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POTENT AND EFFICACIOUS THIENYLAMIDINE-INCORPORATED THROMBIN INHIBITORS

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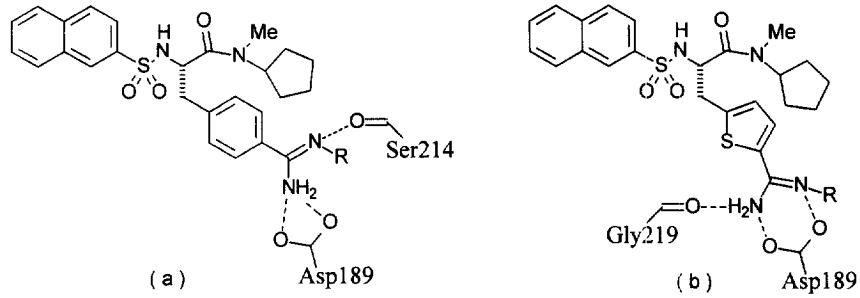
Abstract: Novel thrombin inhibitors incorporating thienylamidine at the P1 position were designed and synthesized. These compounds are potent, trypsin-selective and efficacious in the rat model of venous thrombosis. The proposed P1 binding mode in the thrombin active site was confirmed by X-ray crystallographic analysis.

Thrombin is a trypsin-like serine protease that plays a key role in the blood coagulation process. This enzyme has long been recognized as a central regulator in thrombosis and hemostasis, and its inhibition has thus become a major therapeutic target in the treatment of cardiovascular diseases such as myocardial infarction, deep vein thrombosis and pulmonary embolism.[1] Recently, in our laboratories, the benzamidine **1** and the benzamidrazone **2** (LB30057) were identified as potent and selective inhibitors of thrombin.[2,3] Our previous X-ray crystallographic analysis with **2**-thrombin complex revealed its 4-TAPAP-like[4] binding mode where the P1 amidrazone group interacted with the carboxylate group of the Asp189 through "lateral" salt bridges and hydrogen bonds, as depicted in Figure 1a.[3b]

As part of our research directed towards highly potent and efficacious thrombin inhibitors, we sought to replace the benzamidine portion of **1** with a new side chain that is capable of binding more strongly in the specificity pocket. Our modeling studies based on the structure of **2**-thrombin complex suggested that the substitution of 5-membered aromatic nuclei for the phenylene ring in **1** would place the amidine group facially close to the Asp189, thus resulting in more favorable interaction via "bidentate" hydrogen bonding (Figure 1b).



1, R = H (Ki = 6.8 nM); **2**, R = NH_2 (Ki = 0.38 nM)

3, R = H; **4**, R = NH_2

Figure 1. Schematic drawing of binding modes of inhibitors in the S1 specificity pocket

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On the other hand, the amidrazone function of **4** appeared to be much less favorable in binding due to the steric bulk of the hydrazinyl group. Consequently, we have designed the 2,5-substituted thienyl compound **3** as the thiophene system among heterocycles appeared to have most appropriate size and the 2,5-substitution was most optimal in terms of both bond angle and hydrogen bond length between the amidine and the Asp189.[5]

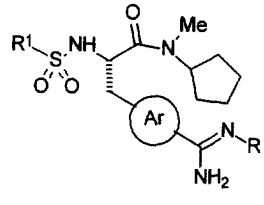
To test this hypothesis, we prepared compound **3** as an initial target and also prepared the amidrazone analog **4** for comparison. As illustrated in Table 1, the thienyl amidine **3** exhibited two-fold increased potency over the benzamidine **1**, while compound **4** displayed only moderate activity that was more than 200-fold weaker than the corresponding phenylene compound **2**. In order to confirm the proposed binding mode, we obtained crystals of the **3**-thrombin complex for X-ray analysis.[7] As shown in Figure 2, the inhibitor was bound in the active site of thrombin in the predicted fashion. The naphthyl and cyclopentyl groups bound in the D- and P-pockets, respectively, remained in the same positions as in the complex of **2**. The thienyl side chain was located in the specificity pocket with both amidinyl nitrogens forming hydrogen-bonds to the carboxylic oxygens of the Asp189 (3.04 and 2.85 Å). The amidine was additionally hydrogen bonded to the backbone carbonyl of Gly219 (2.72 Å) and also indirectly bonded to Phe227 through a water molecule. Despite these fully predicted results of binding mode it is unclear why the thrombin inhibitory activity of **3** is not as improved as expected, while the expected potency were observed with the amidrazone compound **4**. It is presumable that the thiophene ring of **3** has weaker hydrophobic binding affinity in the specificity pocket than that of the phenylene ring of **2**, offsetting the increased binding energy acquired from the bidentate amidine-Asp189 interaction.[8]

Although compound **3** showed somewhat similar thrombin affinity to that of **2**, it demonstrated better antithrombotic activity in the preliminary evaluation *in vivo*. In the rat thromboplastin-induced model of venous thrombosis,[9] **3** was 48% effective in thrombus inhibition at 1 mg/kg iv bolus which is comparable to the highly potent inhibitor **2** (50%[3b]) while marginal efficacy (29%) was seen with **1**.

Having identified a binding mode of **3** and proved its good antithrombotic efficacy, we considered incorporating this new scaffold into other sulfonamide aryl moieties in order to see that thrombin inhibitory activity would be further enhanced. We tentatively chose two aryl replacements, 4-propylphenyl and 6-methoxy-2-naphthyl moieties that were superior in thrombin affinity to the 2-naphthyl group in the series of benzamidrazone-based compounds.[10] Indeed, compounds **6** and **7** afforded 5-6 fold increases over **3** in thrombin inhibition with sub-nanomolar Ki's. These inhibitors also displayed good selectivity against trypsin (250-fold).

Table 1. Thrombin and trypsin inhibitory activities of amidinothienyl alanine derivatives[11]

Compd	R^1	R^2	Ar	Ki_{thr} (nM)[a]	Ki_{tryp} (nM)[b]
1	2-naphthyl	H	1,4-phenylene	6.8	16200
2	2-naphthyl	NH_2	1,4-phenylene	0.38	3200
3	2-naphthyl	H	2,5-thiophene	3.0	153
4	2-naphthyl	NH_2	2,5- thiophene	91	ND
5	4-propylphenyl	H	1,4-phenylene	0.84	2440
6	4-propylphenyl	H	2,5- thiophene	0.60	150
7	6-OMe-2-naphthyl	H	2,5- thiophene	0.49	134



[a] human thrombin. [b] bovine trypsin.

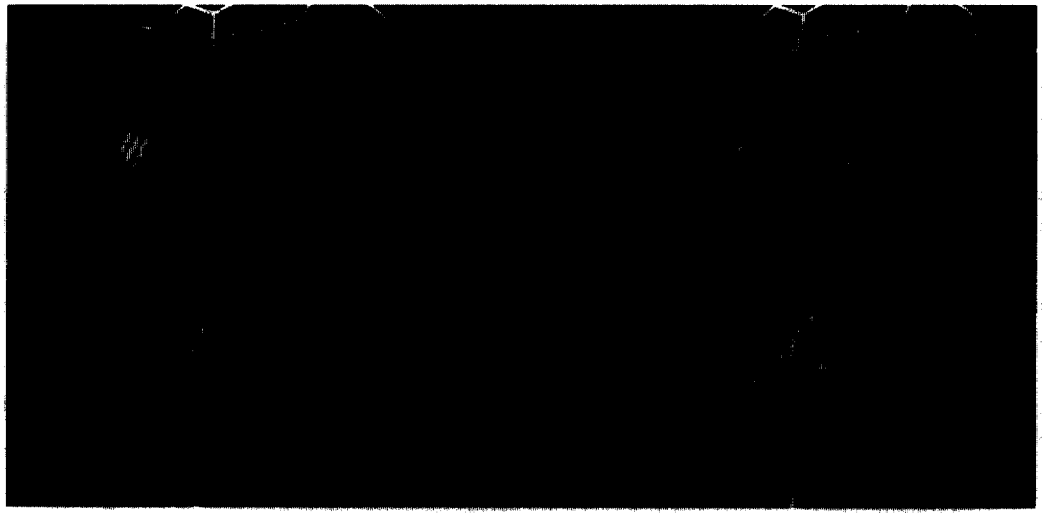
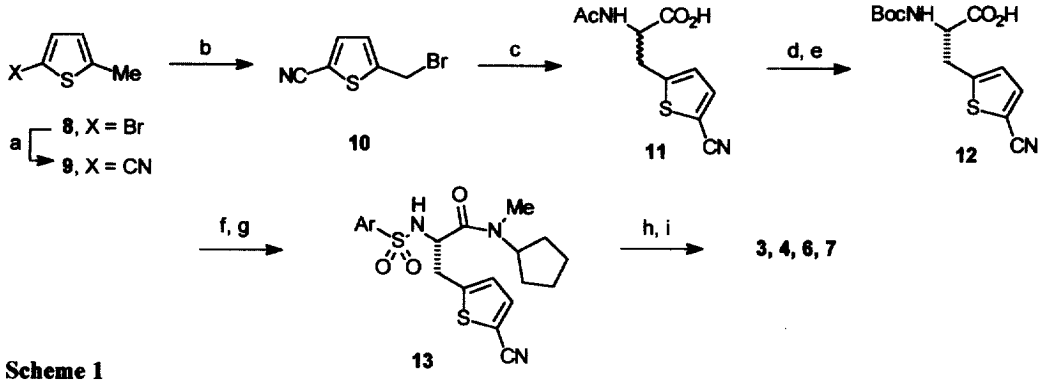


Figure 2. Stereoview of inhibitors **3** (thick) and **5** (thin, purple) bound in the thrombin active site. Some active site residues are omitted for clarity.

Furthermore, 60% of strong inhibition was observed with compound **6** in the rat venous thrombus formation (1 mg/kg iv bolus) while the corresponding benzamidine **5** was only 36% inhibitory.[9] These results indicate not only that the thienylamidines are superior in antithrombotic efficacy to the benzamidines, but also that the potency and efficacy in this series may be further improved by modifying the D-pocket-binding aryl group and possibly employing other P-pocket-binding moieties[12] as well.

In conclusion, we have investigated the 2,5-thienylamidine as a benzamidine surrogate in our TAPAP-like thrombin inhibitors.[13] This new P1 element conferred a strong bidentate interaction with the Asp189 of thrombin and improved *in vivo* antithrombotic activity, while maintaining sufficient trypsin-selectivity. Further optimization and biological activity studies with this series of thrombin inhibitors are currently underway; future communications will describe these results.



Scheme 1

(a) CuCN, DMF, reflux, 76%; (b) NBS, benzoylperoxide, CCl_4, reflux, 86%; (c) i. $EtO_2CCH(NHAc)CO_2Et$, KI (0.05 eq), NaOEt, dioxane, reflux; ii. NaOH (2 eq), H_2O, reflux, 67% 2 steps; (d) acylase, H_2O, pH 6.5, 36 °C; (e) Boc_2O, NaOH, H_2O/dioxane; (f) N,N-cyclopentylmethyl amine-HCl, NMMP, EDC, Hobt, DMF, 32% from **11**; (g) arylsulfonyl chlorides, NMMP, DMF, 88-94%; (h) i. H_2S, py, Et_3N; ii. MeI, CH_3CN, reflux; iii. NH_4OAc (NH_2NH_2 for **4**), MeOH, 67-88%.

Synthesis

The thiophene compounds **3**, **4**, **6**, **7** were prepared employing essentially the same strategy as described in the synthesis of **1** and **2** (Scheme 1).[2,3a] The starting 2-cyanothienyl-3-methyl bromide (**10**) was prepared from bromomethyl thiophene **8** in two steps involving CuCN replacement and subsequent bromination of the methyl group. Condensation with diethyl acetamidomalonate, followed by in situ saponification and decarboxylation provided amino acid **11**. This racemate was enzymatically resolved, and subsequent protection of the free amino group gave **12**. After elaboration of the C- and N-terminals by successive coupling with N,N-cyclopentylmethylamine and arylsulfonyl chlorides, the intermediates of general structure **13** was subjected to a modified Pinner reaction to yield the desired targets. All of the target compounds were obtained as pure acid salts after purification.

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