Characterization of a Class of Peptide Boronates with Neutral P1 Side Chains as Highly Selective Inhibitors of Thrombin

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Z-D-Phe-Pro-boroMpg-OPin (**9a**)[1,2] has been shown previously to be a highly specific inhibitor of thrombin in spite of lacking an arginine-like guanidino group at the P1 site.[1] A range of compounds have been synthesized based upon this lead compound, varying the neutral side chain at the P1 site. Of the 20 examples based upon the structures at P2 and P3 of Z-D-X-Pro (X being Phe or β,β-diphenylalanine), all were found to be effective inhibitors of thrombin (K_i's between 10 and 100 nM). Furthermore all exhibited a high specificity toward thrombin having values for a K_i(trypsin)/K_i(thrombin) ratio of between 10- and 100-fold. High ratio values were found for a number of the compounds tested against a range of serine proteinases (plasmin, factor Xa, kallikrein, urokinase, protein Ca, chymotrypsin, elastase, and cathepsin G).[2] As far as potency toward thrombin, compounds containing the methoxypropyl group at P1 were favored over those with a methoxy grouping on a shorter alkyl chain (**8**) or without the methoxy group (**1–5**). The compounds display potent anticoagulant activity with values for **18** in thrombin time of 0.63 μM and in activated partial thromboplastin time of 2.0 μM. ^{11}B NMR has been used to confirm interaction of the boron atom with the active site. From the high specificity shown with all the compounds we propose that the compounds, constitute a new class of thrombin inhibitors.

Introduction

The impetus for the development of specific inhibitors of thrombin has increased over recent years with the realization that although thrombo occlusive events in the venous circulation are amenable to prophylaxis or treatment by the established pharmaceuticals warfarin and heparin, in the arterial circulation thrombotic processes are less responsive to these drugs. A resistant thrombi[3] may be caused at sites of atherosclerotic stenosis and plaque rupture leading to heart attack and stroke or as a result of treatment for symptomatic atherosclerotic disease, for example, coronary angioplasty (PTCA).[4] More recently such thrombi have been found to be more easily prevented by hirudin,[5] the specific, tight binding inhibitor of thrombin, a 65-amino acid polypeptide isolated from the saliva of leeches, which has become available through recombinant technology. The success in preclinical development of hirudin and its shorter synthetic analogue, hirulog,[6,7] has increased interest in the development of a low molecular weight inhibitor of thrombin, with properties akin to hirudin but administerable orally.

In the development of such inhibitors, we and previous workers have considered the properties of thrombin as a trypsin-like proteinase which preferably cleaves polypeptide substrates at Arg/Lys–Xaa bonds.[8–10] Unlike trypsin and helpful to the development, α-thrombin displays highly restricted specificity at adjacent sites, exhibiting particular preference for hydrophobic P2–P4 residues. The requirements have been used in the design of a number of potent inhibitors of thrombin built around a P1 arginine or arginine analogue, considered to be obligatory for binding to the primary specificity pocket of the enzyme. Indeed a number of agmatine-based inhibitors have been made that retain the positively charged group binding to the specificity pocket, yet, like the so-called tripod-like inhibitors, they do not interact covalently with the active site serine and still retain submicromolar K_i's.[11] However, the inclusion of such a group immediately increases affinity of the inhibitor to other trypsin-like proteinases, trypsin itself with a wide specificity being particularly succeptible to inhibition. This is an important consideration for drugs of potential oral administration, since trypsin inhibition leads to gastric toxicity.[12] The P1 arginal inhibitor D-Phe-Pro-Arg-H[13] has K_i(Try)/K_i(Thr) of only 4.[14] Replacements at P3 had deleterious effects on selectivity against trypsin with Boc-D-Phe having a K_i(Try)/K_i(Thr) ratio of 0.3 and H-D-MePhe with K_i(Try)/K_i(Thr) decreased to 2-fold.[15] Unnatural amino acids at P3 showed higher binding affinity for trypsin than thrombin[16] and lost 10–20-fold activity against thrombin (IC_{50} > 10^{-7} M) on P3 Phe substitution by desamino-Phe[14] and desaminophenylglycine.[16] In selection of candidiate compounds from *in vitro* data that are likely to possess optimum *in vivo* antithrombotic efficacy, potential antifibrinolytic activity is critical. We have demonstrated previously that lack of selectivity to the target proteinase, manifested in inhibition of plasmin and urokinase, has a disastrous effect on the *in vitro* activity, leading to prolongation of fibrinolysis. Recently[17] this has been shown to extend to *in vivo* situations, in a rabbit model of thrombolysis, where arginine-based inhibitors such as Ac-D-Phe-Pro-boroArg-OH (DuP 714)[18] lead to significant fibrinolytic compromise. Since this experiment is representative of potential clinical use of high concentrations of thrombin inhibitors, say during PTCA, selectivity for thrombin is clearly essential in any potential clinical candidate. In the arginal series H-D-Phe[14] and D-MePhe[15] have similar K_i(Pla)/K_i(Thr) of

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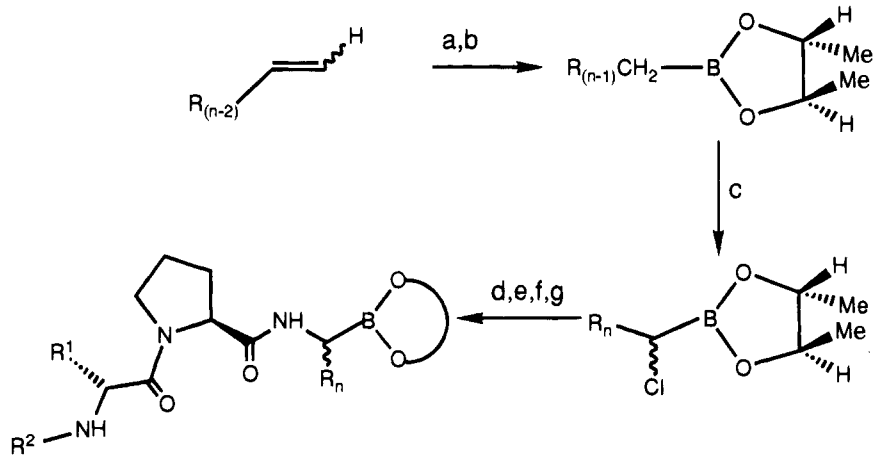
Scheme 1[a]



[a] (a) Heat, catecholborane; (b) butanediol; (c) $LiCHCl_2$, −100 °C; (d) transesterify; (e) $LiN(SiMe_3)_2$, −78 °C; (f) 3 equiv of HCl; (g) dipeptide, *i*-BuCOOCl, NMM, Et_3N.

>40-fold, while the Boc analogue greatly loses selectivity, with a ratio of 1.[16] The competitive inhibitors FOY and FUT-175 have K_i(Try)/K_i(Thr) ratios of 0.001 and 0.03, respectively,[19] and are more potent for protein Ca and plasmin than thrombin and about equipotent for factor Xa and t-PA.[20] The protracted chiral synthesis of the tripod-like inhibitors is unattractive, and the increased binding affinity for thrombin between isomers paralleled that for trypsin[21] so that no clues on selectivity are forthcoming. P1 boroArg inhibitors[18] were equipotent in initial binding affinity for thrombin as trypsin, factor Xa, and plasmin, only showing selectivity against chymotrypsin-like proteinases. It is considered that the arginine group is also responsible for hypotensive effects[22-24] resulting in the limiting toxicities of NAPAP (50 mg/kg iv)[22] and other tripod-like inhibitors. Also highly charged groups disfavor enteral absorption[25-27] such that the cationic guanidino group may contribute to the lack of bioavailability of argatroban.[22]

Considering these factors for inhibitor design, we have recently proposed the possibility of a new approach in describing a thrombin inhibitor with a neutral side chain at P1. The compound Z-D-Phe-Pro-boroMpg-OPin (**9a**) was observed to combine a high binding affinity for thrombin (K_i ~ 10 nM) with high specificity as regards inhibition of a number of other trypsin-like proteinases (trypsin, plasmin, kallikrein). In this present work we have examined the properties of a range of compounds based upon this original structure. We observe that as a group all such compounds show a high specificity toward thrombin and thereby constitute a new class of inhibitors for the enzyme. Such compounds are in general innately specific for thrombin, holding out the possibility of chemical modification to "tune" the properties of the lead compound as regards toxicity and oral availability, without deleterious effects upon potency and specificity.

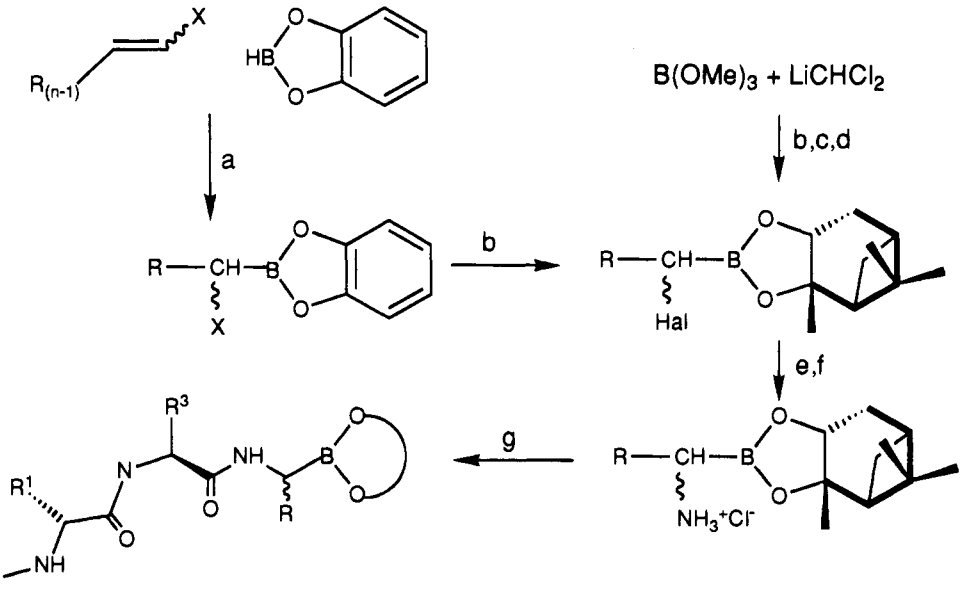
Chemistry

Synthesis of Tripeptide Boronates with Neutral P1 Side Chains. Stereospecific synthesis of the tripeptides **1**, **3**, **4**, **7**−**21**, and **25** with D or L absolute configuration at the P1 α-carbon was conducted by established methodology[28,29] using (+)- or (−)-pinanediol[30,31] as the chiral director. This demonstrated the previously unknown finding that the tripeptide with P1 in the L configuration, **9a**, exhibited 2 orders of magnitude lower K_i for thrombin than the D diastereomer **11**. This was then consistent with the intermediate binding affinity of the racemic boronate, not shown (K_i ~ 41 nM), synthesized via the pinacol α-amino boronate ester, with racemic P1 position. The boroMpg compound **9b** was synthesized as the chiral pinacol ester for the NMR studies. Because of the known refractance of pinanediol esters to transesterification, synthesis was conducted via (2*R*,3*R*)-(−)-2,3-butanediol (Scheme 1) in order to allow conversion to the pinacol ester, and the L-isomer, given in a ratio of ~4:1 (CHα-B: δ 2.63:2.9) to the D isomer, was isolated by chromatography. The configuration was confirmed by comparison to the NMR (e.g., CHα-B: δ 2.63, L-(+)-pinanediol **9a**; δ 2.9, D-isomer **11**) and the optical rotation of $[\alpha]^{24} = 61.1°$ (5 mg/mL in MeCN) of the product from the (+)-pinanediol route. Synthesis of the three ester forms, pinanediol **9a**, pinacol **9b**, and methyl **10**, gave equipotency against thrombin and confirmed that the esters are effectively prodrugs.[32] In order to prepare a wide range of P1 functionalities, our novel route of hydroboronation of α-haloalkenes (Scheme 2) was used,[33] using Rh(I) catalysis[34] for the less reactive olefins required, e.g., for **6** and **24**. For certain inhibitors with sterically crowded β-positions (e.g., **5**, **22**, **23**), not available by the above routes, a third method[33] was synthesis from (dichloromethyl)boronate[35,36] (Scheme 2).

Results

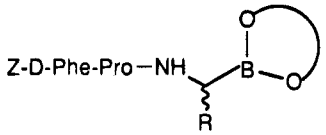
Activity as Thrombin Inhibitors. All of the series of inhibitors bearing the Phe-Pro or Dpa-Pro motif were found to be effective competitive inhibitors of thrombin with K_i values varying from 1 to 100 nM (Tables 1−3). None of the inhibitors exhibited slow tight binding characteristics as was observed for the compound Z-D-Phe-Pro-boroIrg-OPin (**17**) which is similar in structure and properties to the previously described boroArg inhibitors of thrombin. In compound **13** in which the Pro is substituted by Val, there was a 2000-fold loss in binding affinity. Compounds containing Dpa rather than Phe were less potent except in the case of compound **18** (compared to **9a**). The benzyloxycarbonyl group is favored at P3, since substitution by acetyl

Scheme 2[a]



[a] (a) X = Hal, 80–90 °C, ultrasound, or $Rh(PPh_3)Cl$, rt, or X = H, reflux, distill, (+)-pinanediol, $LiCHCl_2$, −100 °C; (b) (+)-pinanediol, THF; (c) −100 °C, THF, 0.5 h, H_3O^+; (d) RMgBr or RLi; (e) $LiN(SiMe_3)_2$; (f) 3 equiv of HCl, −78 °C; (g) Z-D-NHCHR¹CON(R³)CHCO-OH, NMM, *i*-BuCOOCl, Et_3N.

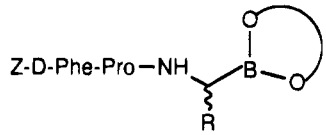
Table 1. D-Phe-Based Thrombin Inhibitors with Apolar P1 Side Chains



no.	R[a]	formula[b]	anal.	K_i(Thr) (nM)
1	CH_2CH_3	$C_{35}H_{46}N_3O_6B$	C,H,N	1000
2	$(CH_2)_4CH_3$	$C_{34}H_{48}N_3O_6B$	C,H,N	26
3	$(CH_2)_4CH_3$	$C_{38}H_{52}N_3O_6B$	C,H,N	19
4[c]	$(CH_2)_7CH_3$	$C_{37}H_{54}N_3O_6B$	C,H,N	43
5[c]	$C(CH_3)_2CH_2CH_3$	$C_{38}H_{52}N_3O_6B$	C,H,N	7
6[c]	CH_2Ph	$C_{36}H_{44}N_3O_6B$	C,H,N	18
7	$CH_2CH_2Ph(p\text{-}Me)$	$C_{38}H_{48}N_3O_6B$	C,H,N	20

[a] All D,L configuration at P1 and pinacol esters except **1** and **5** that are pinanediol esters and **3** which is a chiral pinanediol ester. [b] All compounds show MS and ^1H and ^{13}C NMR consistent with the assigned structures. [c] Presented previously.[28]

Table 2. D-Phe-Based Thrombin Inhibitors with Polar P1 Groups



no.	R	P1[a]	formula[b]	anal.	K_i(Thr) (nM)
8	$(CH_2)_2OMe$	D,L	$C_{36}H_{48}N_3O_7B$	C,H,N	21
9a[c]	$(CH_2)_3OMe$	L	$C_{37}H_{50}N_3O_7B$	C,H,N	7
9b	$(CH_2)_3OMe$	L	$C_{33}H_{46}N_3O_7B$	C,H,N	7
10[d]	$(CH_2)_3OMe$	L	$C_{29}H_{40}N_3O_7B$	C,H,N	7
11	$(CH_2)_3OMe$	D[e]	$C_{37}H_{50}N_3O_7B$	C,H,N	120
12	$(CH_2)_3Br$	L	$C_{36}H_{47}BrN_3O_6B$	C,H,N	7
13[f]	$(CH_2)_3Br$	L	$C_{36}H_{49}BrN_3O_6B$	C,H,N	15 000
14	$(CH_2)_3Br$	D[e]	$C_{36}H_{47}BrN_3O_6B$	C,H,N	130
15	$(CH_2)_2Cl$	D,L	$C_{35}H_{45}ClN_3O_6B$	C,H,N	29
16[g]	$(CH_2)_2CH(OCH_2)_2$	D,L	$C_{39}H_{52}N_3O_8B$	C,H,N	25
17	$(CH_2)_3SC(NH)NH_2$	L	$C_{37}H_{53}30N_5O_6SB.HBr$	C,H,N	1

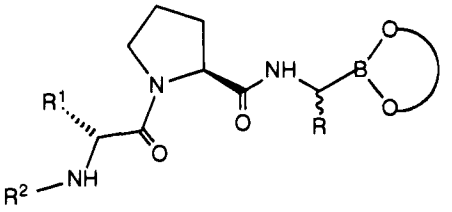
[a] Stereo configuration at P1. [b] All compounds show MS and ^1H and ^{13}C NMR consistent with the assigned structures. [c] Presented previously. [d] Dimethyl ester. [e] (−)-Pinanediol ester. [f] P2 is Val. [g] Presented previously.[28]

caused >100-fold drop in potency (compounds **9a** and **19**, **6** and **21**).

Selectivity for Thrombin against Trypsin. Uniquely, of all previously reported low molecular weight tripeptide-based thrombin inhibitors, the compounds of this series, excluding those with P1 = Phe (**6**, **24**), are more than 10-fold more inhibitory for thrombin than trypsin, and in the best case (compound **9a**), the ratio K_i(Try)/K_i(Thr) is ~100, which is superior by 10-fold to that of NAPAP[37] and only equalled by argatroban.[21] From the initial K_i values of the inhibitor with the positively charged P1 boroIrg side chain, **17**, it is equipotent for thrombin as trypsin. Modification to P3 D-Dpa does still improve selectivity (Scully, unpublished results). Modification from charged **17** to neutral polar side chain **9a**, with retained P3 and P2, increases K_i(Try)/K_i(Thr) by 2 orders of magnitude (Table 5). For compounds with P1 Mbg, change at P3 from D-Dpa (**22**) gives 20-fold K_i(Try)/K_i(Thr), and D-Phe (**5**) and D-β-Nal (**23**) are even better at 36- and 30-fold, respectively. This selectivity against Try is highly significant for a drug designed for oral delivery. Similar aldehyde-based inhibitors (D-MePhe-Pro-Arg-H) manage only 2-fold selectivity, while PPACK has a higher rate of irreversible inhibition of trypsin.[38]

Influence of Variation of P3 Amino Acid on Selectivity toward Serine Proteinases According to Variation in P1 (Table 5, Figure 1). The absolute K_i for each compound against each enzyme is shown in Table 1. Figure 1A shows the the variation in K_i against different serine proteases of compounds with P3 Phe on changing the P1 residue. The inhibitor with P3 Phe and P1 as the positively charged Irg group, **17**, is equipotent for trypsin, plasmin, factor Xa, kallikrein, urokinase, and protein Ca (Figure 1A) (Table 5). Modification of the P1 to the neutral groups Mpg (**9a**), Mbg (**5**), or Pgl (**3**) as noted above increases the specificity for thrombin against trypsin to 100-, 37-, and 17-fold, respectively (Figure 1A). Specificity against all other trypsin-like serine proteinases was increased between 30- and >10 000-fold, and in the case with P1 as Mpg (**9a**),

Table 3. Influence on *in Vitro* K_i(Thr) of P3 and P4 Groups



no.	R[a]	R[1]	R[2] (P4)	formula[b]	anal.	K_i(Thr) (nM)
18	$(CH_2)_3OMe$[c]	$CHPh_2$	Z	$C_{43}H_{54}N_3O_7B$	C,H,N	3
19	$(CH_2)_3OMe$[c]	CH_2Ph	Ac	$C_{31}H_{46}N_3O_6B$	C,H,N	11 000
20[d]	$(CH_2)_4CH_3$	$CHPh_2$	Z	$C_{40}H_{52}N_3O_6B$	C,H,N	42
21	$(CH_2)_4CH_3$	CH_2Ph	Ac	$C_{28}H_{44}N_3O_5B$	C,H,N	100
22[d]	$C(CH_2)_2CH_2CH_3$	$CHPh_2$	Z	$C_{44}H_{56}N_3O_6B$	C,H,N	82
24	CH_2Ph	$CHPh_2$[e]	Z	$C_{42}H_{48}N_3O_6B$	C,H,N	130
25	$CH(CH_3)_2$	$CHPh_2$	Z	$C_{42}H_{53}N_3O_6B$	C,H,N	19

[a] All D,L configuration at P1, except **18** and **19**. [b] All compounds show MS and ^1H and ^{13}C NMR consistent with the assigned structures. [c] L configuration. [d] Presented previously.[28] [e] L configuration.

Table 4. Anticoagulant Activity

no.	TT (μM)[a]	APTT (μM)[b]
3	0.54	2.3
4	3.9	>50
5	1.1	4.0
6	0.53	2.5
9a	2.8	3.4
16	3.4	18
17	0.066	0.069
18	0.63	20
22	21	50
23	16	110
24	5.2	>50

[a] Concentration of inhibitor required to produce a 2-fold prolongation in thrombin time (TT). [b] Concentration of inhibitor required to produce a 2-fold prolongation in activated partial thromboplastin time (APTT).

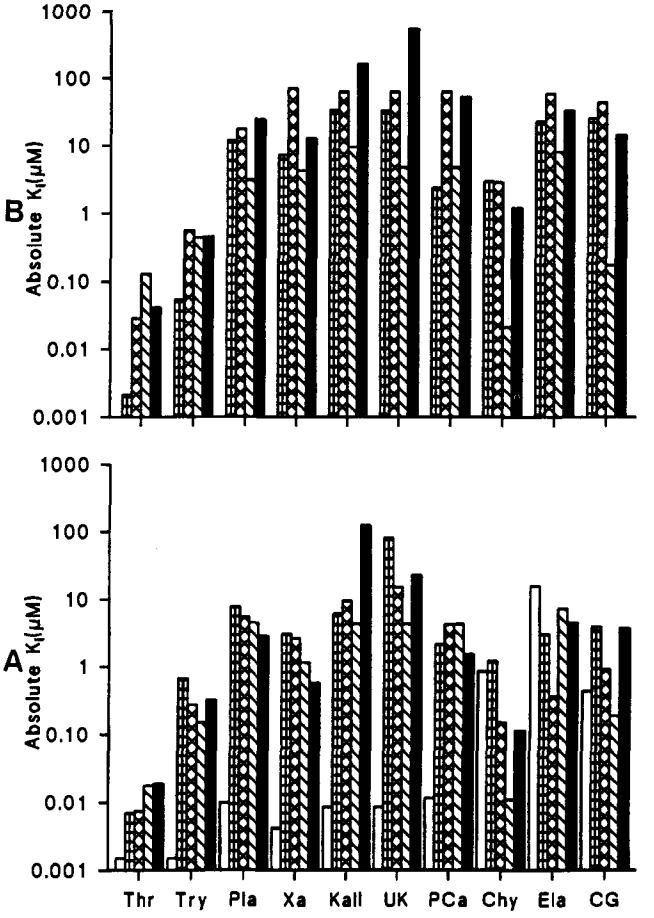


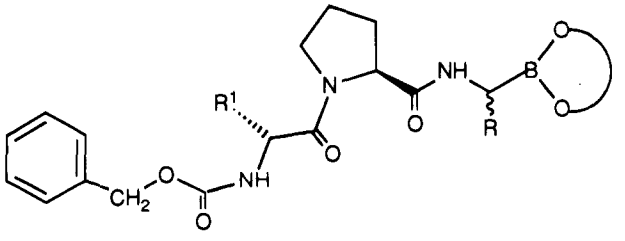
Figure 1. Absolute K_i (μM) of each compound with P3 D-Phe (A) and D-Dpa (B) against the serine proteinase: open bar, P1 BoroIrg compound **17**; +, P1 boroMpg compound **9** (A) and **18** (B); x, for P1 boroMbg compound **5** (A) and **24** (B); hatched bar, P1 boroPhe compound **6** (A) and **24** (B); and filled bars, P1 boroPgl compound **3** (A) and **20** (B).

specificity against plasmin, kallikrein, and urokinase is of the order of 1000–10 000 (Table 5).

Replacement of D-Phe by D-Dpa at the P3 position for the compounds with boroMpg (**18**), boroMbg (**22**), and boroPgl (**20**) at P1 gives generally higher selectivity (300–1000-fold) against serine proteinases other than trypsin (Figure 1B). Although compounds with Phe at P1 (**6**, **24**) are specific against trypsin like serine proteinases, they are not specific against chymotrypsin or cathepsin G (Figures 1B and 2C).

In the case of P3 D-Dpa and P1 boroMpg (**18**), some selectivity for thrombin against trypsin is lost in regard to the parent compound with P3 D-Phe (**9a**). For other

Table 5. Specificity for Thrombin against Other Serine Proteases[a]



no.	R	R[1]	K_i(αThr)	K_i(γThr)	K_i(Pla)	K_i(Xa)	K_i(Kal)	K_i(UK)	K_i(Try)	K_i(PCa)	K_i(Chy)	K_i(Ela)	K_i(CG)
3	$(CH_2)_4CH_3$	CH_2Ph	0.019	0.019	2.9	0.58	120	23	0.32	1.6	0.115	4.56	3.74
5	$C(CH_3)_2CH_2CH_3$	CH_2Ph	0.007	0.024	5.5	2.6	9.5	15	0.27	4.2	0.152	0.365	0.916
6	CH_2Ph	CH_2Ph	0.018	0.011	4.5	1.2	>4.3	>4.3	0.15	>4.3	0.011	7.25	0.187
9a	$(CH_2)_3OMe$	CH_2Ph	0.007	0.028	7.8	3.1	6.1	81	0.67	2.1	1.21	3.08	3.9
17	$(CH_2)_3SC(NH)NH_2$	CH_2Ph	0.0015	0.0065	0.01	0.0042	0.0049	0.0086	0.0015	0.012	0.848	15.8	0.437
18	$(CH_2)_3OMe$	$CHPh_2$	0.002	0.004	12	7.2	34	33	0.054	2.4	2.96	22.8	25.3
20	$(CH_2)_4CH_3$	$CHPh_2$	0.042	0.027	24	13	160	540	0.45	53	1.2	33.1	14.3
22	$C(CH_3)_2CH_2CH_3$	$CHPh_2$	0.028	0.034	17	71	>63	>63	0.56	>63	2.89	59.1	44.1
23	$C(CH_3)_2CH_2CH_3$	CH_2-β-naphthyl	0.082	0.140	13	23	370	140	2.4	32	1.46	2.2	3.85
24	CH_2Ph	$CHPh_2$	0.13	0.059	3.0	4.3	9.5	>48	0.44	>4.8	0.021	8.08	0.174

[a] K_i determined for each inhibitor with each enzyme using chromogenic assay as described (see the experimental). Figures are determined from at least five data points in duplicate (SD = ±5%).

P1 side chains, the binding affinity for trypsin remains approximately constant on introduction of P3 D-Dpa (compounds **18**, **20**, **22**; Figure 1B), and the slight ~3-fold loss of selectivity is due to a decrease in binding affinity for thrombin rather than an increase for trypsin. For inhibitors with P1 boroPhe (**6**, **24**), D-Phe improves the specificity with respect to L-Dpa (Figure 2C).

With P3, D-Phe replacement of the P1 boroMpg with the apolar Pgl group gives compounds with generally reduced specificity against all proteinases (Figure 1A), from a range of 100–1000 down to 10–100. This is true for the compounds with both D-Phe (**3**) and D-Dpa (**20**) at P3 (Figure 1). Interestingly the specificity observed against chymotrypsin-like enzymes observed with P1 boroMpg (**9a**) (Figure 2A) and P3 D-Dpa (**18**) (10 000-fold) is significantly reduced with P1 boroPgl (**2**, **20**) (Figure 2D) to 100–1000-fold.

Influence of Variation of P3 with Constant P1 (Figure 2). For compounds with boroMpg at P1, substitution of D-Dpa (**18**) for D-Phe (**9a**) at P3 increases the specificity against all proteinases other than trypsin (Figure 2A). This is particularly noticed against the chymotrypsin-like enzymes.

Substitution of various hydrophobic groups at P3 for inhibitors with boroMbg at P1 gives specificities for thrombin against trypsin in the range 20–35-fold (Figure 2B). Notably increasing the hydrophobicity at P3 from D-Phe (**5**) to D-Dpa (**22**) to D-β-Nal (**23**) gives K_i(Kal)/K_i(Thr) of 1300-, 2300-, and 4000-fold, respectively. Specificities against other trypsin-like proteinases are generally better than for P1 Pgl (**2**) (Figure 2D) but are not as good as P1 boroMpg (**9a**) (Figure 2A). The differences in the P3 group do not appear to make a significant difference within each proteinase except that the D-Dpa compound **18** gives increased specificity against the chymotrypsin-like enzymes (chymotrypsin, elastase, cathepsin G). For inhibitors with P1 boroPhe (**6**, **24**), D-Phe improves the specificty with respect to L-Dpa (Figure 2C).

Discussion

Lack of inhibitory activity against trypsin is essential for antithrombotics of potential oral administration. Most classes of low molecular weight substrate-like inhibitors previously described demonstrated no greater than a 2-fold K_i(Try)/K_i(Thr) ratio, and this has not been discussed previously. For these reasons design of a specific thrombin inhibitor, with large K_i(Try)/K_i(Thr), has been an immense challenge. Our early observations[39] showed surprisingly that selection of a neutral P1 group for the D-Phe-Pro vector increased selectivity for thrombin more than 1 order of magnitude. Further investigation[28,29,40] confirmed this finding, and preliminary results with the 3-methoxypropyl side chain were used to formulate the hypothesis[41] that specificity was achieved by virtue of the increasing hydrophobicity of the amino acids that constitute the specificity pocket in the sequence plasmin (Ser190-Thr213) to trypsin (Ser 190-Val213) to thrombin (Ala190-Val213). Subsequently our more detailed studies have shown this to be an oversimplification (in preparation). In the present paper we demonstrate that such compounds comprise a new class of potent and highly specific inhibitors of thrombin. The properties of this class of inhibitors differ from previously reported highly specific inhibitors of

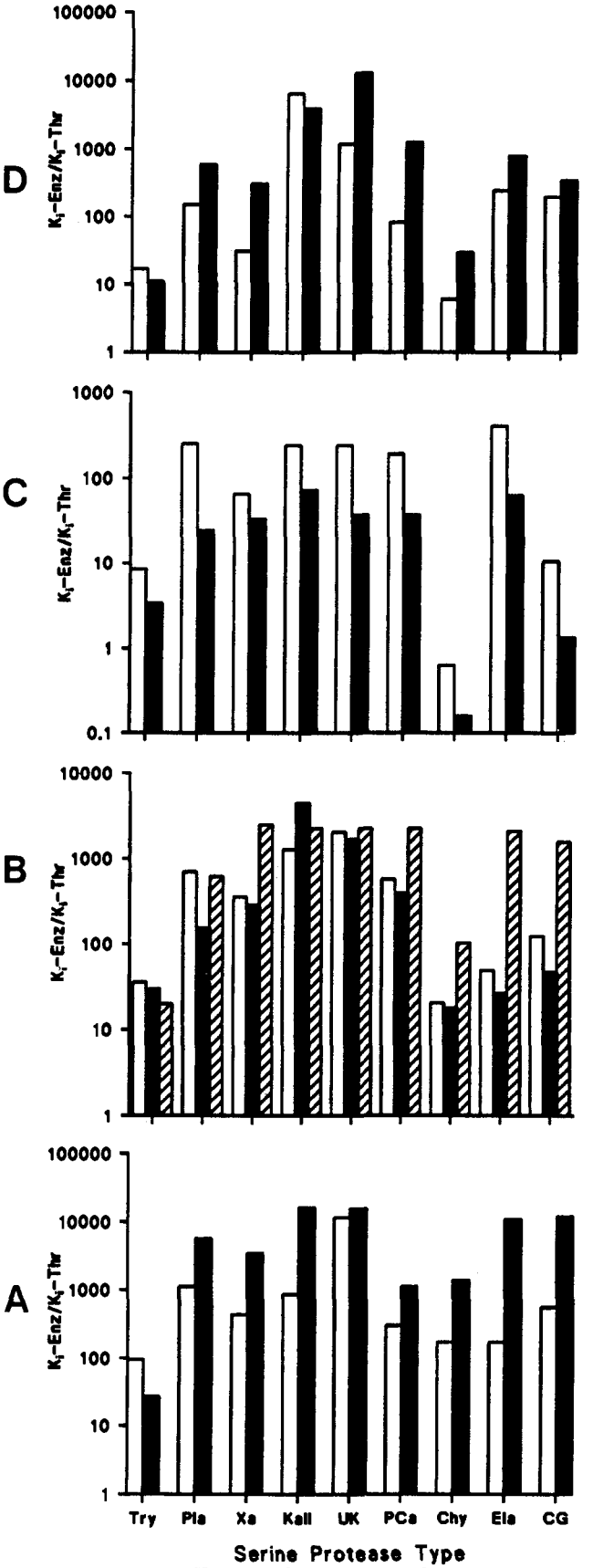


Figure 2. Ratio of K_i for inhibitor against the target enzyme to that against thrombin [K_i(Enz)/K_i(Thr)]: open bars, P3 D-Phe compounds; filled bars, P3 D-Dpa compounds; and hatched bars, for P3 β-Nal compound; i.e., (A), open bar, compound **9**, and filled bar, compound **18**; (B) open bar, compound **5**, filled bar, compound **22**, and hatched bar, compound **23**; (C) open bar, compound **6**, and filled bar, compound **24**; (D) open bar, compound **3**, and filled bar, compound **20**.

thrombin which have been selected as a single example after synthesis and evaluation of a large number of similar compounds (e.g., argatroban, NAPAP).

In contrast to the competitive inhibitors described here (compounds **1**–**16**, **18**–**25**), compounds with positively charged P1 groups (such as compound **17**) display slow binding kinetics, and if activity against the target enzyme is calculated with reference to the final K_i (17.5 pM), such inhibitors appear to be very selective: 1–2 orders of magnitude higher binding affinity with trypsin-like serine proteinases and 4–6 orders of magnitude higher with other proteinases tested.[18,42] However, as the potential use of these compounds is as pharmaceutical agents, to prevent thrombosis, or to control the tendency to thrombosis (the procoagulant state), it is not appropriate to consider specificity in relation to the final K_i. To prevent thrombosis it is necessary to administer anticoagulants at dosages which can control the large pulse of thrombin, up to 0.2 μM,[43] generated locally when clotting is fully activated and measured as the activated partial thromboplastin time (APTT). A dosage of inhibitor must be administered to achieve, at least, an equivalent concentration in the blood to quench the thrombin. Doubling of the APTT by compound **17** is observed at 69 nM (thrombin time, TT = 40 nM), a concentration which exceeds by about 10-fold its K_i with other trypsin-like proteinases. In fact there may also be a contribution to its potency from its influence on other proteinases involved in the intrinsic pathway of coagulation, e.g., kallikrein and factor Xa. The inhibitors described in this study, which are uncharged at P1, double the APTT and TT at concentrations at or below their K_i's with other trypsin-like proteinases, compound **9a**, APTT = 3 μM and TT = 2.8 μM,[1] and **5**, (APTT = 4 μM and TT = 1.12 μM. As significantly even at lower concentrations, such competitive inhibitors tend to shift the balance of the procoagulant state, suppressing the thrombin activity. Equivalent concentrations of slow binding inhibitors which were numerically equipotent *in vitro* would be mopped up by both the target proteinases and others inhibited because of the slow off rates of the EI* complexes formed. For the above reasons, only initial K_i's are considered for discussion of specificity of the compounds. Indications of toxicity with other serine proteinases-targeted inhibitors, also with natural P1 groups,[44] could be indicative of broad inhibitory activity in the liver and other tissues.

We have previously demonstrated that a broad range of serine proteinase inhibition is disastrous for an agent of antithrombotic use, since this leads to prolongation of clot lysis.[1] The model system of urokinase-mediated clot dissolution indicated dose dependent increase in lysis time with the positively charged inhibitor **17**, which inhibits plasmin, urokinase, and protein Ca in the nanomolar range, that is only 5–6 times less strongly than that of thrombin, while no increase was found with the inhibitor **9a** with a neutral polar side chain, and this feature is preserved throughout the series of compound presented here. As well as with boroMpg, it is clear that with P3 as boroPhe, greater than 100-fold specificity with respect to Thrombin is achieved also for P1 as boroMbg, boroPhe, and boroPgl (Figure 1A) against protein Ca, plasmin, and urokinase. For compounds with P3 as Dpa and P1 as boroMpg (**18**), boroMbg (**22**), and boroPgl (**20**), this shows between 100- and 1000-fold selectivity for thrombin against plasmin and protein Ca (Figure 1B) and greater than 10 000-fold for urokinase with P1 as boroMpg (**18**) and boroPgl (**20**). At effective thrombin inhibitory plasma levels (micromolar) of these compounds, inhibition of plasmin, urokinase, and protein Ca would be avoided, since it requires concentrations outside the therapeutic window and fibrinolytic times are unaffected.[45]

Screening this series of thrombin-targeted inhibitors against the family of serine proteinases allows some useful distinctions to be made about the preferred structural features for interaction for each enzyme. Trypsin-like proteinases, which by definition have an Asp189 in the S1 site, bind tightly to inhibitors with positively charged P1 groups (**17**; Table 5), while boroMpg (**9a**) and boroMbg (**5**) show 100-fold higher K_i's for factor Xa, but boroPgl (**3**) and boroPhe (**6**) (Figure 1A) are better accommodated. Introduction of Dpa at P3 improves factor Xa selectivity with the increase of ratio of K_i's to greater than 100-fold for P1 as boroMbg (**22**) (Figure 2B) and boroPgl (**20**) (Figure 2D). Chymotrypsin-like enzymes, e.g., chymotrypsin and elastase which have Ser in the specificity pocket and cathepsin G which has Ala at the equivalent position, lack the charge–charge interaction. Thereby the Irg compound **17** does show selectivity against the latter group of chymotrypsin-like proteinases (Table 2, Figure 1A). Particularly for elastase the S1 site is also further narrowed by residues 216 and 226, and it generally cannot accommodate the bulky side chains investigated here, showing preference for small side chains such as in the inhibitors MeO-Suc-Ala-Ala-Pro-boroAla and boroVal (final K_i's of 0.32 and 0.25 nM, respectively).[42] The boroEtg compound **1** shows equivalent K_i's for elastase (0.56 nM, unpublished results) and thrombin. Takahashi[46] proposed that overlap of a bulky residue from the S1 site could displace the boron of the inhibitor toward His57, but saw no evidence for bonding in the crystal structure. This agrees with the 100-fold poorer K_i for elastase than for chymotrypsin of the boroPhe compounds **6** and **24**. The 5 orders of magnitude specificity for thrombin over elastase for both compound **18** and boroIrg **17** is for different reasons. Within the series the longer aliphatic chains of the boroPgl (**3**) and boroMpg (**9a**) compounds (Figure 1) further reduce the potency toward elastase of the compounds in comparison to the boroMbg (**5**) compound. Activity in the micromolar range retained with the Phe-Pro compounds **1** and **5** is comparable to structure–activity relationship (SAR) data describing Suc-X-Y-Ala-PNA substrates of elastase,[47] where X as a hydrophobic residue and Y as Pro are tolerated. Of the family of enzymes, kallikrein and urokinase are least inhibited by any of the compounds, consistently giving K_i in the millimolar range, and excluding compounds for which P1 is boroPhe (**6**, **24**), the compounds are 3 orders of magnitude more specific for thrombin. For kallikrein and urokinase, the globally low activity of this series of compounds, with over 1000-fold selectivity for thrombin, may reflect an absolute requirement for a positively charged group in the P1 side chain for tight binding, via interaction with their equivalent to Asp189. Since the highest K_i's of all are found with P1 boroPgl (compounds **22**, **23**; Table 5), this may indicate a limit to the geometry of the specificity pockets of these enzymes. In fact plasma

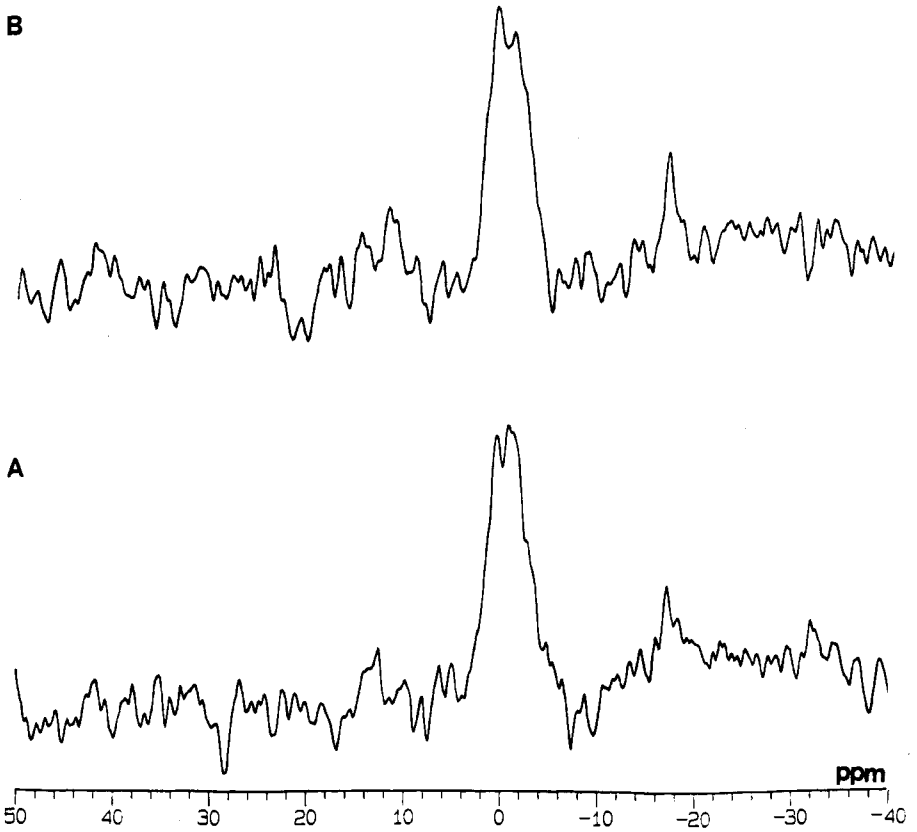


Figure 3. ^{11}B NMR spectrum of compound **9** (pinacol ester) with human α-Thrombin: (A) solution of enzyme and α-thrombin at 2 h and (b) solution of enzyme and α-thrombin at 8 h.

kallikrein favors aromatic groups at P2 and not P3, as in its substrate, H-D-Pro-Phe-Arg-PNa (S-2302). These considerations are indeed reflected in a series of recently reported plasma kallikrein-targeted compounds,[48] with *trans*-4-aminocyclohexanecarbonyl P1 groups. These display selectivity against plasmin in the case with the P2 group being the simplest single aromatic group and favor plasmin binding with a more extended diaryloxy group. However introduction of this latter group reduces the selectivity against thrombin to only 3 times, while in this boronic acid series a minimum of 10-fold is achieved.

Binding Mechanism of Neutral P1 Boronic Acids. Recently members within homologous series of peptidomimetic inhibitors of elastase[49] and thrombin[50] have been shown to utilize different binding modes within the active site of the serine proteinase. Some demonstrate normal binding modes, where the functional group interacts covalently with the catalytic triad, and the molecule forms antiparallel β-hydrogen bonds to Gly216.[51] "Retro" inhibitors are oriented in the reverse direction in the active site, and the free N-terminus of the inhibitors may coordinate to the serine analogously to that of hirudin.[50] Clearly inhibitory activity does not automatically dictate the binding mode.

Furthermore the discrimination between slow binding and competitive inhibition displayed by boron-based inhibitors has yet to be resolved but may be related to formation of a transition-state-like adduct of the boron and Ser195 or His57. This has been investigated with proteinases other than thrombin. An NMR study of peptide boronic acid inhibitors of α-lytic proteinase has shown a dynamic equilibrium between His−$^{15}N^{\epsilon 2}H$ and Ser−OH interactions,[52] and this was seen also in studies of the ^1H NMR spectrum of trypsin and trypsinogen.[53] The type 1 inhibitors, with structures close to those of favored substrates, exhibited slow binding kinetics, while those with other structures, type 2, despite still being potent inhibitors, exhibited competitive binding kinetics. It was considered that type 2 inhibitors form adducts with His57, whereas in type 1 inhibitor−proteinase complexes the boron initially binds to the His57 group followed by a slow transition in which a tetrahedral boron atom is covalently bound to the oxygen of the Ser195. It is known that the chemical shift in ^{11}B NMR is related to the environmental symmetry about the boron,[54,55] and it has been reported that tetrahedral and trigonal enzyme adducts can be distinguished[56-58] in ^{11}B NMR. To investigate these questions in relation to our series of compounds, we observed the ^{11}B NMR (at a field strength of 160.35 MHz) of a D_2O solution of compound **9a** with thrombin. A low-field adduct at −17 ppm, with a line width (~165 Hz) indicative of an enzyme-bound complex, was formed (Figure 3), since a free tetrahedral boron would exhibit a much broader signal (typically of line width >400 Hz). In accordance with the competitive binding nature of the compound, determined kinetically, the spectrum remained unchanged after 7 h of incubation. The same experiment with trypsin showed a similar adduct (Figure 4). A recent report[59] concluded that the boron is tetrahedral in complexes that have been previously shown[60] to be His57 adducts as well as in others that were Ser195 adducts, and so while interaction is confirmed by our study, the locus can not be distinguished. Crystallographic and solid phase NMR studies may reflect the end state of B−O−Ser bond formation, and indeed even competitive inhibitors (such as **6**)[61] achieve

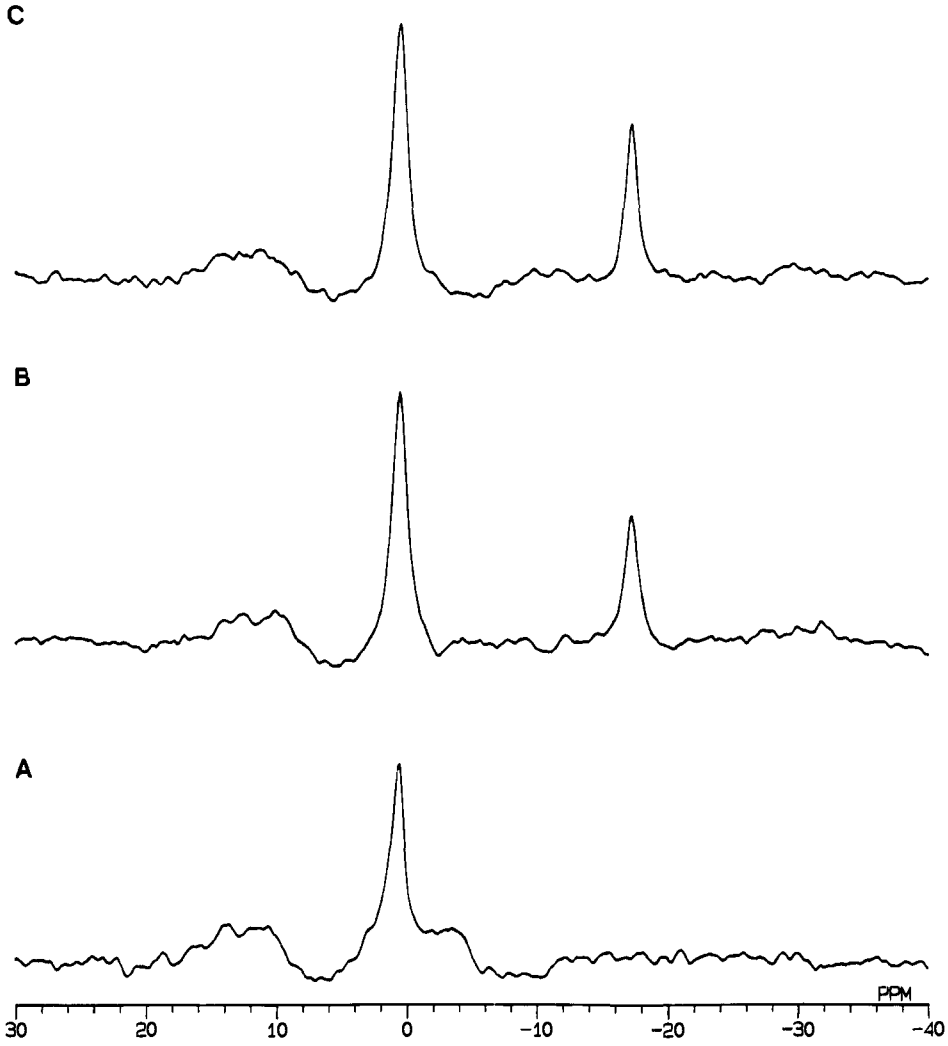


Figure 4. ^{11}B NMR spectrum of compound **9** (pinacol ester) with trypsin: (A) solution of inhibitor, (B) solution of inhibitor and trypsin after 15 min, and (C) solution of inhibitor with trypsin after 7 h.

this tetrahedral configuration. Moreover this validates our interpolation of SAR data from this series on the basis of a "normal" mode of binding to thrombin and the other serine proteinases.

Conclusions

This demonstrates a class of 25 boron-based tripeptide inhibitors of thrombin that are competitive inhibitors with K_i's in the nanomolar range, which in complex form tetrahedral adducts with the active site. We have shown that eliminating the ubiquitous charged arginine-like P1 side chain by replacement with neutral polar and apolar side chains of a wide structural class in combination with the PhePro motif allows greater thrombin specificity. Replacement at P1 with the natural amino acid analogue boroPhe is deleterious. Two separate modes of binding to thrombin are clear, comparing neutral polar compounds **9a** and **18** to apolar compounds **3**, **5**, **22**, and **23**, and the stereochemistry of this interaction is under investigation. Further distinction is that the PhePro scaffold can be favorably modified in P1 as with the boroMpg case, producing the most specific inhibitor, **18**, but results with P1 as boroMbg, boroPgl, or boroPhe are less clear-cut.

Experimental Methods

NMR (proton and ^{13}C [^1H] decoupled and DEPT) were carried out on a Bruker AMX 400 spectrometer; proton and ^{13}C chemical shifts (δ) are reported in parts per million (ppm) using TMS as an internal standard. TLC was performed on precoated silica plates (Merck, F_{254}) in the following system: (a) $CHCl_3$–MeOH (9:1). Mass spectrometry was performed either using a Finnigan 710C spectrometer with an electrospray source or using a fast atom bombardment (FAB) ion source. Microanalysis was carried out by University of London Enterprises, University of North London. All glassware was dried at 150 °C overnight and assembled under argon for air sensitive reactions, as described previously.[31] HPLC systems: (1) 104 × 250 mm, 5 μm, C-18, Hichrom, gradient 50–90% MeOH in H_2O over 22 min; (2) 4 × 150 mm, Hichrom, Sorbsil S5CN, 5 μm particle size, column, gradient 1–40% 1,4-dioxane in hexane over 22 min. TLC system: (A) $CHCl_3$:MeOH, 9:1, using precoated plates (Merck F_{254}). Z-D-β-Nal-Pro, Z-D-Dpa-Pro, and Z-L-Dpa-Pro were synthesized as described previously.[62]

Z-D-Dpa-Pro: ^{13}C NMR δ_{250} 207.0 (CO_2H), 173.0 (quat, O-CO-N), 171.5 (quat), 170 (quat), 156.3 (quat, CH-CO-N), 140.0 (quat, Ph), 139.2 (quat, Ph), 136.4 (quat, Ph), 67.2 (CH_2, Ph-CH_2-O), 59.9 (CH, Pro αCH), 55.9 (CH, Dpa αCH), 54.2 (CH_2, Dpa βCH_2), 47.8 (CH_2, Pro $4CH_2$), 27.9 (CH_2, Pro $2CH_2$), 24.4 (CH_2, Pro $3CH_2$).

Z-L-Dpa-Pro: ^{13}C NMR δ_{250} 206.5 (quat, CO_2H), 172.9 (quat, O-CO-N), 171.5 (quat), 169.2 (quat), 155.6 (quat, CH-CO-N), 141.7 (quat, Ph), 140.6 (quat, Ph), 137.1 (quat, Ph), 65.5 (CH_2, Ph-CH_2-O), 58.8 (CH, Pro αCH), 54.6 (CH, Dpa αCH), 52.6 (CH_2, Dpa βCH_2), 46.7 (CH_2, Pro $4CH_2$), 28.9 (CH_2, Pro $2CH_2$), 24.5 (CH_2, Pro $3CH_2$).

(2*R*,3*R*)-(−)-2,3-Butanediol 3-Methoxypropaneboronate. To 3-methoxypropene (30.1 g, 0.417 mmol) in a flask fitted with a condenser was added, dropwise by dry transfer, catechol borane (50.0 g, 1 equiv), and the mixture was heated at 80 °C

for 24 h. The mixture was allowed to cool to room temperature and distilled, giving as a fraction (bp 65–80 °C/0.2–0.3 mmHg) the expected product catechol 3-methoxypropaneboronate (51.6 g, 64.5%): ^{1}H NMR δ 7.13 (4 H, m, Ph), 3.4 (2 H, m, CH_2-OMe), 3.39 (3 H, s, *OMe*), 1.92 (2 H, m, CH_2), 1.29 (2 H, m, CH_2); ^{13}C NMR δ 148.3 (quat, Ph), 122.5 (CH, Ph), 112.2 (CH, Ph), 73.8 (CH_2), 58.5 (CH_3, OMe), 24.3 (CH_2).

To a solution of catechol 3-methoxypropaneboronate (51.4 g, 0.268 mol) in dry THF (77 mL) was added, at 0 °C, (2*R*,3*R*)-(−)-2,3-butanediol (24.1 g, 1 equiv). The solution was stirred at 0 °C for 30 min and at room temperature for a further 2 h. The solvent was removed under reduced pressure and the residual oil distilled to give the required product (bp 55 °C/0.4 mm Hg) of (2*R*,3*R*)-(−)-2,3-butanediol 3-methoxypropaneboronate (42.0 g, 0.244 mol, 91.0%): ^{1}H NMR δ 3.95 (2 H, m, butanediol), 3.4 (2 H, m, CH_2OMe), 3.35 (3 H, s, OMe), 1.7 (2 H, m, CH_2), 1.3 (6 H, 2 × s, 2 × Me), 0.8 (2 H, m, CH_2); ^{13}C NMR δ 79.9 (2 × CH), 74.1 (CH_2), 58.4 (CH_3, OMe), 24.03 (CH_2), 20.9 (2 × CH_3).

(2*R*,3*R*)-(−)-2,3-Butanediol 4-Methoxy-1-chlorobutaneboronate. DCM (7.67 mL, 1.10 equiv, freshly distilled from P_2O_5) was added to dry THF (131 mL) and the solution cooled to −100 °C, under argon. To the stirred solution was added slowly precooled *n*-BuLi (1.6 N solution in hexane, 74.7 mL, 1.10 equiv), by dry transfer, running down the side wall of the reaction vessel. After 5 min a precooled solution of (3-methoxypropyl)boronate ester (18.7 g, 0.109 mol) in dry THF (131 mL) was added, slowly, to the reaction mixture. Zinc chloride (54.5 mL, 0.5 equiv) was cooled to around 0 °C and added portionwise to the reaction mixture. The reaction mixture was maintained at −100 °C for 30 min and then at −78 °C for 2 h and allowed to warm to room temperature overnight. The solvent was removed under vacuum, and the residue was dissolved in hexane (600 mL) and washed with water (2 × 300 mL). The organic phase was dried ($MgSO_4$) and filtered, and concentrating gave the desired product (56.0 g): ^{1}H NMR δ_{400} 4.0–3.94 (2 H, m, 2 × CH), 3.38–3.34 (2 H, m, CH_2OMe), 3.35 (3 H, s, OMe), 1.72 (2 H, m, CH_2), 1.30 (6 H, d, 2 × Me), 0.83 (2 H, m, CHB).

The crude product was transesterified directly by dissolving in THF (72 mL), at 0 °C, and adding pinacol (30.0 g, 0.253 mol). The reaction mixture was stirred at 0 °C for 30 min and at room temperature for a further 2 h. Solvent was removed under vacuum, the residue dissolved in hexane (600 mL) and washed with water (2 × 300 mL), and the organic phase dried ($MgSO_4$), filtered, and concentrated to give the desired product pinacol 4-methoxy-1-chlorobutaneboronate (20.1 g): Electrospray MS 271 (M + Na), 249 (M^+); ^{1}H NMR δ_{400} 3.47–3.38 (2 H, m, CH_2OMe and CHB), 3.34 (3 H, s, OMe), 2.0–1.62 (4 H, m, CH_2CH_2), 1.35–1.20 (12 H, s, pinacol). Anal. ($C_{11}H_{22}ClO_3B$) C,H,N.

Pinacol 4-Methoxy-1-aminobutaneboronate Hydrochloride. A solution of pinacol 4-methoxy-1-chlorobutaneboronate (20.1 g, 0.081 mol) in THF (25 mL) was added to a solution of lithium hexamethyldisilylamide (1 N in hexane, 81.0 mL, 1 equiv) in THF (40 mL) at −78 °C. The reaction mixture was allowed to warm slowly to room temperature and stirred overnight. Solvent was removed under reduced pressure, and dry hexane (36.1 mL, distilled from P_2O_5) was added to yield a precipitate which was removed by filtration under nitrogen pressure in a closed system. The filtrate was cooled to −78 °C, and HCl (4 N solution in dioxane, 60.8 mL, 3 equiv) was added with stirring. The reaction mixture was allowed to warm slowly to room temperature and stirred for a further 2 h. The resulting product was isolated by filtration under nitrogen in the closed system and collected by washing through with dry $CHCl_3$ (500 mL). The solution was refiltered, as above, and concentrated under reduced pressure to give the required pinacol 4-methoxy-1-aminobutaneboronate hydrochloride (19.9 g, 92.3% yield).

Z-D-Phe-Pro. To a solution of Z-D-Phe (15 g, 0.05 mol) and TBTU (19 g, 1.18 equiv) in DMF (70 mL) under argon were added Pro(O-*t*-Bu) (10 g, 1.16 equiv) and triethylamine, until the pH of the solution was 8.0–9.0. The solution was stirred for 4 h, poured onto $NaHCO_3$ (200 mL, 1 N), and extracted by EtOAc (3 × 100 mL) and $CHCl_3$ (2 × 100 mL). The organic extracts were combined and washed with HCl (2 × 200 mL, 1 N) and the aqueous extracts back-washed with EtOAc (2 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated to give an oily, yellow product. The product was washed several times with hexane to give Z-D-Phe-Pro-(O-*t*-Bu) (95% crude weight). The ester was dissolved in a mixture of TFA:H_2O, 95:5 (v/v), for 2 h at room temperature; the solution was concentrated under reduced pressure and purified, portionwise on a medium pressure column of Lichoprep C18 (Merck), 5 × 22 cm, eluting with a gradient of MeOH in H_2O. The fractions of dipeptide were recrystallized from EtOAc: hexane to give Z-D-Phe-Pro (55% yield based on ester).

Z-D-Phe-Pro-boroMpg-OPinacol (9b). To a solution of Z-D-Phe-Pro (10.8 g, 1 equiv) and *N*-methylmorpholine (3.0 mL, 1 equiv) in THF (62 mL) at −20 °C was added *i*-BuOCOCL (3.5 mL, 1 equiv). The mixture was added by dry transfer to a solution of pinacol 4-methoxy-1-aminobutaneboronate hydrochloride (7.26 g, 0.0273 mol) and triethylamine (3.8 mL, 1 equiv) as a precooled solution in THF (21 mL) and $CHCl_3$ (21 mL). The reaction mixture was stirred at −20 °C for 1 h, allowed to warm to room temperature, and stirred for a further 2 h. The mixture was filtered and concentrated under reduced pressure. The residue was dissolved in EtOAC (500 mL) and washed with HCl (0.2 N, 300 mL), $NaHCO_3$ (1.2 M, 300 mL), and NaCl (saturated aqueous, 300 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated to give the desired crude product as a solid (13.1 g). ^{1}H NMR indicated a mixture of two isomers in a ratio of 80:20. A portion of the crude product (2 g) was passed down a column of Sorbsil, 40–60 μm, S5CN (Hichrom) (5 × 23 cm), at 15 bar. Elution by 1,4-dioxane increasing from 10% in hexane at 160 mL min^{-1} gave the pure product (600 mg): FAB MS *m/z* 608 (M + H); Electrospray MS 629.9 (M + Na), 607.8 (M^+), 522, 511, 507; ^{1}H NMR δ 7.82 (1 H, s, NH), 7.40–7.20 (10 H , m, 2 × Ph), 5.7 (1 H, s, NH), 5.17–5.08 (2 H, dd, J = 7.5 Hz, OCH_2Ph), 4.48–4.44 (2 H, Pro αCH and Phe αCH), 3.46 (1 H, m, Pro 4CH), 3.27 (2 H, m, CH_2OMe), 3.22 (3 H, s, OMe), 2.99 (2 H, m, PhCH_2), 2.63 (1 H, m, CHB), 2.57 (1 H, m, Pro 4CH), 2.23–2.50 (4 H, m, Pro 3CH_2 and Pro 2CH_2), 1.60 (4 H, m, CH_2-CH_2), 1.20 (12 H, s, pinacol, 4 × Me); ^{13}C NMR δ 171 (C, O-*C*O-N), 156 (C, CH-*C*O-N), 136 (Ph, quat), 126–130 (CH, aromatics), 81.5 (C, pinacol, *C*Me$_2$), 73 (CH_2, CH_2OMe), 67.26 (CH_2, PhCH_2O), 58.3 (CH, Pro αCH), 57.94 (CH_3, OMe), 54.46 (CH, Phe αCH), 46.77 (CH_2, Pro 4CH_2), 38.76 (CH_2, PhCH_2CH), 27.4–27.84 (2 × CH_2, $CH_2CH_2CH_2$OMe and Pro 2CH_2), 25.23–24.9 (4 × Me, pinacol major isomer), 24.07 (CH_2, Pro 3CH_2) (Note: due to quadrupolar broadening it is not possible to see the carbon α to boron.); ^{11}B NMR ($CDCl_3$) (MeO_3B) 13. Anal. ($C_{33}H_{46}N_3O_7B$) C,H,N. HPLC gradient 2: t_R 12.4 min. Further elution gave the L-isomer: 150 mg; Electrospray MS 629.9 (M + Na), 608.1 (M^+). Anal. ($C_{33}H_{46}N_3O_7B$) C,H,N. Further elution gave unreacted Z-D-Phe-Pro (0.6 g).

Z-D-Phe-Pro-boroEtg-OPin (1): Synthesized as described previously.[33] Anal. ($C_{35}H_{46}N_3O_6B$) C,H,N.

Z-D-Phe-Pro-boroPgl-OPinac (2): Prepared as described previously.[28,29] Anal. ($C_{34}H_{48}N_3O_6B$) C,H,N.

Z-D-Phe-Pro-boroPgl-OPin (3): Prepared as described previously.[28,29] Anal. ($C_{38}H_{52}N_3O_6B$) C,H,N.

Z-D-Phe-Pro-boroPhe-OPin (6): Prepared as described previously.[33]

Z-D-Phe-Pro-boro-*p*-MehomoPhe-OPin (7): Prepared using the method as described previously,[33] by hydroboration of 1-bromo-3-(*p*-methylphenyl)prop-1-ene. Anal. ($C_{38}H_{48}N_3O_6B$) C,H,N.

Z-D-Phe-Pro-boroMeg-OPin (8): Prepared as described previously.[33] Anal. ($C_{36}H_{48}N_3O_7B$) C,H,N.

Z-D-Phe-Pro-boroMpg-OPin (9a): Prepared as described previously.[1] Anal. ($C_{37}H_{50}N_3O_7B$) C,H,N.

Z-D-Phe-Pro-boroMpg-(OMe)$_2$ (10): The pinacol ester of compound **9b** (0.5 g) was dissolved in MeOH and applied to a column (2 × 60 cm) of Sephadex LH20 (Pharmacia) (100 g). Eluting at 0.5 mL/min over ~24 h gave the dimethyl ester: Electrospray MS 554 (M + H). Anal. ($C_{29}H_{40}N_3O_7B$) C,H,N.

Z-D-Phe-Pro-D-boroMpg-OPin (11): Prepared as described previously,[1] using (−)-pinanediol. Anal. ($C_{37}H_{50}N_3O_7B$) C,H,N.

Z-D-Phe-Val-boroBpg-OPin (13): FAB MS 710 (M^+); ^1H NMR ($CDCl_3$) δ_{250} 7.9 (1 H, s, NH), 7.16–7.37 (10 H, m, 2Ph), 6.37 (1 H, d, J = 9 Hz, NH), 5.52 (1 H, d, J = 5 Hz, NH), 5.14–5.01 (2 H, m, OCH_2Ph), 4.25–4.43 (3 H, m, Pin 2CH, Val αCH, Phe αCH), 3.34–3.39 (2 H, m, CH_2Br), 3.02–3.13 (2 H, m, CH_2Ph), 2.93–3.01 (1 H, m, CHB), 2.26–2.39 (1 H, m, Pin H-3), 2.1–2.25 (2 H, m, CH_2CHB), 2.10–2.15 (1 H, m, Pin H-7), 1.98 (1 H, t, J = 6Hz, Pin H-6), 1.82–1.94 (1 H, m, Pin H-4), 1.72–1.77 (1 H, m, Pin H-3), 1.4–1.6 (3 H, m, CH_2CH_2-CH_2, Val βCH), 1.35 (3 H, s, Pin Me), 1.22 (3 H, s, Pin Me), 0.95 (6 H, s, Val Me), 0.85 (1 H, m, Pin H-7), 0.83 (3 H, s, Pin Me). Anal. ($C_{36}H_{49}BrN_3O_6B$) C,H,N.

Z-D-Phe-Pro-D-boroBpg-OPin (14): Prepared as described previously,[33] using (−)-pinanediol. Anal. ($C_{37}H_{47}BrN_3O_6B$) C,H,N.

Z-D-Phe-Pro-boroEtCl-OPin (15): Prepared as described previously.[33] Anal. ($C_{35}H_{45}ClN_3O_6B$) C,H,N.

Preparation of Z-D-Dpa-Pro-boroMpg-OPin (18): To a solution of Z-D-Dpa-Pro[62] (236 mg, 0.5 mmol) in THF (5 mL) in the presence of triethylamine (70 μL, 0.5 mmol, 1 equiv) was added i-BuOCOCL (65 μL, 0.5 mmol, 1 equiv) at −15 °C, which was stirred at −13 °C for 13 min. After addition of pinanediol 1-amino-4-bromobutaneboronate hydrochloride (183 mg, 0.5 mmol, 1 equiv) in $CHCl_3$ (3 mL) followed by triethylamine (70 μL, 0.5 mmol, 1 equiv), the reaction mixture was stirred at the same temperature for 2 h and then at below 10 °C for 2 h. THF was removed under reduced pressure, and the residue was dissolved in EtOAc (50 mL), which was washed with 1% $NaHCO_3$, water, HCl (0.2 N), and water and then dried over Na_2SO_4. Removal of the solvent gave an oily product in quantitative yield: HPLC gradient 1, t_R 23 min; FAB MS 785.61 (M + H); R_f (system A) 0.29; ^1H NMR ($CDCl_3$) δ_{250} 7.55 (1 H, s, NH), 7.25 (15 H, m, $CHPh_2$, Z), 5.5 (1 H, d, Dpa NH), 5.1 (3 H, m, Dpa αCH, $PhCH_2$), 4.4 (2 H, m, Pro αCH, Dpa βCH), 4.25 (1 H, m, Pin 2CH), 3.75 (1 H, m, Pro 4CH), 3.33 (2 H, m, CH_2Br), 2.8 (1 H, m, CHB), 2.5 (1 H, m, Pro 4CH), 2.4–1.5 (14 H, m, aliphatic envelope), 1.35 (3 H, s, Me), 1.24 (3 H, s, Me), 0.85 (1 H, m, Pin H-7), 0.821 (3 H, s, Me). Anal. ($C_{42}H_{51}N_3O_6BBr$) C,H,N.

The bromo group was converted to methoxy by the method as reported previously for compound **9a**[1,29] to give compound **18**: FAB MS 736 (M + H); HPLC gradient 1, t_R 21.5 min; ^1H NMR δ_{250} 7.6 (1 H, s, NH), 7.25 (15 H, m, Ph), 5.7 (1 H, m, NH), 5.0 (3 H, dd, $PhCH_2O$), 4.95 (1 H, m, Dpa αCH), 4.4 (2 H, m, Pro αCH, Dpa-β-CH), 4.25 (1 H, m, Pin 1CH), 3.65 (1 H, m, Pro 4CH), 3.3 (2 H, m, CH_2OMe), 3.2 (3 H, s, OMe), 2.9 (1 H, m, CHB), 2.0 (1 H, m, Pro 2CH), 2.5–1.5 (12 H, m, aliphatic envelope), 1.35 (3 H, s, CH_3), 1.25 (3 H, s, Me), 0.90 (1 H, m, Pin H-7), 0.83 (3 H, s, Me). Anal. ($C_{43}H_{54}N_3O_7B$) C,H,N.

Ac-D-Phe-Pro-boroMpg-OPin (19): To a solution of Z-D-Phe-Pro-boroMpg-OPin (**9a**) (1 g, 1.51 mmol) in methanol (13 mL) was added palladium on charcoal (10%, 0.45 g). The solution was purged by bubbling argon through in the bomb of the Parr apparatus and then pressurized to a static pressure of 500 psi of H_2, with stirring, for 24 h. The pressure was released and the solution filtered twice through Celite and concentrated under reduced pressure. The residue was dissolved in EtOAc, dried over $MgSO_4$, filtered, and concentrated to give a solid (0.799 g). Crude amine (0.72 g) was dissolved in 1,4-dioxane (2.06 mL) and water (1.02 mL), and Ac_2O (0.483 mL, 5.0 mmol) and $NaHCO_3$ (0.432 g, 5.0 mmol) were added. The reaction mixture was stirred for 20 min at room temperature. EtOAc (50 mL) and water (5 mL) were added and the phases separated, and the organic phase was dried (Na_2SO_4), filtered, and concentrated under vacuum to give a solid (0.493 g). The crude solid was purified by medium pressure Rp chromatography on a column of Lichoprep C18, 5 × 23 cm, in 50% MeOH in water. Elution by an increasing proportion of MeOH, at 20 bar, gave the required product Ac-D-Phe-Pro-boroMpg-OPin: Electrospray MS 590.2 (M + Na), 568.3 (M + H). Anal. ($C_{31}H_{46}N_3O_6B$) C,H,N.

Ac-D-Phe-Pro-D,L-boroPgl-OPinacol (21). To a solution of (1-chloropentyl)boronate pinacol ester (1.24 g, 5.03 mmol) in dry THF (10 mL) was added lithium hexamethyldisilylamide (1 M, solution in hexane, 5.03 mL, 1 equiv) at −78 °C, under argon. The reaction mixture was allowed to warm to room temperature overnight and then recooled to −15 °C, and Ac-D-Phe-Pro-OSu (0.5 equiv, 1.24 g) and TBTU (0.5 equiv, 0.81 g) were added as a precooled suspension in DCM:THF, 1:1 (v:v), 5 mL, and then triethylamine (0.5 equiv, 0.34 mL). The reaction mixture was allowed to warm to room temperature. After 24 h the reaction mixture was concentrated and applied to a silica gel column. Elution by DCM and then DCM:MeOH, 95:5 (v/v), gave a mixture of products (~500 mg). The mixture was applied to LH20 (3 × 30 cm), and elution with MeOH at 1 mL min^{-1}, monitoring at 250 nm, gave the required Ac-D-Phe-Pro-boroPgl-OPinacol (300 mg): FAB MS 536 (M + Na); 22% yield; ^{13}C NMR δ_{250} 173.9 (C, MeCO), 171.5, 171.3, 171.28 (CO), 135.7 (C, Ph), 129.3 (2 CH, o-Ph), 128.7 (2 CH, m-Ph), 127.41 (CH, p-Ph), 81.2,81.4 (2 quat, pinacol), 57.9 (CH, Pro αCH), 53.8 (CH, Phe αCH), 46.7 (CH_2, Pro $4CH_2$), 37.8 (CH_2, Ph-CH_2CH), 31.8 (CH_2, CHCH_2CH_2), 30.8 (CH_2, CH_2CH_2-CH_2), 28.3 (CH_2, Pro $2CH_2$), 27.3 (CH_2, $CH_2CH_2CH_2$), 25.2–24.9 (4 CH_3, pinacol), 23.9 (CH_2), 22.6 (CH_2, Pro $3CH_2$), 14.1 (CH_3, CH_2CH_3). Anal. ($C_{28}H_{44}N_3O_5B$) C,H,N.

Z-D-Dpa-Pro-boroPhe-OPin (24): Prepared as described previously.[29]

Z-D-Dpa-Pro-boroVal-OPin (25): Prepared as described previously.[33] Anal. ($C_{42}H_{53}N_3O_6B$) C,H,N.

Z-D-Phe-Pro-D,L-boroMpg-OPinacol: Prepared as described previously,[29] using pinacol. Anal. ($C_{33}H_{46}N_3O_7B$) C,H,N.

Enzymes. Human α-thrombin was obtained from Dr. J.Freysinnet (Strasbourg) and human γ-thrombin from Dr. J. Fenton (U.S.). The following enzymes were obtained from the following sources: from Choay, human Pla; from Sigma, bovine pancreatic Trp, bovine pancreatic α-Chy, porcine pancreatic Ela, human plasma Kal; from Calbiochem, human plasma PC and human PC activator, human neutrophil CG, human plasma FXa; and from Abbott Labs (North Chicago), human kidney UK.

Determination of K_i. The chromogenic substrates S-2238 (H-D-Phe-Pip-Arg-pNA, Thr), S-2222 (Bz-Ile-Glu-Gly-Arg-pNA, FXa), S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA, α-Chym), S-2288 (H-D-Ile-Pro-Arg-pNA, Kal), S-2444 (Glu-Gly-Arg-pNA, UK), S-2251(H-D-Val-Leu-Lys-pNA, Pla), and S-2366 (Glu-Pro-Arg-pNA, PCa) were obtained from Kabi and used for the relevant enzymes indicated. The substrates Suc-Ala-Ala-Pro-Phe-pNA (CG) and Suc-Ala-Ala-Ala-pNA (Ela) were obtained from Calbiochem Novabiochem (U.K.) Ltd. (Nottingham) and Sigma Chemical Co. Ltd. (Poole), respectively.

Determination of Thrombin Time (TT). To an aliquot of pooled, citrated normal human plasma (150 μL) was added inhibitor solution (20 μL, serially diluted from a stock solution in DMSO by buffer) for 1 min at 37 °C. To the solution was added human α-thrombin (150 μL of a solution of 5 units/mL in 50 mM sodium phosphate, 0.1% PEG at pH 7.4). Concentration required to double the thrombin time was determined in duplicate using a Nach Schnicter und Gross coagulometer.

Determination of Activated Partial Thromboplastin Time (APTT). A solution of pooled, citrated normal human plasma (100 μL), APTT reagent (Organon, Teknika; 100 μL), and inhibitor solution (20 μL) was incubated at 37 °C for 5 min, and then $CaCl_2$ (100 μL of a 25 mM aqueous solution) was added. Concentration required to double the APTT was determined in duplicate using a Nach Schnicter und Gross coagulometer.

Data Analysis. Mechanism of Inhibition of Each Compound. A solution of each enzyme was added to a series of 10-fold dilutions of each inhibitor in the presence of a fixed concentration of the appropriate chromogenic substrate. The velocity of the reaction was measured over 2 min on a Molecular Devices Corp. thermomax plate reader at 25 °C. The percentage inhibition of the enzyme was calculated for each inhibitor concentration, and this data was used to determine the inhibitor concentration necessary to give 50% inhibition of the enzyme. Each experiment was repeated after the enzyme and inhibitor had been preincubated at room temperature for 2 h. A greater than 10-fold reduction in the amount of inhibitor necessary to produce 50% inhibition after preincubation was taken as an indication of slow binding.

For inhibitors that did not show slow binding kinetics, K_i values were obtained from Lineweaver–Burk plots using five substrate concentrations over a 10-fold range around the appropriate K_m for the enzyme substrate interaction. The best fit straight lines were determined by linear regression.

k_2, k_{-2}, and K_i. For inhibitors that exhibited slow binding characteristics, the apparent rate constant, k', was determined by fitting the progress curve to eq 1,[63] by nonlinear regression using the program Enzfitter.[64] Fitting generated values for

$$A = A_o + (v_s t) + (v_o - v_s)(1 - e^{-k't})/k' \quad (1)$$

A_o (initial absorbance), v_o (initial velocity), v_s (final steady state velocity), and k' (apparent rate constant for the transition from v_o to v_s). The slow binding reactions were well defined by eq 1, giving good correlation between the observed and fitted data.

Values of individual rate constants (k_2 and k_{-2}) were calculated by fitting values of k' (the apparent rate constant), generated from the eq 1 at known inhibitor concentration, in accordance with its relation to the inhibitor concentration from the following equation:

$$k' = k_{-2} + k_2([I]/K_i)/(1 + [S]/K_m + [I]/K_i) \quad (2)$$

where [I] is inhibitor concentration, [S] is substrate concentration, K_m is the Michaelis–Menten constant, K_i is the initial dissociation constant of the loose complex, and k_2 and k_{-2} are as defined in eq 3.

A graph of k' against inhibitor concentration ([I]) gives a hyperbolic fit showing that the mechanism of enzyme–inhibitor interaction occurred in accordance with eq 3:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI' \quad (3)$$

The values of k_2, k_{-2}, and K_i, obtained from eq 2, were substituted into the equation:

$$k_2 = [(K_i/K_i')(k_2)] - k_{-2} \quad (4)$$

to calculate the value of K_i' (final inhibition constant).

^{11}B NMR Study of Enzyme–Inhibitor Complex. A 2 mM solution of compound **9b** (pinacol ester) in D_2O was mixed with a 2 mM solution of human α-thrombin or trypsin. The spectra were obtained at 120 °C, with 20–70 000 scans at a frequency of 160.35 Hz. The measured pH of the complex solution within the NMR tube was 6.3 (α-thrombin) and 6.5 (trypsin). Spectra remained unchanged from 15 min to 8 h. The weaker signals obtained with thrombin probably reflect its pH optimum of ~7.4.

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