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STRUCTURE-ACTIVITY RELATIONSHIPS OF INHIBITORS DERIVED FROM 3-AMIDINOPHENYLALANINE

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Thrombin is the key enzyme in coagulation and its inhibitors are of therapeutic interest since they are potential anticoagulants. The most potent inhibitor of the benzamidine type is Nα-(2-naphthylsulfonyl-glycyl)-4-amidinophenylalanine piperidide (NAPAP). However, NAPAP and other substances designed so far do not fulfill the pharmacological requirements. The goal of designing new compounds was to obtain potent inhibitors with improved pharmacokinetic properties, such as absorption after oral application and a sustained elimination. Novel derivatives of 3-amidinophenylalanine as key building block were synthesized. The amidino moiety and both the Nα– and the C-terminal substituents were widely varied. Some of the newly synthesized compounds are potent inhibitors of thrombin and exert improved pharmacokinetic properties.

KEY WORDS: Enzyme inhibitors, thrombin inhibitors, 3-amidinophenylalanine, NAPAP

INTRODUCTION

Today, a great deal of effort is being put into the development of synthetic inhibitors for the coagulation enzymes. In extensive biochemical and pharmacological studies it was shown that inhibitors of thrombin were effective as anticoagulants and antithrombotics. Principal questions about their therapeutic use and possible areas of indication have already been discussed.[1,2]

Due to its potency and selectivity, one must consider the naturally occurring thrombin inhibitor hirudin isolated from the leech *Hirudo medicinalis*, as an "ideal" anticoagulant[3,4]. Nowadays, this inhibitor is available in large amounts for therapeutic uses via recombinant technology. Nevertheless, due to its protein structure, hirudin can only be applied by intravenous administration. If absorbed upon oral application, a synthetic, selectively acting thrombin inhibitor with a sufficient half-life would have certain advantages over hirudin, leading to a decisive progress in prophylaxis and therapy of thrombosis.[4]

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The benzamidine moiety is one of the key structures for the development of inhibitors of trypsin-like enzymes. It mimics the protonated side-chain of the basic amino acids Arg and Lys split off by these enzymes. For some enzymes of the trypsin-family relatively selective inhibitors were found among derivatives of amino acids containing an amidinophenyl alkyl side chain.[5] Starting with 4-amidinophenylalanine we were able to develop very potent and selective thrombin inhibitors such as Nα-(2-naphthylsulfonylglycyl)-4-amidinophenylalanine piperidide (NAPAP).[6]

Even though NAPAP possesses a high antithrombin activity (K_i = 6 nmol/l) and remarkable coagulation inhibiting effectiveness, the compound cannot be further developed for therapeutic use due to its insufficient pharmacokinetic properties: NAPAP is not orally absorbed, and it is rapidly eliminated from the circulation.[7] Early studies showed that variations of the basic structure always led to a drastic loss in inhibitory activity.[8]

However, using HPLC methods we were able to determine the pharmacokinetic behaviour of NAPAP derivatives, which have very low antithrombin activity or none at all.[9] It was found that after i.v.-application the rapid elimination of NAPAP from the circulation was prolonged only when the piperidine ring was replaced with a tetrahydroisoquinoline carboxylic acid residue. After oral application of compounds containing a free carboxyl group in the amide residue a distinct blood level of the compound was detected. Furthermore, absorption was particularly marked when the amidino function was replaced by an amino methyl or an oxamidino group. However, these derivatives which show a certain oral absorption, are inactive as inhibitors.[8,9]

From the X-ray crystal structure of the NAPAP-thrombin complex it was deduced that, due to spatial reasons, the necessary structural changes to be applied in order to improve the pharmacokinetic behaviour, must lead to a loss in inhibitory activity: The NAPAP molecule is bound so ideally to thrombin that there is virtually no further space for additional substituents.[10] Therefore, we looked for new, lead structures. The Nα-tosylated 3-amidinophenylalanine piperidide (3-TAPAP) appeared to be a promising model for the synthesis of new inhibitors because the corresponding inhibitor complex indicated more space available for additional substitutions in the molecule.[11,12] 3-TAPAP which was already described in 1980 does not inhibit thrombin with the potency of NAPAP, but it does have an equivalent selectivity[5] (Table 1).

MATERIAL AND METHODS

Synthetic Methods

The syntheses of inhibitors with the general structure **VII** (Scheme I) were achieved via the corresponding cyano compounds **V** which were synthesized in the following way[13]: 3-Cyano-phenylalanine (**I**) (used either in the racemic, D- or L-form) was converted with the corresponding sulfonyl chloride (R^1-SO_2Cl) to the sulfonylated cyanophenyl-alanine compound **II** with a free carboxyl group. Conversion of **II** into **III** 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 the corresponding nucleophile. The conversion of the cyano function into the amidino

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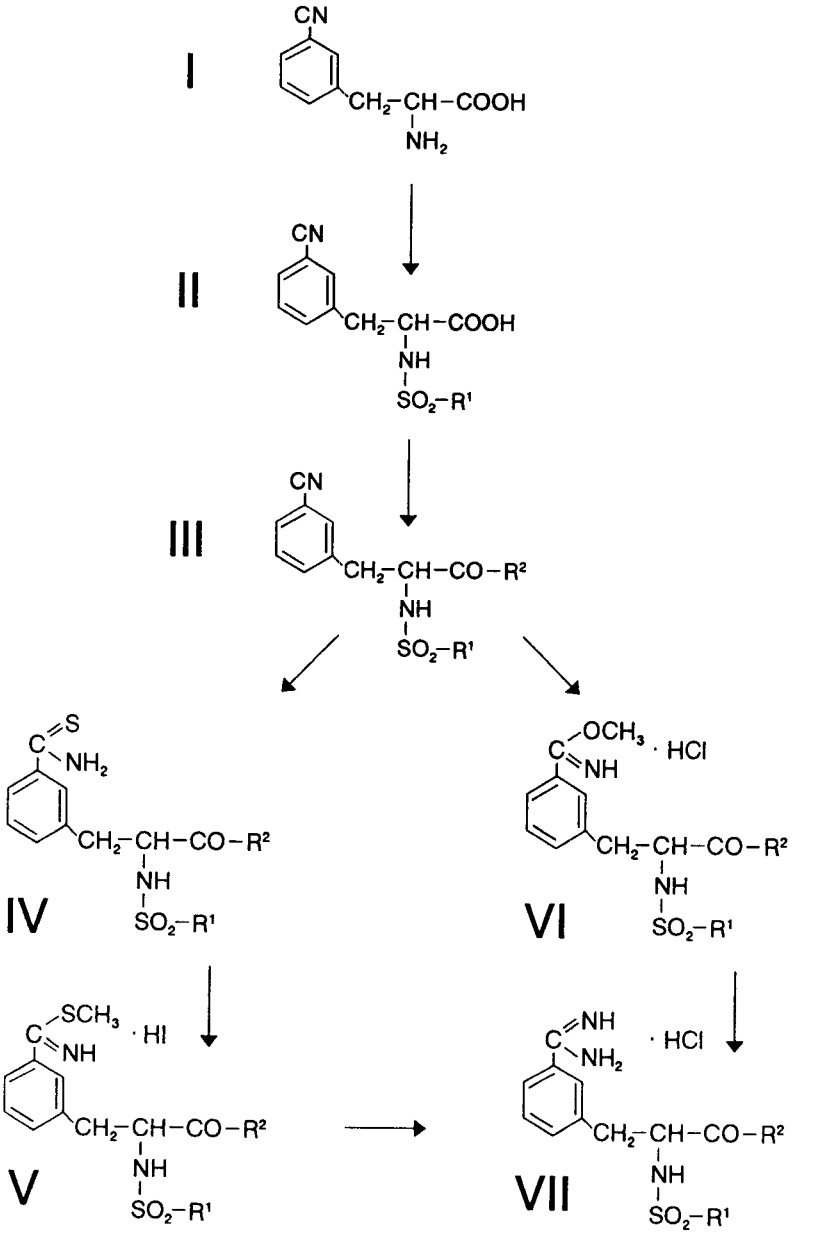

SCHEME 1

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function was achieved using one of two principally known routes. Addition of hydrogen sulfide in the presence of triethylamine in pyridine gave a thioamide (**IV**), which was then S-methylated with iodomethane to give the thioimidate hydrochloride **V** which was further reacted with ammonium acetate in alcohol to give the crude hydroiodide of the end-product **VII**. The end-product was generally purified by chromatography over silica gel 60 or Sephadex LH-20 to give homogeneous products, followed by ion exchange to give the more soluble hydrochlorides of the end-product **VII**.

Another route to the amidino compounds was achieved by converting the corresponding cyano compound **III** to the corresponding imidate hydrochloride **VI** followed by treatment with alcoholic ammonia solution to give the end-products **VII**.

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.

Determination of Inhibition Constants

The measurements were carried out on a microplate reader (MR 5000, Dynatech, Denkendorf, D) at 25°C. The test medium consisted of 200 μl Tris buffer (0.05 mol/l; 0.154 mol/l NaCl, 5% ethanol, pH 8.0), 25 μl aqueous substrate solution and 50 μl enzyme solution. Two concentrations of the substrate and five concentrations of the inhibitor were used. Three min after the addition of the enzyme 25 μl acetic acid (50%) were added to quench the reaction and the optical density was measured at 405 nm. The K_i-values were calculated according to Dixon[14] using a linear regression program. If not indicated, the K_i-values are those of the racemates.

The K_i-values reported are means from at least three determinations. The standard deviation did not exceed 25 per cent (compare Table 5).

Enzymes and Substrates

Thrombin: Bovine thrombin was prepared according to Walsmann.[15] Thrombin was further purified using a hirudin-affinity column (2262 U/mg, final concentration 0.45 U/ml). Substrate was $MeSO_2$-D-hexahydrotyrosyl-Gly-Arg-pNA (Pentapharm Ltd., Basel, CH) at final concentrations of 0.18 and 0.09 mmol/l.

Factor Xa: Bovine factor Xa (5 U/vial, final concentration 0.11 U/ml; Diagnostic Reagents Ltd., Thame, UK) and the substrate $MeSO_2$-D-Nle-Gly-Arg-pNA (Pentapharm Ltd., Basel, CH) at final concentrations of 0.36 and 0.18 mmol/l were used.

Plasmin: Human plasmin (0.67 CTA-U/mg, final concentration 0.06 CTA-U/ml; Behringwerke AG, Marburg, D) was used with the substrate Tos-Gly-Pro-Lys-pNA (Pentapharm Ltd., Basel, CH) at final concentrations of 0.18 and 0.09 mmol/l.

Trypsin: Bovine pancreatic trypsin (42 U/mg, final concentration 0.0038 U/ml; Serva, Heidelberg, D) and the substrate $MeSO_2$-D-hexahydrotyrosyl-Gly-Arg-pNA (final concentrations of 0.18 and 0.06 mmol/l) were used.

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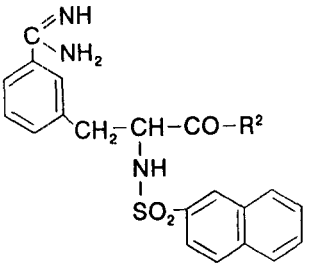
TABLE 1
Inhibition of thrombin, factor Xa, plasmin and trypsin by cyclo-aliphatic amides of β-naphthylsulfonylated 3-amidinophenylalanine



R^2	K_i, μmol/l			
	Thrombin	Factor Xa	Plasmin	Trypsin
Ppd*	0.34	15	12	1.2
Ppd	0.065	38	7.0	0.33
Ppd(2-Me)	0.13	74	13	2.3
Ppd(3-Me)	0.13	32	5.0	0.53
Ppd(4-Me)	0.0062	41	5.2	0.14
Pip-OH	0.26	38	34	0.63
Pip-OMe	0.070	46	10.5	1.9
Nip-OH	1.1	44	19	0.44
Nip-OMe	0.15	18	3.7	0.39
iNip-OH	0.57	43	62	0.58
iNip-OMe	0.017	43	2.4	0.036
Pip(4-Me)-OH	0.12	96	42	1.2
Pip(4-Me)-OMe	0.096	58	26	2.2
Tic-OH	0.018	42	2.9	0.13
Tic-OMe	0.20	27	4.6	1.2
Pzd	0.50	21	10.2	0.22
Pzd(N-Me)	0.036	30	55	1.3
Pzd(N-Ph)	0.52	27	2.6	0.026

*tosyl derivative = 3-TAPAP

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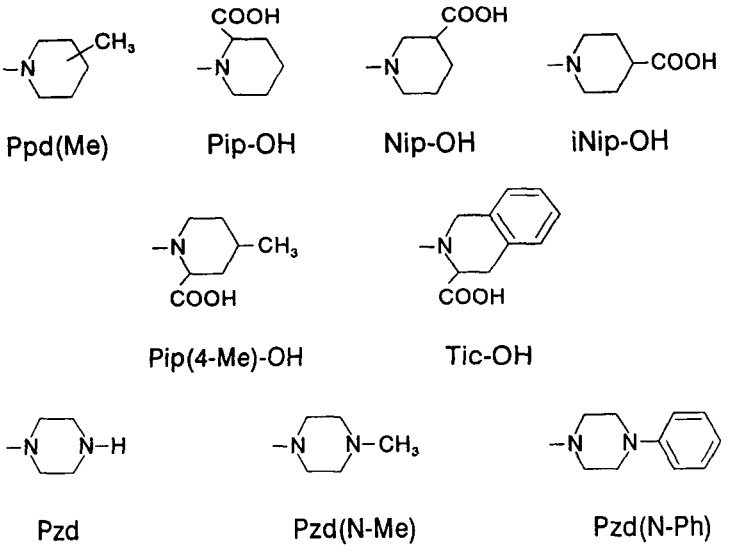

FIGURE 1 Structure of the cycloaliphatic amide residues R^2: Ppd(Me) = methylpiperidide; Pip-OH = pipecolic acid; Nip-OH = nipecotic acid; iNip = isonipecotic acid; Pip(4-Me)-OH = 4-methyl-isonipecotic acid; Tic-OH = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Pzd = piperazide; Pzd(N-Me) = 4-methyl-piperazide; Pzd(N-Ph) = 4-phenylpiperazide; Me = methyl.

RESULTS AND DISCUSSION

Variation of the Piperidine Moiety

Starting from 3-TAPAP as a model a great number of new compounds were synthesized. Table 1 shows selected derivatives which inhibit the enzymes tested in a competitive manner. In contrast to 3-TAPAP, however, the Nα-tosyl blocking group was replaced by a β-naphthylsulfonyl residue leading to a 5-fold increase in antithrombin activity (further variations of the Nα-substituent will be discussed later). Substitution of the piperidine moiety (Figure 1) is tolerated in several compounds, however, *p*-substituted derivatives are preferred for inhibition of thrombin. Therefore, only a 4-methyl group increased the antithrombin activity markedly. Unfortunately, introduction of carboxyl groups, which have been shown to improve bioavailability after oral application[9] reduces the antithrombin activity. The only exception is the Tic-OH derivative with a K_i of 0.018 μmol/l for inhibition of thrombin in which the piperidine ring is replaced by a 1,2,3,4-tetrahydroisoquinoline carboxylic acid residue. This compound was a good candidate recommended for *in vivo* studies in experimental animals.

Esterification, with the exception of the 1,2,3,4-tetrahydroisoquinoline derivative, enhances in all cases the antithrombin activity up to 10-fold. However, these esters

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are not of pharmacological interest. They do not show improved bioavailability and are unstable in biological fluids.

Among the other enzymes, the inhibition of factor Xa is very low, K_i's are in the range of 10–100 μmol/l, where structural variations of the inhibitor do not influence the anti-factor Xa activity. Plasmin as well is inhibited less than thrombin, the K_i's are normally 1 to 2 orders of magnitude lower. Inhibition of plasmin is increased both after substitution in the 3- or 4-position of the piperidine moiety.

Several derivatives inhibit trypsin in the same range as in the case for inhibition of thrombin or maximally ten times lower. The substituents at the piperidine influence the antitrypsin activity in different ways: the inhibitory potency is reduced by substituents at the 2-position; substituents at the 3-position are tolerated without influence on antitrypsin activity; 4-substitution leads to potent inhibitors of trypsin when the substituent is larger than a methyl group. This is especially evident with the piperazides shown in the lower part of Table 1. Whereas introduction of a small methyl group into 4-position leads to potent thrombin inhibition the 4-phenyl derivative has a K_i of 0.026 μmol/l for inhibition of trypsin and reduced affinity for thrombin. This is due to the differences between trypsin and thrombin in the active site region. From the X-ray crystal structure of the complexes of thrombin with benzamidine-derived inhibitors it is obvious that the N-terminal piperidine moiety binds within the S2 cavity buried under the characteristic 60-insertion loop.[16] In contrast, trypsin does not possess this loop, therefore, large substituents in 4-position of the piperidine moiety are not only tolerated by the trypsin molecule, but they allow additional interactions resulting in an increase in inhibitory potency.

It has already been determined that in the case of derivatives of 4-amidinophenylalanine primary amides are poor inhibitors of thrombin in comparison with cycloaliphatic amides.[17] Table 2 confirms this with several amides for compounds of the 3-amidinophenylalanine type. For the inhibition of thrombin, maximum K_i-values in the 10^{-7} mol/l range were found. In every case, inhibition of the other enzymes tested was even lower. Nevertheless, the esters are more potent inhibitors than the primary amides. For the inhibition of thrombin maximal K_i's in the 10^{-8} mol/l range were found. Surprisingly, also factor Xa is inhibited in the micromolar range. This is in agreement with the observation that esters of 3-amidinophenylalanine containing an N-terminal arylsulfonyl-glycyl residue are potent inhibitors of factor Xa (K_i down to 10^{-8} mol/l).[18] In contrast, the N-terminally extended esters inhibit factor Xa more selectively than the compounds shown in Table 2. However, esters are unstable in plasma, therefore, esters and primary amides of 3-amidinophenylalanine are not very useful key structures for the development and design of powerful inhibitors.

Variation of the Nα-Protecting Group

To investigate the influence of the Nα-arylsulfonyl residue on inhibitory activity the 4-methylpiperidide as the most potent inhibitor (see Table 1) was chosen. In general, inhibition of thrombin by the different derivatives is more pronounced than inhibition of factor Xa, plasmin and trypsin (Table 3). Surprisingly, the many varied substitutions of the aryl residue (Figure 2) did not in any case lead to an increase in the

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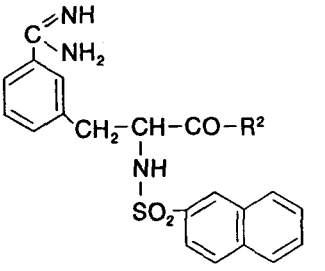
TABLE 2
Inhibition of thromin, factor Xa, plasmin and trypsin by primary amides and esters of β-naphthyl sulfonylated 3-amidinophenylalanine



	K_i, μmol/l			
R^2	Thrombin	Factor Xa	Plasmin	Trypsin
$NHCH_3$	5.7	26	65	25
NHC_3H_7 (i)	6.2	360	15	16
NHC_4H_9 (n)	0.12	47	38	4.0
OH	30	130	67	118
OCH_3	0.28	2.5	5.2	2.5
OC_3H_7 (i)	0.11	3.5	8.4	1.4
OC_4H_9 (n)	0.013	3.1	2.5	0.26
OCH_2Ph	0.024	2.2	1.9	0.52

antithrombin activity (Table 3). However, the influence of the type of the Nα-group on the inhibitory activity is quite different depending on the kind of enzymes used. As a result, differences in the selectivity of the various compounds are obvious. Thus, in the case of trypsin a βNAPS or an ACS residue leads to the highest inhibitory activity, while factor Xa is inhibited predominantly by the ACS- and TIPPS-protected compounds.

Variation of the Amidino Moiety

Studies on the pharmacokinetic behaviour of NAPAP derivatives have shown that absorption after oral application is markedly increased when the amidino function is replaced by an aminomethyl or an oxamidino moiety.[9] Therefore, we have synthesized also the respective 3-amidinophenylalanine derivatives. However, as was the case with the NAPAP derivatives,[8] the change from the strong basic amidino moiety to other substituents resulted in a tremendous loss of efficacy with respect to antithrombin

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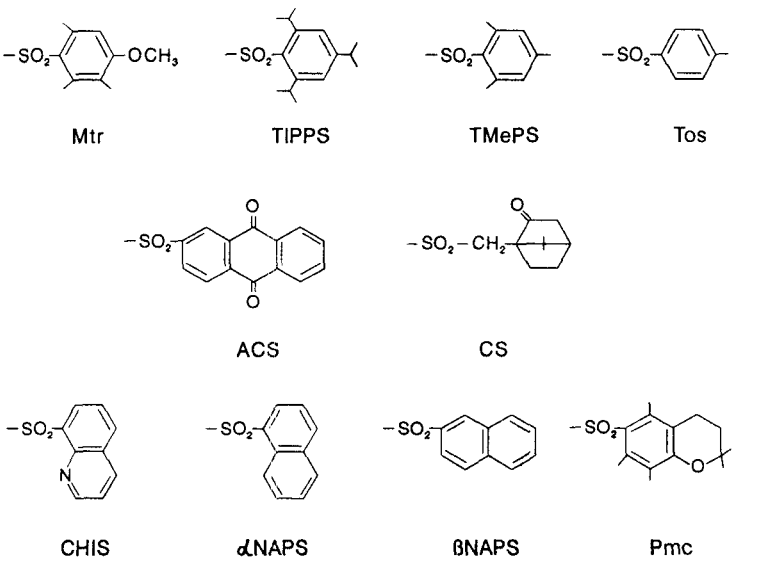

FIGURE 2 Structure of the arylsulfonyl residues R^1: CHIS = quinoline-8-sulfonyl; TIPPS = 2,4,6-triisopropylbenzenesulfonyl; TMePS = 2,4,6-trimethylbenzenesulfonyl; Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tos = tosyl; ACS = anthraquinone-2-sulfonyl; CS = camphor-10-sulfonyl; αNAPS = naphthalene-1-sulfonyl; βNAPS = naphthalene-2-sulfonyl; Mtr = 4-methoxy-2,3,6-trimethylphenylsulfonyl.

activity (Table 4). Surprisingly, besides the 3-amidino derivative the compound with a 3-guanidino group showed the strongest antithrombin activity, whereas besides NAPAP it was the derivative containing a 4-oxamidino moiety (micromolar K_i).[8] Apparently, it is possible with the short 3-substituted compounds to accomodate the bulky guanidino moiety.[11]

With the other enzymes studied the substitution of the amidino function did not affect the inhibitory activity very much: the lower the affinity of the amidino compound for the target enzyme (i.e. factor Xa), the lower the influence of a structural change.

Stereoselectivity of Inhibition

At first it was proven by X-ray crystal structures that for trypsin[11] as well as thrombin[12,19] the insertion of the amidinophenylalanine moiety into the specificity pocket required an L-conformation of the central phenylalanine residue in 3-TAPAP-derived inhibitors, but a D-conformation in inhibitors of the NAPAP type. Although we normally synthesize the racemates we are also able to synthesize the pure enantiomers. A few examples are listed in Table 5.

It is quite evident that the L-derivatives inhibit thrombin and trypsin much more potently than the corresponding D-derivatives. The differences are in every case greater than two orders of magnitude. In the case of the two other enzymes tested, factor Xa and plasmin which are inhibited to a very low extent, the differences are much smaller, maximally one order of magnitude.

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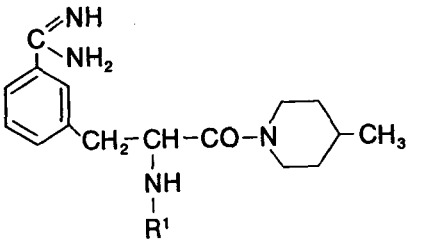
TABLE 3
Inhibition of thrombin, factor Xa, plasmin and trypsin by several Nα-substituted 4-methylpiperidides of 3-amidinophenylalanine



R^1	K_i, μmol/l			
	Thrombin	Factor Xa	Plasmin	Trypsin
H	27	230	350	29
Boc	1.2	64	110	14
CHIS	0.34	180	100	3.6
Mtr	0.089	100	10	0.78
TIPPS	0.075	2.3	4.0	0.69
TMePS	0.059	95	19	4.0
Pmc	0.034	80	32	0.73
Tos	0.026	71	9.1	0.52
ACS	0.026	2.3	7.6	0.074
αNAPS	0.016	42	4.3	0.63
CS	0.014	14	28	0.63
βNAPS	0.0062	41	5.2	0.14

Concluding Remarks

The structure-activity relationships presented are in agreement with the predictions of the X-ray crystal 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 derivatizations since NAPAP is bound so ideally to thrombin that there is no further space for additional substituents.[12,19] Therefore, we used 3-TAPAP as starting molecule because the corresponding inhibitor complex indicates more space for derivatization both at the toluene ring and the piperidine moiety.[11] Indeed, with the

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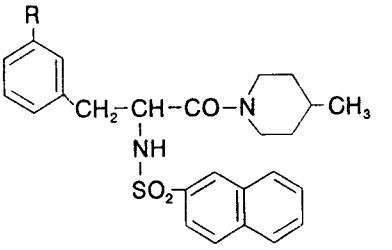
TABLE 4
Inhibition of thrombin, factor Xa, plasmin and trypsin by β-naphthylsulfonylated 4-methylpiperidides of phenylalanine substituted in 3-position with a basic group



R	K_i, μmol/l			
	Thrombin	Factor Xa	Plasmin	Trypsin
$C(NH_2) = NH$	0.0062	41	5.2	0.14
$NH\text{-}C(NH_2) = NH$*	0.4	107	17	4.1
$CH_2\text{-}NH_2$	1.9	500	27	3.4
$C(NH_2) = N\text{-}OH$	2.8	> 1000	140	46
$C(NH_2) = N\text{-}CH_3$	5.7	110	520	47
NH_2	8.9	210	> 1000	> 1000

*piperidide

Nα-naphthylsulfonylated 4-methylpiperidide we succeeded in designing a thrombin inhibitor whose antithrombin activity is equal to that of NAPAP (K_i 6.2 and 6.0 nmol/l, respectively). Other substituents which should theoretically improve the absorption after oral application and the elimination from the circulation could be introduced into the 3-TAPAP molecule without loss of inhibitory activity.

From the determination of the pharmacokinetics of NAPAP derivatives it was found that after i.v.-application the rapid elimination of NAPAP from the circulation is prolonged only with the tetrahydroisoquinoline carboxylic acid derivative. Absorption after oral application was observed with compounds containing a free carboxyl group in the piperidine ring or when the amidino function was replaced by an aminomethyl or an oxamidino group.[9] These NAPAP derivatives which show a certain oral absorption, are inactive as inhibitors.[8,9] In contrast to the 4-amidinophenylalanine derivatives, several compounds derived from 3-amidinophenylalanine exert potent antithrombin activity. Indeed after oral administration the newly synthesized compounds are absorbed to a somewhat higher extent than the benzamidine derivatives studied before. However, the absorption rates were determined in a range with five per cent

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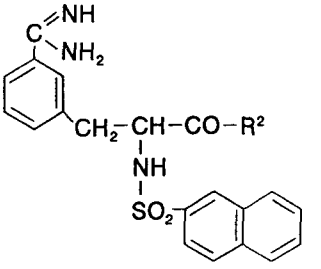
TABLE 5
Inhibition of thrombin, factor Xa, plasmin and trypsin by the enantiomers or diastereomers of several β-naphthylsulfonylated 3-amidinophenylalanine derivatives



Enantiomer	R^2	K_i, μmol/l			
		Thrombin	Factor Xa	Plasmin	Trypsin
D,L	Ppd(4-Me)	0.0062±0.0014	41.3±8.6	5.16±0.30	0.136±0.015
L	Ppd(4-Me)	0.0025±0.0006	29.9±4.6	4.33±0.85	0.0415±0.0027
D	Ppd(4-Me)	0.96±0.13	75.7±10.5	192±60	24.3±3.4
D,L	iNip-OH	0.570±0.021	43.4±8.2	62±18	0.580±0.087
L	iNip-OH	0.192±0.043	25.4±2.6	22.6±3.9	0.219±0.048
D	iNip-OH	100±33	84.6±15.1	> 1000	87.6±20.3
D,L	D,L-Tic-OH	0.0180±0.0044	41.5±2.1	2.87±0.51	0.130±0.030
L	D-Tic-OH	0.0109±0.0015	16.5±5.4	1.05±0.23	0.044±0.010
D	D-Tic-OH	2.01±0.62	39.2±4.2	61.3±7.3	9.12±0.47
D,L	Pzd(N-Me)	0.0360±0.0077	30.1±6.3	55.3±6.8	1.30±0.17
L	Pzd(N-Me)	0.0159±0.0031	18.6±4.2	30.4±2.0	0.47±0.13
D	Pzd(N-Me)	7.1±1.4	83.4±7.8	660±110	73.4±18.6

as maximum.[9] Surprisingly, very high levels of the methylpiperazide derivative (Table 1 and 5) were determined for a prolonged period of time after duodenal application. Furthermore, the plasma levels run in parallel with anticoagulant and antithrombotic activities.[20] Therefore, we have concentrated on the further development of this class of compound. By introducing different substituents on the second nitrogen of the piperazine ring we have been able to synthesize many new derivatives which show improved pharmacokinetic properties.[20] A paper is currently in preparation.

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