

[(Naphth-2-ylsulfonyl)amino]acetic acid (2a): mp 152–154 °C. Anal. ($C_{12}H_{11}NO_4S$·0.01H_2O) C, H, N, S.

(*R*)-2-[(Naphth-2-ylsulfonyl)amino]-3-phenylpropionic acid (2b): mp 150 °C; $[\alpha]^{20}_D$ = +8.5° (c = 1, EtOH). Anal. ($C_{19}H_{17}NO_4S$) C, H, N, S.

(*S*)-3-Indol-3-yl-2-[(naphth-2-ylsulfonyl)amino]propionic acid (2d): mp 173–175 °C; $[\alpha]^{20}_D$ = −59.0° (c = 0.5, EtOH). Anal. ($C_{21}H_{18}N_2O_4S$·0.03H_2O) C, H, N, S.

(*R*)-2-[(Naphth-2-ylsulfonyl)amino]-3-(4-nitrophenyl)-propionic acid (2e): $[\alpha]^{20}_D$ = −14.2° (c = 0.5, EtOH). Anal. ($C_{19}H_{16}N_2O_6S$) C, H, N, S.

(*R*)-3-(4-Aminophenyl)-2-[(naphth-2-ylsulfonyl)amino]-propionic Acid (2f). A mixture of **2e** (7.0 g, 17.5 mmol) and Raney nickel (6 g) in DMF (150 mL) was stirred in a hydrogen atmosphere (1 atm) for 48 h. The catalyst was removed by filtration, and the solvents were evaporated. The residue was treated with H_2O (120 mL), and the insoluble material was collected by filtration and dried (6.2 g). The material so obtained was suspended in hot EtOH and MeOH, filtered, and dried to give **2f** (3.8 g, 59%): mp 211–212 °C; $[\alpha]^{20}_D$ = −7.9° (c = 1.0, HCl); ^{1}H-NMR (DMSO-d_6) δ 2.48–2.66 (m, 1 H), 2.70–2.84 (m, 1 H), 3.82 (m, 1 H), 6.38 (part of AA′BB′, 2 H), 6.5–8.5 (br, 3 H), 6.77 (part of AA′BB′, 2 H), 7.60–7.75 (m, 3 H), 7.94–8.16 (m, 3 H), 8.20–8.34 (m, 2 H); MS m/z (ISP) 371 (M + H). Anal. ($C_{19}H_{18}N_2O_4S$·0.05H_2O) C, H, N, S.

(*R*)-3-[4-(Acetylamino)phenyl]-2-[(naphth-2-ylsulfonyl)amino]propionic Acid (2g). To a solution of **2f** (1.5 g, 4.1 mmol) in pyridine (15 mL) was added acetic anhydride (0.46 mL, 4.9 mmol) under cooling, and the mixture was stirred at room temperature overnight. Then the reaction mixture was diluted with H_2O and acidified with 1 N hydrochloric acid, and the precipitate was collected by filtration. This material was taken up in EtOAc and washed twice with brine, the organic layers were dried, the solvents were evaporated, and the residue was crystallized with EtOH to yield pure **2g** (0.65 g, 39%): mp 199–203 °C; ^{1}H-NMR (DMSO-d_6) δ 2.02 (s, 3 H), 2.58–2.72 (m, 1 H), 2.82–2.94 (m, 1 H), 3.90 (m, 1 H), 7.00 (part of AA′BB′, 2 H), 7.30 (part of AA′BB′, 2 H), 7.54–7.72 (m, 3 H), 7.90–8.08 (m, 3 H), 8.17 (s, 1 H), 8.36 (d, J = 9 Hz, 1 H), 9.75 (s, 1 H), 12.60 (br, s, 1 H); MS m/z (ISN) 411 (M − H). Anal. ($C_{21}H_{20}N_2O_5S$·0.07H_2O) C; H: calcd, 4.89; found, 5.67; N: calcd, 6.79; found, 6.19; S: calcd, 7.77; found, 7.12.

(*R*)-3-[4-[[(4-Methylphenyl)sulfonyl]amino]phenyl]-2-[(naphth-2-ylsulfonyl)amino]propionic Acid (2h). **2f** (1.5 g, 4.1 mmol) was dissolved in 1 N NaOH (4.1 mL) and H_2O (21 mL) and treated with $NaHCO_3$ (1.02 g, 12.1 mmol) and a solution of p-tosyl chloride (0.77 g, 4.1 mmol) in dioxane (18 mL). The mixture was stirred at room temperature overnight, then the solvents were evaporated, and the residue was acidified with 2 N HCl and extracted twice with EtOAc. The organic layers were washed with brine and dried and the solvents evaporated. Chromatographic purification with EtOAc on silica gel yielded **2h** (1.2 g, 57%): ^{1}H-NMR (DMSO-d_6) δ 2.31 (s, 3 H), 2.58–2.72 (m, 1 H), 2.76–2.88 (m, 1 H), 3.76 (m, 1 H), 6.88 (part of AA′BB′, 2 H), 6.98 (part of AA′BB′, 2 H), 7.32 (part of AA′BB′, 2 H), 7.54–7.75 (m, 5 H), 7.88–8.12 (m, 4 H), 8.28 (s, 1 H), 10.11 (s, 1 H), 12.50 (br, s, 1 H); MS m/z (ISN) 523 (M − H). Anal. ($C_{26}H_{24}N_2O_6S_2$·0.16H_2O) C, N; H: calcd, 4.61; found, 5.08; S: calcd, 12.22; found, 11.08.

Compounds **3a**–**e** and **3g**–**l** were prepared by the same procedure as for the preparation of **3f**, using the corresponding sulfonylated amino acids and the appropriate building block **7a**–**c**.

Preparation of (*S*)-1-[3-[[[1-(Aminoiminomethyl)piperidin-3-yl]methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)-amino]propionyl]piperidine-4-carboxylic Acid (1:1 Mixture of Epimers) (19c). **(*S*)-1-[3-(Benzyloxycarbonyl)-2-[(*tert*-butoxycarbonyl)amino]propionyl]piperidine-4-carboxylic Acid Ethyl Ester (15c).** To a solution of Boc-L-Asp(OBzl)-OH (**14a**) (6.46 g, 20 mmol) in DMF (100 mL) were added NEt_3 (5.6 mL, 40 mmol), BOP (8.84 g, 20 mmol), and ethyl piperidine-4-carboxylate (3.14 g, 20 mmol). After the mixture was stirred at room temperature overnight the solvents were evaporated, and the residue was taken up in EtOAc and washed twice with 5% aqueous $KHSO_4/K_2SO_4$ (1:2) solution and brine. The organic layer was separated and dried, and the solvents were removed under reduced pressure. The residue was purified by chromatography (EtOAc–hexane, 2:1) to give slightly yellow **15c** (8.3 g, 90%): ^{1}H-NMR (DMSO-d_6) δ 1.26 (t, J = 7 Hz, 3 H) 1.43 (s, 9 H), 1.54–2.02 (m, 4 H), 2.43–3.31 (m, 5 H), 3.87–4.45 (m, 2 H), 4.15 (q, J = 7 Hz, 2 H), 4.93–5.18 (m, 1 H), 5.12 (s, 2 H), 5.34–5.47 (m, 1 H), 7.34 (s, 5 H); MS m/z (FAB) 406 (M − isobutylene). Anal. ($C_{24}H_{34}N_2O_7$·0.14EtOAc·0.04H_2O) C, H, N.

(*S*)-1-[3-(Benzyloxycarbonyl)-2-[(naphth-2-ylsulfonyl)-amino]propionyl]piperidine-1-carboxylic Acid Ethyl Ester (16c). **15c** (8.1 g, 17.5 mmol) dissolved in EtOAc (75 mL) was cooled to 4 °C and treated with a solution of 4 M HCl in EtOAc (75 mL). After stirring for 4 h at room temperature, the mixture was evaporated to dryness, the residue was redissolved in dioxane (75 mL), and a solution of 1 N sodium hydroxide (17.5 mL) and a solution of 2-naphthalenesulfonyl chloride (2.94 g, 35 mmol) in dioxane (75 mL) were added. The reaction mixture was stirred at room temperature overnight, poured onto ice, and extracted twice with EtOAc. The organic layers were washed twice with aqueous 5% $KHSO_4/K_2SO_4$ (1:2) solution and brine, dried, and evaporated. Purification of the residue by chromatography (EtOAc–hexane, 2:1) afforded colorless **16c** (8.8 g, 90%): ^{1}H-NMR ($CDCl_3$) δ 0.80–1.33 (m, 4 H), 1.33–1.82 (m, 3 H), 2.16–2.70 (m, 2 H), 2.57 (d, J = 6 Hz, 2 H), 2.78–3.00 (m, 1 H), 3.60–4.19 (m, 4 H), 4.66 (br, s, 1 H), 5.04 (d, J = 5 Hz, 2 H), 6.06 (br s, 1 H), 7.32 (s, 5 H), 7.53–7.72 (m, 2 H), 7.75–7.83 (m, 1 H), 7.83–8.01 (m, 3 H), 8.41 (s, 1 H); MS m/z (ISN) 551 (M − H). Anal. ($C_{29}H_{32}N_2O_7S$) C, H, N, S.

(*S*)-4-[4-(Ethoxycarbonyl)piperidin-1-yl]-3-[(naphth-2-ylsulfonyl)amino]-4-oxobutyric Acid (17c). A suspension of **16c** (8.6 g, 15.6 mmol) and 10% Pd/C (1.0 g) in EtOH (500 mL) and CH_2Cl_2 (100 mL) was stirred in a hydrogen atmosphere (1 bar) for 7 h. The catalyst was removed by filtration, a new portion of 10% Pd/C (1.0 g) was added, and the mixture was again stirred in a hydrogen atmosphere (1 bar) overnight. The catalyst was filtered off, the solvents were evaporated, and the residue (7.0 g) was recrystallized from CH_2Cl_2–hexane to give colorless **17c** (3.6 g, 50%): ^{1}H-NMR (DMSO-d_6, rotamers) δ 0.80–1.84 (m, 4 H), 1.15 and 1.18 (2 t, J = 7 Hz, 3 H), 1.98–2.34 (m, 2 H), 2.34–2.80 (m, 2 H), 3.02 (q, J = 10 Hz, 1 H), 3.75–3.96 (m, 2 H), 4.03 and 4.06 (2 q, J = 7 Hz, 2 H), 4.50–4.66 (m, 1 H), 7.60–7.84 (m, 3 H), 8.00–8.22 (m, 3 H), 8.30–8.50 (m, NH) 8.43 (s, 1 H), 11.34 (br, s, 1 H); MS m/z (ISN) 461 (M − H). Anal. ($C_{22}H_{26}N_2O_7S$·0.04H_2O) C, H, N, S.

(*S*)-1-[3-[[[1-(Aminoiminomethyl)piperidin-3-yl]methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)amino]propionyl]-piperidine-4-carboxylic Acid Ethyl Ester Hydrochloride (1:1 Mixture of Epimers) (18c). To a solution of **17c** (1.4 g, 3.0 mmol) in DMF (14 mL) were added 4-ethylmorpholine (1.14 mL, 9.0 mmol), BOP (1.33 g, 3.0 mmol), and **7a** (1.03 g, 4.5 mmol). After the mixture was stirred at room temperature overnight the solvents were removed *in vacuo*, and the residue was purified by RP-18 chromatography (H_2O, increasing acetonitrile gradient). The combined product fractions were evaporated, redissolved in H_2O, and passed through an ion-exchange column (Dowex, Cl^- form) to afford colorless solid

18c (0.65 g, 34%): ^{1}H-NMR (DMSO-d_6, epimers) δ 0.68–1.84 (m, 9 H), 1.15 and 1.18 (2 t, J = 7 Hz, 3 H), 2.00–2.32 (m, 1 H), 2.32–2.74 (m, 4 H), 2.74–3.12 (m, 4 H), 3.58–3.96 (m, 4 H), 4.03 and 4.05 (2 q, J = 7 Hz, 2 H), 4.68 (m, NH), 7.47 (s, $H_2NC{=}NH_2^+$), 7.58–7.84 (m, 3 H), 7.94–8.22 (m, 4 H), 8.22–8.36 (m, 1 H), 8.40 (s, 1 H); MS m/z (ISN) 599 (M − H). Anal. ($C_{29}H_{40}N_6O_6S$·1.0HCl·0.21EtOH·0.22H_2O) C, H, Cl, N, S.

(*S*)-1-[3-[[[1-(Aminoiminomethyl)piperidin-3-yl]methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)amino]propionyl]-piperidine-4-carboxylic Acid (1:1 Mixture of Epimers) (19c). A solution of **18c** (0.48 g, 0.75 mmol) in THF (5 mL) was treated with 1 N LiOH (2.25 mL, 3 equiv) and stirred at room temperature for 90 min. Then 1 N HCl was added and the solvents were evaporated. Chromatographic purification of the residue on RP-18 with an acetonitrile–H_2O gradient afforded **19c** as a colorless solid (0.36 g, 83%): ^{1}H-NMR (D_2O/DCl, epimers) δ 0.57–0.83 (m, 1 H), 1.15–1.62 (m, 4 H), 1.62–2.16 (m, 4 H), 2.25–2.64 (m, 4 H), 2.64–3.37 (m, 5 H), 3.41–3.82 (m, 4 H), 4.51–4.78 (m, 1 H), 7.59–7.78 (m, 3 H), 7.90–8.14 (m, 3 H), 8.42 (s, 1 H); MS m/z (ISP) 573 (M + H). Anal. ($C_{27}H_{36}N_6O_6S$·0.23H_2O) C, H, N, S.

In an analogous procedure, compounds **18a,b** were prepared, using the amines H-A **20a** and **20b**, respectively, instead of **20c**.

Preparation of [[(*S*)-3-[[[(*S*)-1-(Aminoiminomethyl)-piperidin-3-yl]methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)amino]propionyl]cyclohexylamino]acetic Acid (19e). (*S*)-3-[(*tert*-Butoxycarbonyl)amino]-*N*-cyclohexyl-*N*-[(ethoxycarbonyl)methyl]succinamic Acid *tert*-Butyl Ester (15e). To a solution of Boc-L-Asp(OtBu)-OH (**14b**) (2.89 g, 10.0 mmol) in DMF (50 mL) were added 4-ethylmorpholine (3.78 mL, 30 mmol), BOP (4.42 g, 10 mmol), and *N*-cyclohexylglycine **20e** (2.25 g, 12 mmol). After stirring overnight, the solvent was evaporated and the residue was purified by chromatography (EtOAc–hexane, 4:1) to give colorless **15e** (4.5 g, 98%): ^{1}H-NMR ($CDCl_3$, rotamers) δ 0.80–1.53 (m, 4 H), 1.26 (t, J = 7.5 Hz, 3 H), 1.43 (s, 9 H), 1.44 (s, 9 H), 1.57–1.95 (m, 6 H), 2.40–2.80 (m, 2 H), 3.81–3.98 (m, 1 H), 3.93 (s, 2 H), 4.16 (q, J = 7.5 Hz, 2 H), 5.05 (m, 1 H), 5.26 (m, 1 H); MS m/z (FAB) 457 (M + H). Anal. ($C_{23}H_{40}N_2O_7$·0.09H_2O) C, H, N.

(*S*)-*N*-Cyclohexyl-*N*-[(ethoxycarbonyl)methyl]-3-[(naphth-2-ylsulfonyl)amino]succinamic Acid *tert*-Butyl Ester (16e). **15e** (1.4 g, 3.0 mmol), dissolved in acetonitrile (14 mL), was treated with *p*-toluenesulfonic acid monohydrate (1.5 g, 8.0 mmol) for 8 h at room temperature. The solvent was removed *in vacuo* and the residue redissolved in dioxane (45 mL). Then a solution of 2-naphthalenesulfonyl chloride (1.56 g, 7.0 mmol) in dioxane (15 mL), $NaHCO_3$ (1.9 g, 23 mmol) and H_2O (19 mL) were added, and the reaction mixture was stirred overnight. The mixture was taken up in EtOAc, and the organic layer was separated, washed successively with H_2O and brine, dried, and evaporated. Chromatographic purification of the residue (EtOAc–hexane, 4:1) yielded colorless **16e** (0.85 g, 34%): ^{1}H-NMR ($CDCl_3$, rotamers) δ 0.69–1.12 (m, 3 H), 1.12–1.33 (m, 5 H), 1.41 (s, 9 H), 1.47–1.87 (m, 5 H), 2.16–2.37 (m, 1 H), 2.38–2.53 (m, 2 H), 3.46–3.89 (m, 2 H), 4.00–4.19 (m, 2 H), 4.69 (m, 1 H), 5.94 (m, 1 H), 7.56–7.69 (m, 2 H), 7.77–7.99 (m, 4 H), 8.42 (m, 1 H); MS m/z (FAB) 547 (M + H). Anal. ($C_{28}H_{38}N_2O_7S$) C, H, N, S.

[[(*S*)-3-[[[(*S*)-1-(Aminoiminomethyl)piperidin-3-yl]-methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)amino]propionyl]cyclohexylamino]acetic Acid Ethyl Ester Hydrochloride (18e). To a solution of **16e** (4.9 g, 8.96 mmol) in CH_2Cl_2 (50 mL) was added a solution of 4 M HCl in EtOAc (45 mL) under ice cooling. The mixture was stirred for 5 h at room temperature and then evaporated to dryness (**17e**). The residue was dissolved in DMF (50 mL) and treated with 4-ethylmorpholine (5.7 mL, 44.8 mmol), BOP (3.97 g, 8.96 mmol), and **7b** (2.26 g, 9.86 mmol). The dark solution was stirred at room temperature overnight, the solvents were evaporated, and the residue was purified by chromatography on silica gel with EtOAc/AcMe/H_2O/AcOH (16:2:1:1) to give **18e** (4.9 g, 82%). A sample (1.2 g) of this material was taken up in EtOH–H_2O (1:1) and passed through a ion-exchange column (Dowex, Cl^- form) to yield beige **18e** as hydrochloride salt (0.95 g): ^{1}H-NMR (DMSO-d_6, rotamers) δ 0.74–1.82 (m, 17 H), 1.86–2.08 (m, 1 H), 2.10–3.02 (m, 5 H), 3.50–4.32 (m, 8 H), 4.38–4.56 and 4.70–4.84 (2 × m, 1 H, both rotamers), 7.26–7.50 (m, 4 H, $H_2NC{=}NH_2^+$), 7.60–7.94 (m, 4 H), 7.95–8.20 (m, 3 H), 8.22–8.48 (m, 2 H); MS m/z (FAB) 629 (M + H). Anal. ($C_{31}H_{44}N_6O_6S$·1.0HCl·0.12H_2O) H, S, Cl; C: calcd, 55.97; found, 55.24; N: calcd, 12.63; found, 11.71.

[[(*S*)-3-[[[(*S*)-1-(Aminoiminomethyl)piperidin-3-yl]-methyl]carbamoyl]-2-[(naphth-2-ylsulfonyl)amino]propionyl]cyclohexylamino]acetic Acid (19e). A solution of **18e** (0.8 g, 1.0 mmol) in ethanol (6 mL) was treated with 1 N sodium hydroxide (6 mL, 6.0 mmol) and stirred at room temperature overnight. Then 1 N hydrochloric acid (6 mL, 6.0 mmol) was added, the solvents were removed *in vacuo* and the residue was chromatographed on RP-18 with a H_2O–acetonitrile gradient to give colorless, crystalline **19e** (0.25 g, 39%): mp 238–240 °C; ^{1}H-NMR (DMSO-d_6, rotamers) δ 0.84–1.76 (m, 18 H), 1.78–1.96 (m, 1 H, $COCH_2CH$), 2.38–2.64 (m + DMSO, 1 H, $COCH_2CH$), 2.64–3.04 (m, 3 H), 3.32 (d, J = 17 Hz, 1 H), 3.68–4.00 (m, 2 H), 4.04 (d, J = 17 Hz, 1 H), 4.52 (br, s, 1 H), 7.60–7.92 (m, 6 H), 7.98–8.18 (m, 3 H), 8.26–8.56 (m, 3 H); MS m/z (ISP) 601 (M + H). Anal. ($C_{29}H_{40}N_6O_6S$·0.10H_2O + 0.78% residue) C, H, N, S.

The compounds **19d** and **19f–i** were prepared in the way described above, using the amines H-A **20d**,[19] **20f**, **20g**, **20h**, and **20i**,[25,26] respectively.

Determination of Inhibition of Thrombin and Trypsin. Inhibition of thrombin and trypsin was measured spectrophotometrically using chromogenic substrates. To identify inhibitors of thrombin and trypsin with high as well as low affinity, test systems using substrates with low and high K_m have been developed.

The enzymes used in the tests were human thrombin prepared according to Fenton et al.[27] and bovine pancreatic trypsin (code TRL, Worthigton Biochemicals, Pharmacare, Biel, Switzerland). The substrates used were H-D-Phe-Pip-Arg-paranitroanilide (S-2238) or methylsulfonyl-D-Leu-Gly-Arg-paranitroanilide (synthesized in-house) for thrombin and Bz-CO-Ile-Glu-Gly-Arg-paranitroanilide (S-2222) or H-D-Val-Leu-Lys-paranitroanilide (S-2251) both from Kabi Diagnostica (Chromogenix Endotell, Bottmingen, Switzerland) for trypsin. The reaction kinetics between the enzymes and their substrates were linear both with time and with the concentration of the enzyme chosen. The absorbance/min at 405 nm was >0.1 for the control samples. The Michaelis–Menten constants (K_m) for the substrates were determined using the same conditions of temperature, pH, ionic strength with at least five substrate concentrations in the range 0.5–15 K_m (Lottenberg et al.)[28] according to Eadie.[29] Inhibitor solutions were made up at 10^{-2} M in either water, 0.9% NaCl solution, or DMSO and dilutions thereof made in Britton–Robinson buffer[30] pH 7.8 with 0.1% PEG 6000 and 0.02% Tween 80 added. Poly(ethylene glycol) (PEG 6000) was added to the system to avoid loss of enzyme or inhibitor due to absorption to the plastic walls of the cuvettes (Lottenberg et al.).[28] Inhibition of the enzymatic activity by various concentrations of the inhibitors were determined as described below. The IC_{50}, i.e., the concentration of the inhibitor necessary to inhibit, by 50% as compared to the respective solvent–buffer control, the cleavage by the enzyme of paranitroanilide from the chromogenic substrate (S) was derived graphically from concentration response curves using a four parametric iterative computer program for sigmoid curves based on nonlinear regression. The apparent K_i, i.e., the equilibrium dissociation constant, was estimated from the IC_{50} using the formula $K_i = IC_{50}/\{(1 + (S/K_m)\}$ according to Cheng and Prusoff.[31]

Thrombin Inhibition (Low K_m Test). This test was used for potent inhibitors with K_i in the nanomolar range. Inhibition was determined on a COBAS BIO spectrophotometric centrifugal analyzer (Roche Diagnostica, Basel, Switzerland) in a total volume of 300 μL. The instrument pipetted 30 μL of inhibitor or buffer (control–sample) and 20 μL of water and mixed it with 180 μL of thrombin in buffer (2 nM fc) in the test cuvette. After incubation at 25 °C for 240 s, 50 μL of the substrate S-2238 (50 μM fc, K_m 3.33 μM) plus 20 μL of water was added as a start reagent. The ensuing release of paranitroaniline was followed at 405 nm in 10 s intervals for 60 s

in comparison to a water blank. The COBAS BIO needs 7.42 s to deliver one sample. Depending on the number of samples, the delay time was chosen so that the incubation time of the plasma with the enzyme was always 4 min prior to the addition of the chromogenic substrate.

Thrombin Inhibition (High K_m Test). This test was used for weak inhibitors (needles) with K_i in the micromolar range. The inhibition was also determined on the COBAS BIO with the same settings as the low K_m test. However, the final concentration of thrombin amounted to 12 nM and that of the substrate methylsulfonyl-D-Leu-Gly-Arg-paranitroanilide (K_m 167 μM) amounted to 100 μM.

Thrombin Inhibition (Elisa Reader Test). This test was used for the most potent inhibitors with K_i below 1 nM. Inhibition of thrombin was assessed at room temperature using the chromogenic substrate S-2238 in a buffer system using a kinetic microtiter autoreader (Thermomax, Molecular Devices, Menlo Park, CA). For this test, 100 mL of buffer containing the inhibitor or solvent was incubated for 2 min with 50 mL of S-2238 (100 mM fc) before adding 100 mL of thrombin (0.2 nM fc). The velocity (V_{max}) of the reaction was determined by the autoreader from the maximal slope of the linear regression fits of five time points. The IC_{50} and the K_i were derived as described above.

Trypsin Inhibition (High K_m Test). The inhibition of trypsin was determined on the COBAS BIO with settings as described for the low and high K_m thrombin test. However, the final concentration of trypsin used amounted to 7 nM and that of the substrate S-2251 (K_m 380 μM) amounted to 200 μM.

Half-Life of a Thrombin Inhibitor. The half-life of the compound **19f** was determined in the course of an extended pharmacological characterization. The compound was infused into rats for 10 or 60 min at a dose of 1 or 18 mg/kg, respectively. The plasma concentration of active substance was measured using a bioassay after inactivating the endogenous plasma inhibitors with acetone. The bioassay in the treated plasma assessed the inhibition of thrombin toward a chromogenic substrate in the high K_m test described above. The plasmatic activities measured after stopping the infusion were fitted to a two-compartment model using the pharmacokinetic program ELSFIT on a VAX computer. The estimated $t_{1/2}$ of the initial phase amounted to 1.9 and 2.5 min, $t_{1/2}$ of the second phase to 14 and 15 min, respectively.

Crystallography. Thrombin was prepared for crystallization as described.[15,32] In the presence of PEG4000, bar-shaped crystals could be grown, but required seeding. With PEG400, however, small tetragonal bipyramids grew spontaneously. The crystals used here were obtained by optimizing the ratio of PEG4000 to PEG400. Typical reservoir conditions were 25% PEG400, 10% PEG4000, 0.2 M $MgCl_2$, 0.1 M Hepes pH 7.0. Crystals grown either in the presence of NAPAP or of other, weaker inhibitors (not described here) were harvested into 0.5 mL of this buffer and soaked overnight, with two exchanges to fresh buffer, in 1–2 mM concentrations of the inhibitor to be studied. Inhibitor exchange is normally complete as seen from the electron density and the crystals remain isomorphous with the published tetragonal form (space group $P4_32_12$; $A = B = 90.8$ Å, $C = 132.5$ Å). The crystals used here were truncated bipyramids of size 0.35 × 0.35 × 0.25 mm, diffracted to a maximum of 2.5 Å resolution, and oriented in the capillary such that the rotation axis was close to the crystallographic A axis.

Data were collected at 15 °C on a Nicolet/Xentronics area detector mounted on an Elliot GX21 rotating copper anode X-ray generator operated at 36 kV, 83 mA with a 0.3 mm focal spot and a graphite monochromator. The crystal–detector distance was 160 mm; 0.1° frames were measured for 150 s and processed on a VAX9000 computer (Digital Equipment Corp.) using the XDS primary data reduction program.[33,34] Further data reduction and manipulation was performed using the CCP4 crystallographic package (The SERC [UK] Collaborative Computing Project No. 4, A Suite of Programs for Protein Crystallography, Daresbury Laboratory, Warrington, England, 1979).

Table 5. X-ray Data Collection and Refinement Statistics[a]

compd	19d	19g	3c
code	IAM	NBZ	DPH
crystal size, mm	0.45 × 0.45 × 0.25	0.35 × 0.35 × 0.25	0.40 × 0.30 × 0.20
frames	1000	807	1100
observed *hkl*'s	41857	29649	46603
unique *hkl*'s	16836	12962	16462
R_{sym}, %	4.4	4.9	5.0
maximum resolution, Å	2.6	2.8	2.6
completeness (3.0–2.9 Å), %	98.8	74.1	95.5
completeness last 0.1 Å shell, %	68.9	62.2	65.0

[a] Code: three letter code for inhibitor on PDB file. Crystal size: the "NBZ" crystal was wedge shaped. Frames: the "NBZ" data set is not complete. R_{sym} is defined as $\sum_n\sum_i|I_i - \langle I\rangle|/\sum_n\sum_i\langle I\rangle$ where I_i is the ith observation of the nth reflection and $\langle I\rangle$ is the mean of all observations of the nth reflection; at the maximum resolution ~50% of reflections have $I > 3\sigma I$ and the R_{sym} is ~10%.

Table 6. R Factors and Final Geometric Parameters

	19d	19g	3c
R factor, initial, %	23.8	23.8	25.5
R factor, no inhibitor, %	19.0	20.4	20.0
R factor, %	16.7	15.4	16.5
final rms bond length error	0.012	0.011	0.011
rms angle error, deg	1.89	1.91	1.85

Analysis and refinement of the structure were performed with "O",[35] X-PLOR version 3.1.,[36,37] MOLOC,[38–40] and PROCHECK[41] on Indigo 2 workstations (Silicon Graphics Inc.).

Data collection and refinement statistics are given in Tables 5 and 6.

Since no true native data set is available, inhibitor data sets were scaled to a set of structure factors calculated from a "start coordinate set" obtained from other similar thrombin-inhibitor structures currently under investigation (data not given). Initial crystallographic R values were ~25% for all reflections with $F > \sigma F$, (see Table 5), and sank rapidly to ~20% after alternate positional and constrained B factor refinement with X-PLOR, using the target parameters of Engh and Huber.[42] Difference electron density maps were very clean. The inhibitors were built with the MOLOC program and refined with X-PLOR to R factors of ~16% with good geometry as shown in Table 6. The coordinates will be deposited at the Brookhaven Protein Data Bank.[43]

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