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BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

# DIBASIC BENZO[*b*]THIOPHENE DERIVATIVES AS A NOVEL CLASS OF ACTIVE SITE DIRECTED THROMBIN INHIBITORS: 2. SIDECHAIN OPTIMIZATION AND DEMONSTRATION OF IN VIVO EFFICACY

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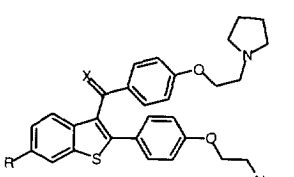
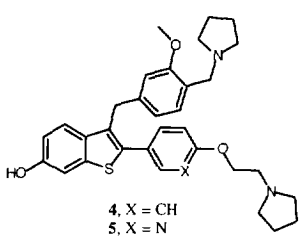
**Abstract:** Potent, subnanomolar thrombin inhibitors **4**, **5**, and **6** are developed through side chain optimization of novel, benzo[*b*]thiophene-based small organic entities **2** and **3** and through SAR additivity studies of the new structural elements identified. X-ray crystallographic studies of **4b**-thrombin complex revealed a hydrophobic and an electrostatic interaction of these new elements with thrombin at the $S_2$ and $S_3$ binding sites. In vitro and in vivo pharmacological studies showed that **4**, **5**, and **6** are potent anticoagulants in human plasma with demonstrated antithrombotic efficacy in a rat model of thrombosis. 

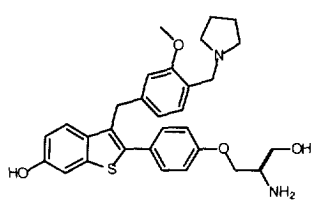
The development of active site directed thrombin inhibitors for antithrombotic therapy has been the subject of extensive research.[2] Most of the recent efforts have been focused on small molecule inhibitors, especially arginine derivatives and their mimics.[3] As part of our continued effort in the development of orally active thrombin inhibitors for the prophylaxis and treatment of venous and arterial thromboembolism,[4] we have identified a new small organic entity **1** ($K_i$ = 374 nM) that distinguishes itself from other reported thrombin inhibitors by its structural novelty, appealing oral bioavailability and exceptional thrombin selectivity.[5] Preliminary structure activity relationship studies have led to clear definition of key structural requirements and to the preparation of the more potent inhibitors **2** ($K_i$ = 234 nM) and **3** ($K_i$ = 22.1 nM).[5] Herein, we wish to report our progress on the sidechain optimization of **2** that has led to the synthesis of **4** ($K_i$ = 0.9 nM), **5** ($K_i$ = 0.5 nM), and **6** ($K_i$ = 0.4 nM) and to the demonstration of their in vivo efficacy in a rat model of thrombosis.




**1**, X = O; R = H
**2**, X = H,H; R = H
**3**, X = H,H; R = OH




**4**, X = CH
**5**, X = N




**6**

**Optimization of the C-3 sidechain of 2.** Other SAR studies suggest that both the aryl ring and the tertiary amine on the C-3 side chain are key structural requirements, since either replacement of the aryl ring

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*PII:* S0960-894X(99)00074-8

with a straight hydrocarbon chain or replacement of the pyrrolidine ring with a cyclopentane ring, a primary or secondary amino group lead to diminished thrombin inhibitory activity.[5] Here we describe the effects of the introduction of additional aryl substituents and the optimal positioning of the tertiary amine functionality on the C-3 side chain. Understanding these effects has been greatly facilitated by the available co-crystal structure of human α-thrombin with **2**, which revealed that the C-3 aromatic ring is positioned at the entrance of the hydrophobic $S_2$ pocket.[6] Consequently, introduction of a bromo substituent at the 3' position results in a seven-fold improvement in binding affinity to thrombin as reflected in an increase in the apparent association constant $K_{ass}$ (Table 1, **2a** vs **2**).[7] Replacement of the bromine with a methyl group provides an equally potent inhibitor (**2b** vs **2a**). However, as the size of the substituent increases, binding affinity drops significantly (**2c–e**), most likely due to unfavorable steric interactions between the substituent and the residues of the $S_2$ binding site, especially when substituents larger than an ethyl group are introduced. While methoxy and nitro substituents enhance potency to a lesser degree than bromine (**2f–g**), hydrogen bond donating substituents compromise the binding affinity significantly (**2h–i**). Introduction of a bromo substituent at the 2' position results in over ten-fold decrease in binding affinity (**2j** vs **2**).

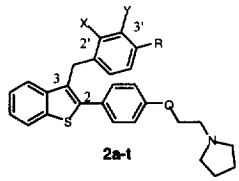
The preference for a tertiary amine on the C-3 side chain[4] suggests that the tertiary amine may be engaged in an electrostatic interaction with thrombin although it was not clear from the crystal structure which residue of the enzyme serves as the anion partner. Cyclization of the amino ethyl portion of the side chain adds no significant effect (**2k–m** vs **2**). Walking the nitrogen around the pyrrolidine ring results in a gradual decrease in binding affinity (**2n–o**), consistent with the electrostatic interaction hypothesis. Chain extension and contraction studies lead to the identification of a methylene linker as providing the optimal spatial relationship between the aryl and pyrrolidine rings (**2p–r**) and result in a four-fold improvement in binding affinity (**2r** vs **2**). Further chain contraction (**2s**) leads to a dramatic drop in binding affinity, apparently due to loss of basicity and the introduction of conformational rigidity which may cause unfavorable interactions between the inhibitor and the enzyme. Replacement of the pyrrolidine in **2r** with dimethylamine (**2t**) also leads to a ten-fold decrease in binding affinity due to loss of lipophilicity and decreased interaction with the lipophilic $S_3$ binding site.

**SAR additivity and X-ray crystallographic studies.** Bridging the structural elements that improve binding affinity provides the following results. Introduction of a hydroxyl group at the C-6 position of the benzo[*b*]thiophene nucleus of **2r** results in a six-fold improvement in $K_{ass}$ (Table 2, **4a** vs **2r** in Table 1), consistent with the observations with **2** and **3**. Introduction of a bromo substituent on the 3' site of **4a** results in an additional eight-fold improvement in $K_{ass}$ (**4b** vs **4a**). As with the SAR studies with **2**, replacement of the bromine with a methyl group leads to an equally potent inhibitor (**4c** vs **4b**). Replacement of the bromine with more hydrophilic groups leads to significant decreases in $K_{ass}$ (**4d–f** vs. **4b**). However, in contrast to the studies with **2a** and **2f**, replacement of the bromine with a methoxy group results in a slight increase in $K_{ass}$ (**4** vs.**4b**). This observation is further supported by the $K_i$ data (Table 2) [8] which suggest that **4** appears to be a more potent inhibitor than **4b** or **4c**. Very likely, a synergistic effect has come into play since the methoxy group is bulkier than either the bromine or methyl substituent and steric repulsion may have helped to orient the pyrrolidinomethyl substituent into its active conformation as suggested by X-ray crystallographic studies (*vide infra*). As shown in Table 2, **4** proves to be a subnanomolar inhibitor, more potent than efegatran (**7**), a potent tri-peptide arginal based thrombin inhibitor which has been shown to be clinically active.[4c]

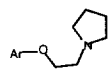
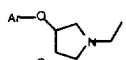
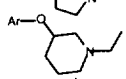
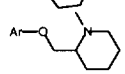
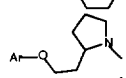
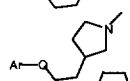
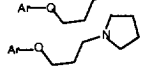
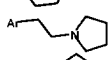
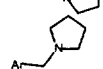
To delineate the nature of the interactions between the C-3 side chain and thrombin, the structure of **4b**/human α-thrombin complex was determined by X-ray crystallography.[9] As shown in Figure 1, **4b** binds to the active site of thrombin in a very similar orientation to the structure disclosed.[5c] The benzo[*b*]thiophene nucleus occupies the $S_1$ specificity pocket with the 6-hydroxyl group being engaged in a hydrogen-bonding interaction with the sidechain carboxyl group of Asp 235 [189].[9b] The C-2 side chain branches away from the active site and is exposed to the solvent. The C-3 side chain occupies the $S_2$ and $S_3$ binding sites and, together

with the C-2 side chain, sandwiches the indole of Trp 86 [60D]. The bromine fits into the hydrophobic pocket formed by the side chains of Leu 132 [99], Tyr 83 [60A], Trp 86 and His 79 [57] ($S_2$ binding site). The C-3 side

**Table 1. SAR summary of modifications on the C-3 sidechain of 2**

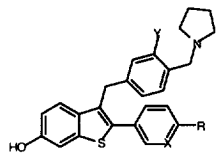


2a-t

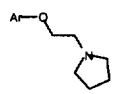
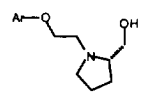
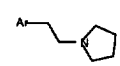
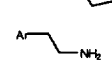
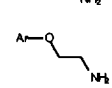
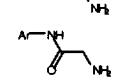
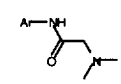
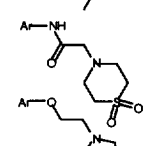
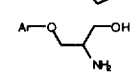
| compd | X | Y | Ar-R | $K_{ass}$[a] ($10^6$ L/mole) |
|---|---|---|---|---|
| 2 | H | H |  | 3.4 ± 0.6 |
| 2a | H | Br | | 25 ± 3 |
| 2b | H | Me | | 27 ± 7 |
| 2c | H | Et | | 6.4 ± 1.6 |
| 2d | H | n-Pr | | 0.67 ± 0.44 |
| 2e | H | n-Bu | | 0.24 ± 0.16 |
| 2f | H | OMe | | 12 ± 2 |
| 2g | H | $NO_2$ | | 9.6 ± 1.7 |
| 2h | H | OH | | 1.1 ± 0.1 |
| 2i | H | $NH_2$ | | 1.2 ± 0.3 |
| 2j | Br | H | | 0.27 ± 0.01 |
| 2k | H | H |  | 3.1 ± 0.2 |
| 2l | H | H |  | 3.1 ± 0.4 |
| 2m | H | H |  | 4.9 ± 0.3 |
| 2n | H | H |  | 2.7 ± 0.0 |
| 2o | H | H |  | 0.85 ± 0.12 |
| 2p | H | H |  | 0.59 ± 0.07 |
| 2q | H | H |  | 5.9 ± 0.1 |
| 2r | H | H |  | 14 ± 5 |
| 2s | H | H |  | 0.04 ± 0.01 |
| 2t | H | H |  | 1.4 ± 0.5 |

[a] Reference 7.

**Table 2. SAR summary of modifications on the C-3 and C-2 sidechains of 4**



4, 4a-m, 5, 6

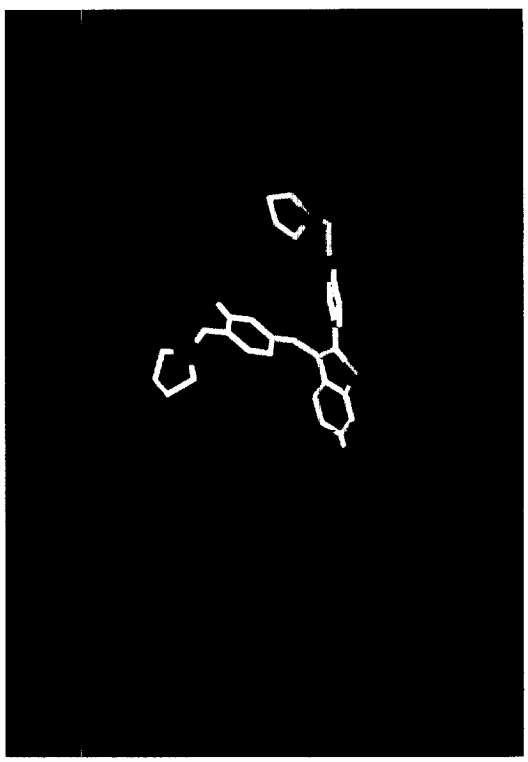
| compd | X | Y | Ar-R | $K_{ass}$[a] ($10^6$ L/mole) | $K_i$[b] (nM) |
|---|---|---|---|---|---|
| 4 | CH | OMe |  | 888 ± 300 | 0.9 |
| 4a | CH | H | | 81.2 ± 6.0 | 4.1 |
| 4b | CH | Br | | 671 ± 177 | 2.1 |
| 4c | CH | Me | | 793 ± 232 | 1.9 |
| 4d | CH | OH | | 39.1 ± 4.7 | 5.1 |
| 4e | CH | CN | | 25.8 ± 6.3 | 7.2 |
| 4f | CH | $CH_2NH_2$ | | 27.5 ± 5.5 | 11 |
| 4g | CH | OMe |  | 1838 ± 1078 | 0.5 |
| 4h | CH | OMe |  | 1071 ± 408 | 0.3 |
| 4i | CH | OMe |  | 84.4 ± 48.6 | 5.1 |
| 4j | CH | OMe |  | 170 ± 51 | 3.8 |
| 4k | CH | OMe |  | 122 ± 27 | 7.0 |
| 4l | CH | OMe |  | 273 ± 7 | 2.3 |
| 4m | CH | OMe |  | 741 ± 405 | 0.9 |
| 5 | N | OMe |  | 1182 ± 482 | 0.5 |
| 6 | CH | OMe |  | 573 ± 277 | 0.4 |
| 7 | Efegatran | | | 668 | 5.5 |

[a] Reference 7, [b] Reference 8.

chain pyrrolidine ring is surrounded by the hydrophobic side chains of Ile 215 [174], Leu 132 and Trp 263 [215] ($S_3$ binding site) and assumes a conformation perpendicular to the C-3 aryl ring. It is not obvious what this basic group may be interacting with. The only polar group in close proximity is the hydroxyl group on the Tyr 83 side chain. Possibly an electrostatic interaction exists between this Tyr 83 hydroxyl oxygen and the pyrrolidine nitrogen as a result of a proton transfer upon inhibitor binding. This could explain not only the requirement for an amine on the C-3 side chain, but also the observation that the contracted side chain binds better than the equivalent extended one. The former structural change positions the pyrrolidine nitrogen 1.0 Å closer to the Tyr 83 hydroxyl oxygen (3.4 Å compared to 4.4 Å).

**Modifications on the C-2 side chain of 4.** Based on the structure of **4b** co-crystallized with thrombin, the C-2 side chain of **4** is partially sandwiched between the side chains of Trp 86 and Glu 238 on the protein surface. Although it is possible that the tertiary amine may be engaged in an electrostatic interaction with the side chain carboxyl group of Glu 238, no specific interactions are observed. Introduction of a hydroxymethyl group at the pyrrolidine ring or contraction of the ethoxy linker to an ethylene linker results in more potent inhibitors (Table 2, **4g** and **4h** vs **4**). Replacement of the pyrrolidine with a primary amine leads to over ten-fold decrease in $K_{ass}$ (**4i** and **4j** vs **4h** and **4**).

**Figure 1.** X-ray crystal structure of **4b** bound to the active site of human α-thrombin. The principal residues that interact with the inhibitor are shown and labeled based on the thrombin numbering system from N-terminal to C-terminal of human α-thrombin.



Interestingly, introduction of a hydroxymethyl group at the α-position of the primary amine of **4j** regains the binding affinity by over three-fold (**6** vs **4j**). Alternatively, replacement of the ether linkage of **4j** with an amide linkage reduces the binding affinity (**4k** vs **4j**). When the primary amine is replaced by dimethyl amine and dioxothiomorpholine, the binding affinity gradually increases to a level equivalent to **4** (**4k–m**). These results appear to suggest that the pyrrolidine hydrocarbons in the C-2 side chain of **4** enhance the binding affinity either through its interaction with the side chain indole of Trp 86 or by "pushing" the inhibitor into the binding site due to its hydrophobic nature. Finally, isosteric replacement of the C-2 aryl group with a pyridine results in a more potent inhibitor (Table 2, **5** vs **4**). Although the C-2 side chain modifications do not result in dramatic improvements in binding affinity, they provide a number of potent thrombin inhibitors comparable to **4** and **7** (efegatran) with a wide range of physicochemical properties which may become significant in subsequent pharmacological studies.

**In vitro anticoagulant activity and in vivo antithrombotic efficacy studies.** In parallel with the thrombin binding studies, the functional relevance of this series of inhibitors was routinely tested in standard clotting assays including the thrombin time (TT), activated partial thromboplastin time (aPTT) and prothrombin

time (PT) assays.[10] Selected results from these studies are given in Table 3. The ability of these inhibitors to bind thrombin with high affinity generally translates into potent anticoagulant activity in human plasma. Thrombin clotting times (using 0.015 μM human thrombin) are prolonged by twofold with 0.02–0.03 μM concentrations of the most potent benzo[*b*]thiophene inhibitors. This stoichiometry reflects the tight-binding nature of these inhibitors. The effects on thrombin time compare very favorably with efegatran (**7**).[4b] The most potent benzo[*b*]thiophene inhibitors prolong the aPTT and PT by twofold at concentrations in the 0.3–0.6 μM range, which are clearly more potent than the aPTT and PT effects produced by efegatran (**7**). Compounds that demonstrate comparable or superior anticlotting potency to efegatran (**7**) are also studied in a rat arterial-venous shunt model of thrombosis.[11] Infusion of **6** at 0.3–5 mg/kg/hr reduces the clot size in a dose-related fashion similar to that observed with efegatran, **7** (Figure 2). In vivo potency ($ED_{50}$ value is defined as the infusion dose that reduces the clot weight by 50%) of **6** approaches that of **7** and exceeds that of **4** and **5** (Table 3).

**Figure 2**. Antithrombotic effect of **6** relative to Efegatran (**7**) in the rat AV shunt model of thrombosis.

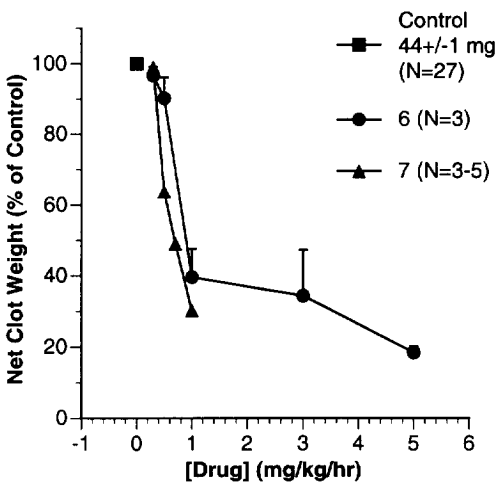



**Table 3.** Summary of the anticoagulant and antithrombotic properties of selected benzothiophene thrombin inhibitors

| compd | 2xTT[a] (μM) | 2xaPTT[a] (μM) | 2xPT[a] (μM) | ED50[b] (mg/kg/h) |
|---|---|---|---|---|
| 4 | 0.026 ± 0.003 | 0.59 ± 0.07 | 0.56 ± 0.17 | 8.8 |
| 4a | 0.098 ± 0.045 | 2.8 ± 0.4 | 2.4 ± 0.7 | |
| 4b | 0.068 ± 0.007 | 2.1 ± 0.6 | 1.8 ± 0.6 | |
| 4c | 0.040 ± 0.014 | 1.0 ± 0.0 | 0.71 ± 0.05 | |
| 4d | 0.16 ± 0.02 | 4.0 ± 0.6 | 3.3 ± 1.5 | |
| 4g | 0.024 ± 0.003 | 0.54 ± 0.12 | 0.44 ± 0.02 | |
| 4h | 0.024 ± 0.004 | 0.42 ± 0.11 | 0.52 ± 0.15 | |
| 4m | 0.033 ± 0.001 | 0.61 ± 0.01 | 0.49 ± 0.05 | |
| 5 | 0.021 ± 0.006 | 0.31 ± 0.02 | 0.42 ± 0.07 | 6.6 |
| 6 | 0.039 ± 0.003 | 0.66 ± 0.05 | 0.60 ± 0.16 | 2.3 |
| 7 | 0.033 | 1.82 | 2.35 | 0.7 |

[a]Reference 10, [b]Reference 11.

In conclusion, side chain optimization and SAR additivity studies of **2** have led to the synthesis of **4**, **5**, **6**, and a number of other dibasic benzo[*b*]thiophene derivatives as potent, active site directed thrombin inhibitors with demonstrated in vitro anticoagulant activity and in vivo antithrombotic efficacy in a rat model of thrombosis. Continued SAR studies of the pharmacodynamics, pharmacokinetics and metabolism of this series of thrombin inhibitors and evaluation of their potential as orally active antithrombotic agents for clinical development are in progress and will be disclosed in due course.

## References and Notes

1. To whom correspondence should be addressed. Corresponding address: Bristol-Myers Squibb Pharmaceutical Research Institute, P.O.Box 5400, Princeton, New Jersey 08543-5400, U.S.A.
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6. Unpublished observations. **2** binds to thrombin in a similar fashion as the structure disclosed in 5c.
7. The binding of the inhibitors to human thrombin was measured as apparent $K_{ass}$ values using the inhibition of thrombin hydrolysis of Bz-Phe-Val-Arg-pNA, as previously described.[4b] Values are given as the mean of triplicate determinations, showing the standard deviation.
8. Jordan, S. P., Waxman, L., Smith, D. E., Vlasuk, G. P. *Biochem.* **1990**, *29*, 11095. The velocity of substrate hydrolysis in the presence and absence of inhibitor was measured in a total volume of 250 μL of 20 mM Tris-Cl pH 7.5, 0.15 M NaCl, 2 mM $CaCl_2$, 0.1%BSA by following the release of *p*-nitroaniline from the substrate S2288 using a Vmax plate reader (Molecular Devices). The thrombin concentration was 0.2 nM. Stock solutions of the inhibitor were prepared in 10% DMSO. The $K_m$ for substrate hydrolysis was determined by nonlinear regression analysis using JMP© statistical software and assuming Michaelis-Menten kinetics. The $K_m$ for S2288 under these conditions was 3.3 ± 0.2 μM. $K_i$s were determined by nonlinear regression analysis using Morrison's equation for tight-binding inhibition.
9. Detailed description of the X-ray structure will be provided in a future publication. X-ray experiment: Crystals of the ternary complex of the thrombin, hirugen and **4b** were obtained by co-crystallization using conditions as described.[9a] Diffraction data set was collected by using RAXIS IIc fuji imaging plate system. The reflections were reduced with the DENZO software package. The intensities were scaled with SCALEPACK. 95% of all reflections that are theoretically possible at 2 Å resolution were obtained with an $R_{merge}$ of 7.7%. Crystallographic refinement was performed using the program X-PLOR. The inhibitor molecule was positioned into the active site based on the difference electron density map. Current crystallographic R-value is 22.4% ($R_{free}$ 28.2%). (a). Chirgadze, N. Y.; Clawson, D. K.; Gesellchen P. D.; Hermann R. B.; Kaiser R. E.; Olkowski J. L.; Sall D. J.; Schevitz R. W.; Smith G. F.; Shuman R. T.; Wery J. P.; Jones N. D. **1992**, American Crystallographic Association Meeting 20: 116 [Abstr. PB311]. (b). The thrombin residue numbering used in this study is a sequential numbering scheme starting from the N-terminus of thrombin. Selected residue numbers based on the numbering system reported for chymotrypsinogen[9c] are given in brackets. (c). Hartley, B. S.; Shotton, D. M. In *The Enzymes*; Boyer, P. D., Ed.; Academic: New York, 1971; Vol. III, pp 323–373.
10. Anticoagulant activities were measured in human plasma, as previously described,[4b] by interpolating effects at different inhibitor concentrations to determine the concentration required to prolong the clotting times by two fold (values are the mean of triplicate determinations, with standard deviation).
11. Experiments with the A-V shunt model were performed with slight modifications of the methods of: Smith, J. R.; White, A. M. *Br. J. Pharmac.* **1982**, *77*, 29. Drug or vehicle was infused for 15 min prior to opening the shunt and was continued for an additional 15 min after the shunt was opened. Net clot weights from drug-treated animals were expressed as a percent of the vehicle-treated animals run in the same experiment.