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Orally active thrombin inhibitors. Part 1: Optimization of the P1-moiety

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Abstract—The synthesis and SAR of novel nanomolar thrombin inhibitors with the common backbone HOOC–CH_2–D-cyclohexylalanyl-3,4-dehydroprolyl–NH–CH_2–aryl-C(=NH)NH_2 are described together with their ecarin clotting time (ECT) prolongation as measure for thrombin inhibition ex vivo. The aryl P1-moiety mimicking the arginine part of the D-Phe-Pro-Arg derived thrombin inhibitors turned out to be a key component for in vitro potency and in vivo activity. Optimization of this part led to compounds with improved antithrombin activity in rats and dogs after oral administration compared to the recently launched anticoagulant melagatran.

Thrombin is a glycosylated trypsin-like serine protease generated from prothrombin by the action of a complex of factors Xa and V, calcium ions, and phospholipids. Thrombin has various biological functions but its main role is to catalyze the transformation of fibrinogen to fibrin, which is the prerequisite for thrombus formation. Thrombin also activates factor XIII to cross-link fibrin and to stabilize the clot, and it promotes and amplifies clot formation by activating other clotting factors. Thrombin plays a central role in blood coagulation and platelet activation and is thus involved in thromboembolic diseases. Therefore, inhibitors of thrombin have long been recognized as potential therapeutic agents for the treatment of a variety of thromboembolic disorders, for example, deep vein thrombosis, pulmonary embolism, atrial fibrillation, and thromboembolic stroke.

Over the last decades many groups spent tremendous efforts in the discovery and development of low molecular weight thrombin inhibitors.[1] Potent antithrombotic efficacy after oral administration was the ultimate goal besides selectivity versus other serine proteases, appropriate half-life, and an improved side effect profile relative to warfarin. Melagatran and its double prodrug Ximelagatran are the most advanced thrombin inhibitors and have recently been launched in a number of European countries for the prevention of venous thromboembolic events in elective hip or knee replacement surgery.[2,3] Nevertheless, it is still an open question whether the extensive clinical use of Ximelagatran can demonstrate a real benefit in terms of efficacy and safety over established antithrombotic therapies with warfarin and heparin. Safety concerns have been raised due to elevated liver enzymes in a number of patients after several weeks' treatment with Ximelagatran.[4]

In a series of publications, we describe our efforts[5,6] towards new and potent thrombin inhibitors derived from the D-Phe-Pro-Arg lead structure. The first part focuses on the systematic modification of the aromatic ring A attached to the amidine function (P1 residue) in HOOC–CH_2–D-cyclohexylalanyl-3,4-dehydroprolyl–NH–CH_2–aryl-C(=NH)NH_2 (cf. Fig. 1), the second part is dedicated to the variation of the P2 position[6a], and the third part describes the optimization of the P3 and P4 moiety.[6b]

In this paper, the SAR data and the synthesis of a number of potent thrombin inhibitors with modifications in the P1 part are presented. It has been found that the activity strongly depends on the nature of the aromatic

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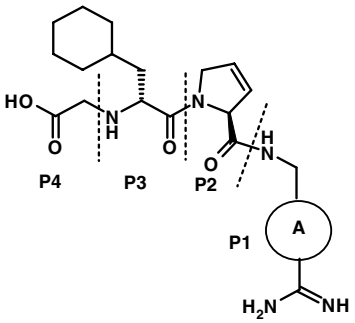


Figure 1. Schematic representation of the thrombin inhibitors.

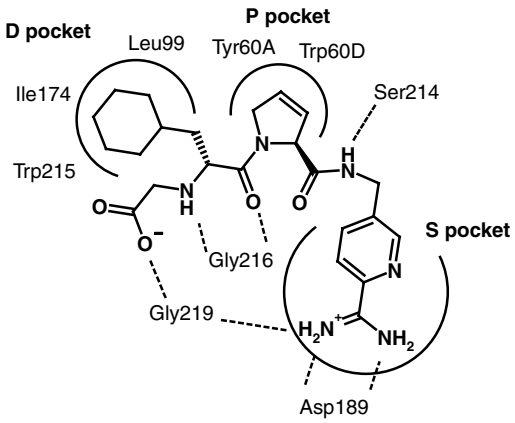


Figure 2. Schematic representation of the inhibitor **8** bound to the active site of thrombin.

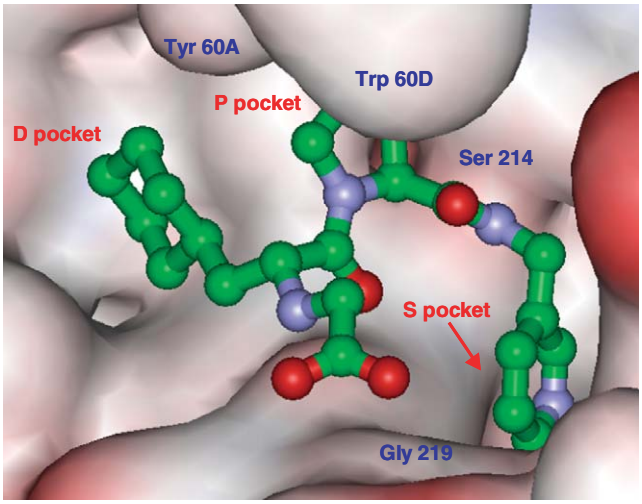


Figure 3. X-ray structure of the inhibitor **8** bound to the active site of thrombin.

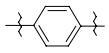
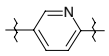
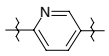
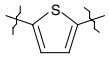
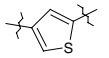
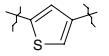
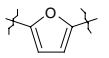
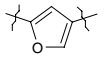
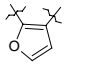
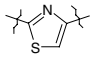
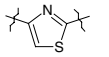
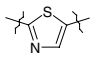
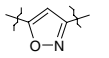
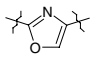
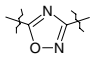
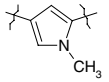
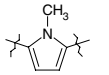
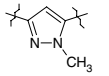
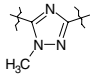
ring in the P1 position and its substitution pattern. This can partly be explained either by electronic effects of the different heterocycles, by favorable or unfavorable interactions of the protein with heteroatoms of the heteroaryl P1 moiety and/or by steric effects. The best compounds were further examined in rats and dogs after oral administration, providing information regarding absorption, metabolic stability, half-life, and antithrombin activity in vivo.

Almost all published thrombin inhibitors with an aryl amidine function in P1 contain a phenyl group in this position (NAPAP, CRC 220, and melagatran).[1] We investigated six- and five-membered heteroaromatic rings as amidine bearing P1 moieties, because we expected slightly different binding modes due to the non-linear arrangement of the ring substituents. Furthermore, the heteroaromatic ring could modify the basicity of the amidine function. For the following SAR discussion, we keep the P4-P3-P2-scaffold in HOOC–CH_2–D-cyclohexylalanyl-3,4-dehydroprolyl–NH–CH_2–aryl-C(=NH)NH_2 constant. This part of the lead structure has been optimized in our laboratories in parallel[6] to the P1 variation efforts and is one of the most suitable P4-P3-P2 building blocks for potent thrombin inhibitors. The P1 modifications are depicted in Figure 1 and Table 1.

The optimization of the P1 scaffold was supported by molecular modeling and by crystal structure determination of thrombin complexed with various inhibitors. All of them revealed a similar binding mode, which is shown in Figures 2 and 3. The cyclohexyl ring of the inhibitor is engaged in hydrophobic interactions in the lipophilic D pocket. The side chains of Ile174, Leu99, and Trp215 come in close contact with the cyclohexyl moiety of D-cyclohexylalanine in **8** (4.5–4.8 Å). The pyrroline ring occupies the P pocket with contacts to Tyr60A, His57, and Trp60D (3.6–4.3 Å). The amidine group at the pyridyl ring of the inhibitor forms strong hydrogen bonds with the carboxylic group of Asp189 at the bottom of the S pocket in thrombin (2.7 and 2.8 Å). This ionic contact seems to be crucial for the high affinity of the ligand to the enzyme, because deviations from the optimal fit of the P1 moiety result in considerable loss of antithrombin activity (cf. Fig. 1 and Table 1). Additional hydrogen bonds are formed between the following residues: The carbonyl group of Ser214 and the NH group of the dehydroproline amide moiety (3.1 Å), the carbonyl and amine function of D-cyclohexylalanine and the amide and carbonyl group of Gly216 (2.7 and 3.0 Å), and the carboxylic group of the P4 moiety and the NH group of Gly219 (2.8 Å). Besides the ionic interaction with Asp189, one nitrogen of the amidine moiety also forms a hydrogen bond with the carbonyl of Gly219 (2.9 Å).

Scheme 1 depicts the general synthesis of these novel thrombin inhibitors. Starting from D-cyclohexylalanine-benzylester N-alkylation with tertiary butyl bromo acetate, N-protection with Boc_2O and subsequent hydrogenation of the benzyl group led to the P4-P3 building block **2**. The derivative **2** was either directly converted in a peptide coupling procedure with the P2-P1 building blocks **4a** or **4b** to the advanced intermediates **6a** and **6b**, respectively, or alternatively was coupled with the P2 moiety 3,4-dehydroproline methyl ester. Subsequent methyl ester hydrolysis and reaction with the P1 moieties **5a–d** provided the P4-P3-P2-P1 scaffolds **6a–d** in moderate to high yield. Amidine formation starting from **6b** was achieved either in three steps via

Table 1. Effect of various aromatic P1 moieties on the in vitro potency and in vivo activity of the thrombin inhibitors (structures depicted in Fig. 1; A as part of $NH–CH_2–A–C(NH)NH_2$)

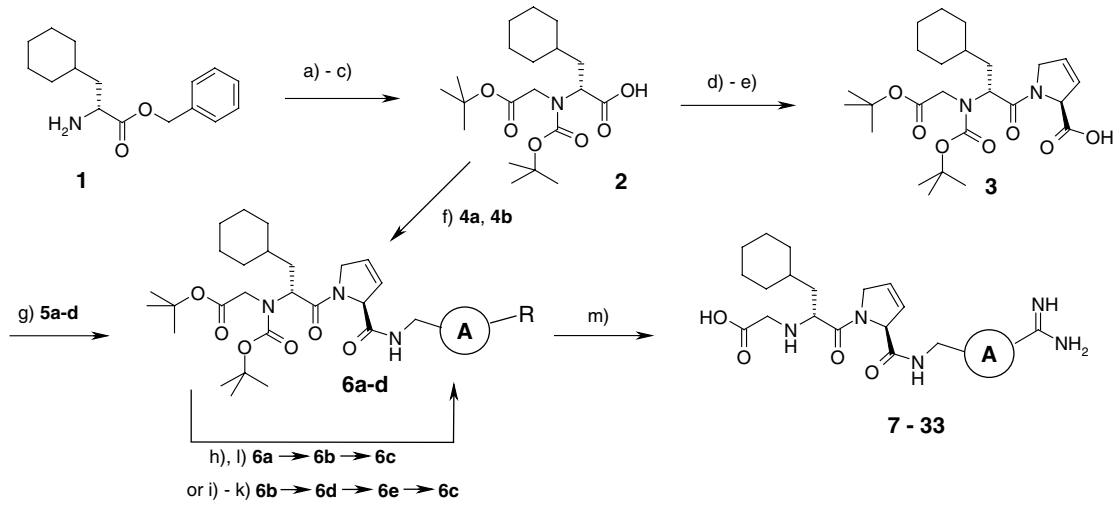
Compound	A	Thrombin-assay IC_{50} (nM)	ECT rat po[a] (s)	ECT rat iv[b] (s)
7		1.68	590	108
8		1.00	649	128
9		51.2	313	104
10		0.98	>800	301
11		6.40	324	81
12		2.70	639	411
13		49.1	220	74
14		1.29	>800	202
15		1430	ND	ND
16		1.39	>800	280
17		44.0	166	41
18		50.9	540	83
19		33.9	395	58
20		20.8	440	102
21		1370	29	33
22		11.2	201	101
23		6.20	372	58
24		1020	45	35
25		232	76	63

(*continued on next page*)

Table 1 (*continued*)

Compound	A	Thrombin-assay IC_{50} (nM)	ECT rat po[a] (s)	ECT rat iv[b] (s)
26		566	ND	ND
27		1.76	132	64
28		4.28	97	48
29		9.88	618	82
30		1080	31	32
31		2780	ND	ND
32		2.15	653	76
33		57.4	ND	ND
Melagatran		69.2	247	98

ND, not determined.
Typical ECT control value, 35 s.
[a] 60 min after po dosing of 21.5 mg kg^{-1}.
[b] 60 min after iv dosing of 1.0 mg kg^{-1}.



Scheme 1. Synthesis of P1 modified thrombin inhibitors: Reagents and conditions: (a) 1.1 equiv $^tBuO_2CCH_2Br$, 10 equiv K_2CO_3, CH_3CN, rt, 3 d; (b) 1.1 equiv Boc_2O, 1.02 equiv K_2CO_3, CH_3CN, rt, 3 d; (c) H_2(1 bar), 3.8 mol% Pd as 10% Pd/C, EtOH, rt, 2 h, 83% over three steps; (d) 1.0 equiv 3,4-dehydroproline methyl ester, 1.3 equiv PPA, 4.3 equiv DIEA, DCM, −10 °C → 0 °C, 2 h, 60%; (e) 2.0 equiv 1 N NaOH, dioxane, rt, 2 h, 94%; (f) 1.0 equiv H-3,4-dehydroprolyl–NH–CH_2–aryl-R (R = $CONH_2$ **4a** or CN **4b**), 1.4 equiv PPA, 5.0 equiv DIEA, DCM, 0 °C → 10 °C, 3 h, 85–95%; (g) 1.05 equiv H_2N–CH_2–aryl-R (R = $CONH_2$ **5a**, CN **5b** or $CSNH_2$ **5d**), 1.1 equiv PPA, 5.0 equiv DIEA, DCM, 0 °C → 10 °C, 3 h, 80–95%; or 1.25 equiv H_2N–CH_2–aryl-$C(NH)NH_2$ (**5c**), 1.0 equiv TOTU, 5.0 equiv NMM, DMF, 0 °C, 1 h, 45%; (h) R = $CONH_2$ (**6a**) → R = CN (**6b**): 4.0 equiv DIEA, 1.8 equiv trifluoroacetic anhydride, DCM, 0–5 °C, 2 h, 48–91%; (i) R = CN (**6b**) → R = $CSNH_2$ (**6d**): H_2S, pyridine/NEt_3 6:1, rt, 2 d, 63–99%; (j) R = $CSNH_2$ (**6d**) → R = $C(NH)SCH_3$ (**6e**): 6.0 equiv CH_3I, DCM, 1 d; (k) R = $C(NH)SCH_3$ (**6e**) → R = $C(NH)NH_2$ (**6c**): 1.0 equiv NH_4OAc (10% in MeOH), CH_3CN, 45 °C, 6 h or rt, 2 d, 35–92% over two steps; (l) R = CN (**6b**) → R = $C(NH)NH_2$ (**6c**): NH_3(g), 1.1 equiv *N*-acetylcysteine, MeOH, 62 °C, 9–15h, 45–81%; (m) 5 N HCl in diethylether, DCM, rt, 1 d, 83–95%.

the corresponding thioamide **6d**[7] or directly by an *N*-acetyl cysteine catalyzed reaction with ammonia.[8] After protective group removal, the thrombin inhibitors **7**–**33** were obtained as white or off-white solids in moderate to good yield. The detailed syntheses of the final compounds and also of their novel building blocks have been described elsewhere.[5,9,10] The synthesis of compound **8** on a technical scale based on the convergent route has recently been reported in detail.[11]

The in vitro potency of the inhibitors was determined as an IC_{50} value for thrombin inhibition in a chromogenic substrate assay (IC_{50}: concentration of inhibitor required to inhibit the amidolytic activity of thrombin by 50% with S-2238 as substrate).[12] The antithrombin activity in vivo was determined after iv or po administration of the test compound by prolongation of the ecarin clotting ex vivo (ECT in rats 60 min after 21.5 mg kg^{-1} po or 1.0 mg kg^{-1} iv application of the inhibitor). A typical ECT control value in rats was 35 s. The ECT prolongation in dogs was determined after oral administration of 4.64 mg kg^{-1}.[13,14]

The thrombin inhibitor **7** with a phenyl ring in P1 was already very potent, as was demonstrated by its nanomolar IC_{50} value and its pronounced prolongation of the ECT after iv and po administration compared to melagatran[15] (cf. Table 1). The pyridine derivative **8** and the corresponding phenyl compound **7** were equipotent, but **8** was slightly more active in vivo. The X-ray structure of **8**[16] confirmed an overall excellent binding of the compound in thrombin, as illustrated in Figure 3. A preferred orientation of the pyridyl nitrogen in the S pocket could not be assigned based on the electron density of the X-ray structure, but the orientation depicted in Figure 3 avoids a close contact of the pyridine nitrogen and the carbonyl oxygen of Gly219 and is therefore more likely. Shifting the nitrogen atom of the P1 pyridyl ring into the isomeric position (compound **9**) resulted in an approximately 50-fold loss of in vitro potency, but unexpectedly only an about 2-fold loss of in vivo activity after oral administration was observed.

A similar sensitivity of the antithrombin activity in vitro and in vivo was observed with compounds comprising a five-membered heteroaromatic ring in P1. The thiophene compound **10** was equipotent to **8** but an unexpected rise in activity with an ECT prolongation of more than 800 s (detection limit) one hour after oral administration of 21.5 mg kg^{-1} in rats was observed. So far, it is one of the most active thrombin inhibitors in our series. The crystal structure of **10** with thrombin revealed a very good fit of the inhibitor in the active site of thrombin.[17] The thiophene isomers **11** and **12** were slightly less potent in vitro and also less active in vivo than the 2,5-substituted thiophene **10**. The five-membered aromatic ring causes some deviation from the linear arrangement of the attached amidine and aminomethyl moieties. In principle, this can result in two different orientations of the aryl amidine group within the S pocket, influencing the amidine/Asp189 interaction and therefore the potency of the inhibitor.

One additional small substituent (methyl, chlorine) at the thiophene ring in **10** hardly affected the potency, but led to a decrease in in vivo activity after intravenous as well as oral administration (cf. **27** and **28** vs **10**). On the other hand, substituted compounds derived from **12**, like **29** and **32**, retained potency and activity at least after oral administration. However, disubstitution of the thiophene as illustrated by **30** was not tolerated.

Replacement of the thiophene by a furan ring completely changed the anticipated SAR. The 3,5-disubstituted furan **14** was much more potent than its corresponding isomer **13** with a 2,5-substitution pattern. As expected the 2,3-substituted furan **15** was inactive.

The substitution pattern in the thiazole series consisting of compounds **16**, **17**, and **18** also strongly influenced the antithrombin activity. This may again be due to the different orientation of the thiazole ring within the S pocket. Compound **16** was one of the most active thrombin inhibitors in this series. The X-ray structure of **16** bound to thrombin[18] revealed a good fit of the inhibitor in the active site of thrombin, which was very similar to that observed for compounds **8** and **10**. The sulfur atom of the thiazole ring pointed toward Gly219, avoiding an unfavorable interaction between the thiazole nitrogen atom and the carbonyl oxygen of Gly219, which would be expected in the opposite orientation of P1. An ortho substituent next to the amidine moiety in the thiazole series led to a substantial decrease in potency (cf. **31** vs **16**), which was not observed in the thiophene series (cf. **27**, **29**). The methyl group in **31** might cause an unfavorable orientation of the P1 moiety in the S pocket, for example, by shifting the nitrogen of the thiazole ring close to the carbonyl oxygen of Gly219. This is in line with the finding that a heteroaromatic residue in P1 with an increasing number of polar atoms led to a pronounced decrease in potency. The oxazoles **19** and **20**, oxadiazole **21**, pyrazole **24**, triazole **25**, thiadiazole **26**, and amino thiophene **33** showed only moderate to weak potency and subsequently low antithrombin activity.

The *N*-methyl pyrroles **22** and **23** exhibited good to moderate in vitro potency and in vivo activity. Unexpected was the gain in thrombin versus trypsin selectivity of **22**, which has been described previously.[19] This effect was attributed to the different orientations of the *N*-methyl pyrrole moieties within the S pocket. In the complex of **22** with thrombin, the *N*-methyl group pointed toward Gly219, causing a rearrangement in the protein structure, which is better accommodated in thrombin than in trypsin. In the case of **23**, however, the *N*-methyl group of the pyrrole ring pointed in the opposite direction, as indicated by the X-ray structures of the inhibitors **22** and **23** with thrombin.[20] A similar increase in thrombin versus trypsin selectivity was observed by adding a methyl group ortho to the amidine moiety in **10** (cf. **27** vs **10**; Table 2).

These examples demonstrate that the trypsin/thrombin selectivity of the inhibitors can be improved by modification of the P1 aryl substitution pattern. Table 2 sum-

Table 2. Potency of selected compounds versus thrombin and other serine proteases

Compound	IC_{50} (nM)				
	Thrombin	Trypsin	tPA	Plasmin	FXa
8	1.0	4.8	540	3270	1390
10	1.0	1.6	70	1240	328
14	1.3	1.9	125	1470	251
16	1.4	21	173	201	995
27	1.8	117	687	>20,000	>45,000
Melagatran	69.2	11.9	ND	3060	17,300

ND, not determined.

marizes the potency of selected thrombin inhibitors of our series in comparison to melagatran for thrombin and some related serine proteases. The selectivity versus proteases like tPA, plasmin, and FXa was in most cases good to excellent.

The encouraging in vitro results and the promising in vivo data in rats prompted us to further investigate the most potent compounds in dogs after oral administration in order to assess their gross pharmacodynamic profile (absorption, metabolic stability, half-life, and antithrombin activity in vivo). Melagatran was also included in this study for the sake of comparison. The PK/PD results indicated by the ECT prolongation are depicted in Figure 4. A similar activity ranking of the compounds in rats and in dogs was observed based on their ECT prolongation after oral administration (cf. Table 1 and Fig. 4).

Thrombin inhibitors with a five-membered heterocyclic P1 moiety like furan **14**, thiophenes **10** and **12**, and thiazole **16** showed the highest level of ECT prolongation (area under ECT curves) that we ever observed with this type of compound in dogs. Their antithrombin activity was even more pronounced than those of the corresponding phenyl or pyridyl derivatives **7** and **8**. The moderate in vitro potency of oxazole **20** and thiophene **11** resulted in only moderate in vivo activity in dogs. Additional factors influencing the oral activity of compounds were their degree of absorption, volume of distribution, metabolic stability, and clearance. The values for oral bioavailability were in the range from 35% to 60% in dogs (cf. melagatran 24%). Pyridine **8** had an oral bioavailability of 42%, a terminal half-life of 3–6 h, and a clearance of 0.5 L h^{-1} kg^{-1}. Thiazole **16** exhibited data comparable to those of **8** with an oral bioavailability of 53%, a terminal half-life of 2–8 h, and a clearance of 0.5 L h^{-1} kg^{-1}. The graph in Figure 4 evidently depicts the overall better performance of these novel thrombin inhibitors compared to the reference compound melagatran.

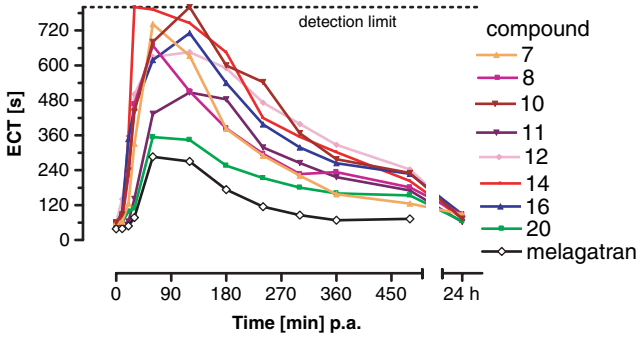


Figure 4. Effect of different P1 scaffolds on the ecarin clotting time in dogs at a dose of 4.64 mg kg^{-1}, po (see Ref. 21).

In summary, the aromatic ring in the amidine bearing P1 moiety of HOOC–CH_2–D-cyclohexylalanyl-3,4-dehydroprolyl–NH–CH_2–aryl-C(=NH)NH_2 was optimized. Five- or six-membered heteroaromatic systems were introduced modifying the basicity of the amidine function and the binding mode of the P1 moiety in the S pocket of thrombin. This optimization program led to thrombin inhibitors with improved potency, which was strongly influenced by the nature of the aromatic ring in P1 and by its substitution pattern. Especially the 2,4-substituted pyridine- (**8**), 2,5- and 3,5-substituted thiophene- (**10** and **12**), 3,5-substituted furan- **14**, and 2,4-substituted thiazole-ring (**16**) in P1 were favorable. The selectivity of the thrombin inhibitors versus trypsin could be improved by additional substituents on P1 (**22**, **27**). The overall improvement in antithrombin activity of these inhibitors compared to melagatran was demonstrated by the remarkably enhanced prolongation of the ecarin clotting time in dogs after oral administration. Selected thrombin inhibitors of this series exhibiting an excellent in vitro and a favorable in vivo profile are very interesting compounds for in-depth pharmacological characterization and suitable candidates for future development.

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10. (a) Synthesis of the building block **5a** for the final compounds **16**, **19**, **30**, (cf. Ref. 5c), and **25** (cf. Ref. 5f); (b) Synthesis of the building block **5b** for the final compounds **8** (cf. Ref. 5e). Compound **9** (Seitz, W.; Mack, H.; Zierke, T.; Böhm, H. J.; Höffken, H. W.; Koser, S.; Pfeiffer, T.; Hornberger, W. WO 9624609, *Chem. Abstr.* **1996**, *125* 276585), compound **10** (Schacht, A. L.; Shuman, R. T.; Smith, G. F.; Wiley, M. R. EP 672658; *Chem. Abstr.* **1995**, *124*, 87791), compounds **11**, **22**, **23**, **27**, **28** (cf. Ref. 5f), compounds **12**, **13**, **14** and **21** (cf. Ref. 5c), compound **18** (cf. Ref. 5e), compound **33** (reaction of 3-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)propanal and malononitrile with subsequent cleavage of the phthaloyl-protecting group; unpublished results); (c) Synthesis of the building block **5c** for the final compounds **22** and **23** (cf. Ref. 5c), compounds **24**, **31** (cf. Ref. 5f); (d) Synthesis of the building block **5d** for the final compounds **17**, **29**, **32** (cf. Ref. 5f), compound **20** (cf. Ref. 5c), and compound **26** (cf. Ref. 5e).
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14. Measurement of ecarin clotting time (ECT): Venous blood is collected from rats or dogs, respectively, at the desired time after administration of the thrombin inhibitor. One hundred microliters of citrated blood is pipetted into a coagulation cuvette and incubated for 2 min at 37 °C in a coagulometer. After the addition of 100 μL of prewarmed (37 °C) ecarin reagent (Pentapharm), the time (ECT) to formation of a fibrin clot is determined.
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16. X-ray structure analysis of thrombin–inhibitor-complex of **8**; X-ray crystallographic data have been deposited with the Brookhaven Protein Data Bank. Deposition code 2ANM.
17. X-ray structure analysis of thrombin–inhibitor-complex of **10**; X-ray crystallographic data have been deposited with the Brookhaven Protein Data Bank. Deposition code 2FES.
18. X-ray structure analysis of thrombin–inhibitor-complex of **16**; X-ray crystallographic data have been deposited with the Brookhaven Protein Data Bank. Deposition code 2FEQ.
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20. The authors have deposited the coordinates with the Brookhaven Protein Data Bank: Deposition code 1O0D (for **23**) and 1NZQ (for **22**).
21. Plasma levels of thrombin inhibitors were determined in dogs by means of ex vivo measurements of ECT in plasma (cf. Ref. 14). The ECT increases linearly with plasma concentration and therefore provides a suitable method for preliminary pharmacokinetic/pharmacodynamic assessment.