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Synthesis and *in vitro* evaluation of new azaphenylalanine derivatives as serine protease inhibitors

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New inhibitors of serine proteases with azaphenylalanine scaffold were synthesized and their activity was evaluated *in vitro*. We studied the effect of different substituents in the part of a molecule that binds in the distal pocket of the thrombin active site. Modifications generally led to decreased activity, however two derivatives are promising lead compounds as new thrombin and dual thrombin-factor Xa inhibitors.

1. Introduction

Thrombin and factor Xa are trypsin-like serine proteases that have a central role in haemostasis, while thrombin is also crucial in the process of thrombosis. Initiation of the blood coagulation cascade, either by the intrinsic or the extrinsic pathway, leads to the conversion of factor X to its activated form, factor Xa. Once produced, factor Xa in combination with factor Va and calcium ions, converts prothrombin to thrombin. In the coagulation cascade thrombin is the key enzyme that proteolytically cleaves fibrinogen and generates fibrin, forming a haemostatic plug (Gresele and Agnelli 2002; Dahlbäck 2000). In recent years factor Xa and thrombin have become important targets for drug design. The main goal is the search for potent, selective and orally bioavailable low-molecular-weight inhibitors of those serine proteases involved in thrombus formation which could be used for treating various cardiovascular disorders. The best studied inhibitors of thrombin are derived from the sequence D-Phe-Pro-Arg as in argatroban, NAPAP and melagatran. Most noncovalent inhibitors currently reported possess a basic functional group that interacts with Asp 189, the central amino acid residue in the specificity pocket, and one or more nonpolar groups for hydrophobic interactions in the proximal and distal pockets of the active site of thrombin (Kimball 1995; Coburn 2001; Menear 1998; Ripka 1997; Rewinkel and Adang 1999; Sanderson 1999). Synthetic inhibitors of factor Xa differ from thrombin inhibitors due to the presence of an additional binding pocket in the active site of an enzyme, wherein interactions between another basic group of inhibitor and the residue of Glu 97 are possible (Hauptmann and Stürzebecher 1999; Vacca 2000; Zega et al. 1999; Rai et al. 2001).

Recently our group has reported a series of azaphenylalanine derivatives with a hydroxyamidino group on position 4 of the aromatic ring. Continuing in this research we have prepared a set of compounds with different substituents that could fit in the distal hydrophobic pocket of thrombin (Zega et al. 2001a, 2001b).

2. Investigations, results and discussion

2.1. Synthesis of the compounds

Compounds **3a–d** were synthesised by activation of *N*-((2-naphthyl)sulfonyl) aminoacids with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in the presence of *N*-methylmorpholine and 1-hydroxybenzotriazol, followed by aminolysis of the activated complex by 2-(1-azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazinium chloride (**2**). Starting compounds were prepared by known procedures (Zega et al. 2001a, 2001b). Compounds **3a–d** were later transformed to their corresponding benzamidoximes **4a–d** by treatment with hydroxylamine, and to benzamidines **5a–d** by reacting ethanolic HCl solution with ammonium acetate or gaseous ammonia (Scheme 1).

Compounds that lack a sulfonamide group were prepared in a way analogous to compounds **3**, using different carboxylic acids as starting material (Scheme 2).

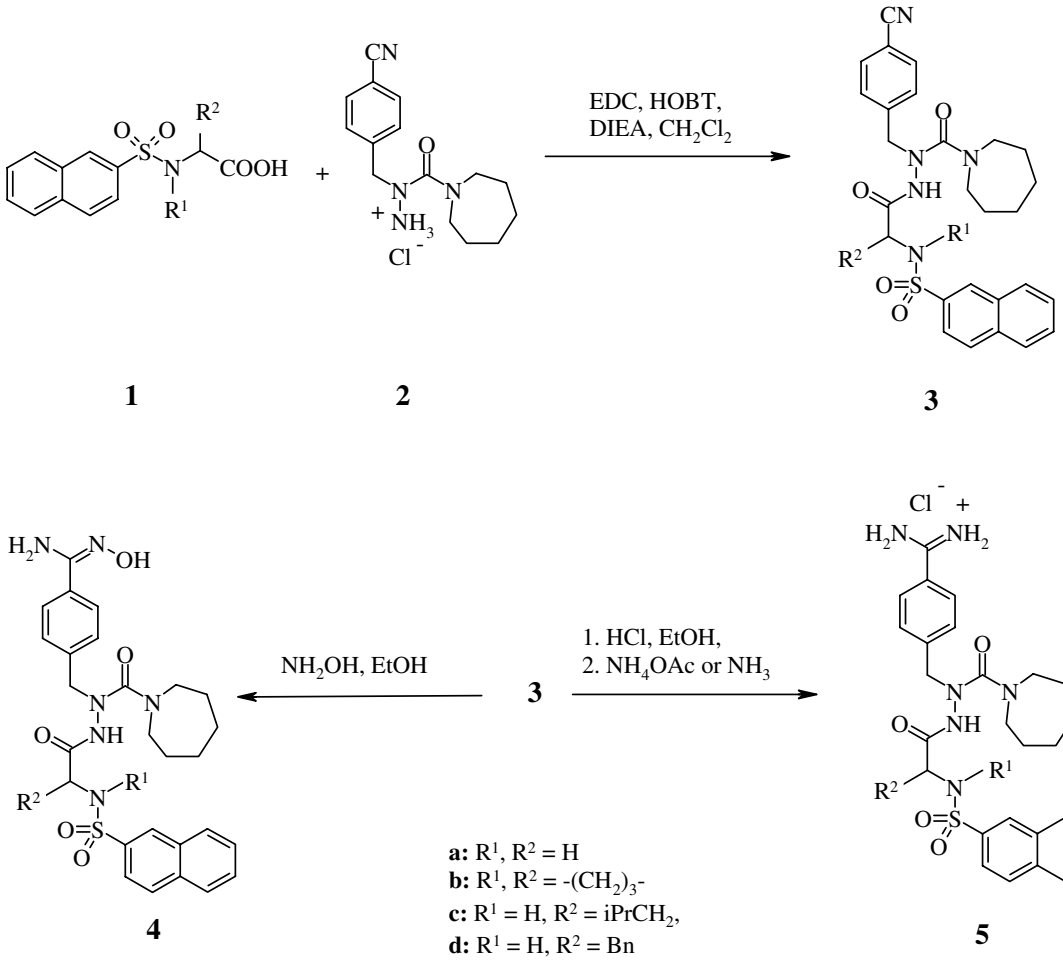
2.2. In vitro activity of synthesized compounds against serine proteases

The activities of compounds **4, 5a–d** and **7a–d** are presented in the Table 1.

Several of the new azaphenylalanine derivatives exhibit moderate activity against thrombin and factor Xa.

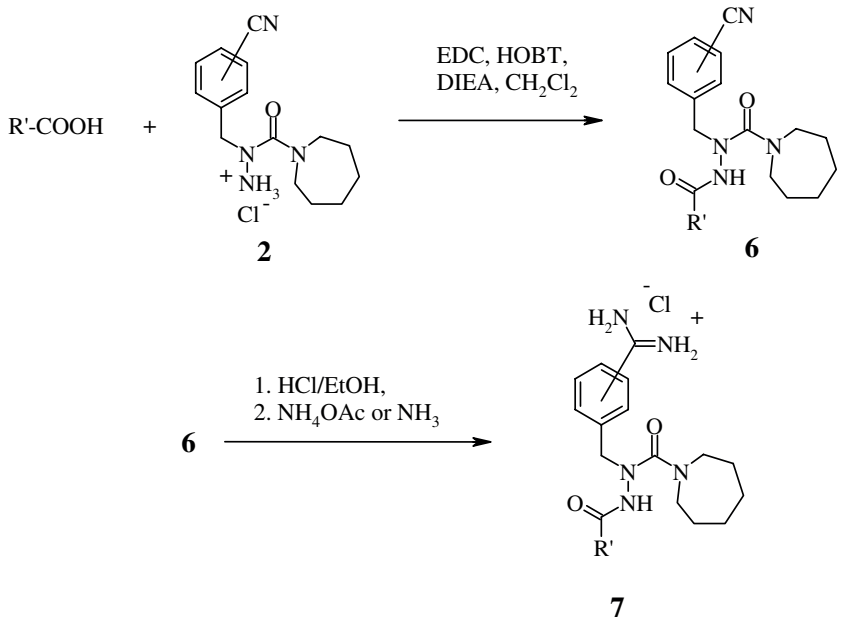
In the first group of compounds, **3–5**, a spacer was introduced between the semicarbazide and naphthylsulfonyl groups, using different amino acid residues. Incorporation of glycine (**4a, 5a**) led to an aza-derivative of NAPAP, but

Scheme 1



1 **2** **3** **4** **5**

Scheme 2



2 **6** **7**

Table: Inhibitory activities of compounds 4, 5a–d and 7a–c against different serine proteases *in vitro*

Compd.	R′	Position on aromatic ring	Ki (μM) thrombin	Ki (μM) trypsin	Ki (μM) factor Xa
4a			>100	>100	>100
4b			>100	>100	>100
4c			>100	>100	>100
4d			9.7	>100	>100
5a			9.0	6.2	37
5b			8.6	2.5	42
5c			10	>100	>100
5d			0.49	1.7	2.9
7a	2-naphthyl	4	3.8	2.4	2.9
7b	(2-naphthyloxy)methyl	4	21	29	38
7c	1-amino-2-phenylethyl	4	4	0.20	11
7d	2-naphthyl	3	0.97	1.11	0.34

with a significant drop in activity. This result was surprising since in our previous studies, the activity of compounds with an α-CH group in the phenylalanine part of the molecule was similar to that of their aza-derivatives in some cases, substitution with nitrogen even enhanced the activity. The rigidity of the structure was increased by the use of amino acids with larger side chains. This change did not affect the activity when proline (**4b**, **5b**) or leucine (**4c**, **5c**) were used, however the introduction of phenylalanine (**4d, 5d**) increased the activity against thrombin and factor Xa, but not trypsin. We suspect that the substitution of the α-CH group with nitrogen in NAPAP analogues results in a change to a conformation that is unfavourable for binding in the active site of thrombin. The presence of the additional benzyl group in **4d** and **5d** probably forced the compound into a more favourable conformation. In all cases amidines were far more active then amidoximes due to the higher basicity and therefore stronger interactions with Asp 189 at the bottom of selectivity pocket. Although amidoximes are potential prodrugs due to their conversion to amidines *in vivo*, we did not synthesize amidoximes from compounds **6a**–**d**. The reason was their supposed inactivity in *in vitro* test systems.

The second step of our research was focused on the substitution of the sulfonamide group with carboxamide. Sulfonamide forms a hydrogen bond with Gly 216 and its replacement results in loss of activity. Introducing an amidino group on position 3 of the aromatic ring (**7d**), leads to significant increase in activity against factor Xa. Compound **7d**, with submicromolar inhibitory constants against both thrombin and factor Xa, is therefore a suitable lead compound for dual inhibitors and will be further investigated.

3. Experimental

^{1}H NMR spectra were recorded on a Bruker avance DPX$_{300}$ (300 MHz) spectrometer, using DMSO-d$_6$ and CDCl$_3$ as solvents and TMS as the internal standard. IR spectra were obtained on a Perkin-Elmer 1600 FT-IR spectrometer. Mass spectra were measured on a VG-Analytical Autospec Q spectrometer. Elemental analyses were made on a Perkin Elmer 2400 CHN analyzer and the results were in an acceptable error range (less than 0.4%). Melting points were measured on a Kofler microscope and are uncorrected. TLC was performed on precoated sheets 60F$_{254}$. All chemicals and solvents were supplied by Merck, Aldrich, Fluca, Acros and Carlo Erba. 2-(1-Azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazinium chloride was prepared according to literature procedures (Zega et al. 2001a). Inhibition of enzymatic activities of thrombin, trypsin and factor Xa was measured by the amidolytic assay using chromogenic substrates S-2238 (for thrombin) and S-2222 (for trypsin and factor Xa, both substrates from Chromogenix). Results were expressed as inhibitory constants (K$_i$), calculated from the relationship between reaction velocity in the absence and presence of compound using the relevant Michaelis constant (K$_m$).

3.1. Synthesis of compounds 3a–d

2-[(2-Naphthylsulfonyl)amino]acetic acid (4.00 mmol) was dissolved, with stirring, in 20 ml of dichloromethane, followed by the addition of 783 mg (4.10 mmol) 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC), 554 mg (4.10 mmol) 1-hydroxy-1 H-benzotriazole (HOBT), 516 mg (4.00 mmol) of *N*-ethyl-*N*,*N*-diisopropylamine (DIEA) and 2-(1-azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazine chloride (1.24 g; 4.00 mmol). The reaction mixture was stirred at room temperature for 2 days.

The solvent was removed under reduced pressure and the residue dissolved in 50 ml of ethyl acetate, then washed with 25 ml 1 M HCl, 25 ml 1 M NaOH, 20 ml distilled water and 20 ml brine. The organic phase was dried over sodium sulphate and the solvent evaporated *in vacuo*. The crude product was re-crystallized from ethanol.

3.1.1. N-{2-[2-(1-Azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazino]-2-oxoethyl}-2-naphthalenesulfonamide (**3a**)

Yield: 31%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.47 (s, 4 H, CH$_2$), 1.67 (s, 4 H, CH$_2$), 3.39 (m, 6 H, CH$_2$), 4.47 (s, 2 H, Ar-CH$_2$), 7.58 (d, 2 H, J = 8.27 Hz, Ar-H), 7.71 (dqu, 2 H, J$_1$ = 6.42 Hz, J$_2$ = 1.51 Hz, Ar-H), 7.80 (d, 2 H, J = 8.27 Hz, Ar-H), 7.83 (dd, 1 H, J$_1$ = 8.70 Hz, J$_2$ = 1.81 Hz, Ar-H), 8.06 (d, 1 H, J = 7.64 Hz, Ar-H), 8.15 (m, 2 H, Ar-H), 8.47 (s, 1 H, Ar-H), 8.81 (s, 1 H, NHSO$_2$), 10.29 (s,1 H, NH) ppm; C$_{27}$H$_{29}$N$_5$O$_4$S; MS (FAB +): m/z (%): 520 (MH$^+$, 42), 126 (100); IR (KBr): 3410, 3253, 2919, 2232, 1708, 1613, 1441, 1344, 1157, 1014, 817, 659, 547 cm^{-1}; m.p. = 149–151 °C.

3.1.2. N′-(1-Azepanylcarbonyl)-N′-(4-cyanobenzyl)-1-(2-naphthylsulfonyl)-2-pyrrolidinecarbohydrazide (**3b**)

Yield: 23%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.47 (s, 4 H, CH$_2$), 1.64 (s, 4 H, CH$_2$), 1.75 (m, 4 H, CH$_2$), 3.24 (m, 4 H, CH$_2$), 3.24 (m,2 H, CH$_2$), 4.12 (m, 1 H, CH), 4.47 (s, 2 H, Ar-CH$_2$), 7.57 (d, 2 H, J = 8.29 Hz, Ar-H), 7.71 (dqu, 2 H, J$_1$ = 7.16 Hz, J$_2$ = 1.50 Hz, Ar-H), 7.80 (d, 2 H, J = 8.29 Hz, Ar-H), 7.84 (dd, 1 H, J$_1$ = 8.67 Hz, J$_2$ = 1.89 Hz, Ar-H), 8.07 (d, 1 H, J = 7.91 Hz, Ar-H), 8.15 (m, 2 H, Ar-H), 8.47 (s, 1 H, Ar-H), 10.29 (s,1 H, NH) ppm; C$_{30}$H$_{33}$N$_5$O$_4$S; MS (FAB+): m/z (%): 560 (MH$^+$, 47), 126 (100); IR (KBr): 3461, 2234, 1708, 1614, 1527, 1428, 1342, 1156, 1082, 1015, 922, 857, 818, 759, 662, 548 cm^{-1}; m.p. = 158–159 °C.

3.1.3. N-(1-{[2-(1-Azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazino]carbonyl}-3-methylbutyl)-2-naphthalenesulfonamide (**3c**)

Yield: 48%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 0.84 (d, 6 H, J = 6.32 Hz, CH$_3$), 1.39 (m, 1 H, CH), 1.49 (m, 4 H, CH$_2$), 1.63 (m, 4 H, CH$_2$), 1.83 (m, 2 H, CH$_2$), 3.26 (m, 4 H, CH$_2$), 4.48 (s, 2 H, Ar-CH$_2$), 4.87 (m, 1 H, CH), 7.49 (d, 2 H, J = 8.31 Hz, Ar-H), 7.68 (d, 2 H, J = 8.28 Hz, Ar-H), 7.75 (m, 3 H, Ar-H), 8.08 (m, 3 H, Ar-H), 8.39 (s, 1 H, Ar-H), 8.73 (s, 1 H, NHSO$_2$), 10.13 (s, 1 H, NH) ppm; C$_{31}$H$_{37}$N$_5$O$_4$S; MS (FAB+): m/z (%): 576 (MH$^+$, 51), 185 (100); IR (KBr): 3246, 2957, 2229, 1789, 1633, 1467, 1329, 1162, 1075, 817, 659, 549 cm^{-1}; m.p. = 75–77 °C.

3.1.4. N-{2-[2-(1-Azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazino]-1-benzyl-2-oxoethyl}-2-naphthalenesulfonamide (**3d**)

Yield: 57%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.44 (m, 4 H, CH$_2$), 1.64 (m, 4 H, CH$_2$), 3.01 (m, 2 H, Ar-CH$_2$), 3.21 (m, 4 H, CH$_2$), 4.45 (s, 2 H, Ar-CH$_2$), 7.18 (m, 5 H, Ar-H), 7.52 (d, 2 H, J = 8.23 Hz, Ar-H), 7.63 (dqu, 2 H, J$_1$ = 8.29 Hz, J$_2$ = 1.53 Hz, Ar-H), 7.81 (d, 2 H, J=8.29 Hz, Ar-H), 7.86 (dd, 1 H, J$_1$=8.63 Hz, J$_2$ = 1.75 Hz, Ar-H), 8.02 (t, 3 H, J = 8.26 Hz, Ar-H), 8.36 (s, 1 H, Ar-H), 8.75 (s, 1 H, NHSO$_2$), 9.32 (s,1 H, NH) ppm; C$_{34}$H$_{35}$N$_5$O$_4$S; MS (FAB+): m/z (%): 610 (MH$^+$, 21), 185 (100); IR (KBr): 3280, 2928, 2228, 1779, 1631, 1418, 1348, 1159, 1074, 817, 749, 657, 546 cm^{-1}; m.p. = 170–172 °C.

3.2. Synthesis of compounds **4a–d**

The corresponding compound **3** (0.77 mmol) was dissolved in anhydrous ethanol, and hydroxylamine (28.0 mg, 0.85 mmol) was added. The reaction mixture was refluxed for 12 h. The solvent was removed under reduced pressure and the product washed with ether and dried.

3.2.1. 4-[(1-(1-Azepanylcarbonyl)-2-{2-[(2-naphthylsulfonyl)amino]acetyl}-hydrazino) methyl]-N′-hydroxybenzenecarboximidamide (**4a**)

Yield: 68%; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.52 (s, 4 H, CH$_2$), 1.78 (s, 4 H, CH$_2$), 2.06 (m,2 H, CH$_2$), 3.49 (q, 4 H, J = 5.54 Hz, CH$_2$), 4.67 (m, 2 H, Ar-CH$_2$), 4.90 (s, 1 H, NOH), 7.41 (d, 2 H, J = 8.17 Hz, Ar-H), 7.61 (d, 2 H, J = 8.17 Hz, Ar-H), 7.73 (m, 3 H, Ar-H), 7.97 (m, 3 H, Ar-H), 8.34 (s, 1 H, Ar-H), 8.50 (s, 2 H, NH$_2$), 8.68 (s, 1 H, NHSO$_2$), 10.42 (s,1 H, NH) ppm; C$_{27}$H$_{32}$N$_6$O$_5$S; MS (FAB+): m/z (%): 593 (MK$^+$, 25), 126 (100); IR (KBr): 3360, 2924, 1783, 1640, 1423, 1346, 1158, 1076, 821, 750, 661, 546 cm^{-1}; m.p. = 104–108 °C.

3.2.2. 4-[(1-(1-Azepanylcarbonyl)-2-{[1-(2-naphthylsulfonyl)-2-pyrrolidinyl]carbonyl} hydrazino)methyl]-N'-hydroxybenzenecarboximidamide (**4b**)

Yield: 76%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.45 (s, 4 H, CH$_2$), 1.66 (s, 4 H, CH$_2$), 1.73 (m, 4 H, CH$_2$), 3.22 (m, 4 H, CH$_2$), 3.27 (m,2 H, CH$_2$), 4.15 (m, 1 H, CH), 4.52 (s, 2 H, Ar-CH$_2$), 5.21 (s, 1 H, NOH), 7.53 (d, 2 H, J = 8.25 Hz, Ar-H), 7.66 (dqu, 2 H, J$_1$ = 7.23 Hz, J$_2$ = 1.74 Hz, Ar-H), 7.79 (d, 2 H, J = 8.29 Hz, Ar-H), 7.85 (dd, 1 H, J$_1$ = 8.73 Hz, J$_2$ = 1.92 Hz, Ar-H), 8.13 (d, 1 H, J = 7.91 Hz, Ar-H), 8.14 (m, 2 H, Ar-H), 8.50 (s, 1 H, Ar-H), 8.93 (s, 2 H, NH$_2$), 10.36 (s,1 H, NH) ppm; C$_{30}$H$_{36}$N$_6$O$_5$S; MS (FAB+): m/z (%): 593 (MH$^+$, 21), 126 (100); IR (KBr): 3358, 2930, 1776, 1650, 1419, 1350, 1083, 768, 692, 552 cm^{-1}; m.p. = 131–135 °C.

3.2.3. 4-[(1-(1-Azepanylcarbonyl)-2-{4-methyl-2-[(2-naphthylsulfonyl) amino] pentanoyl}hydrazino)methyl]-N′-hydroxybenzenecarboximidamide (**4c**)

Yield: 64%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 0.92 (d, 6 H, J = 6.45 Hz, CH$_3$), 1.44 (m, 1 H, leu-CH), 1.52 (m, 4 H, CH$_2$), 1.65 (m, 4 H, CH$_2$), 1.89 (m, 2 H, CH$_2$), 3.31 (m, 4 H, CH$_2$), 4.39 (s, 2 H, Ar-CH$_2$), 4.86 (m, 1 H, CH), 5.46 (s, 1 H, NOH), 7.43 (d, 2 H, J = 8.26 Hz, Ar-H), 7.65 (d, 2 H, J = 8.23 Hz, Ar-H), 7.81 (m, 3 H, Ar-H), 8.12 (m, 3 H, Ar-H), 8.45 (s, 1 H, Ar-H), 8.82 (s, 1 H, NHSO$_2$), 9.12 (s, 2 H, NH$_2$), 10.31 (s,1 H, NH) ppm; C$_{31}$H$_{40}$N$_6$O$_5$S; MS (FAB+): m/z (%): 609 (MH$^+$, 37), 126 (100); IR (KBr): 3326, 2945, 1770, 1638, 1425, 1339, 1211, 923, 770, 645 cm^{-1}; m.p. = 93–96 °C.

3.2.4. 4-[(1-(1-Azepanylcarbonyl)-2-{2-[(2-naphthylsulfonyl)amino]-3-phenylpropanoyl} hydrazino)methyl]-N′-hydroxybenzenecarboximidamide (**4d**)

Yield: 61%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.43 (m, 4 H, CH$_2$), 1.68 (m, 4 H, CH$_2$), 3.17 (m, 6 H, CH$_2$), 4.52 (s, 2 H, Ar-CH$_2$), 5.52 (s, 1 H, NOH), 7.21 (m, 5 H, Ar-H), 7.55 (d, 2 H, J = 8.28 Hz, Ar-H), 7.71 (m, 2 H, Ar-H), 7.80 (d, 2 H, J = 8.32 Hz, Ar-H), 7.91 (m, 1 H, Ar-H), 8.08 (t, 3 H, J = 8.31 Hz, Ar-H), 8.41 (s, 1 H, Ar-H), 8.72 (s, 1 H, NHSO$_2$), 9.23 (s, 2 H, NH$_2$), 10.27 (s, 1 H, NH) ppm; C$_{34}$H$_{38}$N$_6$O$_5$S; MS (FAB+): m/z (%): 643 (MH$^+$, 47), 126 (100); IR (KBr): 3352, 2926, 1776, 1654, 1468, 1237, 1026, 895, 765 cm^{-1}; m.p. = 146–149 °C.

3.3. Synthesis of compounds 5a–d

Compound **3** (1.00 mmol) was dissolved in 25 ml of absolute alcohol and gaseous HCl was bubbled in for 20 min. The solution was then stirred at room temperature for 4 h, followed by the addition of ammonium acetate (85.0 mg, 1.10 mmol). The reaction mixture was left at room temperature for 2 days. Gaseous HCl was again bubbled in for 20 min. After 1 h, the ammonium chloride formed was filtered off and the ethanol evaporated under reduced pressure. The product was suspended in ether and filtered by suction.

3.3.1. Amino{4-[(1-(1-azepanylcarbonyl)-2-{2-[(2-naphthylsulfonyl)amino] acetyl} hydrazino)methyl] phenyl}methaniminium chloride (**5a**)

Yield: 84%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.58 (s, 4 H, CH$_2$), 1.76 (s, 4 H, CH$_2$), 3.04 (m, 4 H, CH$_2$), 3.35 (m, 2 H, CH$_2$), 4.88 (m, 2 H, Ar-CH$_2$), 7.37 (d, 2 H, J = 8.86 Hz, Ar-H), 7.53 (m, 1 H, SO$_2$NH), 7.73 (m, 3 H, Ar-H), 7.92 (d, 2 H, J = 8.86 Hz, Ar-H), 8.09 (m, 3 H, Ar-H), 8.49 (s, 1 H, Ar-H), 9.29 and 9.46 (2s, 4 H, H$_2$N-C=NH$_2^+$), 10.31 (s, 1 H, NH) ppm; C$_{27}$H$_{33}$N$_6$O$_4$SCl; MS (FAB+): m/z (%): 578 ((M−HCl)K$^+$, 12), 100 (100); IR (KBr): 2969, 2759, 1784, 1670, 1452, 1344, 1159, 1076, 822, 750, 661, 546 cm^{-1}; m.p. = 194–196 °C.

3.3.2. Amino{4-[(1-(1-azepanylcarbonyl)-2-{[1-(2-naphthylsulfonyl)-2-pyrrolidinyl]carbonyl} hydrazino)methyl] phenyl}methaniminium chloride (**5b**)

Yield: 40%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.57 (m, 4 H, CH$_2$), 1.76 (m, 4 H, CH$_2$), 2.03 (m, 4 H, CH$_2$), 3.02 (m, 4 H, CH$_2$), 3.32 (m, 2 H, CH$_2$), 3.63 (q, 1 H, J = 3.63 Hz, CH$_2$), 4.96 (s, 2 H, Ar- CH$_2$), 7.52 (d, 2 H, J=8.18 Hz, Ar-H), 7.68 (dqu, 2 H, J$_1$ = 7.14 Hz, J$_2$ = 1.67 Hz, Ar-H), 7.81 (d, 2 H, J = 8.18 Hz, Ar-H), 7.93 (dd, 1 H, J$_1$ = 8.59 Hz, J$_2$ = 2.11 Hz, Ar-H), 8.12 (m, 3 H, Ar-H), 8.49 (s, 1 H, Ar-H), 9.41 (s, 4 H, NH$_2$-C=NH$_2^+$) 9.59 (s,1 H, NH) ppm; C$_{30}$H$_{37}$N$_6$O$_4$SCl; MS (FAB+): m/z (%): 577 ((M−HCl)H$^+$,13), 478 (100); IR (KBr): 3034, 1781, 1681, 1602, 1402, 1347, 1158, 1076, 1010, 823, 748, 660 cm^{-1}; m.p. = 123–127 °C.

3.3.3. Amino-{4-[(1-(1-azepanylcarbonyl)-2-{4-methyl-2-[(2-naphthylsulfonyl)amino] pentanoyl}hydrazino) methyl]phenyl}methaniminium chloride (**5c**)

Yield: 40%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 0.85 (d, 6 H, J = 6.67 Hz, CH$_3$), 1.35 (m, 1 H, CH), 1.52 (m, 4 H, CH$_2$), 1.64 (m, 4 H, CH$_2$), 1.85 (m, 2 H, CH$_2$), 3.24 (m, 4 H, CH$_2$), 4.55 (s, 2 H, Ar-CH$_2$), 4.89 (m, 1 H, CH), 7.50 (d, 2 H, J = 8.27 Hz, Ar-H), 7.69 (d, 2 H, J = 8.29 Hz, Ar-H), 7.75 (m, 3 H, Ar-H), 8.07 (m, 3 H, Ar-H), 8.38 (s, 1 H, Ar-H), 8.56 (s, 1 H, NHSO$_2$), 9.25 (s,1 H, NH), 9.54 and 9.74 (2s, 4 H, H$_2$N-C=NH$_2^+$) ppm; C$_{31}$H$_{41}$N$_6$O$_4$S; MS (FAB+): m/z (%): 595 ((M−HCl)H$^+$, 8), 86 (100); IR (KBr): 3381, 2956, 1786, 1654, 1420, 1275, 1162, 1074, 1020, 860, 749, 659, 548 cm^{-1}; m.p. = 93–95 °C.

3.3.4.Amino{4-[(1-(1-azepanylcarbonyl)-2-{2-[(2-naphthylsulfonyl)amino]-3-phenylpropanoyl}hydrazino)methyl]phenyl}methaniminium chloride (**5d**)

Yield: 37%; ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 1.47 (m, 4 H, CH$_2$), 1.68 (m, 4 H, CH$_2$), 3.13 (m, 2 H, Ar-CH$_2$), 3.31 (m, 4 H, CH$_2$), 4.49 (s, 2 H, Ar-CH$_2$), 7.22 (m, 5 H, Ar-H), 7.55 (d, 2 H, J = 8.27 Hz, Ar-H), 7.65 (m, 2 H, Ar-H), 7.77 (d, 2 H, J=8.33 Hz, Ar-H), 7.90 (m, 1 H, Ar-H), 8.07 (m, 3 H, Ar-H), 8.42 (s, 1 H, Ar-H), 8.86 (s, 1 H, NHSO$_2$), 9.42 (s,1 H, NH), 9.55 and 9.80 (2s, 4 H, H$_2$N-C=NH$_2^+$) ppm; C$_{34}$H$_{39}$N$_6$O$_4$SCl; MS (FAB+): m/z (%): 627 ((M−HCl)H$^+$, 33), 185 (100); IR (KBr): 3435, 3130, 1780, 1661, 1436, 1283, 1081, 1009, 875, 750, 552 cm^{-1}; m.p. = 204–208 °C.

3.4. Synthesis of compounds 6a–d

2-(1-Azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazinium chloride (312 mg, 1.03 mmol), DIEA (2 mL), 144 mg (1.12 mmol) HOBT, 158 mg (1.17 mmol) EDC and 1.11 mmol of the corresponding carboxylic acid were dissolved in dichloromethane (30 mL). The reaction mixture was stirred at room temperature for 2 days. The solvent was removed *in vacuo* and the residue was dissolved in 30 ml ethyl acetate and extracted with 4 × 25 ml 10% citric acid and 30 ml aqueous sodium hydrogen carbonate. The organic phase was further washed with 30 ml water and 30 ml brine, dried over sodium sulphate and the solvent removed to yield the crude product. This was washed with diethylether and further purified by recrystallization from ethanol.

3.4.1. N-(4-Cyanobenzyl)-N′-(2-naphthoyl)-1-azepanecarbohydrazide (**6a**)

Yield: 84%; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.55 (m, 4 H, CH$_2$), 1.73 (m, 4 H, CH$_2$), 3.48 (t, 4 H, J = 5.77 Hz, CH$_2$), 4.68 (s, 2 H, Ar-CH$_2$), 7.51–7.61 (m, 6 H, Ar-H), 7.77 (dd, 1 H, J$_1$ = 8.58 Hz, J$_2$ = 1.71 Hz, Ar-H), 7.95 (m, 3 H, Ar-H), 8.25 (s, 1 H, Ar-H), 8.56 (s, 1 H, NH) ppm; C$_{26}$H$_{26}$N$_4$O$_2$; MS(FAB+): m/z(%): 427 (MH$^+$, 29), 126 (100); IR (KBr): ν 3246, 2926, 2227, 1775, 1674, 1630, 1523, 1444, 1299, 1191, 1129, 866, 829, 750, 544 cm^{-1}; m.p. = 69–71 °C.

3.4.2. N′-(1-Azepanylcarbonyl)-N′-(4-cyanobenzyl)-2-(2-naphthyloxy)acetohydrazide (**6b**)

Yield: 79%; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.55 (m, 4 H, CH$_2$), 1.75 (m, 4 H, CH$_2$), 3.43 (t, 4 H, J = 5.86 Hz, CH$_2$), 4.54 (s, 2 H, CH$_2$), 4.68 (s, 2 H, Ar-CH$_2$), 7.34 (d, 2 H, J = 8.40 Hz, Ar-H), 7.39–7.58 (m, 3 H, Ar-H), 7.47 (d, 2 H, J = 8.40 Hz, Ar-H), 7.68–7.84 (m, 3 H, Ar-H), 8.14 (s, 1 H, Ar-H), 9.06 (s,1 H, NH) ppm; C$_{27}$H$_{28}$N$_4$O$_3$; MS (FAB+): m/z (%): 457 (MH$^+$, 22), 126 (100); IR (KBr): 3228, 2925, 2854, 2227, 1630, 1509, 1426, 1216, 1120, 960, 838, 748, 547 cm^{-1}; m.p. = 142–144 °C.

3.4.3. Ethyl 2-[2-(1-azepanylcarbonyl)-2-(4-cyanobenzyl)hydrazino]-1-benzyl-2-oxoethylcarbamate (**6c**)

Yield: 82%; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.21 (t, 3 H, J = 6.78 Hz, CH$_2$CH$_3$), 1.57 (m, 4 H, CH$_2$), 1.75 (m, 4 H, CH$_2$), 3.01 (m, 2 H, Ar-CH$_2$), 3.43 (t, 4 H, J = 5.80 Hz, CH$_2$), 4.13 (t, 2 H, J = 6.73 Hz, CH$_2$CH$_3$), 4.41 (s, 2 H, Ar-CH$_2$), 4.53 (s, 1 H, CH), 5.04 (s, 1 H, NHCOOEt), 7.29 (m, 5 H, Ar-H), 7.51 (d, 2 H, J=8.20 Hz, Ar-H), 7.66 (d, 2 H, J = 8.33 Hz, Ar-H), 8.48 (s, 1 H, NH) ppm; C$_{27}$H$_{33}$N$_5$O$_4$; MS (FAB+): m/z (%): 492 (MH$^+$, 26), 126 (100); IR (KBr): 3321, 2950, 2225, 1666, 1531, 1421, 1289, 1043, 757, 559 cm^{-1}; m.p. = 133–137 °C.

3.4.4. N-(3-Cyanobenzyl)-N′-(2-naphthoyl)-1-azepanecarbohydrazide (**6d**)

2-(1-Azepanylcarbonyl)-2-(3-cyanobenzyl)hydrazinium chloride was used as a starting compound instead of the *para*-substituted derivative.
Yield: 86%; ^{1}H NMR (300 MHz, CDCl$_3$): δ 1.55 (m, 4 H, CH$_2$), 1.73 (m, 4 H, CH$_2$), 3.49 (t, 4 H, J = 5.84 Hz, CH$_2$), 4.69 (s, 2 H, Ar-CH$_2$), 7.42 (t, 1 H, J = 7.89 Hz, Ar-H), 7.59 (m, 3 H, Ar-H), 7.69 (d, 1 H, J = 7.91 Hz, Ar-H), 7.77 (dd, 1 H, J$_1$ = 8.29 Hz, J$_2$ = 1.88 Hz, Ar-H), 7.81 (s, 1 H, Ar-H), 7.89 (m, 3 H, Ar-H), 8.24 (s, 1 H, Ar-H), 8.47 (s, 1 H, NH) ppm; C$_{26}$H$_{26}$N$_4$O$_2$; MS(FAB+): m/z(%): 427 (MH$^+$, 24), 71 (100); IR (KBr): 3281, 2922, 2236, 1637, 1524, 1289, 1210, 1010, 912, 822, 759, 698, 595 cm^{-1}; m.p. = 139–141 °C.

3.5. Synthesis of compounds 7a–d

Compound **6** (0.50 mmol) was suspended in absolute ethanol (20 mL) and gaseous hydrogen chloride bubbled in for 30 min. The reaction mixture was left at room temperature for 4 h, and the solvent then removed in vacuo. The residue was washed with diethylether (220 mL) and dissolved in absolute ethanol. The solution was treated with gaseous ammonia for 10 min and ethanol was removed under reduced pressure. The crude product was washed with diethylether and crystallized from ethanol.

3.5.1. Amino(4-{[1-(1-azepanylcarbonyl)-2-(2-naphthoyl)hydrazino]methyl} phenyl) methaniminium chloride (**7a**)

Yield: 53%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.59 (m, 4 H, $\underline{CH_2}$), 1.76 (m, 4 H, $\underline{CH_2}$), 3.48 (qu, 4 H, J = 5.27 Hz, $\underline{CH_2}$), 3.93 (s, 2 H, Ar-$\underline{CH_2}$), 7.58–7.65 (m, 6 H, Ar-$\underline{H}$), 7.83 (dd, 1 H, J_1 = 8.64 Hz, J_2 = 1.63 Hz, Ar-$\underline{H}$), 8.04 (m, 3 H, Ar-$\underline{H}$), 8.41 (s, 1 H, Ar-$\underline{H}$), 9.34 (s, 1 H, $\underline{NH}$), 9.41 in 9.58 (2s, 4 H, $\underline{H_2N}-C=\underline{NH_2}^+$) ppm; $C_{26}H_{30}N_5O_2Cl$; MS(FAB+): m/z(%): 444 ((M−HCl)$\overline{H}^+$, 11), 100 (100); IR (KBr): ν 3251, 2970, 2753, 1757, 1668, 1588, 1392, 1099, 1050, 961, 866, 829, 759, 704 cm^{-1}; m.p. = 221–224 °C.

3.5.2. Amino[4-({1-(1-azepanylcarbonyl)-2-[2-(2-naphthyloxy)acetyl]hydrazino}methyl) phenyl]methaniminium chloride (**7b**)

Yield: 50%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.57 (m, 4 H, $\underline{CH_2}$), 1.77 (m, 4 H, $\underline{CH_2}$), 3.05 (t, 4 H, J = 5.74 Hz, $C\underline{H_2}$), 4.20 (s, 2 H, $\underline{CH_2}$), 4.79 (s, 2 H, Ar-$\underline{CH_2}$), 7.29 (d, 2 H, J = 8.34 Hz, Ar-$\underline{H}$), 7.36–7.49 (m, 3 H, Ar-$\underline{H}$), 7.53 (d, 2 H, J = 8.37 Hz, Ar-$\underline{H}$), 7.77–7.89 (m, 3 H, Ar-$\underline{H}$), 8.15 (s, 1 H, Ar-$\underline{H}$), 9.07 (s,1 H, $\underline{NH}$), 9.30 (s, 4 H, $\underline{NH_2}$-C=$\underline{NH_2}^+$), ppm; $C_{27}H_{32}N_5O_3Cl$; MS (FAB+): m/z (%): 474 ((M−HCl)$\overline{H}^+$, 16), 126 (100); IR (KBr): 3404, 2931, 1784, 1664, 1419, 1278, 1179, 1018, 836, 769 cm^{-1}; m.p. = 217–222 °C.

3.5.3. [Amino(4-{[2-(2-ammonio-3-phenylpropanoyl)-1-(1-azepanylcarbonyl)hydrazino] methyl}phenyl)methylene]ammonium dichloride (**7c**)

After the influx of ammonia, the solution was refluxed in 1 M HCl for 1 h to remove the carbamate protection of the amino group.
Yield: 22%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.48 (m, 4 H, $\underline{CH_2}$), 1.58 (m, 4 H, $\underline{CH_2}$), 3.05 (m, 2 H, Ar-$\underline{CH_2}$), 3.23 (t, 4 H, J = 5.62 Hz, $\underline{CH_2}$), 4.48 (s, 2 H, Ar-$\underline{CH_2}$), 4.59 (m, 1 H, $\underline{CH}$), 7.26 (m, 5 H, Ar-$\underline{H}$), 7.51 (d, 2 H, J = 8.29 Hz, Ar-$\underline{H}$), 7.81 (d, 2 H, J = 8.28 Hz, Ar-$\underline{H}$), 9.18 in 9.38 (2s, 4 H, $\underline{H_2N}-C=\underline{NH_2}^+$), 9.55 (s, 1 H, $\underline{NH}$) ppm; $C_{24}H_{34}N_6O_2Cl$; MS (FAB+): m/z (%): 475 ((M−HCl)H^+, 16), 362 (100); IR (KBr): 3312, 2935, 1702, 1663, 1495, 1430, 1270, 1096, 1061, 726, 702 cm^{-1}; m.p. = 221–223 °C.

3.5.4. Amino(3-{[1-(1-azepanylcarbonyl)-2-(2-naphthoyl)hydrazino]methyl} phenyl) methaniminium chloride (**7d**)

Yield: 81%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.58 (m, 4 H, $\underline{CH_2}$), 1.76 (m, 4 H, $\underline{CH_2}$), 3.02 (t, 4 H, J = 5.15 Hz, $\underline{CH_2}$), 3.82 (s, 2 H, Ar-$\underline{CH_2}$), 7.61–7.67 (m, 5 H, Ar-$\underline{H}$), 7.84 (m, 2 H, Ar-$\underline{H}$), 7.98–8.12 (m, 3 H, Ar-$\underline{H}$), 8.42 (s, 1 H, Ar-$\underline{H}$), 9.39 (s, 1 H, $\underline{NH}$), 9.43 in 9.58 (2s, 4 H, $\underline{H_2N}-C=\underline{NH_2}^+$) ppm; $C_{26}H_{30}N_5O_2Cl$; MS(FAB+): m/z(%): 444 ((M−HCl)$\overline{H}^+$, 15), 100 (100); IR (KBr): ν cm^{-1}; m.p. = 247–251 °C.

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References

Coburn AC (2001) Small-molecule direct thrombin inhibitors. Exp Opin Ther Patents 11: 721–737.
Dahlbäck B (2000) Blood Coagulation. Lancet 355: 1627–1632.
Gresele P, Agnelli G (2002) Novel approaches to the treatment of thrombosis. trends pharmacol Sci 23: 25–32.
Hauptmann J, Stürzebecher J (1999) Synthetic inhibitors of thrombin and factor Xa: from bench to bedside. Thromb Res 93: 203–241.
Kimball SD (1995) thrombin active site inhibitors. Curr Pharm Des 1: 441–468.
Menear K (1998) Progress towards the discovery of orally active thrombin inhibitors. Curr Med Chem 5: 457–468.
Rai R, Sprengeler KC, Elrod KC, Young WB (2001) Perspectives on factor Xa inhibition. Curr Med Chem 8: 101–119.
Rewinkel JBM, Adang AEP (1999) Strategies and progress towards the ideal orally active thrombin inhibitor. Curr Pharm Des 5: 1043–1075.
Ripka WC (1997) New thrombin inhibitors in cardiovascular disease. Curr Opin Chem Biol 1: 242–253.
Sanderson PEJ (1999) Small, noncovalent serine protease inhibitors. Inc Med Res Rev 19: 179–197.
Vacca JP (2000) New advance in the discovery of thrombin and factor Xa inhibitors. Curr Opin Chem Biol 4: 394–400.
Zega A, Obreza A, Urleb U (1999) Inhibitorji faktorja X_a. Farm Vestn 50: 53–57.
Zega A, Mlinšek G, Šepic P, Golič-Grdadolnik S, Šolmajer T, Tschopp TB, Steiner B, Kikelj D, Urleb U (2001a) Design and structure-activity relationship of thrombin inhibitors with an azaphenylalanine scaffold: potency and selectivity enhancements via P2 optimization. Bioorg Med Chem 9: 2745–2756.
Zega A, Trampuš-Bakija A, Fortuna M, Stegnar M, Tschopp TB, Steiner B, Urleb U (2001b) Novel thrombin inhibitors with azaphenylalanine scaffold. Pharmazie 56: 683–685.