New Inhibitors of Thrombin and Other Trypsin-like Proteases: Hydrogen Bonding of an Aromatic Cyano Group with a Backbone Amide of the P_1 Binding Site Replaces Binding of a Basic Side Chain[⊥]

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ABSTRACT: Highly effective thrombin inhibitors have been obtained by preparing boronic acid analogues of *m*-cyano-substituted phenylalanine and its incorporation into peptides. The cyano group enhances binding by several orders of magnitude. For example, Ac-(D)Phe-Pro-boroPheOH binds to thrombin with a K_i of 320 nM and the K_i of Ac-(D)Phe-Pro-boroPhe(*m*-CN)-OH is 0.79 nM. Protein crystal structure determination of trypsin complexed to H-(D)Phe-Pro-boroPhe(*m*-CN)-OH indicates that the aromatic side chain is bound in the P_1 binding site and that the cyano group can act as a H-bond acceptor for the amide proton of Gly219. Enhanced binding for inhibitors containing the *m*-cyano group was observed for coagulation factor Xa and for the factor VIIa·tissue factor complex [K_i values of Ac-(D)Phe-Pro-boroPhe-(*m*CN)-OH are 760 and 3.3 nM, respectively]. This result is consistent with the sequence homology of these two enzymes in the P_1 binding site. Two enzymes lacking the strict homology in the P_1 binding site, pancreatic kallikrein and chymotrypsin, did not exhibit significantly enhanced binding.

Considerable effort has been devoted to the design of new inhibitors of trypsin-like proteases as therapeutic agents. Particular attention has been given to thrombin, which is required for blood clotting. Its role in the blood clotting cascade is to form a fibrin clot by hydrolyzing the soluble plasma protein fibrinogen to insoluble fibrin. Low molecular weight inhibitors are expected to be particularly useful in controlling blood clotting and in the prevention of both arterial and venous thrombosis.

A number of strategies have been employed in the design of synthetic inhibitors of thrombin and other serine proteases. The scissile amide bond of peptide substrates have been replaced by electrophilic groups, such as aldehydes (Bajusz et al.,1990), trifluoromethyl ketones (Jones et al., 1995; Neises et al., 1995), ketoamides and esters (Iwanowicz et al., 1992; Lewis et al., 1995), and boronic acids (Kettner et al., 1990; Knabb et al., 1992; Deadman et al., 1995). These peptide inhibitors bind thrombin in a substrate-like manner and form a tetrahedral complex with the active-site serine that mimics the tetrahedral intermediate expected for normal substrate hydrolysis. Our efforts have been focused mainly on the preparation of boronic acid inhibitors of thrombin. In our earlier studies we prepared Ac-(D)Phe-Pro-boroArg-OH (DuP714), Boc-(D)Phe-Pro-boroArg-OH, and H-(D)Phe-Pro-boroArg-OH, which bind thrombin with respective K_i values of 40, 3.6, and ~0.4 pM (Kettner et al., 1990).

Continuing these studies, we have explored structure−function relationships for the P_1 residue in an effort to maximize the selectivity of inhibitors for thrombin. The binding of the amidino analogue of DuP714 and the corresponding analogues where the Arg side chain was replaced with an ornithine, lysine, and homolysine side chain have been evaluated (Weber et al., 1995). In further studies we have prepared analogues of borophenylalanine[1] where the aromatic ring was substituted with a cyano group and the corresponding amidines and aminomethylene. There is a literature precedent for thrombin and trypsin binding phenylalanine analogues substituted with a basic amidine to form an ionic bond with Asp189 in the bottom of the P_1 binding pocket (Stroud et al., 1971; Bode et al., 1990). Thrombin will also bind peptides containing an unsubstituted borophenylalanine and boronic acids containing a methoxypropyl side chain (Deadman et al., 1995). In other studies, N-Me-H-(D)Phe-Pro-TrpCF_2CF_3 was shown to bind thrombin and the tryptophan side chain to occupy the P_1 side-chain pocket (Malikayil et al., 1997). In all cases, the neutral side-chain inhibitors had much less affinity than their basic counterparts. We have made the surprising observation that *m*-cyano-substituted borophenylalanine binds to thrombin with an affinity at least comparable to the corresponding

[⊥] Coordinates for the structure have been deposited in the Brookhaven Protein Data Bank, file name lauj.

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[1] The prefix boro is used to designate the boronic acid analogue of the corresponding amino acid where the -COOH group is replaced by $-B(OH)_2$. The suffix -OH indicates the free boronic acid and $-C_6H_{12}$ and $-C_{10}H_{16}$ indicate the boronic acid pinacol and the pinanediol esters, respectively. -boroPhe(*m*CN)-OH is the abbreviation for the analogue of -boroPhe-OH that is substituted at the *meta* position with a cyano group, and -boroPhe(mCH_2NH_2)-OH is the analogue substituted with an aminomethyl group. Other abbreviations: Boc, *t*-butyloxycarbonyl; Z, carbobenzoxy; Ac, acetyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

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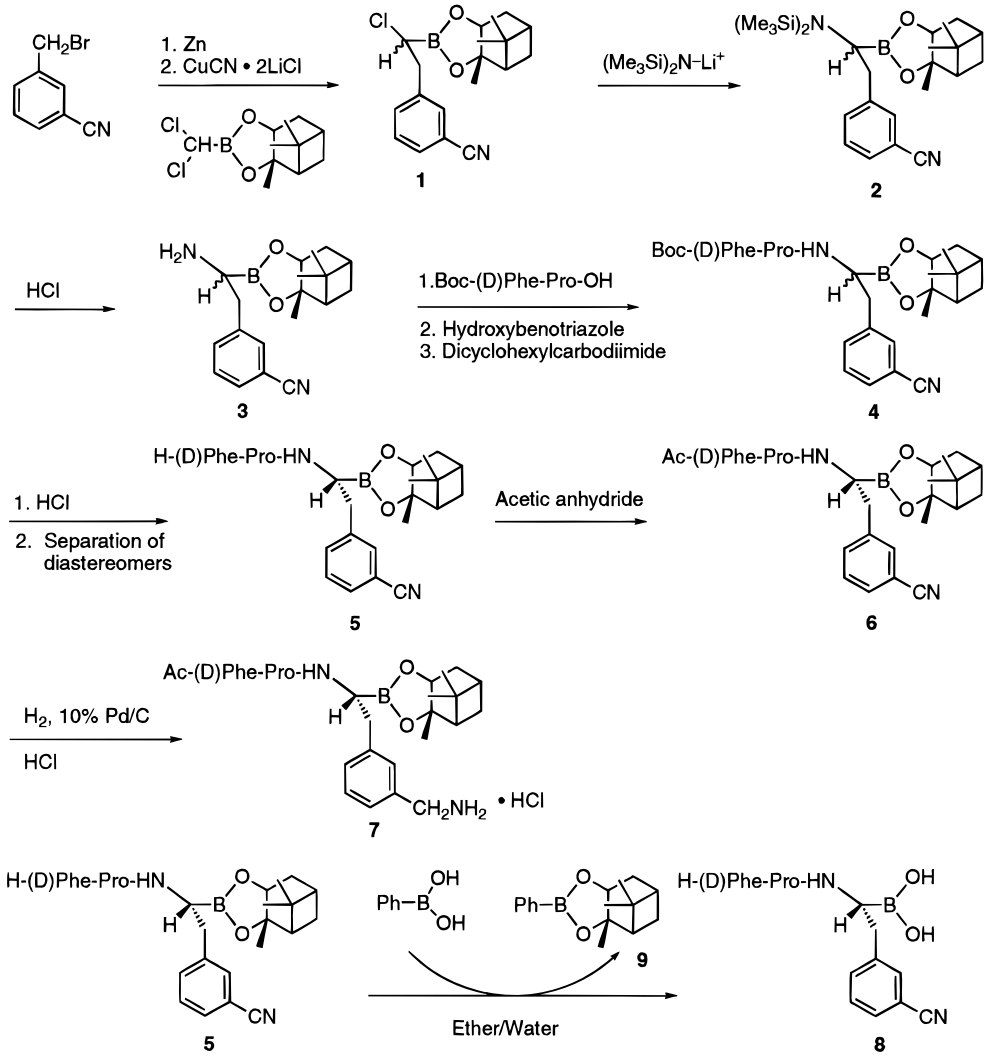


FIGURE 1: Synthetic scheme for the preparation of *m*-cyanoborophenylalanine analogues.

basic amidine and aminomethylene and exceeds the unsubstituted borophenylalanine by 2 orders of magnitude. Trypsin exhibits a similar enhanced affinity for the *m*-cyano-substituted inhibitor. The properties of the *m*-cyano-substituted borophenylalanine inhibitors are the subject of the present paper.

MATERIALS AND METHODS

Preparation of Inhibitors. Protocols used for synthesis of H-boroPhe(*m*-CN)-$C_{10}H_{16}$, H-(D)Phe-Pro-boroPhe(*m*-CN)-OH, and the corresponding Ac and Boc compounds are outlined in Figure 1. A detailed description of the preparation of individual compounds and their characterization is given in the supporting information. The boronic analogue of phenylalanine, H-(D)Phe-Pro-boroPhe-C_6H_{12}, and the corresponding Boc and Ac analogues were prepared as diasteriomeric mixtures of -boroPhe-C_6H_{12} by the method of Kettner and Shenvi (1984).

Enzyme Assays. Protease activity was monitored on an HP 8452 diode array spectrophotometer at 25 °C using *p*-nitroanilide substrates where liberation of *p*-nitroaniline was measured at 404 nm. Assays of human thrombin and factor Xa were performed with the Kabi substrates S2238 and S2222, respectively. Both enzymes were assayed in 0.10 M sodium phosphate buffer, pH 7.5, containing 0.20 M NaCl and 0.5% poly(ethylene glycol) 6000 by the procedure we have described previously (Kettner et al., 1990). Human factor VIIa (lot 852) was obtained from Enzyme Research Laboratories, South Bend, IN. It was assayed by the general procedure described by Lawson et al., (1992) using the Kabi chromogenic substrate S2288. Dade Innovin, Baxter Diagnostic, Inc., was used as source of recombinant, human tissue factor. Levels of tissue factor were based on a reconstituted level of lyophilized material of 25 nM (Richard Jenny, Haematologic Technologies, Essex Junction, VT, personal communication). The assay buffer consisted of 20 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.03% Tween 80. Individual assays contained variable levels of substrate, 0.20 M phosphatidylcholine/phosphatidylserine (75:25), 10 nM tissue factor, and 5.0 nM factor VIIa. Under these conditions the K_m for substrate hydrolysis was 0.83 ± 0.04 mM and the V_m was 1.7 ± 0.6 μM/min. Porcine pancreatic kallikrein (lot 126F-0381) was obtained from Sigma. It was assayed in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.20 M NaCl, 0.5% poly(ethylene glycol) 6000, and 5% DMSO. Z-Lysine thiobenzyl ester was used as a substrate and 0.33 mM Ellman's reagent was used as a chromogen as described previously (Kettner et al., 1980). Michaelis parameters for 10 nM enzyme were K_m = 0.24 ± 0.05 mM and V_m = 5.1 ± 0.8 μM/min. Bovine trypsin (Worthington) was assayed at the 5 nM level in 50 mM Hepes buffer, pH 7.4, containing 0.12 M NaCl, 20 mM

$CaCl_2$, and 0.001% Tween 80 using the Kabi substrate S2222 (20−400 μM). It was hydrolyzed with a $K_m = 0.036 \pm 0.005$ mM and $V_m = 1.0 \pm 0.1$ μM/min. Porcine chymotrypsin was obtained from Worthington and it was assayed by the procedure described previously (Kettner & Shenvi, 1984).

Analysis of Binding. Levels of inhibition were determined by allowing the enzyme to react with substrate and inhibitor for 30 min. K_i values were determined for velocities measured in the time frame of 25−30 min. Initial experiments had confirmed that a steady-state velocity had been reached before this time. In all cases, velocities were readily fit to the equation for competitive inhibition. K_i values were calculated for individual reaction of inhibitors with the enzymes using

$$v_i/v_0 = [K_m(1 + I/K_i) + S]/[K_m + S]$$

This expression is the ratio of the Michaelis equations for velocities measured in the presence of a competitive inhibitor (v_i) and in the absence of inhibitor (v_0). K_m values were determined daily using the method of Lineweaver and Burk, and velocities were measured for at least 5 different substrate concentrations (S). Reported values of K_m and V_m are the average values for different days. Ratios of v_i/v_0 were measured over a range of inhibitor concentrations (I) and for at least two different substrate consentrations. Individual values of K_i were calculated for each value of v_i/v_0 with the aid of a spreadsheet program (Microsoft Excel). For all inhibitors in this study, consistent values of K_i were obtained for different inhibitor levels and also substrate levels as expected for reversible, competitive inhibitors. In general, K_i values calculated for values of v_i/v_0 of 0.2−0.6 were considered more accurate and the reported values are the average (usually 5−10 values) measured in this range.

It should be noted that reversible inhibition can be distinguished for irreversible inhibition by the kinetic measurements we have made. For irreversible inhibitors, the exponential loss of activity with time is observed until complete inhibition is obtained. For this study, steady-state velocities were obtained for all inhibitors as expected for reversible inhibitors. In fact, most inhibitors behaved as rapid equilibrium or non-slow-binding inhibitors. The exceptions were the binding of the *m*-cyanoboroPhe inhibitors to thrombin and the binding of H-(D)Phe-Pro-boroPhe-(*m*-CN)-OH to trypsin. Here, a 3−10-fold difference was measured between initial K_i values measured in the first minutes of the assay and the reported K_i values measured in the 25−30 min time frame.

Crystal Growth and Inhibitor Addition. Bovine trypsin was purchased from Worthington (catalog no. 3707) and used without further purification. Crystals were grown in the presence of benzamidine by the method of Krieger et al. (1974). Since the inhibitor was more active at a lower pH, the crystals were transferred from a Tris buffer (pH 8.2) to a 50 mM MES buffer (pH 6.6). Benzamidine was removed by letting the crystals soak overnight in a buffer with no inhibitor. These crystals were then transferred to a solution containing the inhibitor. The inhibitor solution was prepared by first dissolving 1 mg of inhibitor in 5 mL of DMSO. This was followed by a 40-fold dilution of the inhibitor/DMSO solution into the sitting solution. Data were collected 1 week after inhibitor addition.

Table 1: Data Collection Parameters for Trypsin−Inhibitor Complex

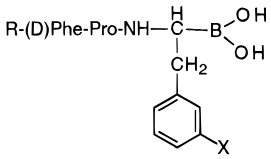
space group	$P2_12_12_1$
unit cell dimensions (Å)	55.0 58.4 67.6 90 90 90
maximum resolution (Å)	2.1
% complete	90
R_{sym}	5.2
R factor	16.9
RMS deviation in bond lengths (Å)	0.011
RMS deviation in bond angles (deg)	1.9
solvent molecules	143

Data Collection and Refinement. A crystal of the trypsin−inhibitor complex was mounted and sealed in a glass capillary. A R-AXIS image plate detector was used for X-ray data acquisition. A Rigaku RU-200 rotating anode X-ray generator operating at 50 kV/100 mA equipped with a graphite monochromator was used for data collection. The trypsin data were collected at 4 °C using an Enraf Nonius cooling device. Data frames of 2° rotation about the spindle axis, ϕ, were collected, with exposure times of 30 min/frame, for total angular rotation ranges about ϕ of 90°. Data were processed using the Raxis data processing software (Molecular Structure Corp.). Data greater than 1σ were used in refinement. The XPLOR (Brunger et al., 1987) program was used for crystallographic refinement. Simulated annealing (at a maximum temperature of 3000 K) was followed by *B*-factor refinement. The refined coordinates of trypsin (Krieger et al., 1974) were used to calculate the initial phases for the enzyme−inhibitor structure. The inhibitor was built with the program QUANTA (MSI, Inc.). Peaks in the difference electron density map that were greater than 3.5σ and that displayed good hydrogen-bonding geometry to the protein were built in as solvent molecules. No major adjustments to the protein model were needed during the course of the refinements. Final *R*-factors as well as other relevant data collection statistics are shown in Table 1.

RESULTS

Synthesis of Peptide Inhibitors. Peptides containing *m*-cyano-substituted borophenylalanine were prepared by the general procedures outlined in Figure 1. Functionalized benzylic anions containing an aromatic cyano substituent were prepared by allowing the benzyl bromide to react with activated Zn metal in THF and then with CuCN·2LiCl using a procedure similar that described by Berk et al. (1990). Dichoromethyl boronic acid pinanediol was prepared by the method of Tsai et al. (1983). It was reacted with transmetalated anion to yield **1** as a mixture of diastereomers. Compound **1** was allowed to react with lithium hexamethylsilazane to yield the bis(trimethylsilane)-protected amine **2**, which was converted to the amine hydrochloride **3** by treatment with anhydrous HCl. Boc-(D)Phe-Pro-OH was coupled to **3** using dicyclohexylcarbodiimide to give a diasteriomeric mixture of peptides **4**. Analytical samples of Boc-(D)Phe-Pro-boroPhe(*m*CN)-$C_{10}H_{16}$ and Boc-(D)Phe-Pro-(D)boroPhe(*m*CN)-$C_{10}H_{16}$ were obtained by HPLC separations. Removal of the Boc protecting group from the diasteriomeric mixture with anhydrous HCl yielded products that were readily separated. Trituration of the mixture with ether gave a single isomer, H-(D)Phe-Pro-(L)boroPhe(*m*CN)-$C_{10}H_{16}$, **5**, as a solid. The tripeptide **5** was also obtained by treating Boc-(D)Phe-Pro-boroPhe(*m*CN)-$C_{10}H_{16}$ (isomer A from HPLC purification) with HCl. Initially, the assumption

Table 2: Binding of Peptide Boronic Acids to Serine Proteases[a]



protease	R	X_1	K_i for X_1 (nM)	X_2	K_i for X_2 (nM)
thrombin	Ac-	-CN	0.79 ± 0.15	-H	320 ± 20
thrombin	Boc-	-CN	0.51 ± 0.10	-H	59 ± 5
thrombin	H-	-CN	0.48 ± 0.09	-H	6.3 ± 0.22
thrombin	Ac-	$-CH_2NH_2$	4.8 ± 1		
trypsin	Ac-	-CN	130 ± 50	-H	11 000 ± 1,000
trypsin	Boc-	-CN	73 ± 8	-H	5400 ± 500
trypsin	H-	-CN	5.6 ± 1.4	-H	1800 ± 170
trypsin	Ac-	$-CH_2NH_2$	5.6 ± 0.8		
factor VIIa[b]	Ac-	-CN	3.3 ± 1.5	-H	360 ± 80
factor Xa	Ac-	-CN	760 ± 250	-H	49 000 ± 9,000
pancreatic kallikrein	Ac-	-CN	8600 ± 500	-H	39 000 ± 2000
chymotrypsin	Ac-	-CN	49 ± 14	-H	100 ± 20

[a] K_i values were determined by the procedure described in the Materials and Methods section. Reported values are the average of at least two separate determinations and they are reported with standard deviations. [b] The factor VIIa·tissue factor complex was used in these studies.

was made that the material was the L-isomer because it has greater biological activity, and later its sterochemistry was confirmed by protein crystallography. Compound **5** was acetylated to give **6** and it was further converted to the amine **7** by catalytic hydrogenation.

Our earlier studies have shown that boronate esters and acids give comparable results in biological assays. In a number of crystallographic studies (Weber et al., 1995), boronic acids were introduced as esters, but they form complexes with the enzyme only as the free boronic acid. This is due to rapid equilibrium between the boronic acid ester and free acid. We estimate that the equilibrium constant is 0.5 mM. However, the boronic acid esters can be converted to the free boronic acid easily by the method we had described earlier (Kettner, 1995; Witayk et al., 1995). For example, **5** was converted to the free boronic acid **8** by treatment with phenylboronic acid in a mixture of ether and water. The free boronic acid was isolated as single component from the aqueous phase. The transesterified phenylboronic acid ester **9** and excess phenylboronic acid were in the organic phase. The driving force for the tranesterification reaction is the difference in solubilities of products since the boronic acid esters are comparable in stability in a single solvent. Compound **8** can be acetylated to yield the free boronic acid of **6**.

Binding of BoroPhe(mCN) Peptides to Thrombin. We have prepared a series of peptides containing -(D)Phe-Pro- in the P_3 and P_2 positions and containing a substituted borophenylalanine residue in the P_1 position. These compounds were first evaluated as inhibitors of thrombin (Table 2). Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH binds thrombin with a K_i of 0.79 nM. Other peptides in the series, Boc-(D)Phe-Pro-boroPhe(*m*CN)-OH and H-(D)Phe-Pro-boroPhe(*m*CN)-OH, have K_i values of 0.51 and 0.48 nM. For comparison with the neutral tripeptide analogues, the basic inhibitor, Ac-(D)Phe-Pro-boroPhe(*m*CH_2NH_2)-OH, was prepared. It was found to bind thrombin with a K_i of 4.8 nM. Also, for comparison in this series, compounds lacking a cyano group were prepared. The Ac-(D)Phe-Pro-boroPhe-OH and the corresponding Boc compound bound thrombin with an affinity 2 orders of magnitude less than the *m*-cyano-substituted inhibitors.

Binding of BoroPhe(mCN) Peptides to Trypsin and Other Serine Proteases. The interaction of trypsin with this series of inhibitors is also shown in Table 2. Trypsin binds Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH with a K_i of 130 nM. It binds the Boc inhibitor 2-fold more tightly and the free amine 20-fold more tightly. As with thrombin, comparison with tripeptide boroPhe inhibitors indicate the *m*-cyano substituent contributes at least 2 orders of magnitude in increased affinity. Finally, the basic inhibitor Ac-(D)Phe-Pro-boroPhe-(*m*CH_2NH_2)-OH binds trypsin with a K_i of 5.6 nM.

Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH provided a highly effective inhibitor of factor VIIa. It was bound with a K_i of 3.3 nM while the compound lacking the cyano group had a K_i of 360 nM. Making the same comparison for factor Xa, K_i values of 760 and 49 000 nM were obtained. Although the overall affinity of this pair of peptides for factor Xa is less, enhanced binding due to the *m*-cyano group is similar in magnitude to the other proteases. Ac-(D)Phe-Pro-boroPhe-(*m*CN)-OH is a micromolar inhibitor of pancreatic kallikrein and the *m*-cyano group contributes less than 5-fold to binding. It inhibits chymotrypsin with a K_i of 49 nM and the cyano group has little influence on binding.

Crystallographic Studies of the H-(D)Phe-Pro-boroPhe-(mCN)-OH·Trypsin Complex. Crystals of trypsin complexed with benzamidine were prepared by the method of Krieger et al. (1974). Soaking these crystals with H-(D)Phe-Pro-boroPhe(*m*CN)-OH gave the crystals for this study. The crystals diffracted to 2.1 Å. *R*-factors and other relevant data collection statistics are given in Table 1. Figure 2 shows the difference electron density map of the trypsin−inhibitor complex.

The *m*-cyanobenzyl group occupies the P_1 binding site and the *m*-cyano group is directed toward the NH of Gly219. The distance between these groups is 3.2 Å, which is in the range for good H-bonds. The remaining portion of the molecule binds in a manner similar to the complex of Ac-(D)Phe-Pro-boroArg-OH with thrombin (Weber et al., 1995) and the complex of Boc-Ala-Val-boroLys-OH with trypsin (Katz et al., 1995). The boronic acid forms a tetrahedral complex with the active-site serine. The NH of boroPhe is H-bonded to the carbonyl of Ser214 and the carbonyl of (D)-Phe is H-bonded to the NH of Gly216. The prolyl residue occupies the P_2 binding site and the (D)Phe residue makes an edge to face interaction with Trp215. The carboxamide of Gln192 is H-bonded to the carbonyl of P_2 proline in a manner similar to that described by Katz et al. (1995). In addition to these interactions, the *m*-cyano group of boroPhe is 3.2 Å from the NH_2 group of H-(D)Phe, indicating an intramolecular H-bond interaction.

DISCUSSION

We have found that neutral compounds containing a *m*-cyano-substituted borophenylalanine residue are highly effective thrombin inhibitors. For example, Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH has a K_i of 0.79 nM. This value approaches that observed for Ac-(D)Phe-Pro-boroArg-OH (DuP714), 0.040 nM. For the latter, interaction of the guanidino group with Asp189 in the bottom of the P_1 binding

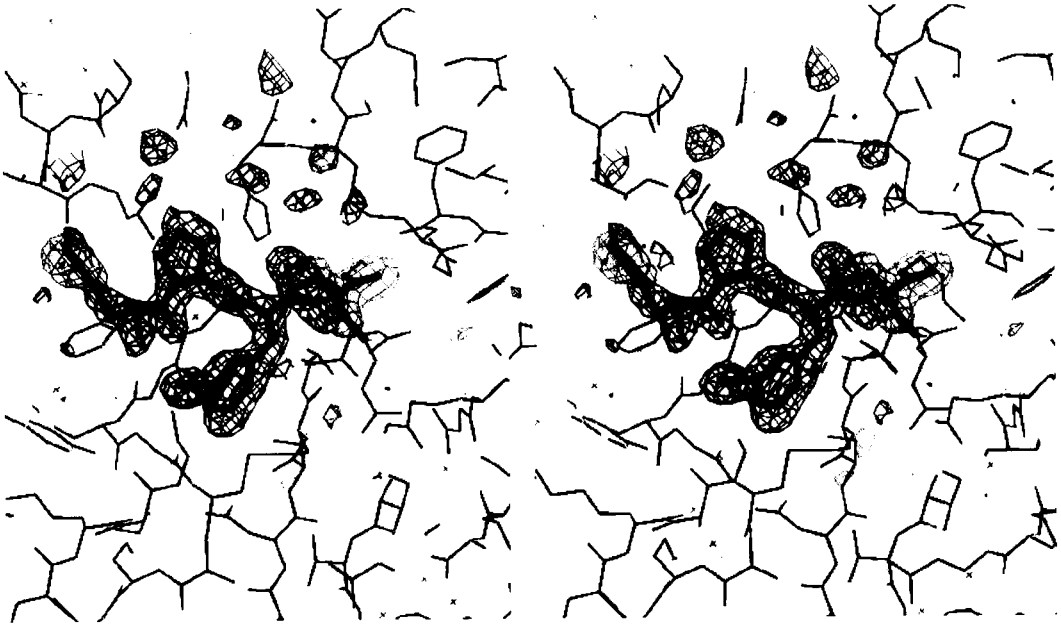


FIGURE 2: Difference electron density map of the trypsin·H-(D)Phe-Pro-boroPhe(*m*CN)-OH complex, generated with coefficients $|F_o| - |F_c|$ and phases calculated from the final model minus the atoms of the inhibitor. The map is contoured at 2.0σ. Inhibitor is displayed in thick bonds. Note the unambiguous density for the cyano group.

pocket contributes significantly to the overall affinity (Weber et al.,1995). This is clearly shown when one compares the binding of the exact analogue of DuP714, where a methoxy group replaces the guanidino group of the arginine side chain. We have measured a K_i of 180 nM for this compound. Binding of the inhibitor lacking the cyano group, Ac-(D)-Phe-Pro-boroPhe-OH, is in the range of this inhibitor. Therefore, the aromatic cyano group contributes ~2 orders of magnitude to binding of thrombin in the tripeptide series. For an additional comparison, the *m*-cyano group was converted to a basic amidine and to a primary amine to yield less effective inhibitors with K_i values of 2−5 nM (data for the amidino compounds is not shown). By analogy with other inhibitors of trypsin-like enzymes, one expects the basic side chain is interacting directly with Asp189 in the bottom of the P_1 binding pocket.

The *m*-cyano group makes a similar contribution to the binding of Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH to trypsin, although its overall affinity is less. Most likely the aromatic cyano group is interacting with similar residues in the P_1 binding pocket of both thrombin and trypsin.

It was our expectation that residues in the P_2, P_3, and P_4 binding sites would have a similar influence on binding for the -boroPhe(mCN)-OH inhibitors as observed for the boroarginine inhibitors. This did not prove to be strictly true. The (D)Phe residue contributes 100-fold to the binding of Ac-(D)Phe-Pro-boroArg-OH (DuP714) to thrombin (Lim et al., 1993), while for Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH the contribution of the (D)Phe residue is 10-fold less (unpublished data). In the boroArg-OH series of tripepides, affinity increases by a factor of 10 in going from Ac- to Boc- and in going from Boc- to H- (Kettner et al., 1990). For the *m*-cyano inhibitors, changing the N-terminal protection group had only marginal effects and one does not achieve the low picomolar inhibition observed for Boc-(D)Phe-Pro-boroArg-OH and H-(D)Phe-Pro-boroArg-OH. The differences between the boroPhe and the boroArg inhibitors are interesting, but they are not the main focus of the present studies.

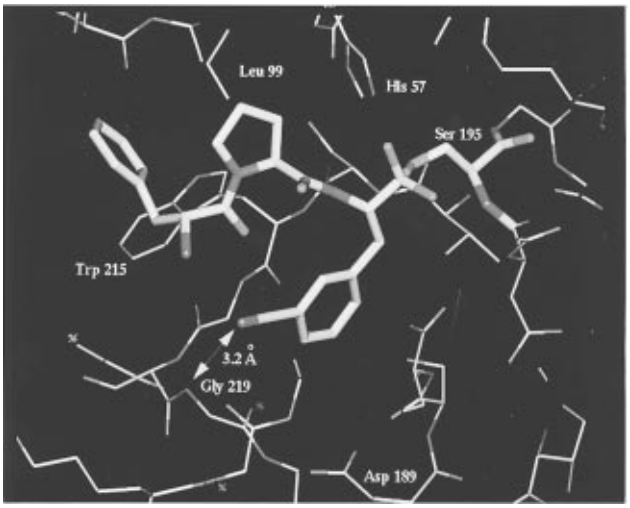


FIGURE 3: Structure of the trypsin·H-(D)Phe-Pro-boroPhe(*m*CN)-OH complex. Selected protein residues are labeled.

To identify specific interactions of the *m*-cyano group in the P_1 binding pocket, we prepared a complex of trypsin with H-(D)Phe-Pro-boroPhe(*m*CN)-OH. A high-resolution X-ray crystal structure at 2.1 Å clearly shows that the *m*-cyano benzyl group occupies the P_1 binding site. The aromatic cyano group clearly points away from Asp189 and towards the backbone nitrogen of Gly219 (3.2 Å) as shown in Figure 3. This H-bond interaction can easily explain the observed enhancement in binding due to the *m*-cyano group. The other observed interactions are very similar to those observed for the binding of Ac-(D)Phe-Pro-boroArg-OH to thrombin (Weber et al., 1995) and of Boc-Ala-Val-boroLys-OH to trypsin (Katz et al., 1995). Figure 4 contrasts the interactions of positively charged P_1 residues of arginine, lysine, or a benzamidine (Bode et al., 1990) interacting with Asp189 with the H-bond of the cyano group to the backbone amide nitrogen of Gly219 we have observed. In the thrombin·DuP714 structure, water is bound in close proximity to Gly219 (Weber et al., 1995). If an analogous binding

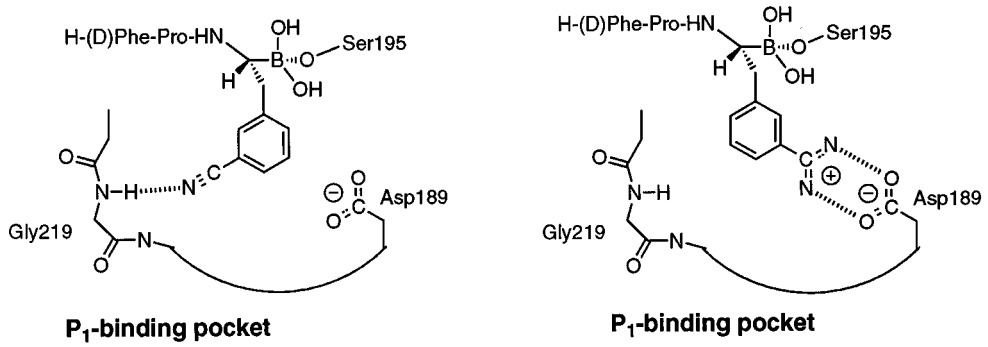


FIGURE 4: Illustration of the binding of -boroPhe(*m*CN)-OH to trypsin and comparison with the corresponding basic inhibitor.

	Amino Acid Residues								
Protease	214	215	216	217	218	219	220	221	221a
Thrombin	Ser	Trp	Gly	Glu		Gly	Cys	Asp	Arg
Trypsin	Ser	Trp	Gly	Ser		Gly	Cys	Ala	Gln
Factor VIIa	Ser	Trp	Gly	Gln		Gly	Cys	Ala	Thr
Factor Xa	Ser	Trp	Gly	Glu		Gly	Cys	Ala	Arg
Kallikrein	Ser	Trp	Gly	His	Thr	Pro	Cys	Gly	Ser
Chymotrypsin	Ser	Trp	Gly	Ser	Ser	Thr	Cys	Ser	

FIGURE 5: Amino acid sequence alignment of residues comprising the P_1 binding sites of serine proteases. Residues are labeled according to the chymotrypsinogen numbering system.

mechanism observed for trypsin occurs for thrombin, this water would be displaced by the cyano group. This was confirmed by crystallographic studies (unpublished structure, see next section).

Our studies have indicated that the *m*-cyano group of boroPhe(*m*CN) plays an almost identical role in the enhanced binding of thrombin. However, structural analysis of thrombin·*m*-cyano inhibitor complexes proved difficult (R. Alexander and P. C. Weber, unpublished results). In the study of the thrombin·Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH complex, good electron density was observed for the inhibitor backbone and P_2 and P_3 side chains but not for the residue in the P_1 binding pocket. In the analysis of the thrombin·Ac-Pro-boroPhe(*m*CN)-OH complex, unambiguous electron density was observed for the inhibitor, but refinement to a model with acceptable stereochemistry for the protein proved difficult. Given that the interactions in the P_1 binding pocket were identical in the thrombin and trypsin complexes and that trypsin crystals diffracted to a higher resolution, we chose to present the trypsin−inhibitor complex.

Our results show the cyano group acts as an H-bond acceptor for the NH proton of homologous Gly219 residues in the P_1 binding pockets of trypsin and thrombin. These results also suggest that this interaction can replace charge−charge interactions of trypsin-like proteases for all enzymes that are homologous to trypsin and thrombin in this series. The sequence of residues comprising the P_1 binding pocket for select serine proteases is shown in Figure 5 (Bajaj & Birktoft, 1993 & Leytus et al., 1987). On the basis of sequence homology, assuming it reflects structural homology, one would expect that enhanced binding would be observed for compounds with a *m*-cyano-substituted benzyl group in the P_1 binding pocket for factor VIIa and factor Xa but not for pancreatic kallikrein or chymotrypsin. For the latter two enzymes, insertion of Thr218 and Ser218, respectively, significantly changes the topology of the P_1 binding site. For kallikrein, Gly219 is replaced with a prolyl residue that cannot act as an H-bond donor. Comparisons of the binding of Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH and Ac-(D)Phe-Pro-boroPhe-OH to these serine proteases gave results consistent with this model. The *m*-cyano group contributes 2 orders of magnitude to the binding of factor VIIa and factor Xa. Its contribution to the binding of pancreatic kallikrein (glandular kallikrein) was less than 5-fold and its contribution to the binding of chymotrypsin was negligible. It should be noted that the K_i of Ac-(D)Phe-Pro-boroPhe(*m*CN)-OH for the factor VIIa·tissue factor complex is 3.3 nM, which compares favorably to the binding of Ac-(D)Phe-Pro-boro-Arg-OH (Dup714), with a K_i of 1.2 ± 0.3 nM (unpublished data).

The principal interaction determining the specificity of trypsin and other trypsin-like enzymes is ionic interaction between the basic side chain for either an arginine or lysine residue and Asp189 in the bottom of of P_1 binding site. In conclusion, we have found that this charge−charge interaction can be replaced by the H-bond interaction between the NH of Gly219 and an aromatic residue containing a H-bond acceptor such as the *m*-cyano group. Preliminary data indicate that this interaction will be observed for most proteases that are structurally homologous to trypsin in the P_1 binding site. Furthermore, this interaction opens new avenues in the design of inhibitors of thrombin and other trypsin-like enzymes that are expected to have improved pharmacological properties.

There is not a clear precedent for a cyano group acting as a H-bond acceptor in enzyme−inhibitor interactions. However, Fitzpatrick et al. (1993) have found that several acetonitrile molecules displaced water molecules present in the aqueous structure when the structure of subtilisin was determined in neat acetonitrile.

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SUPPORTING INFORMATION AVAILABLE

A detailed description of the synthesis of the -boroPhe-(*m*CN)- inhibitors and their characterization (8 pages). Ordering information is given on any current masthead page.

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