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Structural Modification of an Orally Active Thrombin Inhibitor, LB30057: Replacement of the D-Pocket-binding Naphthyl Moiety

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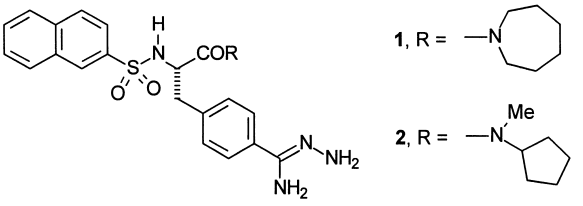
Abstract—An amidrazonophenylalanine derivative LB30057 (**2**) was identified as a potent ($K_i = 0.38$ nM), selective, and orally active thrombin inhibitor. As a continuation of studies into benzamidrazone-based thrombin inhibitors, we have structurally modified compound **2** by replacing the naphthyl group with a variety of hydrophobic moieties. This study led to discovery of several compounds with significantly enhanced potency in thrombin inhibition without sacrificing selectivity against trypsin and oral absorption. The highest activity was obtained with compound **23** ($K_i = 0.045$ nM).

Introduction

Thrombin is a trypsin-like serine protease that plays a key role in the blood coagulation process by catalyzing the conversion of fibrinogen to fibrin. This enzyme has long been recognized as a central regulator in thrombosis and hemostasis, and its inhibition has thus become a major therapeutic target in the treatment of cardiovascular diseases such as myocardial infarction, unstable angina, deep vein thrombosis, and pulmonary embolism.[1]

Direct-acting and small molecule inhibitors of thrombin are of considerable interest,[2] and numerous inhibitors possessing peptidic or non-peptidic structures have been reported.[3] However, they generally suffer from either a lack of selectivity for thrombin against trypsin or poor oral bioavailability. While some selective thrombin inhibitors have been approved as injectable anticoagulants and are in late-stage clinical trials, it is clear that next generation thrombin inhibitors must have both oral bioavailability and selectivity in order to be more clinically effective anticoagulants.[4]

Our work on thrombin inhibitors has been focused on a novel '4-TAPAP-like'[5] structure (e.g. **1**) containing a benzamidrazone residue at the P1 position because of its advantage of excellent selectivity for thrombin over trypsin.[6] In our recent study on these benzamidrazone-based compounds, the *N*,*N*-cyclopentylmethylamine derivative LB30057 (**2**) was found to be optimal in the set of amide derivatives, providing us with a potent ($K_i = 0.38$ nM), selective ($K_{i_{tryp}}/K_{i_{thr}} = 8658$), and orally bioavailable (58% in dogs) compound.[7,8] In order to further enhance the thrombin inhibitory potency of this series, we have undertaken structure-based optimizations, focusing on the aryl moiety of the sulfonamide function that binds in the hydrophobic distal (D-) pocket of thrombin. In this paper, we report a study in which the naphthyl group of sulfonamide **2** is replaced with a variety of hydrophobic moieties, along with studies of the activity of these new compounds.



Key words: Thrombin; inhibitor; oral; selective; amidrazone.
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Results and Discussion

All compounds illustrated in Table 1 were assayed in vitro for their thrombin inhibitory activity; results were expressed as K_i determined using human α-thrombin. The hydrophobic moieties incorporated first in place of 2-naphthyl group in sulfonamide **2** were the 5-*N*,*N*-dimethyl-1-naphthyl, 5-methoxy-1-naphthyl, 6-methoxy-2-naphthyl, 7-methoxy-2-naphthyl, and 6,7-dimethoxy-2-naphthyl groups. These moieties were studied by the Mitsubishi group prior to their argatroban discovery, and reported to enhance thrombin inhibitory activity over the 2-naphthyl group in a series of thrombin inhibitors, the *N*α-arylsulfonyl-substituted argininamide derivatives.[9] Therefore, we prepared compounds **3**–**7** and found that only **7** exhibited an increased thrombin inhibitory activity (sixfold), while others suffered a six- to 28-fold decrease. The large activity difference between the two similar compounds **5** and **7** can be explained by inspecting our earlier X-ray crystal structure[8] of compound **2** bound to thrombin. This structure indicates that there is some space available for substitution of a small hydrophobic group at the 6-position of the naphthyl ring, thus accommodating the methoxy group, while the 7-position is too close to the enzyme wall of Trp215. The 6-ethoxy substituent (**8**) was also quite well tolerated in this space, resulting in only slightly less affinity than the methoxy group. The unsubstituted 1-naphthyl group (**9**) showed a dramatic decrease in affinity.

The 2-tetrahydronaphthyl group (**10**), another case of better binding affinity in the Mitsubishi's study,[9] also led to a three fold improvement in activity over compound **2**. This finding prompted us to investigate simple alkyl-substituted benzenes as non-naphthalene aryl moieties. Furthermore, a molecular modeling study had suggested that a *para*-alkyl substituted phenyl moiety would allow very favorable binding interactions in the hydrophobic D-pocket, and that substituents such as an isobutyl group would have a strong hydrophobic interaction with Leu99, Ile174 and Trp215 in the D-pocket as shown in Figure 1. Accordingly, we prepared a series of *para*-alkyl-substitued phenyl derivatives **12–18** by increasing the chain length or bulk of the alkyl group. In this series, the isobutyl-substituted compound **15**, indeed, showed the best thrombin inhibitory activity (three fold more active than **2**). Propyl-, butyl-, and neopentyl substituents (**13**, **14**, **17**) were also well tolerated, leading to reasonable binding affinities. Additional substitution of a methyl group at the *meta*-position to the sulfonyl had little effect in increasing binding affinity (**19**, **20**) even though the methyl group appeared to have an additional hydrophobic interaction with the residues Ile174 and Trp215. We assume that the isomers which have the methyl group *para* to the sulfonyl in the inseparable mixture contribute less to the activity. Substitution of a cyclohexyl group in this series, which was considered an optimum ring size in modeling, also led to a potent inhibitor in compound **22**.

Further molecular modeling studies concerning the potent inhibitor **10** suggested that homologation of the fused cyclohexenyl ring would lead to more efficient space-filling of the hydrophobic D-pocket. The 2-benzocycloheptenyl compound **23** prepared in this regard showed a three fold increase in inhibitory potency over compound **10**, which is in fact a significant improvement versus **2** (ca. ninefold). We turned to biaryl moieties[10] and prepared biphenyl and pyridothienyl derivatives **24** and **25** primarily because their synthetic starting materials (i.e. sulfonyl chlorides) were commercially available; compounds of both synthons showed good binding affinity for thrombin.

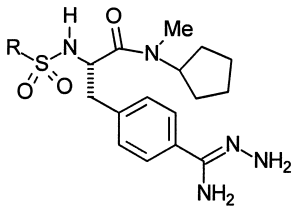
The 2-anthracenyl derivative[11] **26** also exhibited strong thrombin affinity, while the dihydrophenanthrene derivative **27** was slightly less active than **2**. Compound **26**, however, suffers from relatively poor water-solubility compared to other derivatives despite its acid salt form. We have also explored some non-aryl sulfonamide derivatives such as styryl (**28**), benzyl (**29**), linear alkyl (**30**) and bulky alkyl (**31**) groups. Although none of these moieties offered any advantage in affinity over the 2-naphthyl, it is notable that octyl and camphor derivatives **30**, **31** are only eight- and twofold less potent than **2**, respectively, despite their lack of aryl functionality.

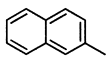
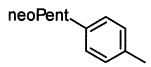
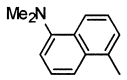
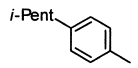
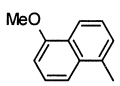
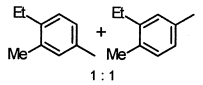
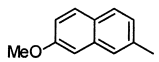
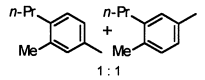
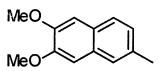
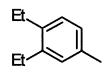
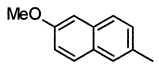
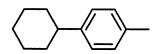
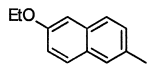
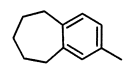
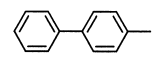
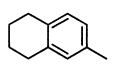
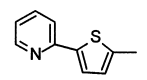
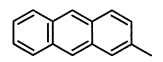
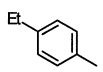
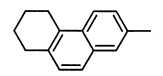
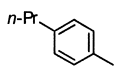
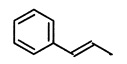
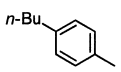
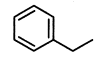
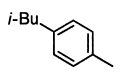
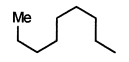
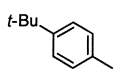
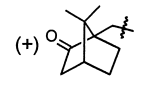
Compounds **7**, **10**, **15**, **23**, **24** and **26**, which were found to have excellent thrombin inhibitory potency, were selected for further evaluation in which their selectivity for thrombin compared to the related serine protease trypsin (Table 2). Most of these compounds displayed micromolar level of binding affinity to trypsin, thus providing excellent selectivity for thrombin. Preliminary evaluation of the oral bioavailability of the selected compounds was also performed. Compounds **7**, **15** and **24** were found to have comparable pharmacokinetic profiles to that of **2** in rats,[12] whereas compounds **10**, **23** and **26** exhibited relatively low oral absorption behavior. An in vivo efficacy study of these compounds is currently underway; the results and details of the pharmacokinetic study will be described elsewhere.

Synthesis

The target compounds listed in Table 1 were synthesized as outlined in Scheme 1. Arylsulfonyl chlorides that were not commercially available were prepared by two different approaches: chlorination of sulfonic acids or their sodium salts (for compounds **4–8**, **24**, **26** and **27**) and direct chlorosulfonylation (for **10–23**). Coupling of

Table 1. Thrombin inhibitory activity of *p*-amidrazonophenyl alanine derivatives



Compd	R	K_i (nM)	Compd	R	K_i (nM)
2		0.38	**17**		0.27
3		0.30	**18**		2.7
4		0.57	**19**		2.0
5		2.5	**20**		0.57
6		10.8	**21**		2.4
7		0.064	**22**		0.21
8		0.15	**23**		0.045
9		8.0	**24**		0.21
10		0.12	**25**		0.60
11		3.8	**26**		0.10
12		0.98	**27**		0.56
13		0.22	**28**		1.55
14		0.46	**29**		18.4
15		0.09	**30**		3.14
16		0.67	**31**		0.65

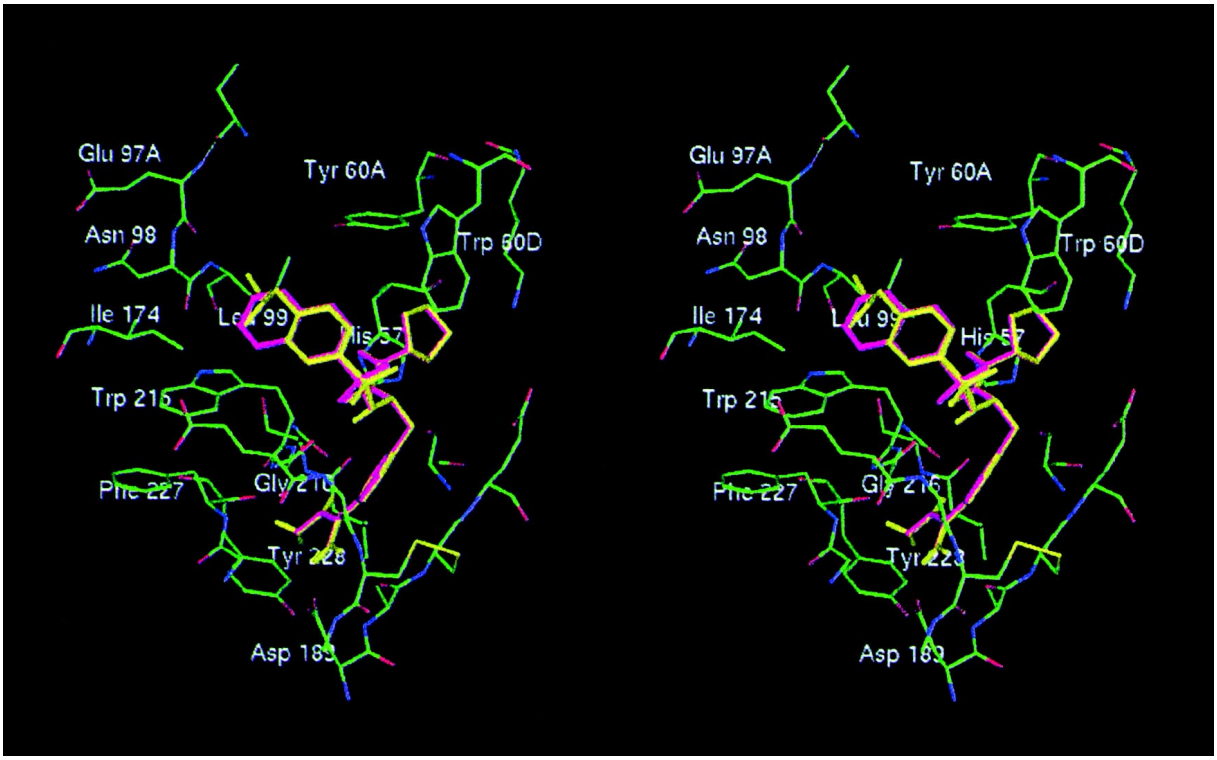


Figure 1. Stereoview of **15** (yellow) modeled in the crystal structure of **2** (magenta)-thrombin complex.

Table 2. Trypsin inhibitory activity and selectivity ratios of *p*-amidrazonophenyl alanine derivatives

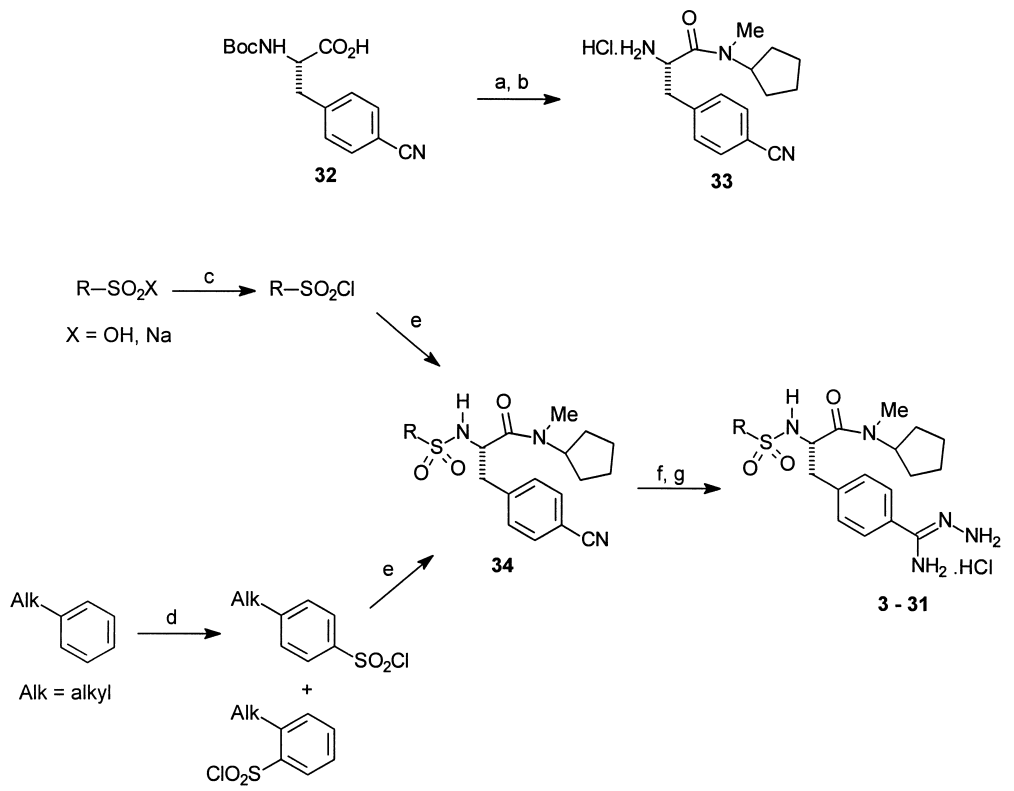
Compd	K_i (μM, trypsin)	K_{itryp}/K_{ithr}
7	9.2	143750
10	4.6	38333
15	6.8	75555
23	5.5	122222
24	6.0	28571
26	0.80	8000

the sulfonyl chlorides with (*S*)-3-(4-cyanophenyl)-*N*-cyclopentyl-*N*-methyl-2-(*t*-Boc-amino)-propionamide hydrochloride (**33**)[6,7] gave intermediates of the general structure **34**. These benzonitrile intermediates were smoothly converted to benzamidrazones **3–31** in good yields by a simple three step sequence involving sequential treatment with hydrogen sulfide, methyl iodide and hydrazine. In the case of monoalkyl benzenes, chlorosulfonylation gave an inseparable mixture of *para*- and *ortho*-substituted sulfonyl chlorides in a 2:1 to 4:1 ratio in generally good yields. However, upon coupling with **33**, a mixture of two regioisomeric sulfonamides that were separable by column chromatography was generated. In the same manner, each of the 2-tetrahydronaphthyl and 2-benzocycloheptenyl sulfonamides was separated as a major product from the corresponding 1-substituted regioisomer. However, asymmetric 1,2-dialkylbenzenes afforded four regioisomeric sulfonamides from which the 4- and 5-sulfonyl isomers were obtained as an inseparable mixture. All of the target compounds described here were obtained as pure acid salts by successive purification involving normal column chromatography, HPLC and ion-exchange chromatography.

In summary, 30 different hydrophobic moieties have been investigated in place of the 2-naphthyl group of the potent oral thrombin inhibitor **2**, and the derivatives were evaluated for thrombin inhibitory activity. Several of these compounds turned out to be more potent than compound **2** itself, with the best compound displaying an order of magnitude enhancement in potency, as well as being highly selective against trypsin. Further structure–activity relationship studies for improving oral bioavailability are currently under investigation; the results of these studies will be published in due course.

Experimental

The starting arylsulfonyl chlorides for the preparation of compounds **3**, **9**, **12**, **13**, **14**, **16**, **18**, **24**, **25**, **28**, **29**, **30** and **31** were purchased from Aldrich and Maybridge,



Scheme 1. (a) *N*-Cyclopentyl-*N*-methylamine, EDC, HOBt, DMF; (b) AcCl, MeOH; (c) $SOCl_2$, DMF; (d) $HOSO_2Cl$, neat; (e) **33**, 4-methylmorpholine, DMF; (f) i. H_2S, pyridine, Et_3N; ii. Mel, CH_3CN; iii. $NH_2NH_2.xH_2O$ (80%), MeOH; (g) i. HPLC, H_2O/MeOH; ii. Dowex 1×8-100 resin.

except biphenylsulfonyl chloride, which was purchased from Fluka. Methoxy- and ethoxy-substituted naphthalenesulfonyl chlorides for compounds **4–8** were prepared from the corresponding hydroxy-substituted naphthalenesulfonic acids and thionyl chloride according to literature procedure.[13] Arylsulfonyl chlorides for compounds **26** and **27** were prepared from the corresponding sulfonic acids which were commercially available (Sigma-Aldrich Rare Chemicals). The solvents employed in these experiments were purchased and were not purified. NMR spectra were recorded on a Jeol 500 spectrometer; chemical shifts are reported in δ (ppm) relative to tetramethylsilane. Column chromatography was carried out with Merck grade 60 silica gel (230–400 mesh). Final purification was achieved by preparative HPLC on a Delta-Pak C18 column, 100-A pore size, with trifluoroacetic acid (0.1%)-H_2O-MeOH solvent systems using various linear gradients. FABMS and HR FABMS were obtained on a Jeol DX300 mass spectrometer.

(*S*)-3-(4-Amidrazonophenyl)-*N*-cyclopentyl-*N*-methyl-2-aminopropionamide hydrochloride (33). To an ice-cooled solution of (*S*)-3-(4-cyanophenyl)-2-(butyloxycarbonylamino)propionic acid[7,14] (7.0 g, 24.1 mmol) in DMF (60 mL) was added EDC (5.08 g, 26.5 mmol) and HOBt (3.58 g, 26.5 mmol), and the mixture was stirred at rt for 7 h. A mixture of *N*-cyclopentyl-*N*-methylamine hydrochloride[7,15] (3.43 g, 25.3 mmol) and 4-methylmorpholine (4.0 mL, 50 mmol) in DMF (50 mL) was added, and the mixture was stirred for another 9 h. After the solvent was removed in vacuo, the residue was dissolved in EtOAc (200 mL), washed with water, dried over $MgSO_4$, and concentrated in vacuo. Purification by column chromatography (hexane: EtOAc, 3:7) afforded (*S*)-3-(4-amidrazonophenyl)-*N*-cyclopentyl-*N*-methyl-2-(butyloxycarbonylamino)propionamide (**32**, 7.43 g, 83%): ^{1}H NMR ($CDCl_3$, rotamers) δ 7.61 (m, 2H), 7.32 (m, 2H), 5.48 (m, 1H), 4.98, 4.90, 4.82 and 4.14 (4m, 2H), 2.79 and 2.64 (2s, 3H), 3.05 (m, 2H), 1.93-1.23 (m, 8H), 1.41 (s, 9H). FAB MS = 372 ($M^+ + H$).

This compound (7.43 g, 20 mmol) was dissolved in MeOH (50 mL) and acetyl chloride (2.36 g, 30 mmol) was added. After the mixture was stirred at rt for 5 h, the solvent was removed in vacuo and the residue was dried under high vacuum to produce compound **33** (5.85 g, 95%) as a white solid: ^{1}H NMR (CD_3OD,

rotamers) δ 7.77 (m, 2H), 7.50 (m, 2H), 4.82, 4.70 and 4.02 (3m, 2H), 3.22 (m, 2H), 2.80 and 2.61 (2s, 3H), 1.98-0.90 (m, 8H). FAB MS = 272 ($M^+ + H$). This product was used directly without further purification.

Synthesis of (*S*)-3-(4-amidrazonophenyl)-*N*-cyclopentyl-*N*-methyl-2-dansylpropionamide hydrochloride (3). To a solution of **33** (2.0 g, 6.5 mmol) in DMF (50 mL) was added 4-methylmorpholine (2.14 mL, 19.5 mmol) and dansyl chloride (1.75 g, 6.5 mmol), and the mixture was stirred for 1 h at rt. After the resulting solution was concentrated in vacuo and the residue dissolved in CH_2Cl_2, the solution was washed with water. The organic layer was dried ($MgSO_4$), concentrated, and purified by column chromatography (hexane: EtOAc, 7:3) to provide the coupling product, (*S*)-3-(4-cyanophenyl)-*N*-cyclopentyl-*N*-methyl-2-dansylpropionamide, as a white solid (3.05 g, 93%): ^{1}H NMR ($CDCl_3$, rotamers) δ 8.54 (m, 1H), 8.16 (m, 2H), 7.71–7.04 (m, 7H), 6.00 (d, 1H), 4.53, 4.40, 4.34 and 3.82 (4m, 2H), 2.90 (m, 8H), 2.50 and 2.26 (2s, 3H), 1.80–0.88 (m, 8H). FAB MS = 505 ($M^+ + H$).

A solution of the coupling compound (0.347 g, 0.69 mmol) in pyridine (15 mL) was saturated with gaseous H_2S. After the mixture was allowed to stand for 2 d, the solvent was removed in vacuo to obtain the thioamide as a yellow solid. To this material was added CH_3CN (15 mL) and iodomethane (0.13 mL, 2.09 mmol), and the mixture was heated at reflux for 1 h. After the solvent was evaporated in vacuo, the resulting methylthioamidate was dissolved in absolute MeOH. To this solution was added portionwise 80% hydrazine hydrate (0.12 mL, 1.98 mmol) over 10 min, and the mixture was stirred at rt. The solution was concentrated and the residue purified by column chromatography using 10% MeOH in $CHCl_3$ to give the title compound which was further purified by preparative HPLC (trifluoroacetic acid (0.1%)-H_2O-MeOH gradient). The pure fractions were passed through an ion-exchange resin column (Dowex 1×8-100) and then lyophilized to give a white solid **3** (0.24 g, 65%) as a HCl salt: ^{1}H NMR (CD_3OD, rotamers) δ 8.50 (m, 2H), 8.21 (m, 1H), 7.68–7.59 (m, 5H), 7.32 (m, 2H), 4.67, 4.43, 4.22 and 3.98 (4m, 2H), 3.11 (s, 3H), 3.08 (s, 3H), 3.00 and 2.87 (2m, 2H), 2.46 and 2.38 (2s, 3H), 1.76–0.90 (m, 8H). HR FABMS calcd for $C_{28}H_{37}N_6O_3S$ 537.2647 ($M^+ + H$), found 537.2658.

In similar procedure to the synthesis of **3**, compounds **4–9**, **12–14**, **16**, **19** and **22–31** were prepared from the corresponding sulfonyl chlorides.

Synthesis of (*S*)-3-(4-amidrazonophenyl)-*N*-cyclopentyl-*N*-methyl-2-(5,6,7,8-tetrahydronaphthalene-2-sulfonyl)-propionamide hydrochloride (10) and (*S*)-3-(4-amidrazonophenyl)-*N*-cyclopentyl-*N*-methyl-2-(5,6,7,8-tetrahydronaphthalene-1-sulfonyl)propionamide hydrochloride (11). To an ice-cooled flask containing chlorosulfonic acid (2.3 mL, 34.6 mmol) was added 1,2,3,4-tetrahydronaphthalene (2.0 mL, 14.2 mmol) dropwise over 10 min. The resulting mixture was stirred for 3 h at rt, poured into ice water and extracted with EtOAc (20 mL×3). The combined extracts were washed with water (20 mL×2), dried over $MgSO_4$ and concentrated in vacuo to give a mixture of 2- and 1–5,6,7,8-tetrahydronaphthalenesulfonyl chlorides (1.3 g, 96%) in a ca. 3:1 ratio as determined by ^{1}H NMR. ^{1}H NMR ($CDCl_3$) major regioisomer, δ 7.74 (d, 1H), 7.40 (d, 1H), 7.27 (d, 1H), 2.89 (s, 4H), 1.85 (s, 4H); minor regioisomer 7.91 (d, 1H), 7.30 (t, 1H), 7.10 (m, 1H), 2.80 (m, 4H), 1.83 (m, 4H).

The crude sulfonyl chloride mixture (1.5 g, 6.52 mmol) was added to a solution of **33** (2.0 g, 6.5 mmol) and 4-methylmorpholine (2.14 mL, 19.5 mmol) in DMF (10 mL) and the mixture was stirred for 1 h at rt. After standard workup, the regioisomeric coupling products were separated by column chromatography (hexane: EtOAc, 3:2). For major product (1.40 g, 46%), (*S*)-3-(4-cyanophenyl)-*N*-cyclopentyl-*N*-methyl-2-(5,6,7,8-tetrahydronaphthalene-2-sulfonyl)propionamide: ^{1}H NMR ($CDCl_3$, rotamers) δ 7.55 (m, 2H), 7.45 (m, 1H), 7.26 (m, 3H), 7.10 (m, 1H), 5.72 (d, 1H), 4.56, 4.49, 4.29 and 3.81 (4m, 2H), 2.97 (m, 2H), 2.80 (bs, 4H), 2.60 and 2.34 (2s, 3H), 1.80–0.90 (m, 12H); FAB MS = 466 ($M^+ + H$). For minor product (0.46 g, 15%), (*S*)-3-(4-cyanophenyl)-*N*-cyclopentyl-*N*-methyl-2-(5,6,7,8-tetrahydronaphthalene-1-sulfonyl)propionamide: ^{1}H NMR ($CDCl_3$, rotamers) δ 7.71 (m, 1H), 7.52 (m, 2H), 7.18 (m, 3H), 7.10 (m, 1H), 5.78 (d, 1H), 4.57, 4.48, 4.28 and 3.78 (4m, 2H), 3.20–2.80 (m, 6H), 2.60 and 2.26 (2s, 3H), 1.90–0.90 (m, 12H); FABMS = 466 ($M^+ + H$).

Each coupling product was sequentially treated with hydrogen sulfide, methyl iodide, and hydrazine to give an amidrazone as described for the preparation of compound **3**. For compound **10** (1.07 g, 67%), ^{1}H NMR (CD_3OD, rotamers) δ 7.65 (m, 2H), 7.42 (m, 4H), 7.15 (m, 1H), 4.55, 4.40 and 4.04 (3m, 2H), 3.05 and 2.90 (2m, 2H), 2.81 (m, 4H), 2.60 and 2.51 (2s, 3H), 1.90–1.00 (m, 12H). HR FAB MS calcd for $C_{26}H_{36}N_5O_3S$ 498.2538 ($M^+ + H$), found 498.2534. For compound **11** (0.38 g, 72%), ^{1}H NMR (CD_3OD, rotamers) δ 7.70 (m, 1H), 7.60 (m, 2H), 7.36 (m, 2H), 7.25 (m, 1H), 7.16 (m, 1H), 4.50, 4.38, 4.34 and 3.91 (4m, 2H), 3.10–2.90 (m, 4H), 2.78 (m, 2H), 2.45 and 2.39 (2s, 3H), 1.85–0.97 (m, 12H). HR FABMS calcd for $C_{26}H_{36}N_5O_3S$ 498.2538 ($M^+ + H$), found 498.2541.

Compounds **15**, **11**, **17**, **18**, **19** and **20–23** were prepared from the corresponding alkylbenzenes in a similar manner to the synthesis of **10**.

Enzyme assays for the inhibition of thrombin and trypsin

The activity of thrombin was measured spectrophotometrically using tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate (Chromozyme TH, Boehringer Mannheim) as a substrate. Thrombin used in the tests was prepared from human plasma according to the protocol of Ngai and Chang.[16] Each compound was dissolved in DMSO to make a 1 mM stock solution and dilutions were made thereof with assay buffer (0.1 M Tris–HCl, 0.15 M NaCl, 0.1% polyethylene glycol 8000, pH 7.8). Different concentrations of inhibitor were mixed with 0.3 NIH units of thrombin in 0.8 mL of the buffer. The mixture was incubated for 10 min at rt before adding 0.2 mL of the substrate to a final concentration of 20 μM. The release of *p*-nitroaniline by hydrolysis of the substrate was monitored for 5 min by measuring the increase in optical density at 381 nm with a UV2100S spectrometer (Shimadzu). A graph for the reciprocal value of initial velocity to the inhibitor concentration was derived from progress curves by fitting the data using a linear regression program. The inhibition constants (K_i values) were then obtained from the Dixon plot equation.[17] Under these conditions, the K_m value for the substrate hydrolysis was 5.2 μM as determined from a non-linear regression analysis of initial rate assuming Michaelis–Menten kinetics.

Inhibition constants for trypsin were determined as described above using 1.2 μg/mL of bovine pancreatic trypsin (Sigma) and 20 μM of *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide hydrochloride (B-4758, Sigma). The Km of the substrate was 10.5 μM.

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