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DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL RIGID AMIDINO-PHENYLALANINE DERIVATIVES AS INHIBITORS OF THROMBIN

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(3S)–(Naphthalene–2–sulfonylamino)–1–[2R–(4–amidinophenyl)–1–piperidinocarbonylethyl]–2–pyrrolidinone (**1a**) is a potent inhibitor of thrombin with an IC_{50} value by 112 times lower than that of NAPAP (racemate). The selectivity versus trypsin can be improved by incorporation of substituents on the naphthyl ring. The mode of binding of the compound was determined by X-ray crystallography.

KEY WORDS: Thrombin, inhibitor, coagulation

INTRODUCTION

The serine protease thrombin is the terminal enzyme in the blood coagulation cascade.[1] After activation of both the intrinsic and the extrinsic coagulation cascade thrombin converts fibrinogen into fibrin thereby starting blood coagulation with a possible thrombus formation. Thrombin further stimulates platelet aggregation. Therefore, thrombin plays a pivotal role in thrombosis and hemostasis. Inhibitors of thrombin are expected to provide a new advantageous route for antithrombotic therapy.

In the present paper, we describe the design, synthesis, biological characterization and X-ray structure determination of a number of novel thrombin inhibitors with rigid amidino-phenylalanine structure.

NAPAP (β-naphthylsulfonyl-glycyl-D,L-4-amidinophenylalanyl-piperidide) is one of the most potent known low-molecular weight inhibitors of thrombin that does not bind covalently to the enzyme.[2] The X-ray structure of thrombin complexes with the inhibitor NAPAP was solved by Brandstetter *et al.*[3] It revealed a number of hydrogen bonds and lipophilic interactions which are shown in Figure 1. The inhibitor forms hydrogen bonds with the amino acids Gly 216, Gly 219 and Asp 189. In addition, the

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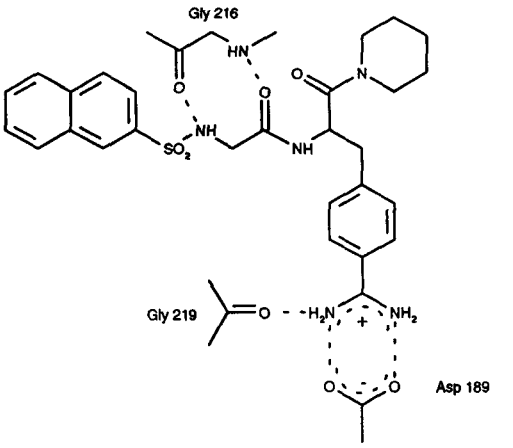


FIGURE 1 Schematic view of the hydrogen bonds formed between NAPAP and thrombin.

carbonyl group of the *p*-amidino-Phe moiety of the inhibitor forms a water mediated hydrogen bond with Lys 60F. There are also extensive lipophilic interactions between the naphthyl and piperidino side chain of NAPAP and several lipophilic side chains of the enzyme.

The analysis of the thrombin-NAPAP complex revealed a number of possible starting points for a synthetic modification of the inhibitor. In the present communication, we describe our work relating to two observations. First, the aryl binding site appears not to be optimally occupied by the naphthyl group of NAPAP. The 3D-structure indicates that there should be space for small substituents at positions 5 or 6 of the naphthyl ring. These substituents should increase selectivity because they would interact with a loop that is present in thrombin but absent in trypsin. Second, the glycine moiety of NAPAP forms a chelate type hydrogen bond with Gly 216. The visual inspection of the 3D-structure of thrombin indicated that there should be enough space available for substituents at both the C_α atom of the glycine moiety and the N-atom of the Phe group. The cyclisation between these two atoms should lead to compounds which bind more tightly to thrombin because they would suffer less loss of configurational entropy when binding to thrombin. Such a cyclisation is possible through formation of a five or six membered lactam as shown in Figure 2.

METHODS

Chemistry

The five-membered lactam derivatives were prepared as follows (Scheme 1). The central step of the synthesis is the base-catalyzed conversion of the S-methylated methionine-*p*-cyanophenylalanine-piperidide **14** into the lactam **15**. This is best achieved using lithium di-isopropylamide as the base.[4] The intermediate **14** is

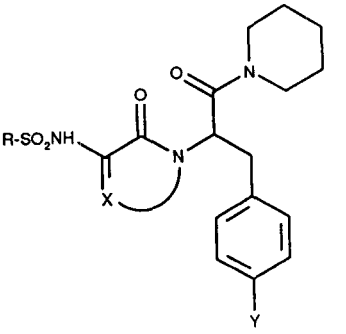
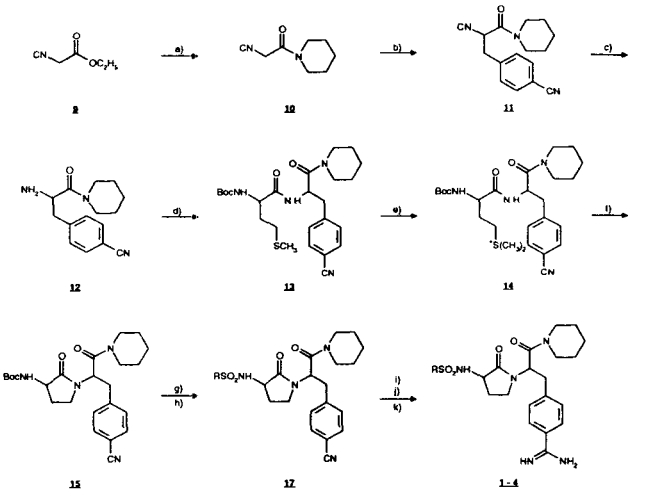


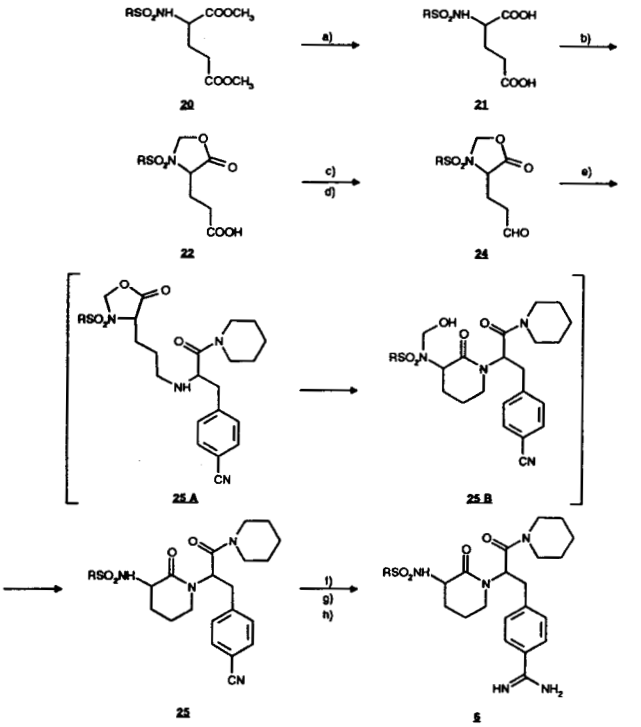
FIGURE 2 General structure of the thrombin inhibitors described in the present paper.

prepared by coupling Boc-methionine with *p*-cyanophenylalanine piperidide **12**, which is prepared from ethyl-isocyanoacetate **9** via isocyanoacetic acid-piperidide **10**, α-isocyano-(*p*-cyanophenyl)-propionic acid-piperidide **11** and *p*-cyanophenylalanine-piperidide **12**. The thrombin inhibitors **1–4** are obtained after eliminating the protective group in **15**, coupling with the aromatic sulfonyl chloride to form **17**, and converting the cyano function into the amidino group.[5] The enantiomerically pure compounds can be prepared either by separating the diastereomers in the final step by means of MPLC (medium pressure chromatography), or else by using the enantiomerically pure educts. In the case of *p*-cyanophenylalanine-piperidide **12**, this can be achieved either by racemate resolution or by using the bislactim ether synthesis method.[6]

SCHEME 1



SCHEME 2

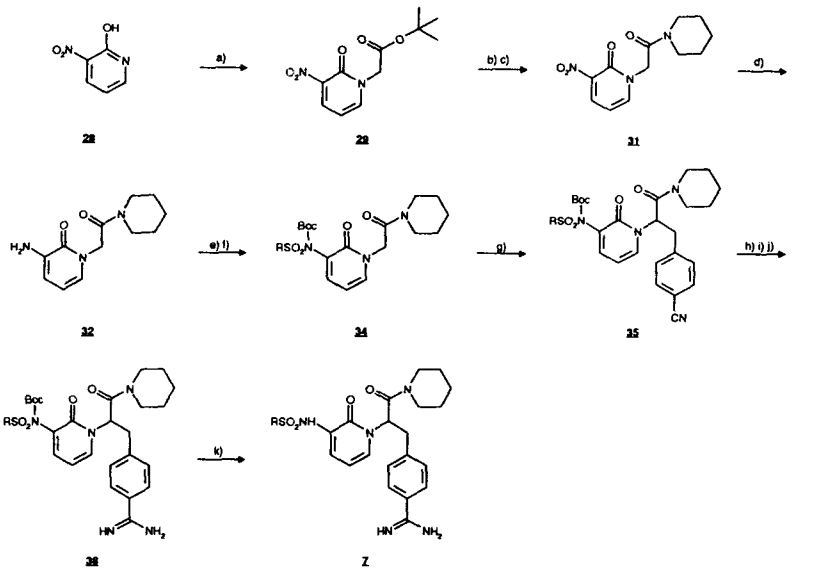


a) NaOH, b) CH_2O, $(CH_3CO)_2O$, $SOCl_2$, c) $(COCl)_2$, DMF, ⇨ **23**, d) CuI, $LiAlH(OC(CH_3)_3)_3$, e) $NaBH_3CN$, f) H_2S, ⇨ **26**, g) CH_3I, ⇨ **27**, h) CH_3COONH_4

The key step in the synthesis of the six-membered lactams is the reductive amination-cyclisation sequence of *p*-cyanophenylalanine-piperidide **12** and the oxazolidinone aldehyde derivative **24** via the instable intermediates **25A** and **25B** (Scheme 2). The thrombin inhibitor **6** is then obtained by conversion of the cyano group into the amidino group. The oxazolidinone-aldehyde derivative **24** can be prepared from dimethyl-*N*-(naphthalene-2-sulfonyl)-glutamate **20** in four steps: saponification of the ester groups, differentiation of the two acid functions by oxazolidinone-ring formation,[7] conversion of the ω-acid to the acid-chloride function and finally reduction of them.[8]

The starting point for the synthesis of the unsaturated six-membered lactam is 2-hydroxy-3-nitropyridine **28**, which, following N-alkylation with tert-butyl bromoacetate and hydrolysis of the tert-butyl group, is converted into the substituted pyridone **31** (Scheme 3). After transformation of the nitro- into an amino-function and reaction with naphthalenesulfonyl chloride and $(Boc)_2O$, the resulting compound **34** can be C-alkylated using **p**-cyanobenzyl bromide. Conversion of the cyano function of **35** into the amidino group is effected in an analogous manner to the preparation of the five-membered lactam thrombin inhibitors, with the six-membered lactam compounds **7** being formed after elimination of the Boc group.

SCHEME 3



a) $BrCH_2$-$COOC(CH_3)_3$, K_2CO_3, b) CF_3COOH, ⇨ 30, c) Piperidine, d) H_2, Pd/C, e) RSO_2Cl, Pyridin, ⇨ 33, f) $(Boc)_2O$, DMAP, g) LDA, $BrCH_2$-C_6H_4-CN, h) H_2S, ⇨ 36, i) CH_3I, ⇨ 37, j) CH_3COONH_4, k) CF_3COOH

All the reactions were carried out under inert gas, preferably nitrogen. The solvents used for the reactions were dried over molecular sieves.

N-Isocyanoacetylpiperidide (**10**) 48.7 g (570 mmol) of dry piperidine were added to 25.8 g (228 mmol) of ethyl isocyanoacetate **9** in 200 ml of dry methanol, and the mixture was stirred at room temperature overnight. It was subsequently evaporated to dryness and crystallized from ether/isopropanol to give 33 g (95% of theory) of pure **10** as a beige solid.

3-(p-Cyanophenyl)-2-isocyanopropionyl-piperidide (**11**) 22.0 g (144.5 mmol) of **10** in 160 ml of THF were added dropwise to 15 g mmol of lithium diisopropylamide in 430 ml of THF at −70°C. Subsequently 28.3 g (144.5 mmol) of *p*-cyanobenzyl bromide dissolved in 300 ml of THF were added dropwise to the mixture at −70°C and, after stirring at this temperature for 3 h (reaction complete according to TLC, mobile phase CH_2Cl_2/CH_3OH 9/1), 70 ml of water was added dropwise. The solution was concentrated under reduced pressure, the residue was acidified with 1N HCl and extracted with methylene chloride, and the organic phase was dried over magnesium sulfate and evaporated. The solid product was mixed with ether, stirred overnight and filtered off with suction. 30.9 g (plus 3.3 g by working up the mother liquor) of **11** were obtained as a white solid (86% of theory).

p-Cyanophenylalanine-piperidide hydrochloride (**12**) 17.4 g (59.2 mmol) of **11** were stirred together with 160 ml of dioxane and 24 ml of concentrated hydrochloric acid

at 60°C for 45–60 min (TLC monitoring, mobile phase CH_2Cl_2/CH_3OH 9/1), and then the mixture was concentrated under reduced pressure. The residue was taken up in water, the aqueous phase was extracted with a little ethyl acetate, mixed with ammonia and extracted with methylene chloride, and the organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was taken up in acetone, and the hydrochloride was precipitated with hydrochloric acid in ether. The precipitate was filtered off with suction and washed with ether to give 16.3 g (83%) of **12** as white solid.

Boc–L–methionyl–D,L–4–cyanophenylalanine-piperidide hydrochloride (**13**) 7.34 g (25 mmol) of **12** were added to a mixture of 15.27 g (125 mmol) DMAP and 6.25 g (25 mmol) Boc-L-methionine in 300 ml methylene chloride. Subsequently 14.38 g (75 mmol) EDC were added and stirred at room temperature for 1 h. The solid phase was filtered off and the filtrate concentrated under reduced pressure. This residue was taken up in water, and extracted with ethyl acetate. The organic phase was washed succesively with 1 N hydrochloric acid, water and brine, dried over sodium sulfate and concentrated under reduced pressure. The mixture of diastereomers remained as a colourless oil (91% of theory), which was used for the next step without further purification.

Boc-L-(S-methyl)methionyl-D, L-4-cyanophenylalanine-piperidide iodide (**14**) 11.2 g (22.92 mmol) of **13** were stirred together with 35 ml of methylene chloride and 45 ml of methyl iodide in a closed flask at room temperature for 4 days. Methylene chloride and excess methyl iodide were removed initially under water pump vacuum and subsequently under high vacuum at room temperature. The residue was used without further purification for the cyclization described below.

3-(Boc-amino)-1-[2-(4-cyanophenyl)-1-piperidinocarbonylethyl]–2–pyrrolidinone (**15**) The product **14** was dissolved in 100 ml of THF and added dropwise to a solution of 65.9 mmol of lithium diisopropylamide in 300 ml of THF prepared at −70°C. After 30 min at −70°C, the mixture was allowed to warm slowly to room temperature and was stirred at this temperature overnight. The mixture was then concentrated under reduced pressure, the residue was taken up in ether, the solution was washed with 1 N hydrochloric acid, the fraction insoluble in ether was removed by filtration, and the ether phase was washed with water, dried over magnesium sulfate and concentrated under reduced pressure to give 8.7 g (86% of theory) of slightly impure **15**, which was used without further purification.

3-Amino-1-[2-(4-cyanophenyl)-1-piperidinocarbonylethyl]-2-pyrrolidinone (**16**) *(as trifluoroacetate)* The crude product **15** was stirred together with 43 ml of methylene chloride and 23 ml of trifluoroacetic acid at room temperature for 90 min. The mixture was then concentrated initially under water pump vacuum and then under high vacuum at room temperature. The resulting slightly impure **16** (as trifluoroacetate) was used without further purification in the next stage.

3–(6,7–Dimethoxynaphthalene–2–sulfonylamino)–1–[2–(4–amidinophenyl)–1–piperidinocarbonylethyl]-2-pyrrolidinone (**17**) The product **16** was dissolved in 25 ml of

methylene chloride, and 5.67 g (19.8 mmol) of 6,7-dimethoxynaphthalene-2-sulfonyl chloride dissolved in 25 ml of methylene chloride were added. Subsequently, 7.6 ml of triethylamine were added, and the mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was taken up in ethyl acetate, and the solution was extracted with 2N hydrochloric acid and subsequently with saturated brine, dried over magnesium sulfate and concentrated. The mixture was purified by column chromatography (250 ml of silica gel 0.063–0.200 mm, methylene chloride with methanol increasing from 0 to 3%). 7.75 g of almost pure **17** (yield over two stages 66%) were obtained.

3-(6,7-Dimethoxynaphthalene-2-sulfonylamino)–1–[2-(4-aminothiocarbonylphenyl)–1–piperidinocarbonylethyl)-2-pyrrolidinone (**18**) 4.6 g (7.78 mmol) of **17** were dissolved in 5 ml of triethylamine and 80 ml of pyridine, and hydrogen sulfide was passed to saturation. The mixture was left to stand overnight and subsequently added dropwise to a mixture of 600 g of ice and 100 ml of concentrated hydrochloric acid. The precipitate was filtered off with suction and dissolved in THF, and the solution was dried over magnesium sulfate and concentrated. The residue was mixed with ether and, after stirring at room temperature for 30 min, the precipitate was filtered off with suction. The slightly impure **18** was used without further purification in the next stage.

3-(6, 7-Dimethoxynaphthalene-2-sulfonylamino)-1-[2-(4-methylthio (imino) methyl-phenyl)-1-piperidinocarbonylethyl]-2-pyrrolidinone (**19**) The crude product **18** was mixed with 10 ml of methyl iodide and stirred at room temperature for 75 min. The mixture was then evaporated to dryness, initially under water pump vacuum and then under high vacuum at room temperature. The slightly impure **19** was used without purification in the next stage.

3-(6,7-Dimethoxynaphthalene-2-sulfonylamino)-1-[2-(4-amidinophenyl)- piperidinocarbonylethyl]-2-pyrrolidinone hydroiodide (**2**) The crude product **19** was stirred together with 1.28 g of ammonium acetate and 80 ml of methanol at 60–65°C for 90 min and subsequently concentrated under reduced pressure. The residue was taken up in 80 ml of methylene chloride, and insolubles were removed by filtration. The filtrate was concentrated under reduced pressure. The residue was taken up in a little ethanol, and the product was precipitated with ethyl acetate. This purification procedure was repeated several times. The collected mother liquors can also be purified in this way. 2.9 g (50.6% of theory over 3 stages) of pure **2** were obtained as a white solid.

The diastereomers of (3S)–(6,7–dimethoxynaphthalene–2–sulfonylamino)–1–[2(R,S)–(4–amidinophenyl)–1–piperidinocarbonylethyl]–2–pyrrolidinone 2 were separated by MPLC.

Dimethyl-N-(2-naphthalenesulfonyl)-L- or D-glutamate (**20**) 28.4 g (134.2 mmol) of dimethyl L- or D-glutamate hydrochloride were introduced into 300 ml of methylene chloride. To this were successively added dropwise 70 ml (51.2 g, 505 mmol) of triethylamine and 25.8 g (113.8 mmol) of 2-naphthalenesulfonyl chloride dissolved in 100 ml of THF at 10–15°C. The mixture was then stirred at room temperature for 1 h and subsequently concentrated under reduced pressure. The residue was taken up in ether/ethyl acetate, and the solution was washed first with dilute aqueous sulfamic

acid solution and then several times with water and was dried over magnesium sulfate and concentrated under reduced pressure to give 33.4 g (76% of theory) of pure **20**.

N-(2-Naphthalenesulfonyl)-L- or D-glutamic acid (**21**) 29.5 g (80.7 mmol) of **20** were dissolved in 500 ml of methanol and stirred with 170 ml (340 mmol) of 2 N sodium hydroxide solution overnight. The pH was adjusted to pH1 with concentrated hydrochloric acid and the solution was evaporated to dryness under reduced pressure. The residue was partitioned between a large volume of methylene chloride and a small volume of water, the methylene chloride phase was dried over magnesium sulfate and evaporated, and the residue was recrystallized from methylene chloride to give 20.3 g (75% of theory) of **21**.

3-[3-(2-Naphthalenesulfonyl-amino)oxazolidin-5-on-4-yl]propionic acid (**22**) 19.3 g (57.2 mmol) of **21** were heated together with 3.4 g (114.4 mmol) of paraformaldehyde, 0.5 g (4 mmol) of thionyl chloride and 6.1 g (60 mmol) of acetic anhydride in 140 ml of glacial acetic acid at 100°C for 5 h. Since the reaction was incomplete, a further 3.4 g (114.4 mmol) of paraformaldehyde, 0.5 g (4 mmol) of thionyl chloride and 6.1 g (60 mmol) of acetic anhydride were added, and the mixture was heated at 100° for 8 h. The solvent was removed under reduced pressure, the residue was taken up in methylene chloride, and the solution was washed with water, dried over magnesium sulfate and concentrated under reduced pressure. The solid was washed several times with water to remove acetic acid. After drying, the product was extracted by boiling with diisopropyl ether, after which the filtrate was evaporated to yield 15.3 g (76% of theory) of almost pure **22**.

3-[3-(2-Naphthalenesulfonyl-amino)oxazolidin-5-on-4-yl]propanal (**24**) 1.4 ml (18.2 mmol) of dry dimethyl-formamide were introduced into 50 ml of methylene chloride and, at 0°C under an inert gas atmosphere, 2.0 ml (28.2 mmol) of oxalyl chloride in 70 ml of methylene chloride were added dropwise. The mixture was stirred at 0°C for 30 min and then the solvent was removed under reduced pressure. The remaining white salt (highly hygroscopic) was suspended in 120 ml of THF/acetonitrile (1:1) and cooled to −30°C. To this were added dropwise 5.6 g (16 mmol) of **22** together with 1.07 ml (16 mmol) of pyridine in 30 ml of THF, and the mixture was then stirred at −20°C for 20 min. The mixture was subsequently cooled to −75°C, 307 mg of cuprous iodide were added, then a solution of 5.6 g (21.8 mmol) of lithium tri-tert-butoxyaluminum hydride in 20 ml of THF was slowly added dropwise, and the mixture was stirred at this temperature for 1 h. Quenching was then carried out at −75°C with 30 ml of 2N hydrochloric acid and, after the mixture had warmed to room temperature, the solvent was removed under reduced pressure. The residue was taken up in methylene chloride, the Cu salt were removed by filtration, and the organic phase was washed three times with water, dried over magnesium sulfate and concentrated under reduced pressure. 5.4 g of slightly impure **24** were obtained which were rapidly processed further because of the low stability.

3-(2-Naphthalenesulfonylamino)-1-[2-(4-cyanophenyl)-1-piperidinocarbonylethyl]-2-piperidinone (**25**) 2.5 g (~7.5 mmol) of **24**, as crude product, in 15 ml of methylene chloride, 2.2 g (7.5 mmol) of **12** hydrochloride in 15 ml of methylene chloride and 1.2 g

(14.6 mmol) of soldium acetate were successively added to 7.5 g of freshly activated molecular sieves (4 Å) in 30 ml of methanol. The mixture was cooled to about 12°C and then 0.9 g (14.3 mmol) of sodium cyanoborohydride dissolved in 20 ml of THF were added dropwise over the course of 35 min, and the mixture was stirred at room temperature for 3 h. The solids were then removed by filtration and washed with methylene chloride. The collected filtrates were evaporated under reduced pressure, the residue was taken up in ethyl acetate, and the solution was washed with dilute aqueous sulfamic acid solution (pH 1) and five times with saturated brine, dried over magnesium sulfate and evaporated under reduced pressure. Chromatography on silica gel (0.063–0.200 mm/eluent methylene chloride/ methanol) resulted in 3.5 g (85% of theory over 3 stages) of pure **25**. The subsequent reactions to prepare **6** the transformation of the cyano into the amidino group, were carried out as described for compound **2**.

*Tert-butyl 2-(3-nitropyrid-2-on-1-yl)acetate (**29**)* 63.3 g (459 mmol) of powdered potassium carbonate were suspended in 100 ml of DMF and cooled to 0°C and, at this temperature, a suspension of 21.5 g (153 mmol) of 2-hydroxy-3-nitropyridine in 100 ml of DMF was added. After stirring at 0°C for 10 min, 23.6 ml (31.4 g, 161 mmol) of tert-butyl bromoaetate in 50 ml of DMF were added dropwise, and the mixture was then stirred for 45 min, during which time an intense red coloration appeared. Potassium carbonate was filtered off with suction, the filtrate was acidified (pH 3) with aqueous hydrochloric acid and extracted with methylene chloride, and the organic phase was washed twice with water, dried over magnesium sulfate and evaporated. After adherent DMF and tert-butyl bromoacetate had been removed under high vacuum, the product was extracted by stirring with diisopropyl ether, resulting in 35.5 g (90% of theory) of **29**.

*2-(3-Nitropyrid-2-on-1-yl)acetic acid (**30**)* 3.5 g (140 mmol) of **29** in 110 ml of methylene chloride were mixed with 107 ml (159.0 g, 1.40 mol) of trifluoroacetic acid and stirred at room temperature for 2 h. The mixture was concentrated under reduced pressure, the residue was stirred with diisopropyl ether, and the solid was filtered off with suction to yield 22.1 g (80% of theory) of **30**.

*2-(3-Nitropyrid-2-on-1-yl)ethyl-piperidide (**31**)* 10 g (50.5 mmol) of **30** were dissolved in 500 ml of THF, cooled to −20°C, 7 ml (6.7 g, 55.6 mmol) of pivaloyl chloride in 30 ml of THF and 7 ml (5.1 g, 50.5 mmol) of triethylamine in 30 ml of THF were added dropwise, and the mixture was then stirred at −20°C for 20 min. Subsequently 5 ml (4.3 g, 50.5 mmol) of piperidine in 40 ml of THF were added dropwise, and the mixture was stirred at −20°C for a further 30 min. The solution was acidified with 1N hydrochloric acid and extracted several times with methylene chloride, and the organic phase was washed with water, dried over magnesium sulfate and concentrated under reduced pressure. The residue was extracted by boiling with hexane several times and filtered hot. This resulted in 11.8 g (88% of theory) of **31** which was still slightly impure with pivaloyl-piperidide.

2-(3-Aminopyrid-2-on-1-yl)acetyl-piperidide hydrochloride (**32**) 22.8 g (86 mmol) of **31** were dissolved in 460 ml of methanol, and 9.8 ml (10.3 g, 172 mmol) of glacial acetic acid and 3.4 g of 10% palladium on active carbon were added. The mixture was hydrogenated with hydrogen under slightly elevated pressure. After the reaction was complete, the catalyst was filtered off with suction and washed with methanol, and the filtrates were concentrated under reduced pressure. The residue was taken up in aqueous sulfamic acid solution, the aqueous phase was extracted twice with methylene chloride (contains mainly pivaloyl-piperidide) then made alkaline and extracted four times with methylene chloride. The extracts were dried over magnesium sulfate, acidified with ethereal hydrochoric acid and concentrated under reduced pressure to give 14.2 g (61% of theory) of **32** as the hydrochloride.

2-[3-(2-Naphthalenesulfonylamino)pyrid-2-on-1-yl]acetyl-piperidide (**33**) 10.3 g (45.5 mmol) of 2-naphthalenesulfonyl chloride in 100 ml of methylene chloride were added dropwise to a solution of 14.2 g (53.5 mmol) of **32** hydrochloride and 43.2 ml (42.3 g, 535 mmol) of pyridine in 250 ml of methylene chloride, and the mixture was stirred at room temperature for 1.5 h. The solution was then acidified with 1N hydrochloric acid, and the organic phase was washed once with water, dried over magnesium sulfate and concentrated under reduced pressure to give 18.4 g (95% of theory) of **33**.

2–[3–(2–Naphthalenesulfonyl (tert-butyloxycarbonyl) amino) pyrid–2–on–1–yl]acetyl–piperidide (**34**) 18.4 g (43 mmol) of **33** were dissolved in 300 ml of methylene chloride and, successively, 9.4 g (43 mmol) of di-tert-butyl dicarbonate and 5.3 g (43 mmol) of dimethylaminopyridine were added, resulting in a homogeneous solution which was then stirred for 40 min. The solution was subsequently adjusted to pH 2.5 with 1N hydrochloric acid, and the organic phase was washed once with water, dried over magnesium sulfate and evaporated under reduced pressure. Extraction by stirring with hexane resulted in 22.0 g (97.3% of theory) of **34**.

3-(2-Naphthalenesulfonyl(Boc)amino)-1-[2-(4-cyanophenyl)-1-piperidinocarbonylethyl] -2-pyridone (**35**) 6.1 ml (10.14 mmol) of 15% strength butyllithium solution in hexane, 4.1 g (7.8 mmol) of **34** in 40 ml of THF and 1.4 g (7.02 mmol) of *p*-cyanobenzyl bromide in 30 ml of THF were successively added dropwise to 1.4 ml (1.03 g, 10.14 mmol) of diisopropylamine in 40 ml of THF at −70°C. The mixture was stirred at −70°C for 2 h and then allowed to warm to room temperature, methylene chloride was added, the pH was adjusted to 2.5 with 1N hydrochloric acid, and the aqueous phase was extracted. The organic phase was washed with water, dried over magnesium sulfate and concentrated under reduced pressure. The resulting crude product was purified by column chromatography (silica gel 0.063–0.200 mm, eluent: methylene chloride/methanol). 2.5 g (56% of theory) of **35** were obtained.

The transformation of the cyano into the amidino group of 3-(2-naphthalene-sulfonyl(Boc)-amino)-1-[2-(4-amidinophenyl)-1-piperidinocarbonylethyl]-2-pyridone **38** was done in a similar way to compound **2**. After elimination of the Boc protective group (trifluoroacetic acid/methylene chloride), the 3-(2-naphthalenesulfonylamino)-1-[2-(4-amidinophenyl)-1-piperidinocarbonylethyl]-2-pyridone **7** was obtained as trifluoroacetate.

Pharmacological characterization

The inhibition of the amidolytic activity of thrombin and other serine proteases was measured in an *in vitro* assay using suitable chromogenic substrates as described in Reference 9. IC_{50} is the inhibitor concentration in mol/l required to reduce the enzyme activity by 50%. The antithrombotic activity *in vivo* was measured in the rat arteriovenous shunt model. Details of this model are given elsewhere.[9]

X-ray structure determination

The crystals of the bovine thrombin and thrombin-**1a** complex were prepared according to Brandstetter *et al.*[3] The complex crystallized isomorphous in the same tetragonal space group $P4_22_12$ with cell constants a=b=88.5Å, c=103.7Å. The crystals contain one complex per asymmetric unit and diffract beyond 2.6Å resolution. Diffraction data were recorded with a Siemens X-100 multiwire area detector mounted on a Rigaku RU-200HB rotating copper anode X-ray generator operated at 50 kV, 90 mA with a 0.3 mm focal spot and graphite monochromator. The crystal to detector distance was 8 cm and all measurements were made at 21°C. The crystal was rotated through 120° and the data frames, each of 0.3°, were processed on a μ-VAX computer (Digital Equipment Corporation) using the XENGEN data processing and reduction suite of programs,[10] which produced a unique list of indexed structure factor amplitudes with standard deviations, with the multiply recorded data suitably scaled and merged.

The bovine thrombin model was subjected to an energy restraint least squares refinement using X-PLOR[11] and the PARAM19X.PRO force field parameters which brought the crystallographic R value down from 0.290 to 0.196. Inspection of the Fo-Fc electron density map near the active site clearly showed strong density for the inhibitor. Subsequently, the model of **1a** was fitted to the density using the computer graphics program O[12] and further refined. Several alternating cycles of positional and restrained B-value refinement reduced the R-value to 0.174. The final model parameters are given in Table 1.

RESULTS AND DISCUSSION

Table 2 summarizes the structures of the lactam compounds with the corresponding *in vitro* data in thrombin assay with chromogenic substrate.

The rigidisation of NAPAP by the lactam moiety leads to compounds with increased binding affinity to thrombin by a factor of 3 to 112 (as compared to the racemic form of NAPAP). The preferred configuration is S in the lactam ring and R in the *p*-amidino-phe moiety. Compound **1a** with a five-membered ring is the best thrombin inhibitor in this series and is about 10 times more potent than the aliphatic six-membered lactam **6** and about 30 times more potent than the unsaturated lactam **7**. The other stereoisomers **1b** and **1c** show weaker thrombin inhibition than NAPAP. The replacement of the naphthyl group is possible within certain limits. All of the substituents R investigated by us appear to be compatible with the aryl binding site of thrombin. Introduction of methoxy substituents **3** increases

TABLE 1
Final model parameters of the thrombin-**1a**-structure. The R-value is defined as $\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$

Parameter	Value
Number of non-hydrogen atoms	2640
Number of solvent molecules	240
rms standard deviations from target values:	
bond lengths	0.012Å
bond angles	3.143
Number of unique reflections ($>2\sigma$)	7780
Resolution range	8.0–2.58Å
R value	0.174

TABLE 2
Rigid NAPAP-derived inhibitors (R, X, Y, as defined in Figure 2)

Compound		Structure			Configuration		Thrombin assay	Selectivity*
		R	X	Y	Phe moiety	Lactam moiety	IC_{50} [nmol/l]	
1	**a**	2-Naphthyl-	$-(CH_2)_2-$	Am	D	L	1.6	22
	b				L	L	660	
	c				D/L	D	330	137
1	**a**	6,7-Dimethoxy-2-naphthyl	$-(CH_2)_2-$	Am	D	L	2.4	25
	b	2-Naphthyl-			L	L	110	103
3		6-Methoxy-2-naphthyl-	$-(CH_2)_2-$	Am	D/L	L	2.6	76
4		2.2.5.7.8-Pentamethyl-6-chromanyl-	$-(CH_2)_2-$	Am	D/L	L	5.3	27
5		2-Naphthyl-	$-(CH_2)_2-$	H	D/L	L	12000	
6		2-Naphthyl-	$-(CH_2)_3-$	Am	D/L	L	12	111
7		2-Naphthyl-	$=(CH)_3-$	Am	D/L	–	44	17
8**		2-Naphthyl-	H, H	Am	D/L	–	180	–

*selectively towards trypsin (IC_{50} trypsin / IC_{50} thrombin)
**NAPAP

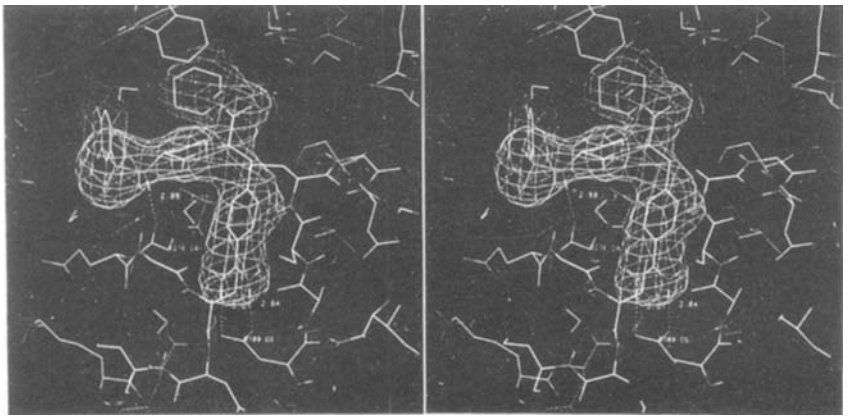


FIGURE 3 Stereo view of the X-ray structure of the thrombin-**1a** complex.

the selectivity versus trypsin from 22 to 76. Comparison of **1** with **5** shows that binding affinity is essentially lost when the amidino group is removed.

The X-ray structure of the complex of thrombin with **1a** is shown in Figure 3. The inhibitor binds to thrombin in a mode very similar to NAPAP. All hydrogen bonds observed in the thrombin-NAPAP complex are also present in the complex with **1a**. As found for NAPAP, **1a** makes extensive lipophilic interactions with the enzyme both with the naphthyl and the piperidino group. In addition, the lactam ring which is not present in NAPAP, is in contact with the side chain of Trp 60D. This additional lipophilic interaction together with a reduced flexibility appears to be responsible for the improved binding of **1a**.

Compound **1a** was also tested *in vivo*. In the rat arteriovenous shunt model, we observed an antithrombotic activity 2–5 times better than that of NAPAP. The duration of action is similar to NAPAP. No antithrombotic activity was observed after oral administration of 100 mg/kg of **1a** to rats.

In summary, we have described the 3D-structure based design, synthesis and biological activity of novel rigid inhibitors of thrombin. The most potent compound binds thrombin with an affinity 112 times higher than that of NAPAP (racemate). Work is in progress to develop thrombin inhibitors with sufficient oral availability.

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