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Exploring the Chiral Space within the Active Site of α-Thrombin with a Constrained Mimic of D-Phe-Pro-Arg — Design, Synthesis, Inhibitory Activity, and X-ray Structure of an Enzyme–Inhibitor Complex

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Abstract—An indolizidinone motif with strategically placed substitutents was designed and synthesized as a constrained mimic of D-Phe-Pro-Arg. Low nanomolar inhibition of α-thrombin validates the design elements in this inhibitor which also exhibits a 20-fold selectivity for thrombin versus trypsin. An X-ray crystal structure of the inhibitor with α-thrombin shows the expected interactions with key amino acids within the active site and some notable changes in positions.

The enzyme thrombin, a member of the serine protease family,[1,2] plays a major role in thrombosis, which is one of the leading causes of cardiovascular disease and morbidity.[3,4] Thrombin is involved in the blood coagulation cascade following vascular injury, eventually leading to cleavage of fibrinogen to fibrin and the activation of platelets.[5] Cognizant of the potential benefits in inhibiting thrombin, intense efforts have been made in search of effective inhibitors (for recent reviews, see refs 6–8).

Of particular significance was the elucidation of the X-ray crystal structure of a number of enzyme–inhibitor complexes.[9–13] This invaluable information, which allows synthetic chemists to literally 'see' the interactions of specific inhibitors within the active site of the enzyme, has instigated a more logic-based approach to the design of new analogues.[1,2,14] In general, inhibitors are designed to incorporate a basic guanidine-like group, which interacts with Asp 189 in the bottom of the S1 pocket, and an electrophilic serine-susceptible group which mimics the oxyanion hole, and interacts with the Ser-His-Asp catalytic triad of the enzyme. The proximal S2-pocket, created primarily by Tyr 60A and Trp 60D of the thrombin specific insertion loop, and the distal S3-pocket, created mainly by the side-chains of Leu 99, Ile 117 and Trp 215, are both of hydrophobic character. Consequently potency is gained by the presence of complementary lipophilic P2 and P3-residues of the inhibitors. These interactions are deduced from X-ray crystal structure data for the complex of thrombin with the chloromethylketone analogue of D-Phe-Pro-Arg (PPACK) **1**, which is believed to mimic a thrombin-sensitive sequence in fibrinogen (Fig. 1).[9]

We reasoned that a constrained mimic consisting of an indolizidinone nucleus[15] with strategically situated substituents as in **2** would be an interesting analogue to probe the fidelity of interactions within the enzyme active site in spite of the absence of the activated electrophilic site as in PPACK (Fig. 1). Superposition of the energy-minimized structure **2** on a truncated D-Phe-Pro-Arg backbone as in **3** showed excellent congruence. Although numerous inhibitors of α-thrombin have been synthesized with impressive activities,[6–8] only a few

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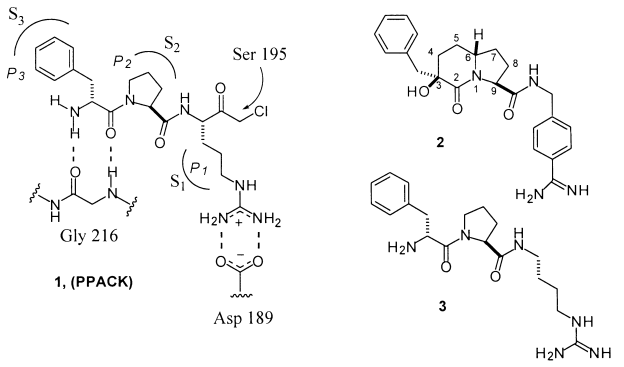
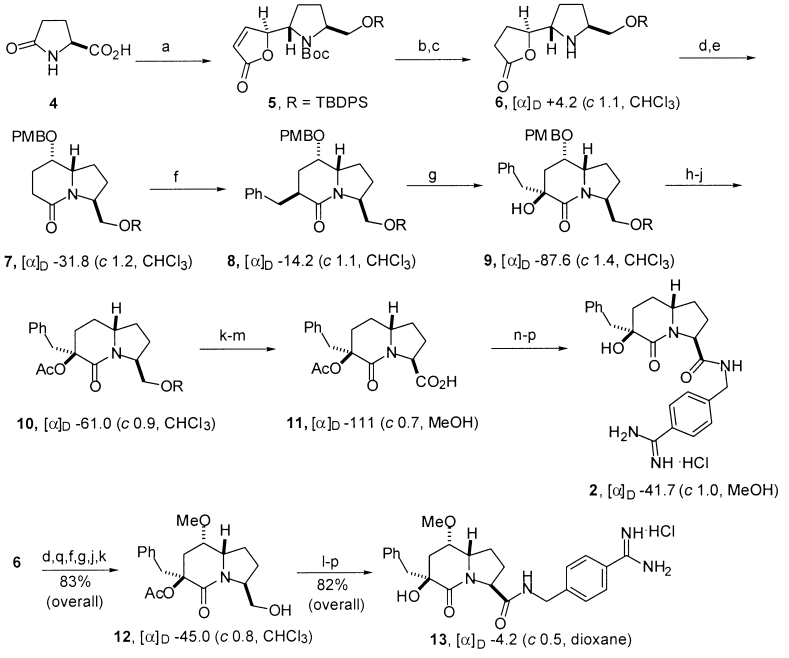


Figure 1. Structures of PPACK **1** (top left); prototype constrained indolizidinone, **2** (top right); and a truncated PPACK (D-Phe-Pro-Arg, **3**).

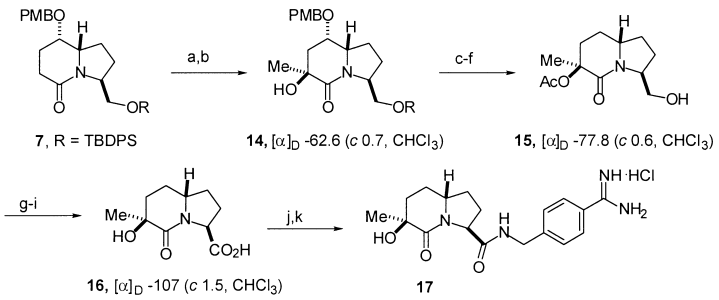
constrained bicyclic heterocyclic analogues have been recently reported.[10,16–22] The synthesis of **2** starts with the readily available L-pyroglutamic acid **4**, which was transformed via six high-yielding steps[23–25] into the enantiopure precursor **5** (Scheme 1). Hydrogenation, removal of the *N*-Boc group with B-bromocatechol borane,[26] lactam formation, and protection of the resulting hydroxyl group led to the desired indolizidinone **7**. The next challenge was to introduce the C-benzyl and the tertiary hydroxyl groups at C-3 with the desired stereochemistry. The α-orientation of the 5-*p*-methoxybenzyl (PMB) ether group was expected to play a stereodirecting role in electrophilic reactions of the lactam enolate from its convex face. Indeed, after investigating several bases, it was found that *t*-butyl lithium[23–25] and benzyl bromide gave the product **8** as a single isomer. Reformation of the enolate under the same conditions and trapping with the Davis oxaziridine reagent[27,28] led to **9** as a single isomer having the desired configuration at C-3 (vide infra). Cleavage of the PMB group with DDQ, Barton–McCombie deoxygenation,[29] and acetylation gave the indolizidinone **10**. Deprotection of the primary silyl ether, and oxidation of the alcohol to the corresponding acid **11** proceeded uneventfully. Deacylation and amide formation gave the desired prototype **2**.

In an effort to probe the chiral space at C-5 in the context of structure **2**, we considered maintaining the oxygen functionality as a methyl ether as in **12**. The synthesis of this analogue was accomplished essentially as described for **2** with equally good stereoselectivity utilizing the methoxy group (Scheme 1). The structure of **12** was confirmed by single crystal X-ray analysis.

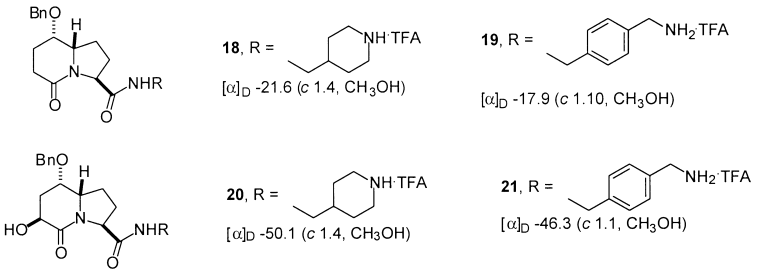
In order to study the importance of the hydrophobic interaction of the P3 subsite, we chose to replace the benzyl group by a methyl group as shown in Scheme 2. Thus, **7** was subjected to a C-methylation–hydroxylation sequence in good overall yield to give **14**. In this series, hydroxylation of the lithium enolate was achieved in the presence of trimethylphosphite and molecular oxygen.[30,31] The same procedure gave a modest yield in the C-benzyl series (ex. **8**→**9**, Scheme 1), hence the use of the Davis oxaziridine reagent.[27,28] The subsequent steps followed the original protocol, which consisted of deprotection, deoxygenation to give **15**, oxidation to **16**, and coupling to give the intended amide **17**. The structures of compounds in this series were corroborated by X-ray crystal analysis.



Scheme 1. (a) Ref 11, six steps, (85% overall); (b) 10% Pd/C, H_2, EtOAc, 96%; (c) *B*-Bromocatecholborane, CH_2Cl_2, 71% (*threo*); (d) NaOMe, MeOH, 98%; (e) PMBCl, Bu_4NI, THF, 86%; (f) *t*-BuLi, BnBr, THF, −78 °C, 88%; (g) *t*-BuLi, Davis' achiral oxaziridine, THF, −78 °C, 63%; (h) DDQ, CH_2Cl_2, H_2O, 79%; (i) NaH, CS_2, MeI, THF, then Ph_3SnH, AIBN, toluene, reflux 30 min, 48%; (j) Ac_2O, Pyr., DMAP, 82%; (k) TBAF, THF, 60%; (l) TPAP, NMO, CH_2Cl_2, 60%; (m) *t*-BuOH, $NaClO_2$, NaH_2PO_4, H_2O, quant.; (n) Cbz-protected 4-aminomethylbenzamidine, BOP, *i*-Pr_2EtN, CH_2Cl_2, 80%; (o) MeONa, MeOH, quant.; (p) H_2, 10% Pd/C, EtOAc, HCl, quant.; (q) MeI, Ag_2O, MeCN, 86%.



Scheme 2. (a)*t*-BuLi, MeI, THF, −78 °C, 97%; (b) *t*-BuLi, $(MeO)_3P$, O_2, THF, −78 °C, 64%; (c) DDQ, CH_2Cl_2, H_2O, 84%; (d) NaH, CS_2, MeI, THF, then Ph_3SnH, AIBN, toluene, reflux, 30 min, 67%; (e) Ac_2O, Pyr., DMAP, 92%; (f) TBAF, THF, 75%; (g) Swern oxidation, 84%; (h) *t*-BuOH, $NaClO_2$, NaH_2PO_4, H_2O, 93%; (i) LiOH, H_2O_2, THF, 63%; (j) Cbz-protected 4-aminomethylbenzamidine, BOP, *i*-Pr_2EtN, CH_2Cl_2, 82%; (k) H_2, 10% Pd/C, EtOAc, HCl quant.



Scheme 3.

In the quest for finding simpler and less substituted analogues, we reasoned that an α-oriented 5-benzyl ether group could possibly simulate the hydrophobic site occupied by the 2-benzyl group in **2**.[32] In this regard, we prepared the 5- and 3,5-substituted indolizidinones **18**, **19** and **20**, **21**, respectively, as shown in Scheme 3 adopting the methods already described above.

Enzymatic Activity

The amide derivatives in the three series shown in Schemes 1–3 were tested for their ability to inhibit α-thrombin.[33] While compounds **18**–**21** were less active ($pIC_{50} = < 4.9$), it was most gratifying to find that the 'designed' constrained analogue **2** was highly active ($pIC_{50} = 7.7$; K_i thrombin = 9 nM). The methoxy analogue **13** and the 3-methyl analogue **17** showed $pIC_{50} = 6.6$ (K_i thrombin = 125 nM).[34] Preliminary tests showed a 20-fold selectivity for thrombin over trypsin with the inhibitor **2**. It is noteworthy to point out that the acyclic dipeptide analogue D-Phe-Pro-*p*-amindinobenzylamine showed excellent activity (K_1 1.5 nM), and a 130-fold selectivity for thrombin over trypsin.[34]

X-ray Crystallography of Thrombin–Inhibitor Complexes

As an important structural feature, we were able to obtain X-ray quality crystals of the α-thrombin–**2** complex. Shown in Figure 2, is the Connolly surface map of the X-ray structure of the α-thrombin–**2** complex at 1.95 Å resolution.[35] Figure 3 depicts the superposition of the α-thrombin–**2** and PPACK X-ray structures. It is of interest that whereas there is excellent overlap for the indolizidinone and the benzamidine portion with PPACK, there is considerable deviation of the C-benzyl group in **2**. Consequently, the Ile 174 residue deviates from its original position compared to the PPACK-α-thrombin structure. The deviation of the Glu 192 residue is due to the larger size of the benzamidine group in **2** compared to the aliphatic counterpart in PPACK (Figure 3, dotted arrows for PPACK, solid arrows for α-thrombin–**2**).

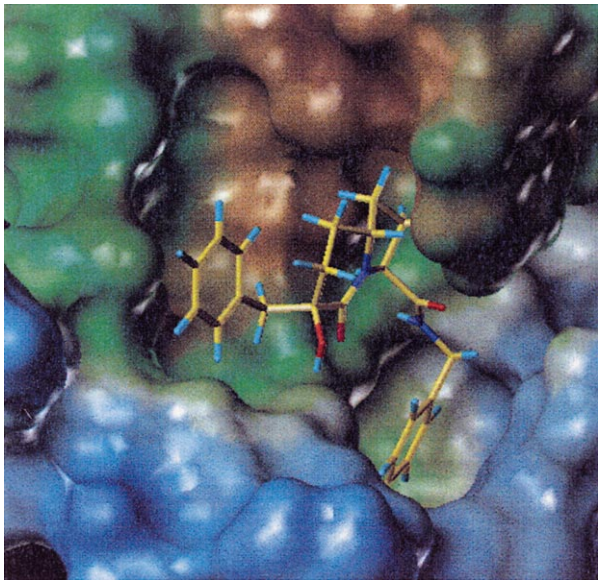


Figure 2. Connolly surface map of the X-ray structure of α-thrombin–**2** complex at 1.95 Å resolution.

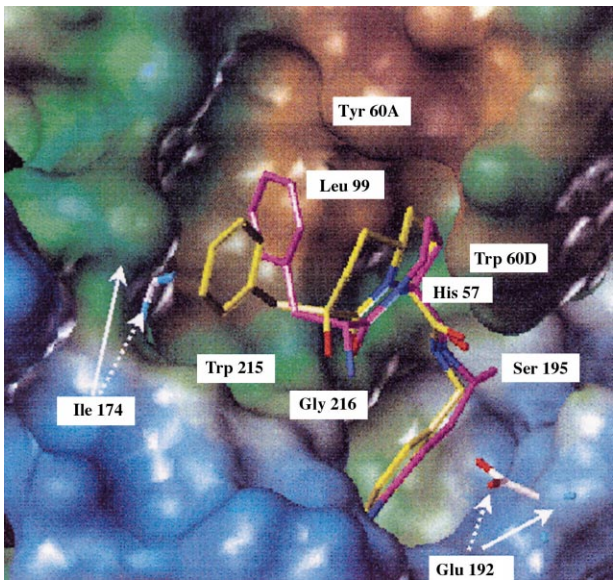


Figure 3. Connolly surface map of the X-ray structure of α-thrombin–**2** complex (yellow) aligned with PPACK (purple). Dotted arrows indicate original positions with PPACK. Solid arrows show deviations with **2**.

An examination of the crystal structure of the α-thrombin–**2** complex reveals the importance of the functional, topological, and stereochemical features of the designed inhibitor (Figs 2 and 3). The choice of a slightly convex indolizidinone motif with the β-hydrogen at C-6 based on modeling studies is also validated. Unlike many other thrombin inhibitors, compound **2** does *not* incorporate a 'serine trap' as a critical functional motif for activity. The presence of a tertiary α-hydroxy lactam group rather than an amine as a H-donor–acceptor unit for Gly 216 is another noteworthy functional feature. Further refinements can now be considered in the light of these results, and they will be reported in due course.

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