Synthesis and Structure−Activity Relationships of Potent Thrombin Inhibitors: Piperazides of 3-Amidinophenylalanine

Jörg Stürzebecher,* Dagmar Prasa, Jörg Hauptmann, Helmut Vieweg,† and Peter Wikström†

Klinikum der Friedrich-Schiller-Universität Jena, Zentrum für Vaskuläre Biologie und Medizin, Nordhäuser Str. 78, D-99089 Erfurt, Germany, and Pentapharm Ltd., CH-4002 Basel, Switzerland

Received September 25, 1996⊗

Thrombin is the key enzyme in the blood coagulation system, and inhibitors of its proteolytic activity are of therapeutic interest since they are potential anticoagulants. The most potent inhibitor of the benzamidine type is N^{α}-[(2-naphthylsulfonyl)glycyl]-4-amidinophenylalanylpiperidide (NAPAP). However, NAPAP and other benzamidine derivatives do not show favorable pharmacological properties; above all, they have very low systemic bioavailability after oral administration. The goal of designing new compounds was to obtain potent inhibitors with improved pharmacokinetic properties. Piperazide derivatives of 3-amidinophenylalanine as the key building block were synthesized. The piperazine moiety opened the possibility to introduce quite different substituents on the second nitrogen using common synthetic procedures. Some of the newly synthesized compounds are potent inhibitors of thrombin and offer an approach to study structure−function relationships for inhibition of thrombin and related enzymes and for the improvement of their pharmacokinetic properties.

Introduction

The currently used anticoagulants meet only some of the criteria for an ideal antithrombotic drug.[1] It has been shown that direct thrombin inhibitors have a number of advantages over the therapeutically used anticoagulants.[2−4] From this point of view, both the naturally occurring thrombin inhibitor hirudin[5] and the synthetic peptide hirulog[6] are promising anticoagulants. However, due to their peptide structure, these agents can only be administered by the parenteral route. In contrast to large peptides and recombinant proteins, synthetic, low molecular weight inhibitors may be absorbed after oral administration. Thus, a synthetic inhibitor selective toward thrombin and with an appropriate half-life after oral administration could be the ideal anticoagulant. Absorption after oral administration has been reported for peptide aldehydes,[7] boronic acid derivatives,[8] and benzamidine-derived inhibitors;[9] however, systemic bioavailability is very low.[10] Rapid elimination and low oral bioavailability of several benzamidine- and arginine-derived thrombin inhibitors were shown to be caused by hepatic uptake and biliary excretion.[11,12] For the first time, relatively high oral bioavailability was shown for agmatine derivatives of D-Phe-Pro-Arg type inhibitors containing a N-terminal acetic acid.[13] However, anticoagulant-active compounds derived from benzamidine with improved pharmacokinetic properties have not yet been described.

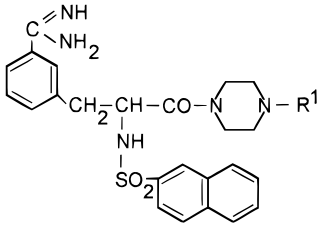
We have designed and systematically synthesized several types of low molecular weight inhibitors derived from benzamidine-containing amino acids. Amidinophenylalanine, in which the benzamidine moiety imitates the guanidinoalkyl side chain of arginine, was used as a key structural element for the development of inhibitors.[14] The most potent thrombin inhibitors of the benzamidine type are N^{α}-[(2-naphthylsulfonyl)glycyl]-4-amidinophenylalanylpiperidide (NAPAP) and its derivatives.[15,16] However, NAPAP, its derivatives, and other benzamidine-derived inhibitors do not yet fulfill the pharmacological requirements for an ideal anticoagulant.[17,18] In the case of NAPAP, most of the structural variations resulted in a drastic loss of antithrombin activity;[19] however, reduction of the basicity of the amidino moiety and introduction of a carboxyl group led to improved pharmacokinetics.[9] From the X-ray crystal structure it was deduced that in the NAPAP-thrombin complex there is no space available for additional substituents.[20,21] However, X-ray crystal structure analysis led to other promising structures, such as N^{α}-tosylated 3-amidinophenylalanylpiperidide (3-TAPAP).[14] The thrombin-3-TAPAP complex indicated more space available for substituents both at the toluene ring and the piperidine moiety.[22,23] A considerable number of new derivatives of 3-amidinophenylalanine were synthesized.[24,25] In this series, the N^{α}-naphthylsulfonylated 4-methylpiperidide shows high antithrombin activity equal to that of NAPAP (K_i 6.2 and 6.0 nM, respectively). Although substituents could be introduced into the molecule which should theoretically improve the absorption after oral application and decrease elimination from the circulation without a dramatic loss of activity, low absorption rates were found for these compounds.[9] Therefore, we looked for alternative derivatives.

The X-ray crystal structure of the complex of thrombin with the N^{α}-naphthylsulfonylated 3-amidinophenylalanyl-4-methylpiperidide shows clearly that the 4-methyl group located on the piperidine ring does not optimally fill the space in the cavity formed by the side chains of His57, Tyr60A, and Trp 60D in thrombin and by the piperidine moiety,[26] so that larger substituents should be tolerated. However, the piperidine moiety does not allow introduction of a wide variety of substituents. In the 3-amidinophenylalanine-type inhibitors we exchanged the piperidine moiety for a piperazine ring to facilitate the incorporation of different substituents on the second nitrogen of the piperazine moiety.[27]

† Pentapharm Ltd.
⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1997.

S0022-2623(96)00668-1 CCC: $14.00

Table 1. Inhibition of Thrombin, Factor Xa, Plasmin, and Trypsin by N′-Substituted Piperazides of N^{α}-2-Naphthylsulfonylated 3-Amidinophenylalanine



		K_i (μM)[a]			
no.	R^1	thrombin	F Xa	plasmin	trypsin
1	H	0.50 (0.09)	21 (5)	10.2 (1.0)	0.22 (0.02)
2	CH_3	0.036 (0.008)	30 (6)	55 (7)	1.3 (0.2)
3	C_2H_4OH	0.058 (0.006)	35 (4)	42 (2)	1.1 (0.3)
4	C_6H_5	0.52 (0.05)	27 (7)	2.6 (0.6)	0.027 (0.004)
5	CO-H	0.037 (0.010)	19 (2)	6.3 (1.3)	0.22 (0.03)
6	CO-CH_3	0.023 (0.004)	48 (4)	8.4 (1.1)	0.14 (0.03)
7	CO-*n*-C_3H_7	1.04 (0.10)	80 (11)	8.6 (2.1)	0.049 (0.012)
8	CO-*n*-C_5H_{11}	6.5 (0.6)	19 (1)	2.6 (0.1)	0.064 (0.013)
9	CO-*c*-C_6H_{11}	36 (9)	58 (4)	10.2 (2.3)	0.072 (0.019)
10	CO-C_6H_5	7.6 (0.7)	16 (1)	7.2 (0.4)	0.14 (0.03)
11	CO-β-$C_{10}H_7$	0.46 (0.11)	7.3 (0.5)	3.0 (0.3)	0.19 (0.05)
12	CO-OCH_3	0.019 (0.005)	77 (18)	2.8 (0.3)	0.020 (0.005)
13	CO-OC_2H_5	0.67 (0.06)	54 (6)	2.5 (0.4)	0.022 (0.003)
14	CO-OC_6H_5	17 (4)	43 (6)	2.3 (0.1)	0.028 (0.006)
15	CO-$N(CH_3)_2$	0.0088 (0.0021)	24 (2)	6.1 (1.2)	0.17 (0.04)
16	CO-$N(C_2H_5)_2$	0.51 (0.10)	12 (2)	14 (3)	0.22 (0.06)
17	SO_2-CH_3	0.0033 (0.0008)	22 (1)	12 (2)	0.19 (0.02)
18	SO_2-C_2H_5	0.044 (0.004)	24 (1)	3.5 (0.9)	0.19 (0.03)
19	SO_2-n-C_3H_7	0.28 (0.04)	22 (6)	7.8 (0.1)	0.21 (0.02)
20	SO_2-n-C_4H_9	0.35 (0.09)	18 (2)	11 (3)	0.18 (0.02)
21	SO_2-C_6H_5	0.041 (0.010)	24 (3)	19 (4)	0.33 (0.07)
22	SO_2-C_6H_4-4-CH_3	0.022 (0.002)	24 (8)	15 (1)	0.22 (0.05)
23	SO_2-β-$C_{10}H_7$	0.024 (0.009)	25 (6)	11 (2)	0.40 (0.13)
24[b]	Ppd	0.065 (0.011)	38 (1)	7.0 (0.4)	0.33 (0.05)
25[b]	Ppd-4-CH_3	0.0062 (0.0014)	41 (9)	5.2 (0.3)	0.14 (0.02)
NAPAP		0.0060 (0.0006)	7.9 (0.7)	30 (4)	0.69 (0.04)

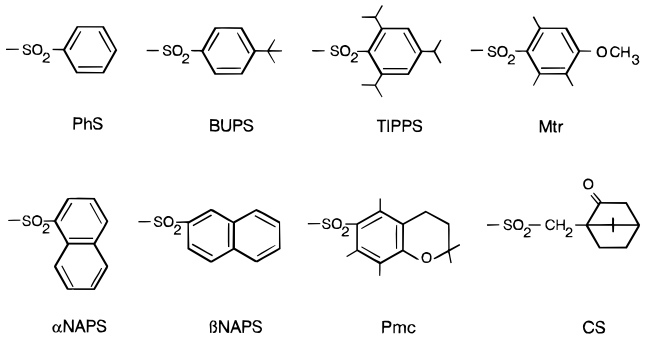
[a] K_i-values were calculated according to Dixon[32] using a linear regression program. Mean values (±SD, $n = 3-5$). [b] These reference compounds contain a C-terminal piperidine ring (Ppd) instead of the piperazine moiety.

We now report that this approach led to the design of novel, potent, and selective thrombin inhibitors.

Results and Discussion

Variation of the N′-Substituent of the Piperazine Moiety. In previous work, we synthesized several derivatives of the N^{α}-(naphthylsulfonyl)-3-amidinophenylalanylpiperidide.[24,25] Substitution in the 4-position of the piperidine moiety was preferred for inhibition of thrombin. However, only a few 4-piperidide derivatives could be obtained due to the cumbersome synthesis. Therefore, the piperazide derivatives **1**, **2**, and **4** (Table 1) were designed opening the possibility for introducing different substituents. The *N′*-methylpiperazide **2** exerts antithrombin activity equal to that of the piperidide **24**; however, it is 10 times less potent than the isosteric 4-methylpiperidide **25**. Despite this, we have synthesized several derivatives with varying N′-substituents. Table 1 shows selected 3-amidinophenylalanine derivatives with a C-terminal piperazide motif which inhibit the enzymes tested in a competitive manner. What was already evident with the alkyl and aryl derivatives (**2**−**4**) has been confirmed with the acyl derivatives: only residues with one or two carbons are compatible with high inhibitory strength toward thrombin. Thus, the formyl and acetyl derivatives (**5** and **6**) inhibit the enzyme with K_i-values in the low micromolar range. In contrast, antitrypsin activity is enhanced slightly with enlargement of the alkyl residue (compounds **7**−**9**) but not with aryl residues (compounds **10** and **11**). Surprisingly, with the β-naphthyl derivative **11**, the antithrombin activity increases again. A similar trend is found among the alkoxy- and phenoxycarbonyl derivatives. Only the methyl carbamate **12** exerts remarkable antithrombin activity, while the inhibitory power decreases drastically with introduction of an ethyl or phenyl residue (compounds **13** and **14**). However, this variation does not influence the high antitrypsin activity. A remarkable compound is the dimethylcarbamoyl derivative **15** with further enhanced antithrombin but lower antitrypsin activity. The variation from the dimethyl derivative **15** to the diethyl compound **16** resulted in a drastic loss of antithrombin activity. Substitution of the piperazide nitrogen with alkyl- or arylsulfonyl moieties is tolerated in several compounds (Table 1). With a K_i of 3.3 nM the methylsulfonyl derivative **17** is the most potent thrombin inhibitor derived from 3-amidinophenylalanine. As shown with the isosteric acyl, alkoxycarbonyl, and carbamoyl derivatives, antithrombin activity is reduced with increasing length of the alkyl residue. However, the N′-arylsulfonylated piperazides (**21**−**23**) are again potent thrombin inhibitors with K_i-values within the 10^{-8} M range.

Chart 1. Structure of the Arylsulfonyl Residues R^2 [a]



[a] PhS = phenylsulfonyl; BUPS = (4-*tert*-butylphenyl)sulfonyl; TIPPS = (2,4,6-triisopropylphenyl)sulfonyl; Mtr = (4-methoxy-2,3,6-trimethylphenyl)sulfonyl; αNAPS = naphthalen-1-ylsulfonyl; *β*NAPS = naphthalen-2-ylsulfonyl; Pmc = (2,2,5,7,8-pentamethylchroman-6-yl)sulfonyl; CS = camphor-10-ylsulfonyl.

From the X-ray crystal structure of the complexes of thrombin with inhibitors of the 3-amidinophenylalanine type, the C-terminal piperazine moiety is expected to bind in the S2 site, which in thrombin is bounded by residues His57, Ser214, Leu99, Tyr60A, and Trp60D and where medium-sized residues, such as piperidine[22] or 4-methylpiperidine,[26] are preferred. In trypsin, this site is much more open, due to the lack of the insertion loop Tyr60A−Trp60D and should therefore be less specific and less sensitive to variations. Thus, the piperazides with a small N′-residue and K_i-values between 10^{-8} and 10^{-9} M should bind optimally into the S2 site of thrombin. However, compounds **21**−**23** with an aromatic moiety at the piperazide nitrogen, which one would expect to be too large to fit in the S2 cavity, also inhibit thrombin within the 10^{-8} M range. Presumably, the N-terminal naphthylsulfonyl group and the hydrophobic aryl moiety form a compact structure that occupies the S2 cavity and the hydrophobic aryl-binding site.[26,28] In contrast, variations at the piperazide nitrogen have little influence on antitrypsin activity, so that the thrombin/trypsin selectivity ratio is improved with increasing thrombin affinity.

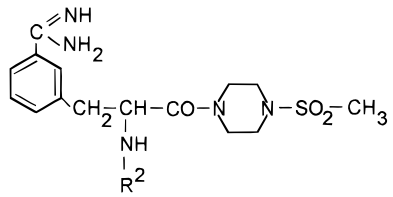
Among the other enzymes, inhibition of F Xa[29] by the piperazides is very low. The K_i-values are in the range of 10−100 μM; structural variations do not influence the anti-F Xa activity. Plasmin as well is inhibited less strongly than thrombin, the K_i-values being mostly near 10 μM.

Variation of the N$^\alpha$-Protecting Group. The *N′*-methylsulfonyl piperazide of 3-amidinophenylalanine (compound **17**, Table 1) was chosen as the most potent inhibitor to investigate the influence of the *N*$^\alpha$-arylsulfonyl residue on the inhibitory activity. In general, inhibition of thrombin by the respective derivatives is more pronounced than inhibition of F Xa, plasmin, and trypsin (Table 2). However, substitution of the α-nitrogen with an arylsulfonyl residue other than the *β*-naphthylsulfonyl protecting group (Chart 1) did not lead to an increase in antithrombin activity (Table 2). In most cases, it resulted in a drastic loss of inhibitory potency. In contrast, the antitrypsin and antiplasmin activities are influenced only to a slight extent by the variations. Only in the case of F Xa did exchange of *β*NAPS for other N$^\alpha$-substituents result in some increase in affinity; however, potent inhibitors of F Xa were not found.

Stereoselectivity. First it was proven by X-ray crystal structure analysis that for trypsin as well as thrombin[20,22] the insertion of the amidinophenylalanine moiety into the specificity pocket required the L-conformation of the central phenylalanine residue in inhibitors derived from 3-amidinophenylalanine but the D-conformation in inhibitors of the NAPAP type. As a rule, the racemates of new compounds were synthesized, as was the case with the inhibitors listed in Tables 1 and 2. However, we were able to synthesize also the pure enantiomers, and a few examples are shown in Table 3. It is evident that the L-derivatives inhibit thrombin and trypsin much more potently than the corresponding D-derivatives. The K_i-values differ by a factor between 50 and 100. In the case of F Xa and plasmin, which are inhibited to a lower extent, the differences are much smaller. Stereoselectivity of inhibition by the piperazide derivatives is found to be similar, as was observed for the derivatives with a C-terminal piperidide structure.[25]

Selectivity of Inhibition. For proposed *in vivo* use as an antithrombotic drug, the inhibitors should be

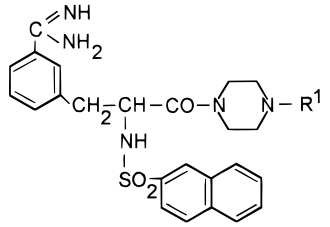
Table 2. Inhibition of Thrombin, Factor Xa, Plasmin, and Trypsin by N$^\alpha$-Substituted *N′*-(Methylsulfonyl)piperazides of 3-Amidinophenylalanine



		K_i (μM)[a]			
no.	R^2	thrombin	F Xa	plasmin	trypsin
17	*β*NAPS	0.0033 (0.0008)	22 (1)	12 (2)	0.19 (0.02)
26	BUPS	0.0043 (0.013)	6.8 (1.1)	14 (2)	0.16 (0.06)
27	Pmc	0.0074 (0.013)	75 (17)	30 (4)	0.76 (0.09)
28[b]	(−)CS	0.012 (0.003)	1.1 (0.1)	14 (1)	0.22 (0.08)
29	αNAPS	0.026 (0.006)	9.7 (0.5)	5.0 (1.1)	0.32 (0.05)
30[b]	(+)CS	0.031 (0.002)	6.7 (1.8)	29 (5)	0.44 (0.12)
31	Mtr	0.032 (0.003)	120 (10)	18 (4)	1.8 (0.5)
32	PhS	0.22 (0.06)	29 (5)	29 (6)	1.0 (0.1)
33	TIPPS	0.28 (0.08)	7.4 (2.1)	6.4 (1.6)	0.98 (0.22)
34	H	13 (2)	> 1000	> 1000	29 (1)

[a] K_i-values were calculated according to Dixon[32] using a linear regression program. Mean values (±SD, n = 3−5). [b] L-Enantiomer.

Table 3. Inhibition of Thrombin and Trypsin by the Enantiomers of Selected N^{α}-2-Naphthylsulfonylated 3-Amidinophenylalanylpiperazides



no.	enantiomer	R^1	K_i (μM)[a] thrombin	trypsin
2	D,L	CH_3	0.036 (0.008)	1.3 (0.2)
	L	CH_3	0.016 (0.003)	0.47 (0.13)
	D	CH_3	7.1 (1.4)	73 (19)
3	D,L	C_2H_4OH	0.058 (0.006)	1.1 (0.3)
	L	C_2H_4OH	0.037 (0.006)	0.60 (0.08)
	D	C_2H_4OH	5.6 (1.0)	39 (4)
5	D,L	CO-H	0.037 (0.010)	0.22 (0.03)
	L	CO-H	0.023 (0.006)	0.073 (0.015)
	D	CO-H	1.6 (0.2)	11.1 (2.1)
6	D,L	CO-CH_3	0.023 (0.004)	0.14 (0.03)
	L	CO-CH_3	0.012 (0.001)	0.044 (0.002)
	D	CO-CH_3	1.9 (0.3)	17 (3)
15	D,L	CO-$N(CH_3)_2$	0.0088 (0.0021)	0.17 (0.04)
	L	CO-$N(CH_3)_2$	0.0040 (0.0007)	0.097 (0.004)
	D	CO-$N(CH_3)_2$	0.35 (0.04)	8.5 (1.9)
17	D,L	SO_2-CH_3	0.0033 (0.0008)	0.19 (0.02)
	L	SO_2-CH_3	0.0021 (0.0005)	0.067 (0.011)
	D	SO_2-CH_3	0.49 (0.01)	13 (2)

[a] K_i-values were calculated according to Dixon[32] using a linear regression program. Mean values (±SD, $n = 3-5$).

highly selective. It is especially important that they do not inhibit enzymes of the fibrinolytic system and protein Ca (APC) which are counterbalancing prothrombotic stimuli.[2,10,30] Therefore, we have determined the inhibitory activity toward different serine proteinases of selected compounds of the series presented. Compared with thrombin, the inhibitory activity toward the clotting enzymes F Xa and F XIIa is low. The same is true for plasma and glandular kallikreins. The anticoagulant-effective enzyme APC is not influenced remarkably. In most cases the K_i-values are more than 3 orders of magnitude higher than that for inhibition of thrombin. The same is true of the inhibition of fibrinolytic enzymes, such as plasmin and the plasminogen activators UK and tPA. In a plasma system, representative derivatives of the series at concentrations higher than 100 μM only impair the clot lysis induced by streptokinase, UK, or tPA.[31] Of the enzymes tested, besides thrombin only trypsin is inhibited with a relatively high inhibitory activity giving K_i-values in the low micromolar range. High potency in trypsin inhibition could lead to side effects especially in the case of oral administration.[10,30] Thus, with the exception of trypsin only, the compounds are highly selective inhibitors of thrombin. Therefore, selectivity with respect to trypsin has to be improved for this class of inhibitor, especially if oral administration is provided.

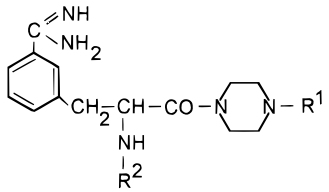
Table 5. Inhibition of Thrombin and Prolongation of Clotting Times of Human Plasma by Selected N^{α}-2-Naphthylsulfonylated 3-Amidinophenylalanylpiperazides and Established Thrombin Inhibitors

no.	K_i (nM)[a]	concentration (IC_{50}, μM)[a] for doubling of TT	aPTT	PT
3, L	37 (6)	0.14 (0.01)	0.56 (0.13)	1.1 (0.3)
5, L	23 (6)	0.063 (0.002)	0.35 (0.07)	0.7 (0.1)
2, L	16 (3)	0.22 (0.04)	1.2 (0.4)	1.8 (0.5)
6, L	12 (1)	0.062 (0.009)	0.28 (0.08)	0.76 (0.14)
15, L	4.0 (0.7)	0.042 (0.005)	0.39 (0.05)	0.72 (0.04)
17, L	2.1 (0.5)	0.037 (0.008)	0.26 (0.11)	0.39 (0.11)
argatroban	9.6 (0.5)	0.062 (0.003)	0.42 (0.06)	0.66 (0.06)
NAPAP	6.0 (0.6)	0.045 (0.002)	0.50 (0.05)	1.0 (0.2)
25, L	2.5 (0.6)	0.24 (0.05)	0.7 (0.1)	1.2 (0.2)
hirudin	0.000027	0.011 (0.001)	0.092 (0.018)	0.27 (0.04)

[a] Mean values (±SD, $n = 3-5$).

Anticoagulant Activity. Some of the new thrombin inhibitors with a C-terminal piperazine moiety exert high anticoagulant activity if they have nanomolar K_i-values (Table 5). Concentrations of 20−60 nM are needed for doubling TT and 0.1−1 μM for doubling aPTT and PT. The anticoagulant potency is higher, or at least in the same range, as that of NAPAP and argatroban. The compounds are similar in potency to hirudin (e.g., compound **17**). For doubling the clotting times the thrombin added (25 nM in the TT assay) or generated (200−400 nM after extrinsic or intrinsic activation in PT and aPTT assays[32]) must be inhibited by nearly 50%. Tight-binding reversible inhibitors need a K_i lower than

Table 4. Inhibition of Various Trypsin-like Enzymes by the L-Enantiomers of Selected N^{α}-Substituted 3-Amidinophenylalanylpiperazides



no.	R^1	R^2	K_i (μM)[a] thrombin	F Xa	F XIIa	APC	PK	GK	plasmin	UK	sc-tPA	trypsin
2, L	CH_3	βNAPS	0.016	19	280	94	35	>1000	30	110	>1000	0.47
3, L	C_2H_4OH	βNAPS	0.037	22	400	39	27	>1000	21	140	180	0.60
5, L	CO-H	βNAPS	0.023	9.7	53	10.3	3.3	>1000	3.3	34	110	0.073
6, L	CO-CH_3	βNAPS	0.012	34	200	12	15	>1000	5.2	32	410	0.044
15, L	CO$N(CH_3)_2$	βNAPS	0.0040	16	61	13.5	7.3	>1000	4.9	28	>1000	0.097
17, L	SO_2-CH_3	βNAPS	0.0021	19	52	15.2	58	>1000	9.0	28	190	0.067
27, L	SO_2-CH_3	Pmc	0.0053	41	>1000	180	97	>1000	10.3	58	470	0.32
28, L	SO_2-CH_3	(−)CS	0.012	1.1	55	33	22	>1000	14	49	20	0.22

[a] K_i-values were calculated according to Dixon[32] using a linear regression program. Standard deviation did not exceed 25%.

Table 6. Plasma Level of the Acetylpiperazide **6** (L-Enantiomer) and NAPAP in Anesthetized Rats after iv Administration of 1 mg/kg and Oral Administration of 50 mg/kg

time after injection (min)	concentration in plasma (μg/mL)[a]			
	compound **6**, L		NAPAP	
	iv	oral	iv	oral
2	3.55 ± 1.22 (3)[b]	nd[c]	2.43 ± 0.10 (3)	nd
5	2.08 ± 0.97 (4)	0.23 ± 0.15 (3)	0.98 ± 0.08 (3)	nd
15	0.61 ± 0.27 (4)	0.41 ± 0.14 (3)	0.24 ± 0.02 (3)	nd
30	0.27 ± 0.14 (4)	0.45 ± 0.11 (3)	0.07 ± 0.02 (3)	0.10 (2)
45	0.17 ± 0.09 (4)	0.41 ± 0.14 (3)	0.04 (2)	nd
60	0.13 ± 0.08 (4)	0.38 ± 0.10 (3)	<0.04 (3)	0.08 (2)
90	0.10 ± 0.05 (4)	0.27 ± 0.09 (3)	<0.04 (3)	nd
120	0.09 ± 0.04 (3)	0.26 (2)	<0.04 (3)	0.08 (2)
180	<0.04 (3)	0.30 (2)	<0.04 (3)	0.09 (2)
240	<0.04 (3)	nd	<0.04 (3)	0.09 (2)

[a] Data are mean ± SD. [b] Number of experiments in parentheses. [c] Not determined.

20 nM for nearly total blockade of the thrombin active site.[33] Therefore, if the inhibitor reacts exclusively with the thrombin active site, a concentration of 10−20 nM is necessary for doubling TT and 100−200 nM for doubling aPTT and PT. Corresponding effective inhibitor concentrations (IC_{50}) in this range were found with hirudin and the potent synthetic inhibitors. The methylpiperazide **2** and the methylpiperidide of 3-amidinophenylalanine **25** are exceptions to this rule. Probably, binding to plasma proteins of these more hydrophobic compounds is the reason for their higher effective concentrations.

Pharmacokinetic Studies. The plasma levels of selected inhibitors were followed after intravenous administration at a dose of 1 mg/kg of body weight to anesthetized rats. Compound **6** was studied in comparison to NAPAP. Table 6 shows the plasma level over a period of 2 h. The plasma level of NAPAP rapidly declines reaching the detection limit 60 min after administration. There is almost no distinction between distribution and elimination phases. For **6**, there are somewhat higher values in the initial distribution phase followed by a slower decline of the plasma level. For comparison, on a molar basis, **6** shows plasma levels 5 times that of NAPAP after 30 and 45 min, whereas the difference in dose was 1.26-fold. The time course of the plasma level derived from a clotting time assay (experiments in a separate group of animals, data not shown) is in accord with those of the HPLC assay, although the numerical values are lower by a factor of about 1.4. HPLC did not give evidence of metabolites in plasma. The results in rats on NAPAP confirm the finding of a very short half-life first found in rabbits.[17] The present results show a comparatively prolonged time course of plasma levels of **6** in rats. After oral administration, **6** reaches higher plasma levels than NAPAP. More detailed studies are under way on the oral bioavailability and metabolism of **6** and other compounds of this series.

Chemistry

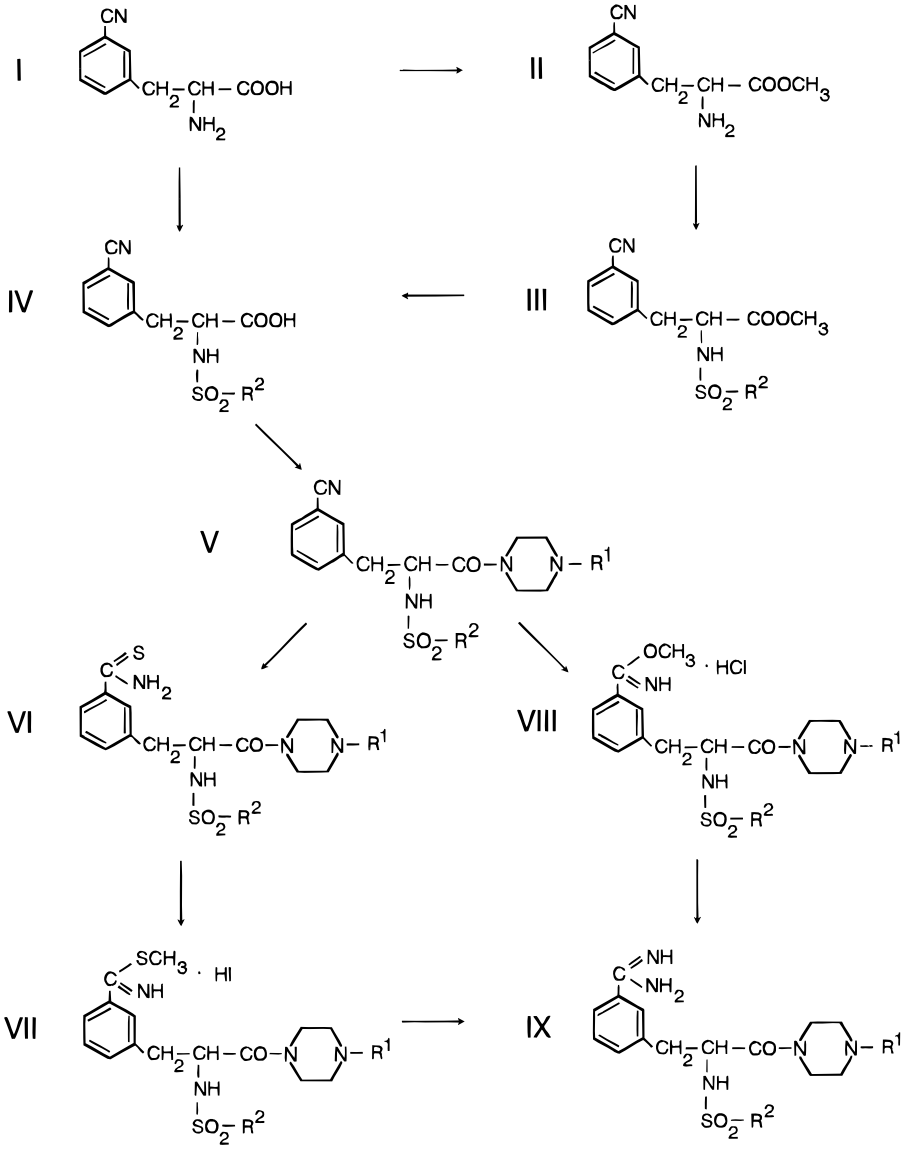
The syntheses of N^{α}-sulfonylated 3-amidinophenylalanylpiperazides with the general structure **IX** (Scheme 1) were achieved via the corresponding cyano compounds **V** which were synthesized in the following way.[27] Starting from *N*-acetyl-D,L-3-cyanophenylalanine, 3-cyanophenylalanine (**I**) was synthesized and used for further syntheses in either the racemic, D-, or L-form. The enantiomers were obtained from the racemate of the acetyl derivative via acidic deacetylation using acylase.

Conversion with the corresponding sulfonyl chloride (R^2-SO_2Cl) led to the sulfonylated cyanophenylalanine compound **IV** with a free carboxyl group. However, because of the low yield the methyl ester **II** was prepared, sulfonylated to **III**, and hydrolyzed yielding the sulfonylated cyanophenylalanine **IV**. Conversion of **IV** into **V** was carried out via a general peptide-coupling reaction between a reactive intermediate (such as the corresponding acid chloride or an activated ester with DCC/HOBt) and a piperazide substituted at the 4-nitrogen with different residues (R^1). The conversion of the cyano function into the amidino function was achieved using one of two known routes. Addition of hydrogen sulfide in the presence of triethylamine in pyridine gave a thioamide (**VI**) which was then S-methylated with iodomethane to give the thioimidate hydrochloride **V** which was further reacted with ammonium acetate in methanol to give the crude hydroiodide of the end product **IX**. The end product **IX** was generally purified by chromatography over silica gel or Sephadex LH-20 to give homogeneous products followed by ion exchange to give the more soluble hydrochlorides of the end product **IX**. Another route to the amidino compounds was achieved by converting the corresponding cyano compound **VI** to the corresponding imidate hydrochloride **VIII** followed by treatment with ethanolic ammonia solution to give the end products **IX**. The syntheses of esters of derivatives containing a carboxyl group were achieved by esterification of the amidino compounds with the corresponding alcohols in the presence of gaseous hydrogen chloride or *p*-toluenesulfonic acid. Preparation of the N^{α}-(2-naphthylsulfonyl)-3-amidino-L-phenylalanyl-4-(2-hydroxyethyl)piperazide (**3**) and the N^{α}-(2-naphthylsulfonyl)-3-amidino-L-phenylalanyl-4-(methylsulfonyl)piperazide hydrochloride (**17**) is exemplified in detail in the Experimental Section.

Conclusions

The structure−activity relationships presented for the new thrombin inhibitors are in agreement with the predictions from the X-ray crystal structure analysis of thrombin−inhibitor complexes. From the structure of the NAPAP-thrombin complex it was deduced that the NAPAP molecule is not a suitable candidate for deriva-

Scheme 1. Syntheses of N$^{\alpha}$-Arylsulfonylated 3-Amidinophenylalanylpiperazides



tizations since NAPAP fits so ideally to thrombin that there is no further space for additional substituents.[20,21] Therefore, we used the N$^{\alpha}$-tosylated 3-amidinophenylalanylpiperidide (3-TAPAP) as a prototype for further synthesis because the corresponding inhibitor complex indicates more space for substituents.[22] Indeed, within a first series of compounds, we found with the N$^{\alpha}$-naphthylsulfonylated 4-methylpiperidide of 3-amidinophenylalanine **25** a thrombin inhibitor whose antithrombin activity is equal to that of NAPAP (K_i 6.2 and 6.0 nM, respectively);[27] however, the compounds do not show improved pharmacokinetics.[9] With the piperazide derivatives described in this paper, potent thrombin inhibitors with sufficient selectivity were found. Some of the compounds possess remarkable anticoagulant activity. As is shown with the acetylpiperazide **6**, the piperazides are eliminated more slowly than NAPAP and 3-amidinophenylalanine derivatives containing a C-terminal piperidide. Furthermore, first results indicate enhanced enteral absorption. Therefore, we are engaged in establishing structure-function relationships of the pharmacokinetic properties of derivatives containing quite different substituents of the second nitrogen of the piperazine ring in order to improve the oral bioavailability. Results will be reported in due course.

Experimental Section

Chemistry. Reagent-grade solvents were used without further purification. Evaporation refers to 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. TLC plates coated with silica gel 60 F_{254} used were from E. Merck AG (Darmstadt, Germany); detection was by UV (254 nm). Normal-phase silica gel used for flash chromatography was Kieselgel-60 (230−400 mesh); ion-exchange resin used was Amberlite IRA-420 (Cl^- form, 16−50 mesh); both were supplied by Fluka (Buchs, Switzerland). Optical rotations $[\alpha]^{20}_D$ were determined with a Perkin-Elmer 243B polarimeter, *c* in g/100 mL. All ^{1}H NMR spectra were recorded on a Bruker AMX 600 spectrometer. Fast atom bombardment mass spectra (FAB/MS; Finnigan 4516) and TOF/MS (Voyager RP) were recorded.

***N*$^{\alpha}$-(2-Naphthylsulfonyl)-3-cyano-L-phenylalanine Methyl Ester (III in Scheme 1, R^2 = *β*-naphthyl) (3a).** 3-Cyano-L-phenylalanine methyl ester hydrochloride (24.1 g, 0.1 mol) was suspended in 200 mL of absolute dioxane, 20.6 g (0.204 mol) of NMM was added under stirring, and a solution of 23.6 g (0.104 mol) of 2-naphthalenesulfonyl chloride in 200 mL of ethyl acetate was added dropwise. The mixture was stirred for 16 h at room temperature, the precipitated *N*-

methylmorpholine hydrochloride was filtered off, and the solvent was evaporated under reduced pressure. The formed precipitate crystallized from methanol/water: yield 35.6 g (90%); ^{1}H NMR (DMSO) δ 8.60 (1 H, d, NH), 8.15 (1 H, m, phenyl-H), 8.05−7.20 (10 H, m, phenyl-H, naphthyl-H), 4.11 (1 H, m, Cα-H), 3.31 (s, 3 H, CH_3), 3.00/2.75 (dd, 2 H, CH_2-phenyl); TOF/MS m/z $(M + 2H)^+$ 396.73 ($C_{21}H_{18}N_2O_4S$, M_r = 394.45); mp 118 °C; $[\alpha]_D$ = +11.8°, c = 1 (MeOH). Anal. ($C_{21}H_{18}N_2O_4S$) C, H, N.

***N*$^\alpha$-(2-Naphthylsulfonyl)-3-cyano-L-phenylalanine (IV in Scheme 1, R^2 = β-naphthyl) (3b).** Methyl ester **3a** (15 g) was allowed to reflux for 2 h in a mixture of 75 mL each of 1 N HCl and acetic acid. Upon cooling to room temperature, water was added until a turbid solution resulted and crystallization took place. The crystals were filtered off, washed with 30% acetic acid in water and vacuum-dried over KOH: yield 13.3 g (92%); ^{1}H NMR (DMSO) δ 12.90 (1 H, s, COOH), 8.30 (1 H, d, NH), 8.17 (m, 1 H, phenyl-H), 8.05−7.18 (10 H, m, phenyl-H, naphthyl-H), 4.00 (m, 1 H, Cα-H), 3.02/2.75 (2 H, dd, CH_2-phenyl); TOF/MS m/z $(M + 2H)^+$ 382.52 ($C_{20}H_{16}N_2O_4S$, M_r = 380.43); mp 192−193 °C; $[\alpha]_D$ = +11.9°, c = 1 (MeOH). Anal. ($C_{20}H_{16}N_2O_4S$) C, H, N.

***N*$^\alpha$-(2-Naphthylsulfonyl)-3-cyano-L-phenylalanyl-4-(2-hydroxyethyl)piperazide (V in Scheme 1, R^2 = β-naphthyl, R^1 = CH_2CH_2OH) (3c).** *N*$^\alpha$-(2-Naphthylsulfonyl)-3-cyano-L-phenylalanyl chloride was prepared from compound **3b** (5 g) by addition to 15 g of thionyl chloride and refluxed for 1 h. The corresponding acid chloride was precipitated by the addition of petroleum ether; 5.1 g (12.8 mmol) of the acid chloride was dissolved in 75 mL of THF and added dropwise over 15 min to a solution of 3.5 g (26.8 mmol) of 1-(2-hydroxyethyl)piperazide in 45 mL of THF. It was stirred for 1 h and filtered and the solvent evaporated under reduced pressure. The residue was dissolved in 30 mL of methanol, water was added until turbidity set in, and the mixture was allowed to stand overnight. The free base of **3c** precipitated out as an oil. The solvent was decanted and the oil dissolved in 150 mL of ethyl acetate. The organic phase was washed with saturated NaCl solution and dried over $MgSO_4$, and the solution was concentrated to ca. 100 mL and acidified by the addition of 2 N HCl in ethyl acetate. The hydrochloride of **3c** was precipitated by the addition of diethyl ether, filtered off, and dried: yield 4.95 g (73%); ^{1}H NMR (DMSO) δ 8.35 (1 H, d, NH), 8.15 (1 H, m, phenyl-H), 8.09−7.32 (10 H, m, phenyl-H, naphthyl-H), 5.35 (1 H, bs, OH), 4.58 (1 H, m, Cα-H), 4.35−4.00 (2 H, m, ethyl-H), 3.75 (2 H, m, ethyl-H), 3.50−3.25 (3 H, m, piperazide-H), 3.10 (1 H, m, piperazide-H), 2.95/2.75 (2 H, dd, CH_2-phenyl), 2.75−2.65 (3 H, m, piperazide-H), 2.30 (1 H, m, piperazide-H); TOF/MS m/z $(M + 2H)^+$ 494.1 ($C_{26}H_{28}N_4O_4S$, M_r = 492.60); mp 155 °C; $[\alpha]_D$ = −5.4°, c = 1 (MeOH). Anal. ($C_{26}H_{28}N_4O_4S$) C, H, N.

***N*$^\alpha$-(2-Naphthylsulfonyl)-3-amidino-L-phenylalanyl-4-(2-hydroxyethyl)piperazide Dihydrochloride (IX in Scheme 1, R^2 = β-naphthyl, R^1 = CH_2CH_2OH) (3).** Cyano compound **3c** (4.2 g, 7.95 mmol) was dissolved in a mixture of 22 mL of absolute methanol and 30 mL of absolute dioxane and cooled to 0 °C, and 15.6 g (0.43 mol) of dried HCl gas was introduced. The reaction solution was kept at 2 °C for 4 days and poured into 400 mL of diethyl ether, and the resulting precipitate was triturated with 100 mL of THF until crystallization took place. The crystals were washed with diethyl ether and dried; 4.32 g (7.24 mmol) of the methylimidate dihydrochloride was suspended in 90 mL of methanol, and ethanolic ammonia solution was added under stirring until a clear solution was obtained (pH 8.7). The mixture was heated to 60 °C for 4 h in a water bath. The solvent was evaporated, the residue was dissolved in 45 mL of absolute ethanol and filtered, and 3 mL of 2 N HCl in ethyl acetate was added. The amidine dihydrochloride was precipitated with addition of diethyl ether and chromatographed over Sephadex LH-20: yield 2.8 g (73.5%); ^{1}H NMR (DMSO) δ 9.25 (1 H, bs, Imin-H), 8.35 (1 H, d, NH), 8.15 (1 H, m, phenyl-H), 8.09−7.32 (10 H, m, phenyl-H, naphthyl-H), 4.61 (1 H, m, Cα-H), 4.40−3.95 (2 H, m, ethyl-H), 3.72 (2 H, m, ethyl-H), 3.52−3.25 (3 H, m, piperazide-H), 3.10 (1 H, m, piperazide-H), 2.95/2.75 (2 H, dd, CH_2-phenyl), 2.75−2.65 (3 H, m, piperazide-H), 2.31 (1 H, m, piperazide-H); FAB/MS m/z $(M + H)^+$ 510.4 ($C_{26}H_{31}N_5O_4S$ formula 509, M_r = 509.633); mp 219 °C; $[\alpha]_D$ = +15.4°, c = 1 (MeOH). Anal. ($C_{26}H_{33}N_5O_4SCl_2 \cdot 1.5\ H_2O \cdot 0.75NH_4Cl$) C, H, N, O, S, Cl.

***N*$^\alpha$-(2-Naphthylsulfonyl)-3-cyano-L-phenylalanyl-4-(methylsulfonyl)piperazide (V in Scheme 1, R^2 = β-naphthyl, R^1 = SO_2CH_3) (17a).** Compound **3b** (5.4 g, 14.2 mmol), 2.32 g (15.6 mmol) of HOBt, and 3.22 g (15.6 mmol) of DCC were dissolved in 70 mL of DMF and stirred for 1 h. A mixture of 3.12 g (15.6 mmol) of 1-(methylsulfonyl)piperazide hydrochloride and 1.72 mL (15.6 mmol) of NMM in 30 mL of DMF was added, and the mixture was stirred for another 20 h at room temperature. The precipitated dicyclohexylurea was filtered off and the solvent evaporated; the resulting residue was chromatographed over silica gel with chloroform as the eluent: yield 6.4 g (85%); ^{1}H NMR (DMSO) δ 8.35 (1 H, bs, NH), 8.15 (1 H, m, phenyl-H), 8.09−7.32 (10 H, m, phenyl-H, naphthyl-H), 4.50 (1 H, m, Cα-H), 3.00/2.85 (2 H, dd, CH_2-phenyl), 3.50−3.35 (3 H, m, piperazide-H), 3.15 (1 H, piperazide-H), 2.75−2.68 (3 H, m, piperazide-H), 2.72 (s, 3 H, SO_2CH_3), 2.32 (1 H, m, piperazide-H); TOF/MS m/z $(M + 2H)^+$ 528.08 ($C_{25}H_{26}N_4O_5S_2$, M_r = 526.64); mp 157 °C; $[\alpha]_D$ = +47.3°, c = 1 (MeOH). Anal. ($C_{25}H_{26}N_4O_5S_2$) C, H, N.

***N*$^\alpha$-(2-Naphthylsulfonyl)-3-amidino-L-phenylalanyl-4-(methylsulfonyl)piperazide Hydrochloride (IX in Scheme 1, R^1 = β-naphthyl, R^2 = SO_2CH_3) (17).** The cyano compound **17a** (5.8 g) was dissolved in 70 mL of pyridine, 1 mL of TEA was added, and the solution was saturated by a 10-min introduction of gaseous H_2S. The mixture was allowed to stand at room temperature for 2 days. The solvent was evaporated under reduced pressure and the residue dissolved in ethyl acetate, whereby the thioamide crystallized out: yield 5.9 g (95%). The obtained thioamide (5.9 g, 10.5 mmol) was dissolved in 8 mL of warm DMF and diluted with 100 mL of acetone, 14.2 g (0.1 mol) of methyl iodide was added, and the mixture was kept in the dark for 20 h at room temperature. The product was precipitated by the addition of diethyl ether, filtered off, and dried: yield 6.5 g (88%). A 9.3-mmol portion of methylthioimidate hydroiodide was dissolved in 220 mL of methanol, 1.2 g (15.5 mmol) of ammonium acetate was added, and the mixture was heated for 3 h at 60 °C in a water bath. The solvent was evaporated under reduced pressure; the residue (amidine hydroiodide) was dissolved in methanol and converted to the corresponding hydrochloride by passing the solution over Amberlite IRA-410 (Cl^- form). The resulting hydrochloride salt was precipitated from methanol by the addition of diethyl ether. The crude product was chromatographed over Sephadex LH-20: yield 3.78 g (71%); ^{1}H NMR (DMSO) δ 9.25 (1 H, bs, Imin-H), 8.12 (1 H, m, phenyl-H), 8.15−7.32 (10 H, m, phenyl-H, naphthyl-H), 4.58 (1 H, m, Cα-H), 3.45−3.35 (3 H, m, piperazide-H), 3.10 (1 H, m, piperazide-H), 3.00/2.85 (2 H, dd, CH_2-phenyl), 2.75−2.65 (3 H, m, piperazide-H), 2.72 (s, 3 H, SO_2CH_3), 2.31 (1 H, m, piperazide-H); FAB/MS m/z $(M + H)^+$ 544.6 ($C_{25}H_{29}N_5O_5S_2$ formula 543, M_r = 543.67); mp 220 °C; $[\alpha]_D$ = +70.0°, c = 1 (MeOH). Anal. ($C_{25}H_{30}N_5O_5S_2Cl \cdot 0.6H_2O$) C, H, N, O, S, Cl.

Determination of Inhibition Constants. The measurements were carried out on a microplate reader (MR 5000, Dynatech, Denkendorf, Germany) at 25 °C. The test medium consisted of 200 μL of Tris buffer (0.05 M; 0.154 M NaCl, 5% ethanol, pH 8.0), 25 μL of aqueous substrate solution, and 50 μL of enzyme solution. Two concentrations of the substrate and five concentrations of the inhibitor were used. Three minutes after the addition of the enzyme, 25 μL of acetic acid (50%) was added to quench the reaction, and the optical density was measured at 405 nm. The K_i-values were calculated according to Dixon[34] using a linear regression program. If not otherwise indicated, the K_i-values are those of the racemates. The K_i-values reported are means from at least three determinations.

Enzymes and Substrates for K_i Determination. The following enzymes and the respective substrates were used at the final concentrations indicated: bovine thrombin prepared according to Walsmann[35] (2262 U/mg, final concentration 0.45 U/mL), substrate $MeSO_2$-D-hexahydrotyrosyl-Gly-Arg-pNA (final concentrations 0.18 and 0.09 mM); bovine F Xa (5 U/vial,

0.11 U/mL; Diagnostic Reagents Ltd., Thame, U.K.), substrate $MeSO_2$-D-Nle-Gly-Arg-pNA (0.36 and 0.18 mM); human F XIIa (0.2 U/vial, 0.0023 U/mL; RD Laboratorien, Martinsried, Germany), substrate H-D-hexahydrotyrosyl-Gly-Arg-pNA (0.36 and 0.18 mM); human APC (1 mg/vial, 1.13 μg/mL; Kordia Lab. Supplies, Leiden, The Netherlands), substrate H-D-Lys(Cbo)-Pro-Arg-pNA (0.36 and 0.18 mM); human PK (1 U/vial, 0.0088 U/mL; RD Laboratorien, Martinsried, Germany), substrate Bz-Pro-Phe-Arg-pNA (0.36 and 0.18 mM); GK isolated from porcine pancreas (500 KU/vial, 1.5 KU/mL; RD Laboratorien, Martinsried, Germany), substrate H-D-Val-cyclohexylalanyl-Arg-pNA (0.18 and 0.09 mM); human plasmin (0.67 CTA-U/mg, 0.06 CTA-U/mL; Behringwerke AG, Marburg, Germany), substrate Tos-Gly-Pro-Lys-pNA (0.18 and 0.09 mM); human UK (500 000 U/vial, final concentration 150 U/mL; Ribosepharm GmbH, Haan, Germany), substrate Bz-βAla-Gly-Arg-pNA (0.18 and 0.09 mM); sc-tPA purified from CHO cells[36] (4.1 mg/mL, 0.0031 μg/mL), substrate $MeSO_2$-D-hexahydrotyrosyl-Gly-Arg-pNA (0.54, 0.27, and 0.145 mM); bovine pancreatic trypsin (42 U/mg, 0.0038 U/mL; Serva, Heidelberg, Germany), substrate $MeSO_2$-D-hexahydrotyrosyl-Gly-Arg-pNA (0.18 and 0.06 mM). The substrates were supplied by Pentapharm Ltd., Basel, Switzerland.

Clotting Assays. For determination of prothrombin time (PT), 0.1 mL of thromboplastin (Dade, Unterschleissheim, Germany) and 0.1 mL of inhibitor dissolved in $CaCl_2$ (0.025 M, 5% ethanol) were incubated at 37 °C for 2 min. Coagulation was started by the addition of 0.1 mL of citrated human plasma. For determination of activated partial thromboplastin time (aPTT), citrated human plasma (0.1 mL) was incubated at 37 °C with 0.1 mL of PTT reagent (Boehringer Mannheim GmbH, Mannheim, Germany). After 3 min, 0.1 mL of inhibitor dissolved in $CaCl_2$ (0.025 M, 5% ethanol) was added. For determination of thrombin time (TT), citrated human plasma (0.1 mL) was mixed with 0.05 mL of inhibitor dissolved in NaCl (0.154 mM, 5% ethanol), and coagulation was started by the addition of 0.05 mL of thrombin (10 U/mL). Clotting times were determined in duplicate using the coagulometer Thrombotrack 8 (Immuno GmbH, Heidelberg, Germany). Inhibitor concentrations required to double the respective clotting times (IC_{50}) were read from lin/log graphs of clotting times versus inhibitor concentrations.

***In Vivo* Elimination Study.** NAPAP and compound **6** were administered to anesthetized (urethane, 1.5 g/kg ip) rats in aqueous solution intravenously in a dose of 1 mg/kg and orally (by gastric tube) in a dose of 50 mg/kg. Citrated blood was drawn at various times after administration from the cannulated carotid artery and centrifuged, and the plasma was poured onto Chromabond C18 solid-phase exctraction columns (Macherey-Nagel, Düren, Germany). The concentrations in plasma were measured by HPLC (2700 HPLC Systems, Bio-Rad, München, Germany) using Nucleosil 7 C18 columns (Macherey-Nagel, Düren, Germany). The mobile phase was acetonitrile/water/perchloric acid (30/70/0.04; flow rate 1 mL/min); the compounds were detected by fluorescence ($\lambda_{ex} = 232$ nm, $\lambda_{em} = 343$ nm).

Acknowledgment. We would like to express our thanks to Ute Altmann, Christa Böttner, and Gabriele Riesener for their excellent technical assistance.

References

(1) Sixma, J. J.; de Groot, P. G. The ideal anti-thrombotic drug. *Thromb. Res.* **1992**, *68*, 507−512.
(2) Powers, J. C.; Kam, C.-M. Synthetic substrates and inhibitors of thrombin. In *Thrombin. Structure and Function*; Berliner, L. J., Ed.; Plenum Press: New York, 1992; pp 117−158.
(3) Harker, L. A.; Hanson, S. R.; Kelly, A. B. Antithrombotic benefits and hemorrhagic risks of direct thrombin antagonists. *Thromb. Haemostas.* **1995**, *74*, 464−472.
(4) Stone, S. Thrombin inhibitors - A new generation of antithrombotics. *Trends Cardiovasc. Med.* **1995**, *5*, 134−140.
(5) Markwardt, F. The development of hirudin as an antithrombotic drug. *Thromb. Res.* **1994**, *74*, 1−23.
(6) Maraganore, J. M.; Bourdon, P.; Jablonski, J.; Ramachandran, K. L.; Fenton, J. W., II Design and characterization of hirulogs: a novel class of bivalent peptide inhibitors of thrombin. *Biochemistry* **1990**, *29*, 7095−7101.
(7) Bagdy, D.; Barabas, E.; Szabo, G.; Bajusz, S.; Szell, E. In vivo anticoagulant and antiplatelet effect of D-Phe-Pro-Arg-H and D-Me-Phe-Pro-Arg-H. *Thromb. Haemostas.* **1992**, *67*, 357−365.
(8) Hussain, M. A.; Knabb, R.; Aungst, B. J.; Kettner, C. Anticoagulant activity of a peptide boronic acid thrombin inhibitor by various routes of administration in rats. *Peptides* **1991**, *12*, 1153−1154.
(9) Stürzebecher, J.; Prasa, D.; Bretschneider, E.; Bode, W.; Bauer, M.; Brandstetter, H.; Wikström, P.; Vieweg, H. New developments in the field of benzamidine-derived thrombin inhibitors. In *DIC - Pathogenesis, Diagnosis and Therapy of Disseminated Intravascular Fibrin Formation*; Müller-Berghaus, G., Madlener, K., Blombäck, M., ten Cate, J. W., Eds.; Excerpta Medica: Amsterdam, London, New York, Tokyo, 1993; pp 183−190.
(10) Kimball, S. D. Challenges in the development of orally bioavailable thrombin active site inhibitors. *Blood Coag. Fibrinol.* **1995**, *6*, 511−519.
(11) Hauptmann, J.; Kaiser, B.; Paintz, M.; Markwardt, F. Biliary excretion of synthetic benzamidine-type thrombin inhibitors in rabbit and rats. *Biomed. Biochim. Acta* **1987**, *46*, 445−453.
(12) Hauptmann, J.; Kaiser, B. In vitro and in vivo comparison of arginine- and benzamidine-derived highly potent synthetic thrombin inhibitors. *Pharmazie* **1991**, *46*, 57−58.
(13) Eriksson, U. G.; Renberg, L.; Vedin, C.; Strimfors, M. Pharmacokinetics of inogatran, a new low molecular weight thrombin inhibitor, in rats and dogs. *Thromb. Haemostas.* **1995**, *73*, 1605.
(14) Markwardt, F.; Wagner, G.; Stürzebecher, J.; Walsmann, P. Nα-Arylsulfonyl-ω-(4-amidinophenyl)-α-aminoalkylcarboxylic acid amides - novel selective inhibitors of thrombin. *Thromb. Res.* **1980**, *17*, 425−431.
(15) Stürzebecher, J.; Markwardt, F.; Voigt, B.; Wagner, G.; Walsmann, P. Cyclic amides of Nα-arylsulfonylaminoacylated 4-amidinophenylalanine - tight binding inhibitors of thrombin. *Thromb. Res.* **1983**, *29*, 635−642.
(16) Reers, M.; Koschinsky, R.; Dickneite, G.; Hoffmann, D.; Czech, J.; Stüber, W. Synthesis and characterisation of novel thrombin inhibitors based on 4-amidinophenylalanine. *J. Enzyme Inhib.* **1995**, *9*, 61−72.
(17) Kaiser, B.; Hauptmann, J.; Weiss, A.; Markwardt, F. Pharmacological characterization of a new highly effective synthetic thrombin inhibitor. *Biomed. Biochim. Acta* **1985**, *44*, 1201−1210.
(18) Dickneite, G.; Seiffge, D.; Diehl, K. H.; Reers, M.; Czech, J.; Weinmann, E.; Hoffmann, D.; Stüber, W. Pharmacological characterization of a new 4-amidinophenylalanine thrombin inhibitor (CRC 220). *Thromb. Res.* **1995**, *77*, 357−368.
(19) Stürzebecher, J.; Horn, H.; Walsmann, P.; Voigt, B.; Markwardt, F.; Wagner, G. Synthetische Inhibitoren der Serinproteinasen. 34. Mittg.: Einfluss der Abwandlung der Amidinofunktion bei Nα-substituierten 4-Amidinophenylalaninamiden auf die Inhibitoraktivität. (Synthetic inhibitors of serine proteinases. Part 34: Influence of modification of the amidino function of Nα-substituted 4-amidinophenylalanine amides on the inhibitory activity.) *Pharmazie* **1988**, *43*, 782−783.
(20) Bode, W.; Turk, D.; Stürzebecher, J. Geometry of binding of benzamidine and arginine-based inhibitors NAPAP and MQPA to human α-thrombin: X-ray crystallographic determination of the NAPAP-trypsin complex and modeling of NAPAP-thrombin and MQPA-thrombin. *Eur. J. Biochem.* **1990**, *193*, 175−182.
(21) Bauer, M.; Brandstetter, H.; Turk, D.; Stürzebecher, J.; Bode, W. Crystallographic determination of thrombin complexes with small synthetic inhibitors as starting point for the receptor-based design of antithrombotics. *Semin. Thromb. Hemostas.* **1993**, *19*, 352−360.
(22) Turk, D.; Stürzebecher, J.; Bode, W. Geometry of binding of the Nα-tosylated piperidides of m-amidino-, p-amidino- and p-guanidino phenylalanine to trypsin and thrombin complexes. *FEBS Lett.* **1991**, *287*, 133−138.
(23) Brandstetter, H.; Turk, D.; Hoeffken, H. W.; Grosse, D.; Stürzebecher, J.; Martin, P. D.; Edwards, B. F. P.; Bode, W. Refined 2.3 Å X-ray crystal structure of bovine thrombin complexes formed with the benzamidine and arginine-based thrombin inhibitors NAPAP, 4-TAPAP and MQPA. *J. Mol. Biol.* **1992**, *226*, 1085−1099.
(24) Stürzebecher, J.; Vieweg, H.; Wikström, P. WO 92/08709, 1991.
(25) Stürzebecher, J.; Prasa, D.; Wikström, P.; Vieweg, H. Structure-activity relationships of inhibitors derived from 3-amidinophenylalanine. *J. Enzyme Inhib.* **1995**, *9*, 87−99.
(26) Bergner, A.; Bauer, M.; Brandstetter, H.; Stürzebecher, J.; Bode, W. The X-ray crystal structure of thrombin in complex with Nα-2-naphthylsulfonyl-L-3-amidinophenylalanyl-4-methyl-piperidide: the beneficial effect of filling out an empty cavity. *J. Enzyme Inhib.* **1995**, *9*, 101−111.
(27) Stürzebecher, J.; Vieweg, H.; Wikström, P. WO 94/18185, 1993.
(28) Bode, W.; Turk, D.; Karshikov, A. The refined 1.9 Å X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human α-thrombin: Structure analysis, overall structure, elecctrostatic

properties, detailed active-site geometry, and structure-function relationships. *Protein Sci.* **1992**, *1*, 426−471.
(29) Abbreviations: F Xa, factor Xa; F XIIa, factor XIIa; APC, activated protein C; PK, plasma kallikrein; GK, glandular kallikrein; UK, urokinase; sc-tPA, single-chain tissue-type plasminogen activator; NMM, *N*-methylmorpholine; HOBt, hydroxybenzotriazole; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; TEA, triethylamine; THF, tetrahydrofuran.
(30) Deadman, J. J.; Elgendy, S.; Goodwin, C. A.; Green, D.; Baban, J. A.; Patel, G.; Skordalakes, E.; Chino, N.; Claeson, G.; Kakkar, V. V.; Scully, M. F. Characterization of a class of peptide boronates with neutral P1 side chains as highly selective inhibitors of thrombin. *J. Med. Chem.* **1995**, *38*, 1511−1522.
(31) Authors, unpublished results.
(32) Hemker, H. C.; Willems, G. M.; Béguin, S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb. Haemostas.* **1986**, *56*, 9−17.
(33) Prasa, D.; Svendsen, L.; Stürzebecher, J. The ability of thrombin inhibitors to reduce the thrombin activity generated in plasma on extrinsic and intrinsic activation. *Thromb. Haemostas.* **1997**, *77*, 498−503.
(34) Dixon, M. Determination of enzyme inhibitor constants. *Biochem. J.* **1953,** *55*, 170−171.
(35) Walsmann, P. Über die Reinigung von Thrombinpräparaten. (On the purification of thrombin preparations.) *Pharmazie* **1968**, *23*, 401−402.
(36) Stürzebecher, J.; Neumann, U.; Kohnert, U.; Kresse, G.-B.; Fischer, S. Mapping of the catalytic site of CHO-t-PA and the t-PA variant BM 06.022 by synthetic inhibitors and substrates. *Protein Sci.* **1992**, *1*, 1007−1013.

JM960668H