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Communications to the Editor

Potent and Selective Thrombin Inhibitors Incorporating the Constrained Arginine Mimic L-3-Piperidyl(*N*-guanidino)alanine at P_1

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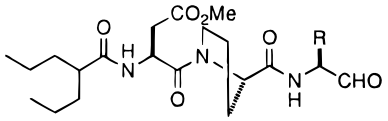
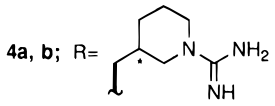
Thrombin, a serine protease with trypsin-like specificity, plays a central role in the coagulation cascade by mediating both the final conversion of fibrinogen to fibrin and the activation of platelets.[1] The prominent anticoagulant therapeutic agents currently available, heparin and its low molecular weight derivatives, and the indirectly acting orally bioavailable coumarins suffer from many side effects and limited efficacy.[2] Considerable efforts to develop safer and novel thrombin inhibitors are presently underway.[3]

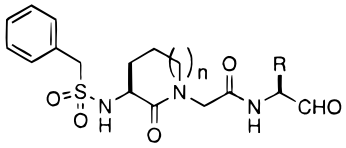
Recently, in our laboratories, compound **1** (CVS 1123) was identified as a potent transition-state thrombin inhibitor which demonstrated good oral bioavailability and selectivity profiles (see Figure 1).[4] Molecular-modeling considerations and structure−activity relationship (SAR) studies on **1** and related serine protease inhibitors led to the design of compounds **2** (CVS 1578) and **3** (CVS 1778),[5] which incorporated a six- and a seven-membered lactam sulfonamide moiety at P_3−P_4,[6] respectively. The lactam template was based upon the pioneering work of Freidinger.[7] Both molecules displayed a high degree of selectivity for the inhibition of thrombin over trypsin (Table 1).



1, CVS 1123; R = -$(CH_2)_3HNC(=NH)NH_2$

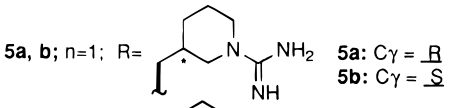


4a, b; R=



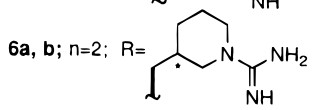
2, CVS 1578; n=1; R = -$(CH_2)_3HNC(=NH)NH_2$

3, CVS 1778; n=2; R = -$(CH_2)_3HNC(=NH)NH_2$



5a, b; n=1; R=

5a: Cγ = R
5b: Cγ = S



6a, b; n=2; R=

Figure 1.

Table 1. In Vitro IC_{50} Values (nM) and Selectivity Ratios of Compounds **1**−**6** against Thrombin (FIIa), FXa, and Trypsin[a]

compd	FIIa[b]	trypsin[c]	FXa[b]	trypsin/FIIa	FXa/FIIa
1	1.2	1.2	301	1	250.8
2	12.4	1550	>2500	125	>201.6
3	0.71	329	20.6	463.4	29
4a	1.7	113	>2500	66.5	>1470.6
4b	1.5	69.7	>2500	46.5	>1666.7
5a	28.8	60700	>25000	2107.6	>868
5b	0.67	68400	>25000	102090	>37313.4
6a	1.8	15100	>2500	8388.9	>1388.9
6b	0.57	45800	>25000	80351	>43859.6

[a] Concentration (nM) of **1**−**6** required to inhibit enzymatic cleavage of the chromogenic substrates described in reference 13 by 50%. [b] Human thrombin and FXa were used. [c] Bovine trypsin was used.

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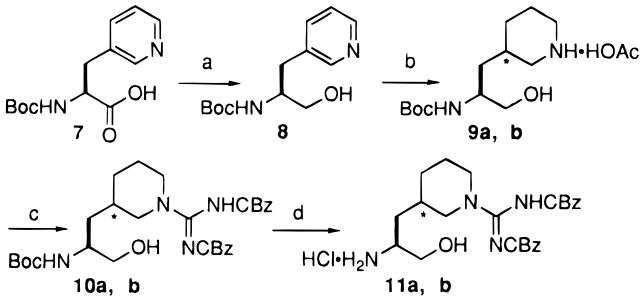
Scheme 1[a]



[a] (a) BH_3, THF, 84%; (b) H_2 (40 psi), 10% PtO_2, MeOH, HOAc, quant; (c) bis-CBz-*S*-methylisothiourea, NMM, THF, 82%; (d) HCl, MeOH, quant.

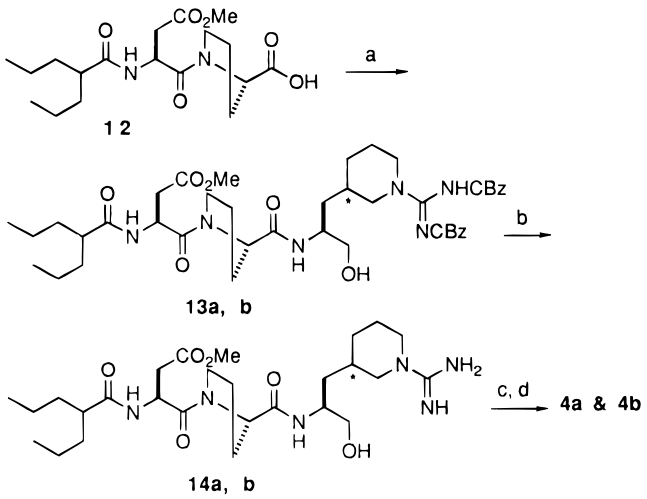
In our search for novel oral thrombin inhibitors with selectivity against other enzymes including trypsin, we investigated conformationally constrained arginine mimics, in particular the novel L-3-piperidyl(*N*-guanidino)-alaninal for P_1.[8] This communication describes the syntheses, biological activities, and crystal structures of several thrombin inhibitors which contain this novel residue at P_1 (compounds **4**−**6,** Figure 1). Since the bis-CBz protected L-3-piperidyl(*N*-guanidino)alaninols (**11a** and **11b**) were prepared nonstereoselectively at the Cγ center, two diastereomers were obtained from each P_1 substitution. In Figure 1 each isomer is labeled as "a" and "b" according to the order of elution of the diastereomeric pair on a reverse phase C18 HPLC column. ^{1}H NMR studies have shown that each of the two diastereomeric aldehydes exists predominantly in the hydrate form. Furthermore, the HPLC chromatograms show single peaks. Unlike argininals **1**−**3**, the conformationally constrained mimics are incapable of undergoing cyclic aminal formation.[9]

The preparation of the 3-piperidyl(*N*-guanidino)alanine moiety is illustrated in Scheme 1. Commercially available Boc-L-3-pyridylalanine **7** (purchased from Synthetech Inc.) was reduced to Boc-L-3-pyridylalaninol **8** with BH_3·THF in high yields. Catalytic hydrogenation with PtO_2 in methanol generated Boc-L-3-piperidinylalaninols **9a, b** quantitatively, as an equal mixture of diastereomers. Guanylation of the resulting piperidine ring nitrogen atom with bis-CBz-protected *S*-methylisothiourea,[10] (compounds **10a,b**) and deprotection of the Boc group afforded the corresponding hydrochloride salts **11a,b**, which were then coupled to the desired P_2−P_4 peptide segments.

The syntheses of the P_2−P_4 fragments for **4a,b**[4a,8c] **5a**,**b**, and **6a**,**b**[5a,8d] are reported elsewhere. A representative synthesis for the diastereomeric pair **4a**,**b** is shown in Scheme 2. Segment condensation of the P_2−P_4 fragment **12,** with **11a,b**, furnished the fully protected peptide alcohols **13a**,**b** in 81% yield. Hydrogenation of the bis-CBz protecting groups in the presence of palladium on carbon in methanol resulted in compounds **14a,b** in high yields. A modified Moffat oxidation[11] utilizing the water soluble carbodiimide (EDC), DMSO, and dichloroacetic acid in toluene converted the alcohols **14a,b** to the aldehydes **4a,b**. The diastereomers were separated during final purification by HPLC.[12] The diastereomeric pairs **5a,b** and **6a,b** were prepared analogously to **4a,b**, by condensation of **11a,b** with the corresponding P_2−P_4 fragments.

The compounds prepared are listed in Table 1 with the IC_{50} values for the inhibition of thrombin (FIIa),

Scheme 2[a]



[a] (a) **11a**, **b**, EDC, HOBt, NMM, CH_3CN, 81%; (b) H_2 (40 psi), 10% Pd/C, MeOH, quant; (c) EDC, DMSO, $CH(Cl)_2CO_2H$, toluene, 68%; (d) HPLC, separation of diastereomers.[12]

trypsin, and factor Xa (FXa) amidolytic activity.[13] Selectivity ratios of trypsin/FIIa and FXa/FIIa are also tabulated. Other proteases such as FVIIa, FXIa, FXIIa, plasmin, urokinase, and activated protein C (aPC) were also screened; however, all new targets were inactive (IC_{50} > 2.5 μM) on these enzymes.

As exemplified by compounds **2** and **3,** incorporation of a six- or a seven-membered lactam ring at P_3 with a natural arginine side chain at P_1 increased the trypsin selectivity by ~125- and 463.4-fold, respectively, compared to **1**. As shown with compounds **4a** and **4b** (Table 1), replacement of the arginine side chain in **1** (selectivity ratio = 1) with that of the more rigid 3-piperidyl-(*N*-guanidino)alaninal led to an increase in the desired thrombin versus trypsin selectivity ratio (66.5 and 46.5, respectively). Thus, L-3-piperidyl(*N*-guanidino)alaninal in P_1 represents an additional structure modification to the lactams in P_3 which confers selectivity for thrombin over trypsin. The discovery of two independent structural features which enhance selectivity led us to combine these features to give compounds **5** and **6,** in the hope that further increases in selectivity would be obtained. As shown in Table 1, this goal was in fact realized in that analogs **5** and **6** exhibited greatly enhanced thrombin inhibitory potencies over trypsin. Compound **5b**, which incorporates both the novel P_1 residue and the six-membered lactam at P_3, has a selectivity ratio of 102 090 and represents the most selective transition-state inhibitor of thrombin described to date. A similar gain in selectivity was observed for compounds **5a**, **6a**, and **6b**. It is evident from this data that the introduction of the L-3-piperidyl(*N*-guanidino)-alaninal moiety at P_1 in conjunction with a lactam ring at P_3 resulted in a cooperative effect on selectivity. This selectivity was achieved by abolishing the trypsin activity while retaining the thrombin potency in these compounds.

Compounds **4a,b** and **5a,b** were evaluated for antithrombotic efficacy in a rat model of acute arterial thrombosis.[14] Three of the compounds **4b, 5a** and **5b** showed a dose dependent reduction in the incidence of thrombotic occlusion in this model, consistent with their potent in vitro inhibition of thrombin. Additionally, compound **5b** was also demonstrated to be adsorbed

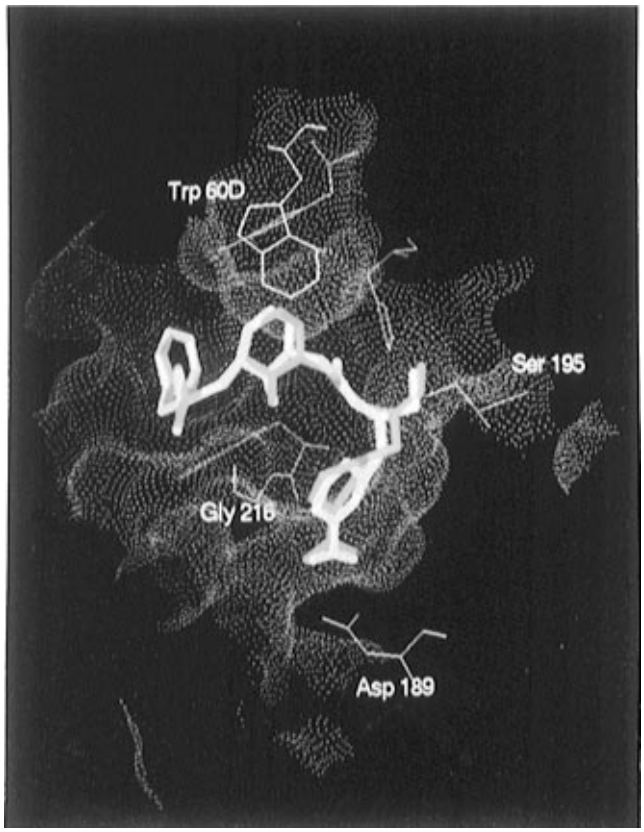


Figure 2. Superimposed structures of inhibitors **5a,b** bound in the thrombin active site.

following oral administration in conscious dogs to a level of approximately 15%.[15]

The preliminary crystal structures of human α-thrombin complexed with **5a** and **5b** have been determined, and the absolute configuration of each isomer at P_1-Cγ has been assigned.[16] Thus, **5a** and **5b** have the (*R*)- and the (*S*)-configuration at Cγ, respectively. X-ray crystallographic analysis further revealed that both inhibitors bind to thrombin in a substrate-like manner, as expected, with the L-3-piperidyl(*N*-guanidino)-alaninal occupying the S_1 site and the carbonyl and amine NH of the P_3 residue hydrogen bonding with the thrombin Gly 216 N,O atoms. Both structures were conformationally very similar at the catalytic site and at the S_2 and S_3 subsites (see Figure 2). The positioning of the **5a** and **5b** lactam rings in the enzyme site was also very similar to the structure of the lactam in the argininal analog **2**.[5b] The trypsin/thrombin selectivity imparted by the L-3-piperidyl(*N*-guanidino)alaninal residue does not appear to be due to a differences in hydrophobicity between the S_1 sites of trypsin and thrombin since the hydrophobic $-(CH_2)_3-$ loop of the rigid arginine mimic is oriented out of the S_1 site into the solvent.

In summary, we have discovered novel, highly potent and selective transition-state thrombin inhibitors which incorporate the constrained arginine mimic, L-3-piperidyl(*N*-guanidino)alaninal, at P_1. While the inhibitory potencies of these compounds were comparable to the potencies achieved by their arginine-containing counterparts, selectivity for thrombin over other serine proteases such as trypsin and FXa was greatly increased. Compound **5b** with a trypsin/thrombin potency ratio of 5 orders of magnitude is the most selective transition-state thrombin inhibitor reported to date. Further biological and pharmacological evaluation of these compounds and additional details of structural requirements for their unusual selectivity will be described in a future paper.

Acknowledgment. We thank Terence K. Brunck, Ph.D., and Marguerita Lim-Wilby, Ph.D., for useful discussions. Many thanks to Suzanne M. Anderson, Peter W. Bergum, and George P. Vlasuk, Ph.D., for all pharmacological studies and Thomas G. Nolan, Ph.D., Steven A. Dixon, Malissa M. Lods, Michael G. Ma, and Huan S. Tran for all analytical support.

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(12) Purification and separation of the diastereomers were performed on a reverse phase HPLC column. Analytical runs were performed with a 0.46 × 25 cm VYDAC column containing a C-18 resin comprised of 5 μm gel particles with a 300 Å pore size. The analytical column was eluted with a water (containing 0.1% trifluoroacetic acid)/acetonitrile gradient. The gradient was run from 15% to 30% acetonitrile for **4a** and **4b** (retention times 15.6 and 16.7 min, respectively); 10% to 20% for **5a** and **5b** (retention times 15.7 and 18 min, respectively); 5% to 25% for **6a** and **6b** (retention times 20 and 21 min, respectively). Diastereomeric ratios: **4a/4b** = 1.6/1; **5a/5b** = 1/1.5; **6a/ 6b** = 1/1.7. All final compounds were characterized by mass and ^{1}H NMR spectroscopy. Results of the high-resolution mass spectra for the purified peptides are as follows: **4a** and **4b** (MH^+ expected 551.3557, obtained 551.3563 and 551.3572, respectively); **5a** and **5b** (MH^+ expected 507.2390, obtained 507.2398 and 507.2393, respectively); **6a** and **6b** (MH^+ expected 521.2546, obtained 521.2551 and 521.2560).

(13) **Enzyme assays: IC_{50} Determinations.** Human thrombin was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN); its concentration was predetermined by the supplier from the absorbance at 280 nM and the extinction coefficient. The activity of this material was 3806 NIH units/mg. The potency of inhibitors (IC_{50}) was determined from the inhibition of the enzymatic (amidolytic) activity of thrombin at 22 °C, using the chromogenic substrate Pefachrome tPA (CH_3SO_2-D-hexahydrotyrosylglycylarginine-*p*-nitroanilide·HOAC, Pentapharm, Ltd, Basel, Switzerland), obtained from the American distributor Centerchem, Inc. (Tarrytown, NY). The substrate was reconstituted as a 4.0 mM stock with ultrapure water (18 mΩ-cm). The enzymatic reactions were monitored in the wells of a microtitre plate (Dynatech) by measuring the increase in absorbance at 405 nm, using a Thermomax microplate reader; this change in absorbance directly reflected thrombin's cleavage of Pefachrome tPA and the release of pNA. A stock solution of inhibitors (1 mM) in ultrapure water was diluted to the desired range of 12 concentrations with Hepes-buffered saline containing BSA (HBSA), 10 mM Hepes, 150 mM NaCl, and bovine serum albumin, 0.1%, w/v, pH 7.5. All reactions were performed in triplicate in a final volume of 200 μL of HBSA, containing at final concentration 0.5 nM thrombin and 250 μM Pefachrome tPA. To individual wells was added 50 μL of inhibitor or, in the case of the control, 50 μL of HBSA, followed by 50 μL of HBSA and 50 μL of human thrombin. After a 30 min incubation, the reaction was initiated by the addition of 50 μL of Pefachrome tPA. All reactions were under steady-state conditions, where less than 3% of the substrate was consumed. The increasing absorbance was measured at 10 s intervals over 5 min, and the values were stored by a dedicated computer, using Softmax software. From the data, the software allowed for the calculation of the velocity (change in absorbance per min); the averaged velocity for a triplicate sample was plotted against the inhibitor concentration. The data were then fit to a curve described by the four-parameter equation: $Y = (A - D)/(1 + (X/C)^B + D$ where the IC_{50} is represented by term C in the equation. The selectivity of the inhibitors was examined against 11 additional serine proteinases, including trypsin. The IC_{50}'s were calculated as outlined above for thrombin. The concentration of trypsin and substrate S-2222 (phenacylisoleucylglutamyl(γ-*O*-methyl)glycylarginine−*p*-nitroanilide·HCl, 250 μM, chromogenix) employed were 0.5 nM and 250 μM, respectively.

(14) Compounds **4a,b** and **5a,b** were evaluated for antithrombotic efficacy using a modification of the rat $FeCl_3$-induced model of acute arterial thrombosis originally described by Kurz et al. (Kurz, K. D.; Main, B. W.; Sandusky, G. E. *Thromb. Res.* **1990**, *60*, 269−280). Each compound was administered to anesthetized rats as a single intravenous bolus 5 min prior to the application of the thrombogenic stimulus ($FeCl_3$). The incidence of total vascular occlusion as monitored by ultrasonic flowtometry was used as the primary end point. Details of this model will be published elsewhere.

(15) The absolute systemic bioavailability (% F) for compound **5b** was determined in fasted, conscious, purpose-bred beagle dogs (two males and two females), following separate intravenous (5 mg/kg) and oral (20 mg/kg) administration and collection of plasma samples over a defined time course covering 6 h. The determination of plasma levels was accomplished using HPLC following postcolumn fluorogenic derivitization using methodologies that will be published elsewhere. The area under the plasma concentration versus time curves (AUC[0-∞]) for the oral (AUC[oral]) versus the intravenous (AUC[iv]) dosing regimens were calculated by linear trapezoidal estimation using a noncompartmental model and were used to calculate %F (AUC[oral]/AUC[iv] × 100). Further details on the pharmacokinetic and pharmacodynamic profile of these compounds will be published elsewhere.

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