Expedited Articles

Design of Novel, Potent, Noncovalent Inhibitors of Thrombin with Nonbasic P-1 Substructures: Rapid Structure−Activity Studies by Solid-Phase Synthesis

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Study of surface representations of the inhibitor-bound thrombin P-1 pocket revealed a lipophilic recess in this pocket which is not occupied by any known inhibitor. Solid-phase synthesis was used to generate benzylamides of D-diphenylAlaPro by aminolysis of Boc dipeptide Kaiser resin. The resulting amides inhibited thrombin in the range $IC_{50} = 3-13\,000$ nM, and the structure−activity relationships and molecular modeling suggest a unique fit of the benzyl side chain into P-1 with the meta substituent occupying the recess.

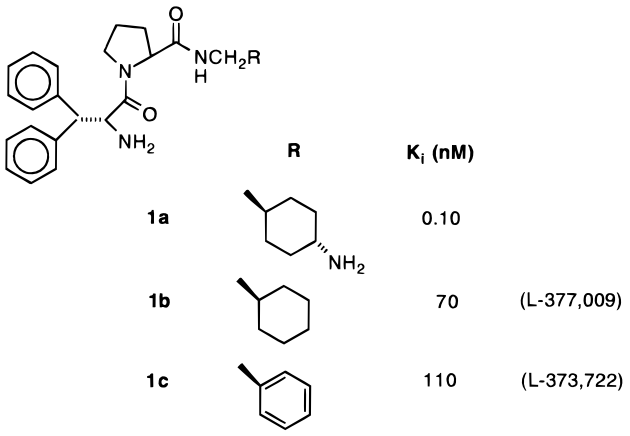
Thrombin is a serine protease which acts in the blood coagulation cascade to initiate clotting. It catalyzes the conversion of fibrinogen to fibrin and also converts factor XIII to factor XIIIa which cross-binds fibrin. Thrombin also activates factors V and VIII which accelerate the clotting cascade (thrombin synthesis). It is known[1] that inhibitors of thrombin based on the active site binding tripeptide D-PhePro-Arg have an anticoagulant effect in in vivo models of thrombosis after iv administration. Thus, an orally active thrombin inhibitor would be an excellent candidate for an anticoagulant in pathological states characterized by thrombosis, such as deep vein thrombosis and stroke.

The development of orally active thrombin inhibitors has presumed a requirement for a basic group to be present on the molecule in order to bind the Asp 189 in the bottom of the thrombin "recognition pocket" (P-1). Thrombin inhibitors with guanidine and amidine moieties in P-1 have been associated with poor oral absorption, hypotension and other side effects.[1,2]

The potent noncovalent thrombin inhibitor **1a**[3] (Chart 1) illustrates the importance of a basic P-1 amino group for binding potency. Deletion of the amino group results in a 700 times reduction in potency (**1b**). The benzylamide (**1c**) is 1110 times less potent.[4] Nevertheless these results encouraged us to further explore the P-1 pocket for lipophilic binding in search of a potent, neutral thrombin inhibitor since the absolute potency of the desamino compounds was sufficient for a lead compound.

Examination of the P-1 pocket (Figure 1) of thrombin bound to **1a** in a hydrophobic surface representation revealed a potential binding pocket for hydrophobic groups in a trans, axial relationship to the amino function. Thus it was decided to explore the lipophilic binding potential of the pocket by generating benzylamides using solid-phase synthesis.

Chart 1



Kaiser oxime resin was coupled to BocProOH using dicyclohexylcarbodiimide (DCC)[5] (Scheme 1). Deprotection with trifluoroacetic acid followed by HOBt/EDC coupling with Boc-D-diphenylAlaOH gave the dipeptide resin which was cleaved with a collection of RCH_2NH_2 (chosen for chemical diversity) and triethylamine at room temperature. The released amides were deprotected with TFA to give the desired inhibitor in 50−90% average yield based on **1a** as a reference sample. HPLC IC_{50} purity and mass spectra (data not shown) of crude products were used to estimate relative concentrations for interpreting thrombin inhibition IC_{50} data as shown in Table 1. Absolute potencies were gauged by comparison of HPLC area percent relative to the nearly quantitative yield of **1a** included as a standard.

Structure−activity relationships from Table 1 suggested that 2,5-lipophilic substituents were optimal for benzyl P-1 moieties. The 2-substituent adds only a 3−4 times factor in potency and may serve to cap the highly

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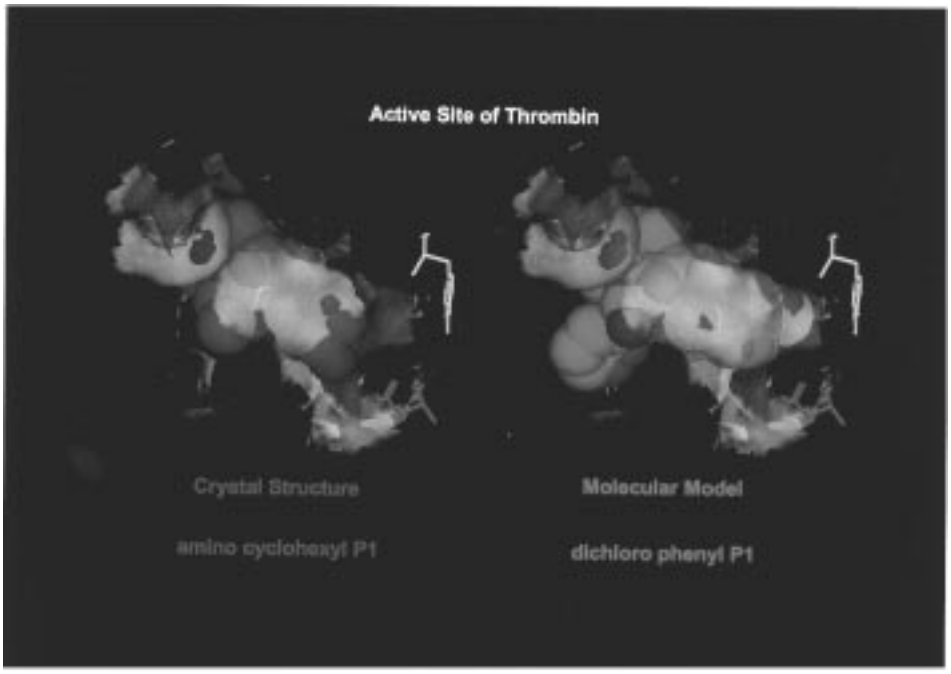
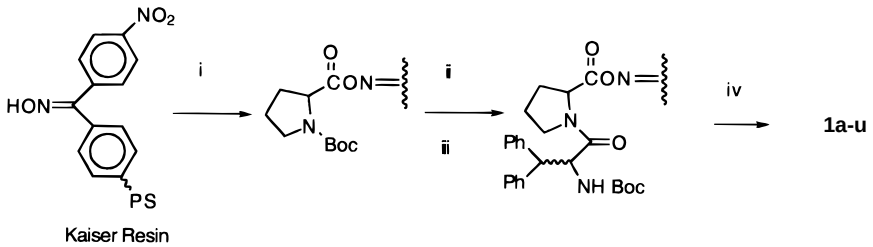


Figure 1. Left: Surface representation of the P-1 pocket of thrombin bound to compound **1a**. Right: Same surface with modeled fit of compound **1u**.

Scheme 1[a]



[a] (i) DCC, CH_2Cl_2; (ii) CF_3CO_2H, CH_2Cl_2; (iii) Boc(D)diPhe, DCC, CH_2Cl_2; (iv) $ArCH_2NH_2$, 3−6 days, 25 °C, then TFA.

Table 1. Benzylamides **1c**−**u** (**1c**: R = R2−R6−Ph)

compd	R2−R6	IC_{50} (nM)	HPLC (major %)	FAB $(M + H)^+$
1a		0.100	97.5	451 (C,H,N)
1c	nil	130	95	428 (C,H,N)
1d	2,5-CH_3-4-CH_2NH_2	39	33	485
1e	2,3,5,6-CH_3-4-CH_2NH_2	11300	48	513
1f	2,5-CH_3	38 (K_i = 40)	93	456
1g	4-$N(CH_3)_2$	4000	98	471
1h	4-SO_2NH_2	13000	86	507
1i	2,3-Cl	260	56	496/498
1j	2,3-CH_3	310	31	456
1k	2,3-OCH_3	>10000	30	488
1l	3,5-Cl	450	46	496/498
1m	2,6-OCH_3	>10000	35	488
1n	3,5-OCH_3	50	21	488
1o	2-CH_3	600	58	442
1p	3-CH_3	100	64	442
q	3-OCH_3	36	69	458
1r	3-Cl	12	59	462
1s	3-Br	10	67	506
1t	3-OH	160	49	444
1u	2,5-Cl	3 (K_i)		

solvated P-1 channel. Polar substituents were not well-tolerated in the 3−6 positions. The 2,5-dichloro analogue **1u** (Table 1) was synthesized by conventional means (i.e., not on resin) and shown to be optimally potent among simple benzylamides, being only 20 times less potent than **1a**, which makes an ionic bond in P-1.[6,7] Furthermore, **1u** is >40 times as potent as the unsubstituted benzylamide **1c**.

The rationale for beginning this study came from the recognition that an aminocyclohexyl moiety in P-1 fails to take advantage of a recess in this pocket which projects axially from the cyclohexyl ring toward a boundary created by the Tyr 228 side chain (Figure 1, left). The potency of **1r,s** prompted docking of **1u** in this site (Figure 1, right). In this model, the benzene fits nearly perpendicular to the central plane of the cyclohexylamine ring.[8] The present study confirms the predictive power of spatial analysis of X-ray structures of "filled" pockets of liganded enzymes. It also illustrates the power of rapid solid-phase synthesis for exploration of additional binding space. Extensions of the work which have led to low-picomolar, nonbasic thrombin inhibitors will be reported in future publications.

Experimental Section

General. All resin-substrate substitutions were performed in Fisher 12- × 75-mm borosilicate glass disposable culture tubes (stoppered) with constant vortexing during the reaction term. Empty borosilicate glass solid-phase extraction columns (J.T. Baker) with 20-μm Teflon frits in conjunction with a 12-port vacuum manifold were used to filter/wash the resin/product mixtures. The amines used for substitutive deblocking were acquired from the Merck sample collection and Aldrich

(Milwaukee, WI). Solvents and reagents were the highest quality available commercially. Instrumentation included an Eppendorf mixer 5432 vortexer, Savant SpeedVac concentrator, and Hewlett-Packard HP1090 HPLC. Spectral analyses were conducted on a VG 7070E Sector magnetic 70-eV mass spectrometer and Varian XL-300 nuclear magnetic resonance spectrometer.

Preparation of Dipeptide Resin. A. Kaiser resin (2.0 g, 0.5 mequiv/g; Novabiochem) was stirred in a solution of Boc ProOH (2 mmol; BACHEM) in 20 mL of CH_2Cl_2. A solution of dicyclohexylcarbodiimide (4 mL, 0.5 M) in CH_2Cl_2 was added and the suspension shaken for 24 h at room temperature. The resin was filtered and washed with alternating CH_2Cl_2/EtOAc and then suction-dried.

B. The BocPro resin was suspended in 15 mL of trifluoroacetic acid and 30 mL of $MeCH_2Cl_2$ for 1.5 h at room temperature then filtered and washed with alternating CH_2Cl_2/2-propanol and then CH_2Cl_2, and dried.

C. A solution of Boc-D-diphenylalanine (1.023 g, 3.0 mmol) in CH_2Cl_2 was treated with 3 mL of 0.5M DCC in CH_2Cl_2. The resulting precipitate was removed by filtration and the filtrate added to a suspension of the resin from step B in 20 mL of CH_2Cl_2 containing 0.15 mL of triethylamine. The suspension was shaken for 18 h at room temperature. The resin was filtered and washed with a large amount of CH_2Cl_2 and dried. Amino acid analyses of the resin showed 214.8 μmol/g Pro and D-diphenylalanine.

Preparation of Compounds. Displacement of the resin with *trans*-4-Boc-aminocyclohexylamine followed by Boc removal was used to validate the method. The resulting product **1a** (IC_{50} = 120 pM) yield was used to calibrate the relative IC_{50}'s, assuming comparable yields with other amines when HPLC indicated one major displacement product. Approximately 1 mL of CH_2Cl_2, 10 μmol equiv of resin (based on resin-substrate coupling), 2 equiv of TEA, and a slight excess of the appropriate amine were combined. Progress was monitored by HPLC (210 nm). The resin plus reaction medium were filter/washed with 1 mL of CH_2Cl_2 (2×), 1 mL of EtOAc (2×), and 1 mL of CH_2Cl_2 (2×), and the resultant filtrate was dried in vacuo overnight (no heat). To deblock, the Boc-protected compound was steeped in approximately 2 mL of 20% TFA/CH_2Cl_2 at an optimum of 4 h (ambient temperature). The solvent was again removed in vacuo overnight; the resultant compound residue was examined by HPLC (percent purity), ^{1}H NMR, and mass spectrometry (spectrum match) and then submitted for bioassaying as a 5 mM solution in DMSO. Preparation of L-373,102-001C was used as a control in all runs. A blank was added to each run and consisted of all conditions except no displacing amine was added. Yields were "adjusted" relative to the bioactivity of control versus standard (L-373,102-001C). A similar procedure was used for all compounds synthesized in this study, except that as much as 10 equiv of TEA and 2−4 equiv of amine were added if the substituting amines were in salt form or sterically hindered or if reaction progress was slow. Reaction times varied from 24 to 72 h.

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(6) This work is the subject of a patent, WO97/15190; see also: De Grado, W. F.; Kaiser, E. T. *J. Org. Chem*. **1980**, *45*, 1295.
(7) Several other research teams have reported *covalent* inhibitors with neutral P-1 moieties (see refs 1 and 2).
(8) Modeling methods are described fully in ref 3. X-ray structural studies will be published elsewhere.

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