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Communications to the Editor

Dibasic Benzo[*b*]thiophene Derivatives as a Novel Class of Active Site-Directed Thrombin Inhibitors. 1. Determination of the Serine Protease Selectivity, Structure−Activity Relationships, and Binding Orientation

Daniel J. Sall,* Jolie A. Bastian, Stephen L. Briggs, John A. Buben, Nickolay Y. Chirgadze, David K. Clawson, Michael L. Denney, Deborah D. Giera, Donetta S. Gifford-Moore, Richard W. Harper, Kenneth L. Hauser, Valentine J. Klimkowski, Todd J. Kohn, Ho-Shen Lin, Jefferson R. McCowan, Alan D. Palkowitz, Gerald F. Smith, Kumiko Takeuchi, Kenneth J. Thrasher, Jennifer M. Tinsley, Barbara G. Utterback, Sau-Chi B. Yan, and Minsheng Zhang

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

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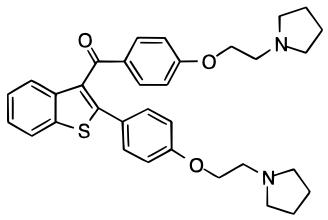
Introduction. The biochemistry of thrombus formation and the thrombotic disorders that can result from aberrant coagulation have been well documented.[1,2] The serine protease thrombin is an arginine endopeptidase and plays a pivotal role in thrombosis through fibrin formation and the activation and recruitment of platelets.[3] Accordingly, inhibitors of thrombin have become an intensely pursued therapeutic target for the treatment of thrombotic complications. On the basis of the limitations of warfarin,[4] a vitamin K antagonist and the only currently available oral anticoagulant, we have had a long standing interest in the discovery of oral, direct-acting thrombin inhibitors that can be used in the chronic treatment of thrombotic disorders.

A number of recent approaches to the discovery of orally active thrombin inhibitors have their origins in the tripeptide sequence D-Phe-Pro-Arg. Incorporation of a C-terminal electrophile in the form of an aldehyde was first accomplished by Bajusz *et al.* in 1978 leading to the transition state analog D-MePhe-Pro-Arg-H.[5] With the aid of X-ray crystallography and molecular modeling,[6] great strides have been made recently in the design of more potent and selective inhibitors.[7,8] However, there have been very few reports describing the progression of these basic, peptidic agents into clinical trials as oral anticoagulants. For example, it was reported from phase I clinical trial data that the profile for the tripeptide arginal (*R*)-1-Piq-Pro-Arg-H ((*R*)-1-Piq: (1*R*)-*cis*-(4a*S*,8a*S*)-perhydroisoquinoline-1-carboxylic acid) was not optimal for an oral agent.[9] Due to the apparent limitations of this class of agents and the unmet need for a direct-acting, orally active anticoagulant, the search for novel structural classes of thrombin inhibitors has continued. In this communication, we present the identification, thrombin selectivity, structure−activity relationships (SAR), and oral bioavailability of a series of 2,3-disubstituted benzothiophene derivatives that represent a novel class of inhibitors. The interaction of these agents with the active site of thrombin was characterized by X-ray crystallography and will be discussed as well.

Results and Discussion. In an effort to identify novel classes of thrombin inhibitors, screening efforts at Lilly Research Laboratories were undertaken. Among the hits in a thrombin amidase screen[10] was the benzo[*b*]thiophene derivative **1**, consisting of a benzo[*b*]-thiophene nucleus with basic side chains at both the C-2 and C-3 positions.[11] Although compound **1** exhibits



1
K_{ass} = 0.43 ± 0.007 L/mole x 10^6
K_i = 373.5 ± 38.6 nM

only weak thrombin inhibition (K_{ass} = 0.43 ± 0.07 × 10^6 L/mol),[12] its novel structure is intriguing due to the lack of an active site director such as a guanidine or amidine and its nonpeptidic nature, two hallmarks of

* To whom correspondence should be addressed.

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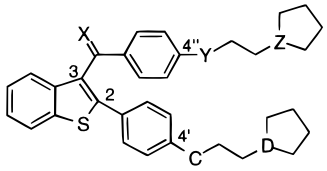
Table 1. Serine Protease Inhibition and Thrombin Specificity Profile for Inhibitor **1**

serine protease	K_{ass}[a,b] ($\times 10^6$ L/mol)	K_{ass}(thrombin)/ K_{ass}(serine protease)
human α-thrombin	0.43 ± 0.07	
bovine trypsin	0.003	143
human fXa	0.01	43
human plasmin	0.003	143
human nt-PA	0.03	14
human urokinase	<0.0009	>400

[a] Represents the apparent association constant as measured by the methods of Smith *et al.*[12] [b] K_{ass} value for each serine protease, except thrombin, is the average of two determinations, where the variation in the assay is ±10%. For thrombin, K_{ass} is a mean of $n = 3$, showing the standard deviation.

Table 2. Thrombin Inhibition by Derivatives **2**−**7**: Effect of Heteroatoms in the C-2 and C-3 Side Chains



2-7

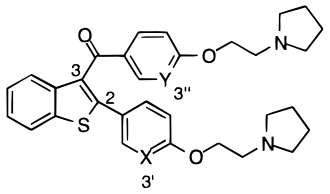
entry	C	D	X	Y	Z	K_{ass}[a] ($\times 10^6$ L/mol)
1	O	N	O	O	N	0.43 ± 0.07
2	O	CH	O	O	N	<0.005
3	O	N	O	O	CH	0.01 ± 0.001
4	CH_2	N	O	O	N	0.09 ± 0.008
5	O	N	O	CH_2	N	0.84 ± 0.07
6	O	N	CH_2	O	N	0.77 ± 0.07
7	O	N	H, H	O	N	3.43 ± 0.55

[a] Represents the apparent association constant as measured by the methods of Smith *et al.*[12] K_{ass} values are the mean of $n = 3$, showing the standard deviation.

classical active site-directed inhibitors. In light of these attributes, this novel thrombin inhibitor (**1**) was characterized pharmacologically. Despite its structural uniqueness, inhibitor **1** binds in a competitive fashion, as confirmed by examination of the double-reciprocal plot of $1/v$ vs $1/S$ ($K_i = 373.5 \pm 38.6$ nM),[13] indicating interaction at the active site of the enzyme. Realizing that thrombin selectivity relative to other physiologically relevant serine proteases is a necessary criteria for a chronic, oral anticoagulant, we determined the enzyme specificity profile of inhibitor **1**. As indicated in Table 1, agent **1** selectively inhibits thrombin relative to other serine proteases, including trypsin, other coagulation factors, and the fibrinolytic enzymes.[12] For the purposes of determining the potential usefulness of the series as oral agents, derivative **1** was studied *in vivo* and was found to be 15% bioavailable in the rat.[14]

Early SAR studies focused on identifying the critical structural elements responsible for thrombin inhibition. Beginning with the C-2 and C-3 side chains, the role of each heteroatom was determined (Table 2). Replacing either of the basic nitrogens with a neutral carbon (**2** and **3**) reduces potency at least 40-fold, suggesting that the two basic side chains are optimal for thrombin inhibitory activity. While substitution of carbon for the ether oxygen in the C-4′ side chain also reduces potency (**4**), this same structural manipulation in the C-4″ arm affords a 2-fold more potent derivative in compound **5** (Table 2). Conversion of the C-3 ketone to the corresponding olefin (**6**) results in 2-fold higher activity, indicating that a hydrophilic and/or hydrogen bond-

Table 3. Thrombin Inhibition by C-2 and C-3 Pyridyl Derivatives **8** and **9**



8, 9

entry	X	Y	K_{ass}[a] ($\times 10^6$ L/mol)
1	CH	CH	0.43 ± 0.07
8	N	CH	0.13
9	CH	N	0.63 ± 0.19

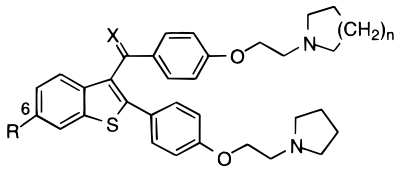
[a] Represents the apparent association constant as measured by the methods of Smith *et al.*[12] K_{ass} values are the mean of $n = 3$, showing the standard deviation except for compound **8** which is the mean of $n = 2$, where the variability of the assay is ±10%.

accepting atom is not critical at this site. Reductive deoxygenation to the methylene derivative **7** increased potency by 8-fold, possibly suggesting that steric factors or conformational effects due to the hybridization state at the carbon atom are important for binding. Pyridyl derivatives **8** and **9** were prepared to study the impact of incorporating hydrophilic nitrogens into the C-2 and C-3 phenyl rings (Table 3). While incorporation of the heteroatom in the C-2 phenyl resulted in a 4-fold loss in activity (**8**), pyridyl analog **9** was modestly more active. Tracking the impact of the C-2 and C-3 side chain modifications on oral bioavailability, *in vivo* studies showed derivative **7** to be 52% bioavailable.[14]

Studies aimed at defining the critical structural elements within the benzo[*b*]thiophene nucleus were directed toward both the 6-membered A-ring and the 5-membered B-ring. Elimination of the A-ring to afford the 2,3-disubstituted thiophene (**10**, $K_{ass} < 0.005 \times 10^6$ L/mol) or saturation to the 4,5,6,7-tetrahydrobenzo[*b*]-thiophene analog (**11**, $K_{ass} = 0.006 \times 10^6$ L/mol) resulted in at least 70-fold losses in potency relative to lead compound **1**. Concentrating on the 5-membered B-ring, removal of the sulfur atom resulted in the more flexible phenyl derivative **12** which decreased potency 40-fold ($K_{ass} = 0.01 \times 10^6$ L/mol). Reversal of the substitution pattern at C-2 and C-3 as in derivative **13** caused at least a 200-fold loss in activity ($K_{ass} < 0.005 \times 10^6$ L/mol). To determine the role of the sulfur atom in the 5-membered ring, the corresponding benzofuran, indole, naphthyl, and 1,2-dihydronaphthyl derivatives were prepared and evaluated. Each was at least 15-fold less potent than the parent benzo[*b*]thiophene.[15] Taken together, the data would indicate that the benzo[*b*]-thiophene nucleus with the appropriate 2,3-disubstitution pattern is a preferred structural element for thrombin inhibition within the series.

The unsubstituted A-ring of the benzo[*b*]thiophene nucleus provides a number of sites for substitution. Studying the effect of aromatic substituents on thrombin inhibitory activity, we targeted the C-6 position since oxidative metabolism is known to occur at this site. Incorporation of a halogen (**14**; Table 4) or a methoxy group (**15**) resulted in 4−20-fold losses in activity. The hydrogen bond-donating hydroxyl (**16**), however, increased potency 4-fold. Reductive deoxygenation of the C-3 ketone increased activity 10-fold (**17**). Introduction

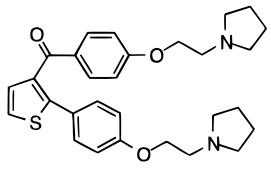
Table 4. Thrombin Inhibition by Derivatives **14**−**22**: Effect of C-6 Substitution



14-21

entry	R	*n*	X	K_{ass}[a] ($\times 10^6$ L/mol)
1	H	1	O	0.43 ± 0.07
14	Cl	1	O	0.024 ± 0.001
15	OMe	1	O	0.12 ± 0.01
16	OH	1	O	1.86 ± 0.14
17	OH	1	H, H	20.2 ± 0.8
18	$CONH_2$	2	O	0.026
19	CH_2NH_2	2	H, H	0.35
20	CH_2OH	2	H, H	<0.005
21	H	2	O	0.16 ± 0.01
22	H	2	H, H	1.88

[a] Represents the apparent association constant as measured by the methods of Smith *et al.*[12] K_{ass} values are the mean of $n = 3$, showing the standard deviation, except for compounds **18**−**20** and **22** which are the mean of $n = 2$, where the variability of the assay is ±10%.



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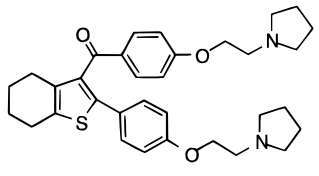

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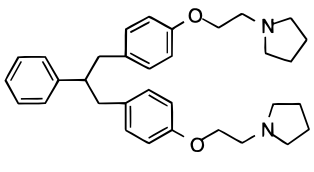

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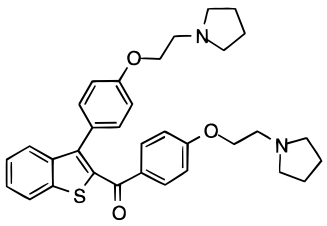


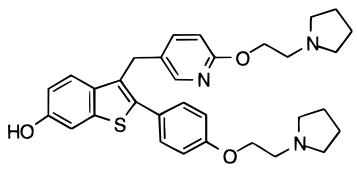
13

of other hydrophilic groups such as the primary amide (**18**), aminomethyl (**19**), or hydroxymethyl (**20**) afforded compounds of lower potency relative to the parent compounds **21** and **22**.

The inhibitory activity of the screening hit (**1**) was enhanced through a variety of structural modifications. Combining each of the advantageous structural features described above into a single molecule led to derivative **23** (Table 5). Its activity ($K_{ass} = 24.3 \times 10^6$ L/mol; Table 5) represents a 50-fold enhancement over the lead structure **1,** suggesting that the SAR for the series is additive. This improvement in potency was achieved with increased levels of thrombin selectivity relative to the screening hit **1** (Table 5). Additionally, derivative **23** displayed competitive kinetics ($K_i = 9.9 \pm 1.2$ nM)[13] and was 9% bioavailable.[14] Comparison of the K_i values shows that compound **23** is 35-fold more potent than lead structure **1**. The difference between this value and the value of 50-fold in the comparison between K_{ass} numbers could be due to the different thrombin substrates that are used in each assay.

X-ray Crystallographic Studies.[16] To gain a better understanding of the interaction of this series with the enzyme, derivative **23** was cocrystallized with human

Table 5. Thrombin Inhibitory Activity and Serine Protease Selectivity for Derivative **23**



23

serine protease	K_{ass}[a,b] ($\times 10^6$ L/mol)	K_{ass}(thrombin)/ K_{ass}(serine protease)
human α-thrombin	24.3 ± 2.0	
bovine trypsin	<0.009	>2700
human fXa	0.015	1620
human plasmin	<0.009	>2700
human nt-PA	<0.009	>2700
human urokinase	<0.009	>2700

[a] Represents the apparent association constant as measured by the methods of Smith *et al.*[12] [b] K_{ass} value for each serine protease, except thrombin, is the average of two determinations, where the variation in the assay is ±10%. K_{ass} values for thrombin is the mean of $n = 3$, showing the standard deviation.

α-thrombin. Crystals of the ternary complex between human α-thrombin, hirugen, and compound **23** were obtained using conditions similar to those previously described.[17] The crystals were isomorphous with those reported. A set of X-ray diffraction data was collected using a RAXIS IIc defractometer.[18] The reflections were reduced using the HKL software package;[19] 98% of all reflections that are theoretically possible at 2.1 Å resolution were obtained with a R_{merge} of 7.0%. Crystallographic refinement was performed using the program X-PLOR.[20] The inhibitor molecule was positioned in the active site based on the difference electron density map. The crystallographic *R*-value was 21.3%.

The interaction of inhibitor **23** with the principal active site residues of thrombin is shown in Figure 1.[21] Unexpectedly, the hydrophobic benzo[*b*]thiophene nucleus binds in the specificity pocket (S_1) of the enzyme, the site at which the basic side chain of arginine within D-Phe-Pro-Arg-H interacts. This S_1 pocket appears to have specific shape requirements for accepting hydrophobic groups. The thiophene analog **10** likely does not bind as deeply as the benzo[*b*]thiophene derivative **1**, leading to decreased potency. The lower activity of the 4,5,6,7-tetrahydrobenzo[*b*]thiophene relative to its aromatic counterpart could be due to unfavorable steric interactions, although aminocyclohexanes and *N*-guanidinopiperidines have been successfully employed at P_1.[7a,c,d] The orientation of the benzo[*b*]thiophene nucleus in the S_1 binding site allows for the formation of a hydrogen bond between the C-6 hydroxyl and the carboxyl group of Asp189.[22] The distance between the oxygen atoms of the C-6 hydroxyl and the Asp189 γ-carboxyl is 2.7 Å, suggesting that the decreased activity of analogs **18**−**20** is possibly due to overcrowding caused by the additional steric bulk of the substituent. The C-3 side chain of inhibitor **23** spans the S_2 and S_3 pockets. This arrangement positions the pyridyl ring near the entrance of the hydrophobic S_2 pocket and places the pyrrolidine in the S_3 binding region. Due to the similarities in the electron densities of carbon and nitrogen, it was not possible to definitively assign the nitrogen atom in the C-3 pyridyl ring. In Figure 1, the pyridyl nitrogen was arbitrarily assigned so that it resided in the more hydrophilic environment. Although

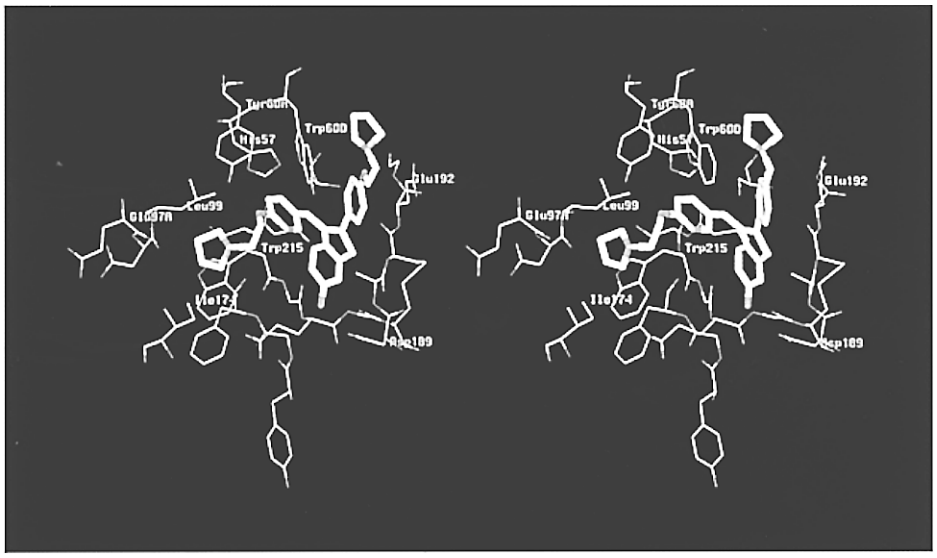


Figure 1. Stereoview of the X-ray crystal structure in which inhibitor **23** is bound in the active site of human α-thrombin.[22] The principal interactions between the inhibitor and the active site residues are shown. The proximal pocket (S_2) is formed by thrombin residues Trp215, Leu99, His57, Tyr60A, and Trp60D, while the distal binding site (S_3) is composed of residues Trp215, Ile174, and Glu97A−Leu99.[22]

not specifically resolved, the critical presence of the pyrrolidine nitrogen in the C-3 side chain could be attributed to a π-cationic interaction with the indole side chain of Trp215 or to a hydrogen bond interaction with the phenolic OH of the Tyr60A side chain. The C-2 chain of the inhibitor branches away from the active site and is located between the side chains of Trp60D and Glu192. The interactions of the C-2 pyrrolidine with the enzyme are not clear since it is predominantly exposed to solvent. Together, the aromatic rings of the C-2 and C-3 side chains begin to sandwich the indole ring of Trp60D.

The observed binding orientation for inhibitor **23** is quite novel when compared to the traditional active site-directed thrombin inhibitors such PPACK, MD-805, NAPAP, and the "retro-binders".[6] The most notable differences include the lack of a basic group at the P_1 position, minimal occupancy of the hydrophobic S_2 site, incorporation of a basic group in the broad hydrophobic S_3 site, and no apparent hydrogen bonding with the protein backbone of residues Ser214−Gly216. A detailed structural comparison of this series and other thrombin active site inhibitors is in preparation.[16]

Conclusion. The dibasic benzo[*b*]thiophene derivatives described in this communication represent a structurally novel class of active site-directed thrombin inhibitor. Preliminary studies have shown that they are thrombin selective and orally bioavailable. Initial SAR studies have identified structural elements that are critical for interaction with the enzyme leading to a 50-fold enhancement in thrombin inhibition over the initial screening hit. The interaction of this series with the thrombin active site has also been characterized by X-ray crystallography. Its binding orientation is distinct from any previously reported active site-directed inhibitors. Future communications will describe the optimization of this series of novel thrombin inhibitors leading to *in vivo* efficacy in animal models of thrombosis.

Acknowledgment. We would like to thank the Physical Chemistry Department of Lilly Research Laboratories for their careful characterization of the compounds reported in this manuscript. In particular, we are grateful to John Paschal and Larry Spangle for making the regiochemical assignments for various intermediates using ^{1}H NMR.

Supporting Information Available: *In vitro* biological data for the benzofuran, indole, naphthyl, and 1,2-dihydronaphthyl derivatives of lead structure **1** as well as the spectroscopic and analytical data for all of the compounds of this study (12 pages). Ordering information is given on any current masthead page.

References

(1) For a general discussion of the role of thrombin in thrombus formation, see: (a) Machovich, R. Thrombin and Hemostasis. In *The Thrombin*; Machovich, R., Ed.; CRC Press, Inc.: Boca Raton, FL, 1984; Vol 1, pp 1−22. (b) Hirsh, J.; Salzman, E. W.; Marder, V. J.; Colman, R. W. Overview of the Thrombotic Process and Its Therapy. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 2nd ed.; Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W., Eds.; J. B. Lippincott Co.: Philadelphia, PA, 1987; pp 1063−1072.

(2) (a) Hirsh, J.; Salzman, E. W. Pathogenesis of Venous Thromboembolism. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 2nd ed.; Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W., Eds.; J. B. Lippincott Co.: Philadelphia, PA, 1987; pp 1199−1207. (b) Barnett, H. J. M. Thrombotic Processes in Cerebrovascular Disease. *Ibid*.; pp 1301−1315.

(3) (a) Colman, R. W.; Marder, V. J.; Salzman, E. W.; Hirsh, J. Overview of Hemostasis. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 3rd ed.; Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W., Eds.; J. B. Lippincott Co.: Philadelphia, PA, 1994; pp 3−18. (b) Mann, K. G. Prothrombin and Thrombin. *Ibid*.; pp 184−199. (c) Colman, R. W.; Cook, J. J.; Niewiarowski, S. Mechanisms of Platelet Aggregation. *Ibid*.; pp 508−523. (d) Edit, J. F.; Allison, P.; Noble, S.; Ashton, J.; Golino, P.; McNard, J.; Buja, L. M.; Willerson, J. T. Thrombin is an Important Mediator of Platelet Aggregation in Stenosed Canine Coronary Arteries with Endothelial Injury. *J. Clin. Invest.* **1989**, *84*, 18. (e) Heras, M.; Chesbro, J. H.; Penny, W. J.; Bailey, K. R.; Badimon, L.; Fuster, V. Effects of Thrombin Inhibition on the Development of Acute Platelet-Thrombus Deposit or During Angioplasty in Pigs. Heparin vs Hirudin, a Specific Thrombin Inhibitor. *Circulation* **1989**, *79*, 657−665.

(4) (a) O'Reilly, R. A. Vitamin K Antagonists. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 2nd ed.; Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W., Eds.; J. B. Lippincott Co.: Philadelphia, PA, 1987; pp 1367−1372. (b) Hirsch J. Oral Anticoagulants. *N. Engl. J. Med.* **1991**, *324*, 1865−1875.

(5) (a) Bajusz, S.; Barabas, E.; Szell, E.; Bagdy, D. Peptide Aldehyde Inhibitors of the Fibrinogen-Thrombin Reaction. In *Peptides: Chemistry, Structure and Biology, Proceedings of the Fourth American Peptide Symposium*; Walter, R., Meienhofer, J., Eds.;

Science Publishers Inc.: Ann Arbor, MI, 1975; p 608. (b) Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Dioszegi, M.; Fittler, Z.; Szabo, G.; Juhasz, A.; Tomori, E.; Szilagyi, G. Highly Active and Selective Anticoagulants: D-Phe-Pro-Arg-H, a Free Tripeptide Aldehyde Prone to Spontaneous Inactivation, and Its Stable *N*-Methyl Derivative, D-MePhe-Pro-Arg-H. *J. Med. Chem.* **1990**, *33*, 1729−1735.

(6) (a) Bode, W.; Mayr, I.; Baumann, U.; Huber, R.; Stone, S. R.; Hofsteenge, J. The Refined 1.9 Å Crystal Structure of Human α-Thrombin: Intreraction with D-Phe-Pro-Arg Chloromethylketone and Significance of the Tyr-Pro-Pro-Trp Insertion Segment. *EMBO J.* **1989**, *8*, 3467−3475. (b) Banner, D. W.; Hadvary, P. Crystallographic Analysis at 3.0 Å Resolution of the Binding to Human Thrombin of Four Active Site-Directed Inhibitors. *J. Biol. Chem.* **1991**, *266*, 20085−20093. (c) Malley, M. F.; Tabernero, L.; Chang, C. Y.; Ohinger, S. L.; Roberts, D. G. M.; Das, J.; Sack, J. S. Crystallographic Determination of the Structures of Human α-Thrombin Complexed with BMS-186282 and BMS-189090. *Protein Sci.* **1996**, *5*, 221−228.

(7) For recent accounts on the design of tripeptide-based thrombin inhibitors; see: (a) Tucker, T. J.; Lumma, W. C.; Mulichak, A. M.; Chen, Z.; Naylor-Olsen, A. M.; Lewis, S. D.; Lucas, R.; Freidinger, R. M.; Kuo, L. C. Design of Highly Potent Noncovalent Thrombin Inhibitors That Utilize a Novel Lipophilic Binding Pocket in the Thrombin Active Site. *J. Med. Chem.* **1997**, *40*, 830−832. (b) Lyle, T. A.; Chen, Z.; Appleby, S. D.; Freidinger, R. M.; Gardell, S. J.; Lewis, S. D.; Li, Y.; Lyle, E. A.; Lynch, J. J., Jr.; Mulichak, A. M.; Ng, A. S.; Naylor-Olsen, A. M.; Sanders, W. M. Synthesis, Evaluation, and Crystallographic Analysis of L-371,912: A Potent and Selective Active-Site Thrombin Inhibitor. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 67−72. (c) Semple, J. E.; Rowley, D. C.; Brunck, C. K.; Ha-Uong, T.; Minami, N. K.; Owens, T. D.; Tamura, S. Y.; Goldman, E. A.; Siev, D. V.; Ardecky, R. J.; Carpenter, S. H.; Ge, Y.; Richard, B. M.; Nolan, T. G.; Hakanson, K.; Tulinsky, A.; Nutt, R. F.; Ripka, W. C. Design, Synthesis and Evolution of a Novel, Selective, and Orally Bioavailable Class of Thrombin Inhibitors: P1-Argininal Derivatives Incorporating P3-P4 Lactam Sulfonamide Moieties. *J. Med. Chem.* **1996**, *39*, 4531−4536. (d) Levy, O. E.; Semple, J. E.; Lim, M. L.; Reiner, J.; Rote, W. E.; Dempsey, E.; Richard, B. M.; Zhang, E.; Tulinsky, A.; Ripka, W. C.; Nutt, R. F. Potent and Selective Thrombin Inhibitors Incorporating the Constrained Arginine Mimic L-3-Piperidyl(*N*-guanidino)alanine at P1. *J. Med. Chem.* **1996**, *39*, 4527−4530. (e) Costanzo, M. J.; Maryanoff, B. E.; Hecker, L. R.; Schott, M. R.; Yabut, S. C.; Zhang, H.-C.; Andrade-Gordon, P.; Kauffman, J. A.; Lewis, J. M.; Krishnan, R.; Tulinsky, A. Potent Thrombin Inhibitors That Probe the S_1' Subsite: Tripeptide Transition State Analogues Based on a Heterocycle-Activated Carbonyl Group. *J. Med. Chem.* **1996**, *39*, 3039−3043. (f) Dominguez, C.; Carini, D. J.; Weber, P. C.; Knabb, R. M.; Alexander, R. S.; Kettner, C. A.; Wexler, R. R. S_1 Heterocyclic Thrombin Inhibitors. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 79−84. (g) Galemmo, R. A., Jr.; Fevig, J. M.; Carini, D. J.; Cacciola, J.; Wells, B. L.; Hillyer, G. L.; Buriak, J., Jr.; Rossi, K. A.; Stouten, P. F. W.; Alexander, R. S.; Hilmer, R.; Bostrom, L.; Abelman, M. M.; Lee, S.-L.; Weber, P. C.; Kettner, C. A.; Knabb, R. M.; Wexler, R. R. (N-Acyl-N-alkyl)-glycyl *Boro*lysine Analogs: A New Class of Potent Thrombin Inhibitors. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2913−2918.

(8) For recent reviews on approaches to the design of active site-directed thrombin inhibitors, see: (a) Edmunds, J. J.; Rapundalo, S. T. Thrombin and Factor Xa Inhibition. *Annu. Rep. Med. Chem.* **1996**, *31*, 51−60. (b) Das, J.; Kimball, S. D. Thrombin Active Site Inhibitors. *Bioorg. Med. Chem.* **1995**, *3*, 999−1007. (c) Scarborough, R. M. Anticoagulant Strategies Targeting Thrombin and Factor Xa. *Annu. Rep. Med. Chem.* **1995**, *30*, 71−80.

(9) Shuman, R. T.; Gesellchen, P. D. Development of an Orally Active Tripeptide Arginal Thrombin Inhibitor. *Integration of Pharmaceutical Discovery and Development*: *Case Histories*, of the series *Pharmaceutical Biotechnology*; Plenum Publishing Co.: New York, in press.

(10) Inhibition of thrombin amidase activity was detected by monitoring the decrease in absorbance (405 nm), relative to control, caused by the release of *p*-nitroanilide when bovine thrombin (19.3 nm; Parke-Davis) catalyzed the hydrolysis of Bzl-Phe-Val-Arg-pNA (0.2 mM).

(11) Jones, C. D.; Suarez, T. 2-Phenyl-3-aroylbenzothiophenes Useful as Antifertility Agents. U.S. Patent 4,133,814.

(12) Inhibitor binding affinities for human α-thrombin and other serine proteases were measured as apparent association constants (K_{ass}) which were derived from inhibition kinetics as previously described in Smith, G. F.; Gifford-Moore, D.; Craft, T. J.; Chirgadze, N.; Ruterbories, K. J.; Lindstrom, T. D.; Satterwhite, J. H. Efegatran: A New Cardiovascular Anticoagulant. In *New Anticoagulants for the Cardiovascular Patient*; Pifarre, R., Ed.; Hanley & Belfus, Inc.: Philadelphia, PA, 1997; pp 265−300. Briefly, enzyme inhibition kinetics were performed in 96-well polystyrene plates, and reaction rates were determined from the rate of hydrolysis of appropriate *p*-nitroanilide substrates at 405 nm using a Thermomax plate reader from Molecular Devices (San Francisco, CA). The same protocol was followed for all enzymes studied: 50 μL buffer in each well followed by 25 μL of inhibitor solution and 25 μL of enzyme; within 2 min, 150 μL of chromogenic substrate was added to start the enzymatic reactions. The rates of Bzl-Phe-Val-Arg-pNA hydrolysis reactions provide a linear relationship with human α-thrombin such that free thrombin can be quantitated in reaction mixtures. Data were analyzed directly as rates by the Softmax program to produce [free enzyme] calculations for tight-binding K_{ass} determinations. For apparent K_{ass} determinations, 5.9 nM human thrombin or 1.4 nM bovine trypsin was used to hydrolyze 0.2 mM Bzl-Phe-Val-Arg-pNA, 3.4 nM human plasmin with 0.5 mM H-D-Val-Leu-Lys-pNA, 1.2 nM human nt-PA with 0.81 mM H-D-Ile-Pro-Arg-pNA, 0.37 nM urokinase with 0.30 mM pyro-gfsGlu-Gly-Arg-pNA, and 1.34 nM human factor Xa with 0.18 mM Bzl-Ile-Glu-Gly-Arg-pNA.

(13) K_i values were determined as follows. The velocity of substrate hydrolysis (S2288, H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroaniline dihydrochloride; Chromogenix) by human α-thrombin (Enzyme Research Labs) 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*-nitroanilide using a V_{max} 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 and the K_i for each inhibitor under these conditions were 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. The double-reciprocal plots of $1/v$ vs $1/S$ are supplied in Supporting Information.

(14) Bioavailability studies were conducted as follows. Compounds were administered as aqueous solutions to male Fisher rats, intravenously at 5 mg/kg via tail vein injection and orally to fasted animals at 20 mg/kg by gavage. Serial blood samples were obtained at 5, 30, 120, and 240 min postdose following intravenous administration and at 1, 2, 4, and 6 h after oral dosing. Plasma was analyzed for drug concentration using an HPLC procedure involving C8 Bond Elute (Varion) cartridges for sample preparation and a methanol/30 nM ammonium acetate buffer (pH 4) gradient optimized for each compound. Oral bioavailability (F) was calulated by the following equation: $\%F = [(AUC_{oral} \cdot dose_{iv})/(AUC_{iv} \cdot dose_{oral})] \cdot 100$.

(15) The *in vitro* biological data on the benzofuran, indole, naphthyl, and 1,2-dihydronapthyl derivatives are contained in the Supporting Information.

(16) A detailed account of the X-ray crystallography studies performed on this series of thrombin inhibitors will be provided in a future publication.

(17) Chirgadze, N. Y.; Sall, D. J.; Klimkowski, V. J.; Clawson, D. K.; Briggs, S. L.; Hermann, R.; Smith, G. F.; Gifford-Moore, D. S.; Wery, J.-P. The Crystal Structure of Human α-Thrombin Complexed with LY178550, a Non-Peptidyl, Active Site Directed Inhibitor. *Protein Sci.* **1997**, *6*, 1412−1417.

(18) Shibata, A. Diffraction Data Collection with R-AXIS II, an X-ray Detecting System Using Imaging Plate. *Rigaku J.* **1990**, *7*, 28−32.

(19) (a) Minor, W. XDSPLAYF Program, Purdue University, 1993. (b) Otwinowski, Z. Oscillation Data Reduction Program. In *Proceedings of the CCP4 Study Weekend: "Data Collection and Processing"*; SERC Daresbury Laboratory: England, 1993; pp 56−62.

(20) *X-PLOR Brünger AT. 1992. X-PLOR, version 3.1: A system for X-ray Crystallography and NMR*; Yale University Press: New Haven, CT.

(21) The image depicted in Figure 1 was generated using the QUANTA software package (version 4.1.1) from Molecular Simulations Inc., 9685 Scranton Rd., San Diego, CA.

(22) The numbering for the thrombin residues discussed in this study is based on topological equivalence to chymotrypsinogen; see ref 6a.

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