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(N-ACYL-N-ALKYL)GLYCYL *BORO*LYSINE ANALOGS : A NEW CLASS OF POTENT THROMBIN INHIBITORS

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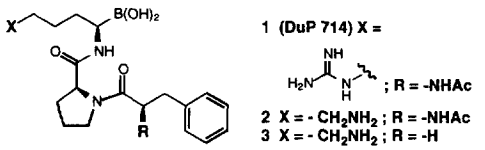
Abstract: In this report the structure-activity relationships of a series of novel (N-acyl-N-alkyl)glycyl *boro*lysine thrombin inhibitors are described. This work culminates in the discovery of (N-3-phenylpropanoyl-N-phenethyl)glycyl *boro*lysine (**12j**), a potent, orally active inhibitor with a binding conformation in which the N-phenethyl group occupies the aryl binding pocket of thrombin.

Thrombin's role in intravascular clot formation as the enzyme responsible for the conversion of fibrinogen to fibrin, the unmasking of the clot cross-linking agent factor XIII, and the feedback amplification of the coagulation cascade, has made this enzyme an important target for the design of novel antithrombotic agents.[1] One of the first small molecule thrombin inhibitors with high affinity for the thrombin active site (K_i = 41 pM) and selectivity over the fibrinolytic enzymes plasmin (K_i = 5100 pM) and t-PA (K_i = 5700 pM) was the NAc-*D*-PhePro *boro*arginine, DuP 714 (**1**, Figure 1).[2] The determination of the X-ray crystal structure of **1** and its *boro*lysine analog **2** bound to thrombin[3] provides an opportunity to use structure-based design techniques to develop structurally diverse alternatives to **1** with good potency, a less peptide-like nature and potentially improved pharmacokinetic and toxicological properties. In this report we describe the discovery of a novel class of inhibitors incorporating a less basic *boro*lysine at P_1 with a binding motif differing from that reported for the *D*-PheProAA class of inhibitor.

Thrombin is a trypsin-like serine protease, its amidolytic activity is dependent upon the enhanced nucleophilicity of the active site Ser^{195} side-chain hydroxyl group catalyzed by the side-chain imidazole of His^{57} (chymotrypsinogen numbering).[4] The substrate specificity of this enzyme for cationic amino acids at the scissile position is determined by the anionic carboxylate supplied by Asp^{189} located at the bottom of the primary recognition site (S_1). The thrombin crystal structures of the *D*-PheProAA inhibitors **1** and **2** shows that these compounds bind to the subsidiary recognition sites of this enzyme in

Figure 1. DuP 714 and its *boro*lysine analogs.

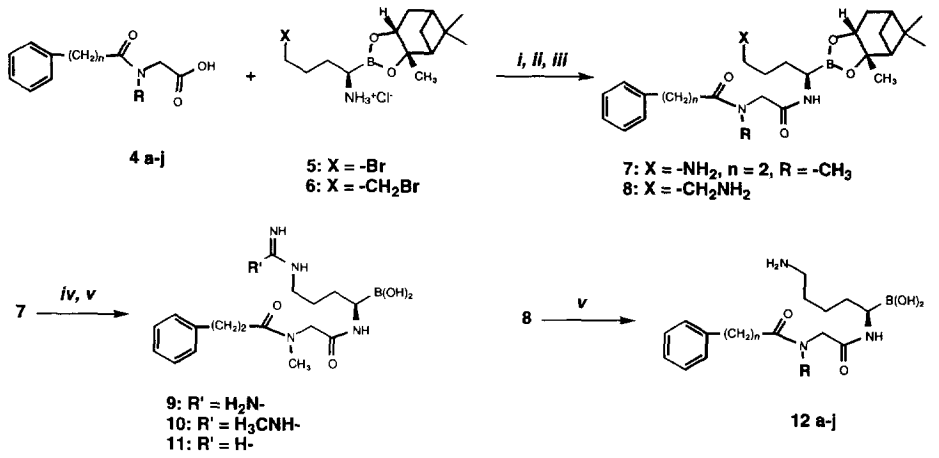


a fashion typical for this class of inhibitor.[3] The pyrrolidine ring of the P_2 Pro is nestled within a lipophilic S_2 binding pocket, defined by the insertion peptide sequence $Tyr^{60a}Pro^{60b}Pro^{60c}Trp^{60d}$. The phenyl of the P_3 *D*-Phe forms an edge-to-face stacking interaction with Trp^{215} in an adjacent aryl binding pocket and a hydrogen bonding network is formed between the NH and carbonyl of Gly^{216} of thrombin and the carbonyl and α-acetamide NH of the *D*-Phe. The boronic acid group of both **1** and **2** forms a near-tetrahedral adduct with the active-site serine mimicing the tetrahedral intermediate expected for normal substrate hydrolysis. The P_1 side chain of both compounds adopts an extended conformation, in the case of **1** the terminal guanidinium group forms a bidentate interaction with the carboxylate side chain of Asp^{189}, while the butylammonium side chain of *boro*lysine **2** interacts with Asp^{189} indirectly through a bridging water molecule.[3]

Our objective was to develop a series of less peptide-like inhibitors with *boro*lysine at P_1. It has been our experience that, compared to *boro*arginine analogs, *boro*lysines have a diminished tendency to produce elevations in serum transaminase levels and hypotension in vivo.[5] Modeling suggested to us that the structures of **1** and **2** could be simplified by replacing the P_3 *D*-Phe with a 3-phenylpropanoyl group[6] to give compound **3**;[7] a further simplification would result if the P_2 *L*-Pro was exchanged for an asymmetric N-alkyl Gly. This hypothesis is exemplified by the model for the *boro*lysine analog **12a** in Figure 3;[8] the N-methyl group of the P_2 Gly substituent is accommodated by the S_2 pocket, while the phenyl ring of the 3-phenylpropanoyl adopts an edge-to-face interaction with Trp^{215}. In this series, the two hydrogen bonds formed by the *D*-Phe carbonyl and acetamide of **1** and **2** with Gly^{216} is reduced to a single hydrogen bond between the carbonyl of the 3-phenylpropanoyl and the amide NH of Gly^{216}.

Synthesis: The compounds in this study were prepared by the methods outlined in Scheme 1. The optically pure *boro*arginine precursor **5** was synthesized by a previously described procedure[2,9] and *boro*lysine precursor **6** was obtained by an adaptation of this route.[3] The P_3P_2 substituents **4a**, **4e-j** were prepared by the acylation of the appropriate N-alkyl Gly ester[10] with 3-phenylpropanoyl chloride ($CHCl_3$, Et_3N, 0 °C) or, in the case of compounds **4b-d**, with benzoyl-, phenylacetyl- and 4-phenylbutanoyl chloride, respectively. Following saponification of the ester functionality, **4a-j** was coupled to either **5** or **6** (HBTU, N-methylmorpholine, DMF, 0 °C, 45 min) whereupon the resulting bromide was displaced with sodium azide (3 equiv, DMF, 100 °C, 1 h). The azide was reduced to the amine **7** or **8** (H_2, 1 atm, 10% Pd-C, MeOH) and isolated as the hydrogen chloride salt. At this point the free boronic acids **12a-j** were obtained by removing the pinanediol ester by transesterification of **8** with phenylboronic acid (5 equiv, 1:1 water:ethyl ether).[11] Compound **3** was obtained by acylation of **6** with the mixed anhydride of N-(3-phenylpropanoyl)proline (1 equiv isobutylchloroformate, 2 equiv N-methylmorpholine, THF, -20 °C); the acylation product was then treated by the same sequence described for preparing *boro*lysines **12a-j**. *Boro*arginine analogs **9-11** were synthesized by reaction of amine **7** with a guanidinylation or amidination reagent (formamidine sulfonic acid to prepare **9**, N-methylformamidine sulfonic acid to prepare **10** and ethylformimidate•HCl to give **11**, in EtOH, 2 equiv DMAP, reflux) followed by transesterification to give the free boronic acids.

Scheme 1. Preparation of the study compounds.

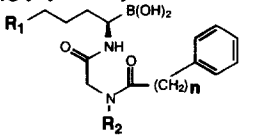


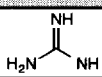
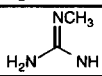
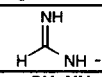
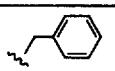
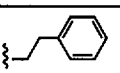
Reagents : (i) HBTU, NMM, DMF; (ii) NaN_3, DMF; (iii) H_2, Pd-C, MeOH; (iv) for 9: $H_2NC(=NH)SO_3H$, DMAP, EtOH; for 10: $H_2NC(=NCH_3)SO_3H$, DMAP, EtOH; for 11: HC(=NH)OEt•HCl, DMAP, EtOH; (v) $PhB(OH)_2$, Et_2O, H_2O.

Results and Discussion: Our experience with this series is summarized in Table 1. Examination of the data for the (N-(3-phenylpropanoyl)prolyl)*boro*lysine **3** demonstrates that exchanging the P3 *D*-Phe of **2** for a 3-phenylpropanoyl group results in only a 3-fold decrease in the inhibitory constant (K_i),[12a] while substituting the P_2 Pro of **3** for the N-methyl Gly of **12a** gives a 2-fold decrease in K_i. For both cases, in our in vitro measure of anticoagulation, thrombin time,[12b] little variation in activity is observed within the limits of that assay. Comparison of **1** with **9** and **2** with **12a** demonstrates that a modest 4- to 7-fold decrease in inhibitory constant and a small change in thrombin time results when these replacements for P_2 and P_3 are combined in one molecule. Compounds **10** and **11** are alternative modifications of the cationic P_1 side chain. These analogs proved to be less effective than the corresponding boroarginine **9** or borolysine **12a** in either enzyme inhibition or in vitro anticoagulation. The IC_{50} values[12c] obtained for the thrombin inhibition of **12a** and **13** (Figure 2) in a preliminary assay demonstrates the importance of N-methyl substitution in this series. This finding parallels a similar relationship observed between **1** and its P_2 Gly analog,[13] and supports the assumption that the N-methyl group would interact with the S_2 pocket as a surrogate for the pyrrolidine ring of **1** and **2**. The effectiveness of *boro*lysine analog **12a** in the thrombin time assay prompted us to explore the structure-activity relationships of this P_1 variant .

The data for compounds **12b-d** confirms the prediction of the binding model for **12a** (Figure 3) that effective interaction with the aryl binding pocket requires a 3-phenylpropanoyl group. As illustrated in Figure 3, lower and higher homologs either cannot be extended to make an effective edge-to-face interaction with the indole π-system of Trp^{215} or must adopt a high energy conformation to do so. The N-alkyl group in the P_2 position of **12a** was varied over a range of substituents to give compounds **12e-j**. The lipophilic S_2 pocket of thrombin is capped by the $Tyr^{60a}Pro^{60b}Pro^{60c}Trp^{60d}$ insertion sequence. This portion of the 9-residue insertion loop has been reported to be very rigid and rarely varies position by more than 1 Å in the thrombin crystal structures reported to date,[4] thereby providing a well-defined

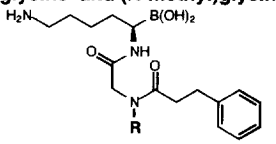
Table 1. Discovery of the (N-acyl-N-alkyl)glycyl *boro*lysine thrombin inhibitors.



	R_1	R_2	n	Thrombin K_i nM	Thrombin Time nM	Trypsin K_i nM (selectivity)*	Plasmin K_i nM (selectivity)	tPA K_i nM (selectivity)
1				0.041	55	0.045 (1.1 x)	5.1 (120 x)	5.7 (140 x)
2				0.24	200	-	-	-
3				0.80	100	-	-	-
9		- CH_3	2	0.16	100	0.61 (3.8 x)	460 (2880 x)	51 (320 x)
10		- CH_3	2	2.1	1500	18 (8.6 x)	14000 (6670 x)	-
11		- CH_3	2	9.1	400	21 (2.3 x)	> 10000 (> 1000 x)	7000 (770 x)
12a	- CH_2NH_2	- CH_3	2	1.71±0.38	150	2.8 (1.6 x)	250 (150 x)	3800 (2240 x)
12b	- CH_2NH_2	- CH3	0	123	1000	-	-	-
12c	- CH_2NH_2	- CH3	1	16	500	-	-	-
12d	- CH_2NH_2	- CH3	3	5.4	350	-	-	-
12e	- CH_2NH_2	- $CH(CH_3)_2$	2	1.6±0.0	125	3.8 (2.4 x)	140 (90 x)	3370 (2100 x)
12f	- CH_2NH_2		2	0.69 ±0.10	200	1.1 (1.6 x)	160 (230 x)	750 (1100 x)
12g	- CH_2NH_2		2	4.9±2.1	600 **	8.4 (1.7 x)	250 (50 x)	1100 (220 x)
12h	- CH_2NH_2		2	1.4±0.10	150	-	230 (160 x)	280 (200 x)
12i	- CH_2NH_2		2	4.68±0.48	350	-	-	-
12j	- CH_2NH_2		2	0.42±0.07	200	2.5 (6 x)	170 (400 x)	680 (1620 x)

*Selectivity computed by the ratio of $K_{i\ enzyme}$ / $K_{i\ thrombin}$. ** Tested as the pinanediol ester of the boronic acid.

Figure 2. Comparison of glycine and (N-methyl)glycine at P_2



12a : R = -CH_3
Thrombin IC_{50} = 1.5 μM
13 : R = H
Thrombin IC_{50} = 90.0 μM

limit along the upper face of the S_2 pocket. The model of these analogs suggested that the N-isopropyl (**12e**) and N-cyclopropyl (**12f**) would be accommodated by the S_2 pocket while the larger substituents for **12g-h** would not be. This prediction is not supported by the data of Table 1; it is apparent from the good enzyme inhibition and reasonable thrombin time for even the bulky N-cyclohexyl analog **12g** that little selection is observed based on substituent size. The flexible nature of these compounds, and the increasing negative contribution of desolvation energy to the free energy of binding as substituent size is

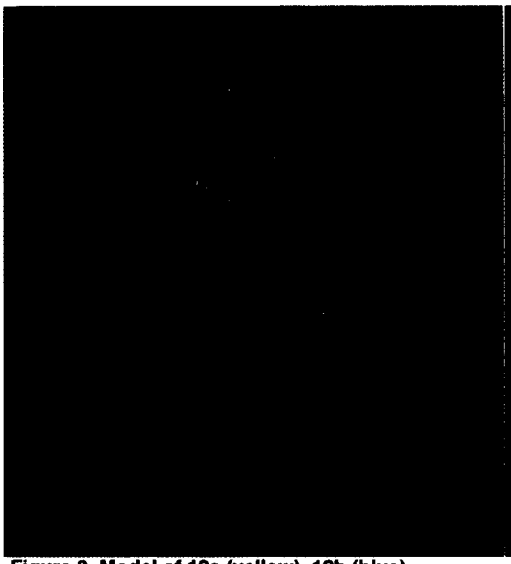


Figure 3. Model of 12a (yellow), 12b (blue), 12c (pink) and 12d (orange) bound to thrombin.

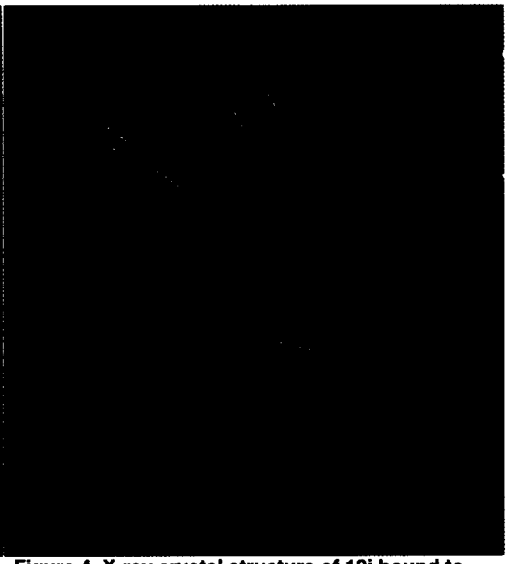


Figure 4. X-ray crystal structure of 12j bound to thrombin.

increased, makes qualitative computational approaches to this series poorly predictive.

We have found the *boro*arginine and *boro*lysine thrombin inhibitors have poor selectivity over trypsin, however, substantial improvements over **1** were observed for selectivity against the anticoagulant enzymes plasmin and tPA.[12a] Examination of the selectvity ratios for the *boro*arginines **9** and **1** suggests that much of this improvement is due to the departure from the *D*-PhePro P_3P_2. Comparison of **9** with the corresponding *boro*lysine **12a** indicates that the *boro*lysine P_1 is less selective for thrombin against plasmin but more selective against tPA. This selectivity can be modified, as variation of the N-alkyl group among *boro*lysines **12a**, **12e-h** and **12j** demonstrates. The N-phenethyl analog **12j** represents the most selective and potent borolysine of this series. This compound is orally active in dog with aPTT elevated > 2-fold for 2h when dosed at 5 mg/kg, po.

To better understand the mode of binding of **12j** an X-ray crystal structure was obtained (Figure 4).[14] As in the case of **2**,[3] the *boro*lysine of **12j** adopted the expected extended conformation into the S_1 pocket with a strong water mediated hydrogen bond to Asp^{189} and an apparent covalent interaction between the boronic acid group and Ser^{195} hydroxyl. The backbone of the P_3P_2 substituent of **12j** adopts a turn conformation anti-parallel to the $Ser^{214}Trp^{215}Gly^{216}$ sequence defining the S_1 pocket of thrombin with two hydrogen bonds to the carbonyl of Ser^{214} and NH of Gly^{216}. While only a small portion of the S_2 pocket is occupied by the ethyl linker of the N-phenethyl group, the aryl binding pocket is filled by an edge-to-face stacking interaction between the aryl ring of the N-phenethyl substituent with the indole ring of Trp^{215}. The 3-phenylpropanoyl group packs against the side chain of Glu^{217}. There is clear electron density for only a single binding conformation for **12j**, in contrast to the binding mode reported for the thrombin complex of the inhibitor (N-(*D*-homoprolyl)-N-(phenethyl)-glycyl) arginal by others .[15]

Compound **12j** is the protoype for a potent series of thrombin inhibitors with a binding motif differing from the D-PheProAA class of inhibitor. The simplified structure lends itself to modification and has served as the basis for a more selective class of enzyme inhibitor devoid of the hypotension and serum transaminase enzyme elevation often associated with thrombin inhibitors.[5] Future reports will describe our efforts in this area.

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References and Notes

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8. Each ligand was modeled in various conformations and orientations into the thrombin active site and attached covalently to Ser195 with a tetrahedral, negatively charged carboxylate as a boronic acid surrogate. Energy minimizations with the Discover program and the CVFF force field (Molecular Simulations, Inc., Burlington, MA 01803-5297) were carried out for these different binding models keeping the protein rigid, but allowing the ligand to be completely flexible. The model with the lowest energy was assumed to represent the most probable binding mode.
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