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# NOVEL THROMBIN INHIBITORS THAT ARE BASED ON A MACROCYCLIC TRIPEPTIDE MOTIF

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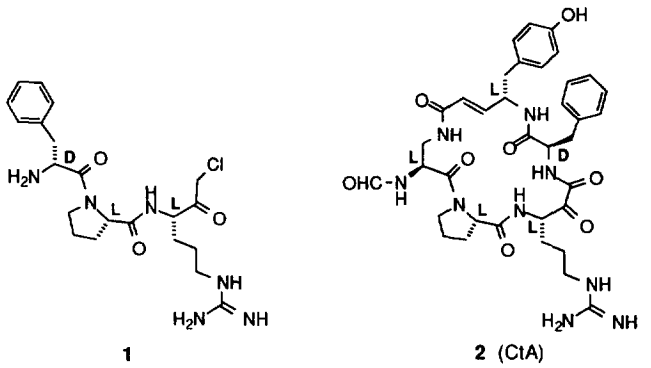
**Abstract.** A series of macrocyclic α-keto amides containing the D-Phe-Pro-Arg (fPR) motif were synthesized and evaluated in vitro as inhibitors of human α-thrombin and bovine trypsin. Structure-function studies, relating ring size and modifications at the P3 and P1' positions to enzyme inhibition, are described. An X-ray crystallographic study was performed on a ternary complex formed from **3i**, thrombin, and hirugen.


Since thrombin is a trypsin-like serine protease with a central role in the bioregulation of thrombosis and hemostasis, selective active-site-directed inhibitors represent potentially useful therapeutic agents for the management of thrombotic disorders.[1-4] Various research groups have directed efforts to achieving a better understanding of thrombin and its interactions with ligands. The application of NMR[5] and X-ray crystallographic[6] techniques has helped to define the mechanism by which thrombin proteolytically cleaves fibrinogen to generate fibrin monomer with the associated release of fibrinopeptides A and B. Also, X-ray crystallographic determinations of thrombin complexed with inhibitor molecules, as well as studies of related serine protease complexes, have provided the basic tools for pursuit of structure-based drug design.[1] For example, the X-ray structure of PPACK (**1**) complexed with human α-thrombin depicts details of the binding mode of the archetypal D-Phe-Pro-Arg ("fPR") class of thrombin inhibitors.[7] This enhanced understanding has contributed to the design of potent, active-site-directed thrombin inhibitors involving various tripeptide motifs.[8]

We have been involved in studies with cyclotheonamide A (**2**), a macrocyclic pentapeptide thrombin inhibitor isolated from the marine sponge *Theonella* sp.[9] Given the interactions observed for this α-keto amide Pro-Arg molecule, we extended the macrocycle concept into the design of a novel class of synthetic thrombin inhibitors. In this paper, we describe the synthesis and evaluation of a series of macrocyclic, tripeptide-based, active-site-directed thrombin inhibitors represented by **3** and **4** (Table 1), some of which have single-digit nanomolar potency.



**1** **2** (CtA)

**Chemical Synthesis**. The general synthetic route to **3a-3j** is exemplified for **3c** in Scheme 1. 8-Aminooctanoic acid, protected as the *N*-Cbz derivative, was coupled to Phe-Pro-O-*t*-Bu followed by hydrogenolysis of the adduct to afford **5**. Coupling between **5** and hydroxyhomoarginine derivative **6**[9c] provided **7**, which was deprotected, and cyclized with BOP-Cl to afford macrocycle **8**. Oxidation of **8** to the keto amide, followed by removal of the tosyl protecting group yielded the final target, **3c**. Analogous chemistry was used to prepare the remainder of the compounds listed in Table 1.[10]

Scheme 1

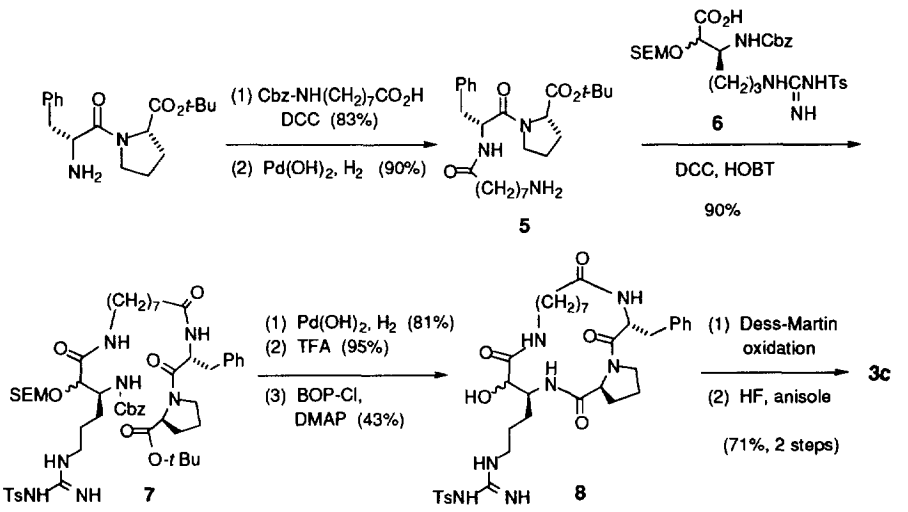
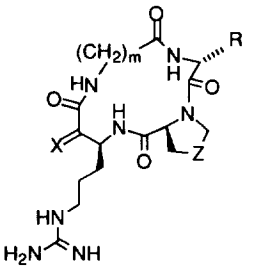
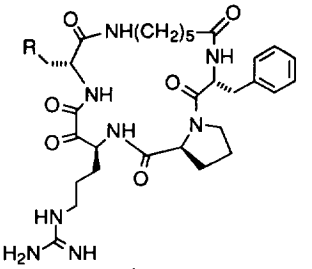


Table 1. Chemical Properties and Biological Data for Macrocyclic Thrombin Inhibitors.[a]



3



4

| compd | m | X | Z | R | mp,°C[c] | $K_i$ thrombin[b] | $K_i$ trypsin[b] |
|---|---|---|---|---|---|---|---|
| 3a | 5 | O | $CH_2$ | $CH_2Ph$ | 160-165 (dec) | 473 ± 23 (2) | 710 ± 260 (3) |
| 3b | 6 | O | $CH_2$ | $CH_2Ph$ | 74-84 | 1800 ± 1000 (6) | 280 ± 20 (6) |
| 3c | 7 | O | $CH_2$ | $CH_2Ph$ | 162-172 (dec) | 24 ± 4.2 (6) | 10.0 ±1.0 (6) |
| 3d | 9 | O | $CH_2$ | $CH_2Ph$ | 50(s)-130 (dec) | 9.9 ± 0.7 (7) | 2.1 ± 0.5 (5) |
| 3e | 10 | O | $CH_2$ | $CH_2Ph$ | 130-135 | 15 ± 1.6 (6) | 25 ± 12 (4) |
| 3f | 7 | O | $CH_2CH_2$ | $CH_2Ph$ | 60(s)-160(m) | 86 ± 5 (3) | 66 ± 15 (3) |
| 3g | 7 | O | cis-CHOMe | $CH_2Ph$ | 75-115 (dec) | 159 ± 22 (9) | 13 ± 2.4 (6) |
| 3h | 7 | O | $CH_2$ | Ph | 105-111 | 163 ± 21 (3) | 11 ± 4 (2) |
| 3i | 7 | O | $CH_2$ | $CH_2CH_2Ph$ | 50-60 | 20 ± 2.8 (5) | 14 ± 2.4 (5) |
| 3j[d] | 7 | H/OH | $CH_2$ | $CH_2CH_2Ph$ | 130-140 | IA[e] | |
| 4a | | | | $CHPh_2$ | 132-138 | 5.3 ± 1.2 | 2.5 ± 0.4 |
| 4b | | | | $CH_2Ph$ | 142-150 | 3.1 ± 0.4 | 5.2 ± 1.2 |
| cyclotheonamide A[f] | | | | | | 4.1 ± 1.9 (4) | 1.0 ± 0.1 (2) |
| efegatran[11] | | | | | | 10.0 ± 5.0 (6) | 3.9 ± 1.2 (6) |

a. All new compounds were isolated and purified by reverse-phase HPLC (Bondpack C18, 15-20 mm) and lyophilized as hydrated trifluoroacetate adducts. All new compounds were characterized by $^1H$ NMR, mass spectroscopy, and elemental analysis. b. Data are reported in nM units. Standard error is given for N measurements, indicated in parentheses. c. Melting points are corrected. All compounds are amorphous white powders; s = softens; m = melts. d. Single, undefined stereoisomer at the hydroxyl bearing carbon. e. IA = inactive, as defined by the inability to inhibit thrombin at a concentration of 50 μM. f. exhibits slow-binding characteristics; see ref 9c.

**Biological Evaluation.** As a measure of selectivity for thrombin vs. other serine proteases, we routinely evaluated compounds for their ability to inhibit thrombin and trypsin.[12] Compounds **3a-3j** (Table 1) provide general trends relating macrocycle ring size and P3 substituents to activity. Macrocyclic ring sizes of 19, 21, and 22 members appear to be in the optimal range for thrombin inhibition (cf. **3c-3e** with **3a** and **3b**). Compounds **3f** and **3g**, in which the proline ring has been altered to probe potential binding interactions within the hydrophobic S2 subsite, offer no advantages in either potency or selectivity. Interestingly, substitution of the D-Phe residue of **3c** with a phenylglycine residue as in **3h** resulted in a 7-fold decrease in thrombin inhibition. This result, in contrast to the acyclic Boc-D-Phe-Pro-Arg-H series in which a similar substitution resulted in a 3-fold increase in thrombin inhibition,[13] suggests that the macrocycle confers a degree of constraint with respect to the positioning of the P3 substituent within the corresponding apolar binding site of thrombin. Compound **3i**, which extends the phenyl substituent by a methylene relative to **3c**, is essentially equipotent to **3c**. The importance of the keto amide functionality is demonstrated by the loss of biological activity that occurs on changing the keto amide of **3i** to the hydroxy amide of **3j**. Positioning hydrophobic residues in the P1' position, as in **4a** and **4b**, increases potency for the inhibition of thrombin and trypsin. This result is consistent with the hydrophobic nature of the S1' subsite both enzymes.[14]

**X-ray Crystallography.** A ternary complex was formed by adding a 10-fold molar excess of **3i** to a complex of hirugen and human α-thrombin;[15] crystals of the complex were grown as described previously for CtA.[9a,16,17] Overall, the D-homo-Phe-Pro-Arg segment of **3i** exhibits binding interactions typical of the fPR class of inhibitors (Fig. 1). The D-homo-Phe residue occupies the corresponding apolar binding site of thrombin, while the Pro-Arg residues interact at the S2 "P pocket" and S1 specificity pocket, respectively. The keto amide carbonyl forms a tetrahedral hemiketal with the hydroxyl of Ser-195 in a manner analogous to CtA•thrombin.[9a] The carbonyl oxygen of the hemiketal is hydrogen-bonded to Gly-193 and Ser-195, while the keto oxygen of the α-keto amide forms a hydrogen bond with His-57. The seven-methylene hydrocarbon segment, which is disordered in the crystal structure, does not influence the tripeptide array that adopts an extended conformation similar to that observed for acyclic fPR-based inhibitors.

**Conclusion.** We have examined a new class of thrombin inhibitors that incorporates the prototypical fPR tripeptide motif into a macrocyclic, α-keto amide structure. The more potent compounds contain a D-Phe or D-homo-Phe residue at P3 and have ring sizes ranging from 19-22. Combination of the fPR motif, a D-Phe residue at P1', and a 20-membered ring gave the most potent thrombin inhibitor (**4b**, $K_i$ = 3.1 nM). X-ray crystallography of a representative member of the series, **3i**, complexed with thrombin revealed standard interactions of the fPR sequence in an extended conformation, but with the hydrocarbon linker segment disordered. The results reported here, together with our studies of CtA, form a basis for the further design of macrocyclic serine protease inhibitors.

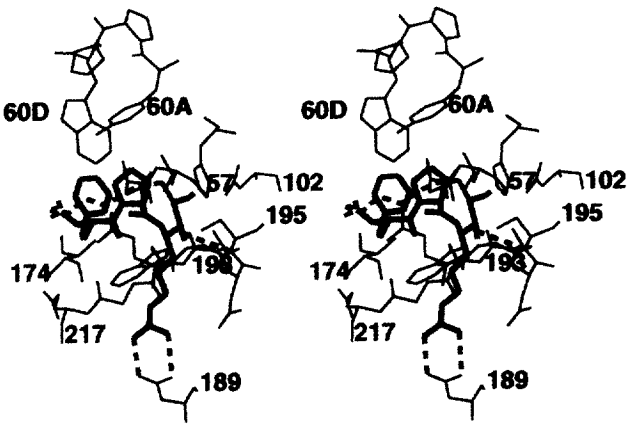



Figure 1. Stereoview of **3i** in the active site of thrombin. Thrombin (blue) and **3i** (red) are color coded; disordered methylene groups are modeled in broken black lines, and hydrogen bonds as broken blue lines.

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10. Synthesis of **4a** and **4b** required coupling of di-D-PheO-*t*-Bu and D-PheO-*t*-Bu, respectively, to **6** prior to incorporation of **5** into the sequence.
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12. Procedure for enzyme assays: The rate of increase in absorbance at 405 nM due to hydrolysis of synthetic chromogenic peptide substrates (S: 5-100 μM H-D-HHT-L-Ala-L-Arg-pNA for thrombin, 0.1-3 μM Cbo-Gly-D-Ala-Arg-pNA for trypsin), was measured in the presence and absence of inhibitors (I) with a microplate reader at 37 °C. The enzyme reaction is started by the addition of enzyme (E: 0.9 nM human α-thrombin, 3.2 U/mL bovine trypsin). Data was collected over a period of 30 min and the initial rate of substrate hydrolysis [$V_O$ (mOD/min)] was calculated. Following Michaelis-Menten kinetics, the affinity of the enzyme for the substrate, in the absence of the inhibitor ($K_m$) and in the presence of an inhibitor ($K_p$), is determined to be the negative inverse x-intercept of Lineweaver-Burk plots. The $K_i$ values were calculated by using the equation: $K_i = (K_m[I])/(K_p - K_m)$. Trypsin-catalyzed hydrolysis rates were measured using the same type of procedure. Bovine type 1 trypsin (Sigma) and Spectrozyme® TRY (Cbo-Gly-D-Ala-Arg-pNA•AcOH; American Diagnostics) replaced their thrombin equivalents in a concentration range of 3.2 U/mL trypsin and 0.1-0.3 μM Spectrozyme.
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16. X-rays (CuKα radiation) were generated with a Rigaku fine-focus RU200 rotating-anode generator operating at 50 kV and 100 mA; diffraction intensity data were measured with an R-AXIS imaging plate detector. The crystal X-rays scattered to about 1.8 Å resolution (48% observed with $I/\sigma(I) > 2.5$ in (2.0-1.8) Å resolution shell), and is isomorphous (a = 71.08, b = 72.14, c = 72.82 Å, β = 100.8°, space group C2, 4 molecules per unit cell) with crystals of hirugen-thrombin[14] and CtA-hirugen-thrombin.[9a] A total of 47,859 reflections were measured and averaged to give 25,408 independent reflections (74.2% of theoretical) with R merge = 4.5% ($I/\sigma(I) > 2.0$). The structure was solved using the coordinates of the hirugen-thrombin structure and it was refined by restrained least squares methods with the program PROLSQ. The refinement converged at R = 16.6% including 135 water molecules in the calculations.
17. X-ray coordinates have been deposited in the Brookhaven Protein Data Bank; access number BN12004.

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