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Inhibition of HIV-1 Protease by a Boron-Modified Polypeptide

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ABSTRACT. Six boronated tetrapeptides with the carboxy moiety of phenylalanine replaced by dihydroxyboron were synthesized, and their activities against human immunodeficiency virus 1 (HIV-1) protease subsequently investigated. The sequences of these peptides were derived from HIV-1 protease substrates, which included the C-terminal part of the scissile bond (Phe–Pro) within the gag–pol polyprotein. Enzymatic studies showed that these compounds were competitive inhibitors of HIV-1 protease with K_i values ranging from 5 to 18 μM when experiments were performed at high enzyme concentrations (above 5×10^{-8} M); however, at low protease concentrations inhibition was due in part to an increase of the association constants of the protease subunits. Ac-Thr-Leu-Asn-PheB inhibited HIV-1 protease with a K_i of 5 μM, whereas the non-boronated parental compound was inactive at concentrations up to 400 μM, which indicates the significance of boronation in enzyme inhibition. The boronated tetrapeptides were inhibitory to an HIV-1 protease variant that is resistant to several HIV-1 protease inhibitors. Finally, fluorescence analysis showed that the interactions between the boronated peptide Ac-Thr-Leu-Asn-PheB and HIV-1 protease resulted in a rapid decrease of fluorescence emission at 360 nm, which suggests the formation of a compound/enzyme complex. Boronated peptides may provide useful reagents for studying protease biochemistry and yield valuable information toward the development of protease dimerization inhibitors. BIOCHEM PHARMACOL **60**;7:927–936, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. HIV-1; HIV-1 protease; inhibitors of HIV-1 protease; associative enzyme inhibitors; boron-modified peptides

HIV-1‖ protease specifically processes gag and gag–pol polyproteins into mature viral structural proteins (p6, p7, p17, and p24) and replication enzymes (reverse transcriptase, integrase, and protease) in infected cells [1–3]. HIV-1 protease is required for the production of infectious virions; thus, it is an effective target for antiviral intervention. Currently, five peptidic protease inhibitors (saquinavir, indinavir, ritonavir, amprenavir, and nelfinavir) are available in clinics [4–9], and several approaches for the therapy of AIDS involve a combination of two reverse transcriptase inhibitors with one protease inhibitor. Combination therapy can reduce viremia to unquantifiable levels [4, 5]. However, 30–50% of patients are failing antiviral therapy, presumably due to patient non-adherence and/or resistance development. Therefore, there exists an urgent need for the development of protease inhibitors that require less frequent dosing, that are less toxic, and that exhibit distinct resistance profiles.

HIV-1 protease is a homodimeric enzyme with a molecular mass of 22 kDa that contains a single active site [10]. Inhibition of this enzyme can be achieved either by binding a substrate analog to the enzyme active site (transition state inhibitors) or by interfering with the association of the two enzyme subunits (association inhibitors) (Fig. 1). All currently developed HIV-1 protease inhibitors are analogs of HIV-1 polypeptide substrates that replace the scissile bond of the viral polyproteins, for example Phe–Pro, with transition state mimics such as hydroxyethylene, hydroxyethylamine, or phosphinate groups [11]. In addition, non-peptide-based HIV-1 inhibitors, such as bromperidol, have been identified through crystallographic and/or modeling studies [12, 13]. Furthermore, small peptides that mimic the N- and C-termini of HIV-1 protease have been reported to be weak association inhibitors of HIV-1 protease activity, exhibiting IC_{50} values ranging from 0.08 to 1.5 mM [14]. These association inhibitors presumably exert their activity

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‖ *Abbreviations*: HIV-1, human immunodeficiency virus 1; DTT, dithothreitol; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxanilide; THF, tetrahydrofuran; and CC_{50}, 50% cytotoxic concentration.

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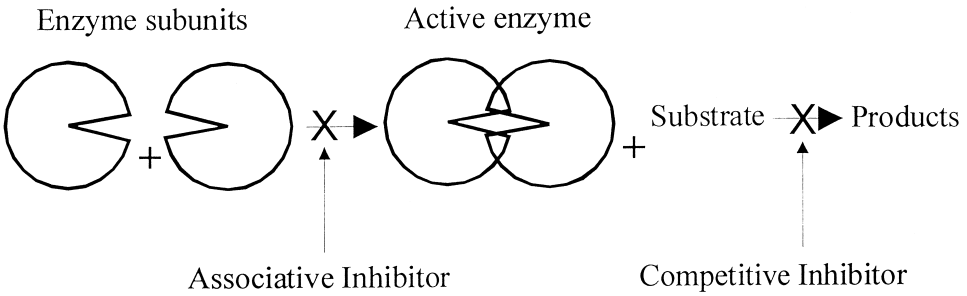
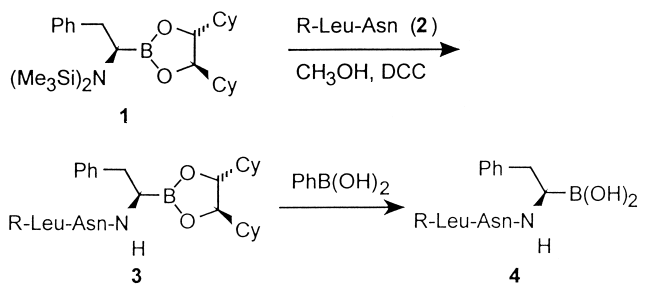


FIG. 1. Schematic representation of sites of inhibition of dimeric enzymes.

by increasing the association constant for HIV-1 protease subunits, thus resulting in a decrease in the pool of active enzyme complexes. In general, the activity of enzyme association inhibitors is affected by enzyme concentration, rather than by substrate concentration as for competitive inhibitors.

Boron-modified peptides have been reported to be effective inhibitors of mammalian and bacterial serine proteases with K_i values of 1–100 pM [15–17]. The inhibition of serine proteases by boron-modified peptides usually is due to a covalent reaction of the boron with the serine residue of proteases. It is also believed that the boron substitution leads to an increase of the affinity of compounds for the enzyme active site. HIV protease is an aspartic protease; however, it is possible that boron modification of its substrates may also increase the affinity of these noncleavable substrates toward the enzyme, and thus the formation of an inactive complex cannot be ruled out. In this paper, we report the preparation of six tetrapeptides, which are substrate analogs of HIV-1 protease (Thr-Leu-Asn-Phe or Ser-Leu-Asn-Phe) with the carboxylic acid of Phe replaced by boronic acid (Fig. 2). These compounds are designed from knowledge of the composition of the HIV-1 cleavage sites located between protease and reverse transcriptase in the HIV pol region; therefore, the peptide-based inhibitors are analogs of the C-terminus of the Phe–Pro scissile bond of HIV-1 protease. As mentioned above, HIV-1 protease C-terminal peptides have been reported to inhibit HIV-1 protease dimerization [14]. We found that the boronated tetrapeptides exhibit inhibitory activity against HIV-1 protease that is more potent than that observed with the nonsubstituted control peptide. The peptides seem to exert their activity through both competitive active site inhibition and dimerization interference. We further demonstrated significant activity against a mutant protease that is resistant to many other HIV protease inhibitors.



R: **a**, BOC-Ser; **b**, BOC-Thr; **c**, Ac-Ser; **d**, $C\Gamma\ H^+$-Ser; **e**, $C\Gamma\ H^+$-Thr;

Cy = cyclohexyl, Ph = phenyl

FIG. 2. Chemical structure and synthesis of boronated tetrapeptides.

MATERIALS AND METHODS

Materials

Purified wild-type HIV-1 protease used in the initial study was provided by Dr. M. Otto (DuPont Merck Pharmaceutical Co.). Later studies of HIV-1 protease were performed with a preparation described below. α-Aminophenylethylboronic acid–pinacole ester was obtained from Boron Biologicals Inc. and converted into the boron-modified tetrapeptide Ac-Thr-Leu-Asn-PheB-pinacole by the Peptide Technologies Corp. Ac-Ser-Gln-Asn-Try-Pro-Val-Val (substrate 1) was obtained from the Sigma Chemical Co., and Ser-Gln-Asn-Try-Pro-Ile-Val (substrate 2) from Peptides International. Ac-Thr-Leu-Asn-Phe was synthesized by W. M. Keck of the Foundation Biotechnology Resource Laboratory (Yale University). Ritonavir, indinavir, nelfinavir, and saquinavir were extracted and purified at Bristol-Myers Squibb from the prescription drugs. The modified HIV octapeptide substrate DABCYL-SQNYPIVQ-EDANS was purchased from Bachem, Inc. Other materials were the highest commercial grade available.

Synthesis of Boronated Peptides

(R)-DICHED BENZYLBORONATE [(R,R)-4,5-DICYCLOHEXYL-2-(PHENYLMETHYL)-1,3,2-DIOXABOROLANE]. Dimethyl benzylboronate (16.4 g, 100 mmol), prepared as previously described [18], was mixed with (*R,R*)-DICHED [(*R,R*)-1,2-dicyclohexyl-1,2-ethanediol] (22.6 g, 100 mmol) in diethyl ether (200 mL) at room temperature. The reaction mixture was stirred for 15 hr. Concentration under vacuum yielded oily (*R*)-DICHED benzylboronate (32.6 g, 100%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.87–1.74 (m, 22), 2.31 (s, 2), 3.82 (m, 2), 7.03–7.24 (m, 5); 75 MHz ^{13}C-NMR ($CDCl_3$) δ 19.2 (broad, C-B), 25.7, 25.8, 26.3, 27.2, 28.1, 42.6, 83.3, 124.6, 128.0, 128.8, 138.6. HRMS calc. for $C_{21}H_{31}BO_2$:

326.2417. Found: 326.2413. Anal. Calc. for $C_{21}H_{31}BO_2$: C 77.30; H, 9.58; B, 3.31. Found: C, 77.10; H, 9.60; B, 3.15.

(*R*)-DICHED (*S*)-1-CHLORO-2-PHENYLETHYLBORONATE [[2(1*S*),4*R*,5*R*]-4,5 DICYCLOHEXYL-2-(1-CHLORO-2-PHENYLETHYL)-1,3,2-DIOXABOROLANE]. (Dichloromethyl)lithium (72.6 mmol) was made from butyllithium and dichloromethane at −100° and treated with (*R*)-DICHED benzylboronate (19.56 g, 60 mmol) followed by anhydrous zinc dichloride (8.17 g, 60 mmol) in the usual manner [18, 19]. After 24 hr at 25°, followed by concentration, aqueous workup, and concentration, viscous liquid (*R*)-DICHED (*S*)-1-chloro-2-phenylethylboronate was obtained (21.99 g, 98%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.77–1.83 (m, 22), 3.13 (m, 2), 3.62 (t, 1), 3.89 (m, 2), 7.23 (m, 5); 75 MHz ^{13}C-NMR ($CDCl_3$) δ 25.7, 25.8, 26.3, 27.0, 27.9, 40.5, 83.9, 126.6, 128.2, 129.0, 138.2. HRMS calc. for $C_{22}H_{32}BClO_2$: 374.2184. Found: 374.2192. Anal. Calc. for $C_{22}H_{32}BO_2$: C, 70.51; H, 8.54; B, 2.88, Cl, 9.46. Found: C, 70.66; H, 8.48; B, 2.60, Cl, 9.32.

(*R*)-DICHED (*R*)-[1-BIS(TRIMETHYLSILYL)AMINO-2-PHENYLETHYL]BORONATE [[2(1*R*),4*R*,5*R*]-4,5-DICYCLOHEXYL-2-[1-BIS(TRIMETHYLSILYLAMINO)-2-PHENYLETHYL]-1,3,2-DIOXABOROLANE] (1). This compound was prepared in the same manner as the analogous pinanediol ester, which has been described previously [19, 20], from a solution of lithiohexamethyldisilazane (50 mmol) and (*R*)-DICHED (*S*)-1-chloro-2-phenylethylboronate (19.70 g, 50 mmol) yielding **1** (23.6 g, 95%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.15 (s, 18), 0.7–1.9 (m, 22), 2.66 (m, 1), 2.85 (m, 1), 3.02 (m, 1), 3.72 (m, 2), 7.25 (m, 5); 75 MHz ^{13}C-NMR ($CDCl_3$) δ 2.9, 25.8, 26.1, 26.4, 27.5, 28.8, 42.5, 43.1, 44.1 (broad, C-B), 83.9, 125.6, 127.8, 129.6, 141.3. HRMS calc. for $C_{28}H_{49}BNO_2Si_2$ (M - 1): 498.3395. Found: 498.3409.

BOC-SER-LEU-ASN (2A). A water solution of Ser-Leu-Asn (0.110 g, 0.345 mmol) in triethylamine (500 μL, 1.5 eq.) and water (1 mL) was mixed with a solution of "BOC-ON" [2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile] (0.093 g, 1.1 eq.) (Aldrich Chemical Co.) in 1,4-dioxane (1.5 mL). After 24 hr at 20–25°, addition of water, extraction of by-products with ethyl acetate, and concentration of the aqueous phase under vacuum yielded **2a** (0.120 g, 76%); 300 MHz ^{1}H-NMR (D_2O) δ 0.75 (dd), 1.09 (t), 1.25 (s), 1.46 (d), 1.80 (s), 2.50 (m), 3.00 (q), 3.62 (d), 4.02 (t), 4.29 (m), 4.65 (s), 7.35 (d), 7.60 (d).

BOC-THR-LEU-ASN (2B). The procedure described in the preceding paragraph was used for the preparation of **2b**; 300 MHz ^{1}H-NMR (D_2O) δ 0.86 (d), 1.24 (m), 1.40 (s), 1.51 (d), 1.61 (d), 1.92 (d), 2.63 (m), 3.15 (m), 4.42 (d), 4.39 (m), 4.80 (s).

BOC-SER-LEU-ASN-PHEB[(*R*)-DICHED] [[2(1*R*),4*R*,5*R*]-4,5-DICYCLOHEXYL-2-[1-[(]N-*T*-BUTOXYCARBONYLSERYLLEUCYLASPARAGINYL)AMINO]-2-PHENYLETHYL]-1,3,2-DIOXABOROLANE] (3A). A solution of BOC-Ser-Leu-Asn (**2a**) (0.240 g, 0.573 mmol) and Ph-$CH_2CH(NSIMe_3)_2$-B[(*R*)-DICHED] (**1**) (0.280 g, 0.573 mmol) in THF (20 mL) was treated with anhydrous methanol (0.0278 mL, 1.2 eq.). After a few minutes of stirring, 1,3-dicyclohexylcarbodiimide (1 mL of a 1 M solution in dichloromethane) was added. After 24 hr under an inert atmosphere, the reaction was quenched with water (1 drop initially, 1 mL 30 min later). The solvent was distilled under vacuum, and ethyl acetate (20 mL) and water (10 mL) were added; the white precipitate (dicyclohexylurea) was filtered, and the ethyl acetate phase was dried (magnesium sulfate) and concentrated under vacuum to yield **3a** (0.290 g, 66%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.79–1.93 (m), 2.04 (t), 2.50 (dd), 2.70 (m), 2.85 (m), 3.58 (m), 3.95 (q), 4.13 (m), 4.30 (m), 4.76 (d), 5.43 (d), 7.11 (m).

BOC-THR-LEU-ASN-PHEB[(*R*)-DICHED] (3B). By the method described for the preparation of **3a**, BOC-Thr-Leu-Asn (**2b**) (0.403 mmol) was converted to **3b** (0.110 g, 35%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.59–1.80 (m), 2.52 (dd), 2.71 (q), 2.89 (dd), 3.03 (m), 3.56 (d), 3.65 (m), 3.92 (m), 4.42 (m), 7.04 (m).

AC-SER-LEU-ASN-PHEB[(*R*)-DICHED] (3C). By the method described for the preparation of **3a**, Ac-Ser-Leu-Asn (**2c**) (0.105 g, 0.268 mmol) was converted to **3c** (0.110 g, 58%); 300 MHz ^{1}H-NMR ($CDCl_3$) δ 0.88–1.91 (m), 2.80 (d), 2.87 (t), 3.16 (m), 3.44 (m), 3.60 (q), 3.81 (q), 3.93 (q), 4.34 (d), 4.55 (s), 4.91 (s), 6.14 (s), 6.20 (s), 7.26 (m).

SER-LEU-ASN-PHEB[(*R*)-DICHED] HYDROCHLORIDE (3D). A solution of **3a** (0.290 g) in 3 M hydrochloric acid in 1,4-dioxane (1 mL) was stirred under argon for 24 hr at 20–25°. Concentration under vacuum overnight yielded solid **3d** (0.189 g, 72%), which was converted directly to derivatives without purification. Thr-Leu-Asn-PheB[(*R*)-DICHED] hydrochloride **(3e)** was prepared similarly.

AC-SER-LEU-ASN-PHEB(OH)$_2$ [(*R*)-[1-(N-ACETYLSERYLLEUCYLASPARAGINYL)AMINO]-2-PHENYLETHYL]BORONIC ACID] (4C). A solution of **3c** (0.110 g, 0.151 mmol) and phenylboronic acid (0.147 g, 1.21 mmol) in ethyl acetate (8 mL) was stirred under argon for 24 hr. For workup, more ethyl acetate (5 mL) and water (5 mL) were added, the two-phase mixture was filtered to remove foamy precipitate, the aqueous phase was extracted three times with ethyl acetate (3 x 2 mL), and the organic phase was washed with water (3 mL). Concentration of the aqueous phase under vacuum yielded crystalline **4c** (0.010 g, 13%); 300 MHz ^{1}H-NMR (D_2O) δ 0.88 (m), 1.23 (m), 1.59 (d), 1.74 (q), 1.93 (m), 3.09 (m), 3.59 (m), 3.79 (m), 4.35 (m), 7.48 (m), 7.71 (d). MALDI–MS calc. for $C_{23}H_{36}BN_5O_8$: 521.266. Found: 519.295.

SER-LEU-ASN-PHEB(OH)$_2$ HYDROCHLORIDE (4D). The method described for preparation of **4c** was used to convert

3d (0.189 g, 0.273 mmol) to **4d** (0.027 g, 20%); 300 MHz ^{1}H-NMR (D_2O) δ 0.71–1.79 (m), 1.92 (s), 2.67 (m), 3.23 (t), 3.46 (s), 3.55 (d), 3.58 (d), 3.78 (m), 3.97 (m), 4.20 (m), 7.20 (m), 7.56 (d).

THR-LEU-ASN-PHEB(OH)$_2$ HYDROCHLORIDE (**4E**). The method described for preparation of **4c** was used to convert **3e** (0.125 g, 0.173 mmol) to **4e** (0.030 g, 33%); 300 MHz ^{1}H-NMR (D_2O) δ 0.75–1.79 (m), 2.47–2.68 (m), 2.83 (m), 2.95 (m), 3.30 (m), 3.48 (s), 3.67 (m), 3.88 (m), 4.22 (m), 4.78 (s), 4.88 (t), 7.25 (m), 7.59 (d).

HIV-1 Protease Purification

Both wild-type and resistant HIV-1 RF proteases were expressed in *Escherichia coli* strain BL21 (DE3)ply S using the pET24 vector from Novagen, Inc., and the enzyme was purified as described [21, 22] with minor modifications. Briefly, the inclusion bodies prepared from an actively growing culture were dissolved in a buffer containing 10 mM Tris (pH 7.5), 8 M urea, 10 mM DTT and passed through a Q-Sepharose column previously equilibrated with the same buffer. The fractions of the breakthrough peak, which contained HIV-1 protease, were pooled and dialyzed against the buffer containing 10 mM sodium acetate (pH 3.5), 1 mM DTT, 1% glycerol overnight. The dialyzed sample then was loaded onto an FPLC Mono S column previously equilibrated with the buffer containing 50 mM 2-[*N*-morpholino]ethane sulfonic acid (pH 6.5), 1 mM DTT, 1 mM EDTA, 5% glycerol and eluted with a linear gradient of 0–500 mM NaCl in the same buffer. The fractions containing the HIV-1 protease were pooled and stored at −70°.

HIV-1 Protease Assay

The assay was performed in a volume of 75 μL containing 100 mM sodium acetate at pH 5.5, 0.1 to 10 μg/mL of HIV-1 protease, 1 M sodium chloride, 10% DMSO as a drug solubilizer, 2.5 mM DTT, 0.1 to 2.5 mM HIV-1 protease substrates (Ac-Ser-Gln-Asn-Try-Pro-Val-Val or Ser-Gln-Asn-Try-Pro-Ile-Val), and various concentrations of inhibitors. The reaction mixtures were incubated for 30–90 min at 37°, and were analyzed immediately by HPLC or quickly frozen and stored at −70° for future HPLC analysis. The area of the individual peaks during HPLC analysis consisting of remains of the initial substrate and products formed during the reaction was used for the calculations.

HPLC Analysis of the Products of HIV-1 Protease Assay

The reaction products formed in 70 μL of the reaction mixture after incubation were analyzed by HPLC using a C_{18} reverse-phase column (Radial Pack, Waters Chromatographic Division of the Millipore Co.). The reaction products were separated by a 30-min gradient of acetonitrile from 2.5 to 22.5% in 0.05 M ammonium phosphate, pH 4.5, at a flow rate of 1.5 mL/min. The separated products of the HIV-1 protease assay were detected and quantified by UV absorbance at 263 nm.

HIV-1 Protease Inhibition Assay

To determine the IC_{50} values for each protease inhibitor, purified HIV-1 RF wild-type protease (2.5 μM) was incubated at 37° with a 10 μM concentration of fluorogenic substrate (Bachem No. M-1865) in reaction buffer (1 M NaCl, 1 mM EDTA, 0.1 M NaAc, pH 5.5, and 0.1% polyethylene glycol 8000) in the presence or absence of inhibitor. Cleavage of the substrate was quantified by measuring an increase in fluorescent emission at 490 nm after excitation at 340 nm [9] using a Cytofluor 4000 (PerSeptive Biosystems). Substrate cleavage was monitored continuously (5-min intervals) for 30 min. Cleavage rates then were determined for each sample at early time points in the reaction, and IC_{50} values were calculated. Next K_i values were derived from the equation $K_i = IC_{50}/(1 + [S]/K_m)$, where the K_m of the substrate was determined to be 0.5 mM.

Non-HIV-1 Protease Assays

Standard assays with chymotrypsin, trypsin, and peptidase were performed using Na-benzoyl-L-tyrosine ethyl ester, Na-benzoyl-L-arginine ethyl ester, and L-leucine-β-naphthylamine, respectively, with or without boronated tetrapeptides in a volume of 0.2 mL at pH 7.8, 7.6, and 7.1, respectively. The reaction was monitored by measuring the absorbance at 251 nm.

Anti-HIV Activity and Cytotoxicity Assays

Two compounds, Ser-Leu-Asn-PheB and Ac-Thr-Leu-Asn-PheB, were evaluated against HIV-1 RF infection of CEM-SS cells using an XTT assay [29]. Briefly, CEM-SS cells were infected with HIV-1 RF at a multiplicity of infection of 0.16, and incubated at 37° in the presence of serial dilutions of the compounds. Five days later, XTT and *N*-methylphenazonium methosulfate were added to each well, and plates were incubated at 37° for 4 hr to allow for XTT formazan production. Cell viability was quantified by light absorbance at 450 and 650 nm as a reference wavelength. The EC_{50} was calculated as the concentration of drug that increased the viability of virus-infected cells to 50% of that of untreated control cells. The cytotoxicity of these compounds was evaluated in CEM-SS cells using the same XTT assay except for the absence of virus infection. The CC_{50} was calculated as the concentration of drug that decreased the percentage of formazan production in drug-treated cells to 50% of that produced by untreated cells.

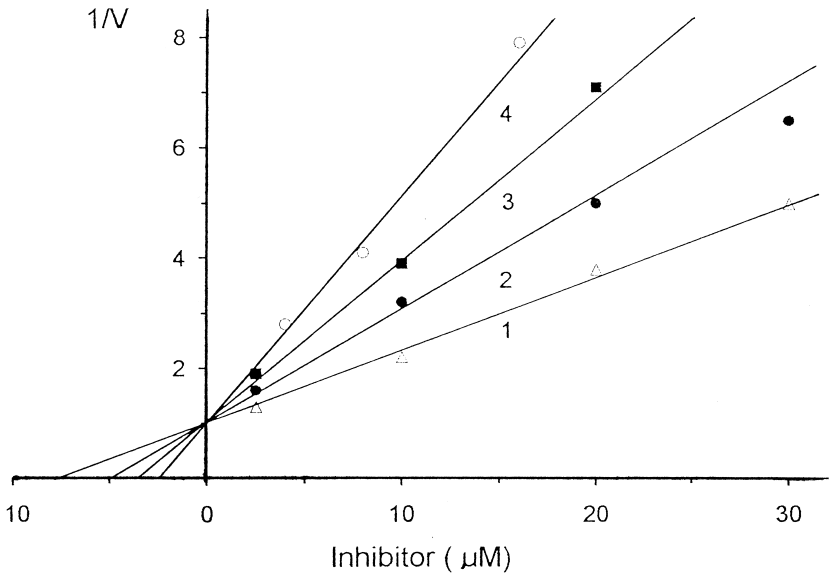


FIG. 3. Effect of substrate and inhibitor concentration on the inhibition of HIV-1 protease activity by Ac-Thr-Leu-Asn-PheB. HIV-1 protease assay and HPLC analysis were performed as described in Materials and Methods. The concentration of HIV-1 protease was 10 μg/mL; the concentrations of substrate 1 (Ac-Ser-Gln-Asn-Try-Pro-Val-Val) were 2.5 mM (line 1) and 1.25 mM (line 2), and for substrate 2 (Ser-Gln-Asn-Try-Pro-Ile-Val) were 2.5 mM (line 3) and 1.25 mM (line 4).

Fluorescence Analysis of HIV-1 Protease

The fluorescence analysis of HIV-1 protease was performed at 25° in 1.25 mL buffer containing 50 mM sodium acetate, pH 6.0, 1 M sodium chloride, 10% DMSO, 2.5 mM DTT, 100 μg HIV-1 protease, and various amounts of boron-modified tetrapeptide. The effect of the substrate and boron-polypeptide on the fluorescence was monitored using a Hitachi F-3010 fluorescence spectrofluorimeter at an excitation wavelength of 283 nm and an emission wavelength of 360 nm.

RESULTS

Synthesis of Boronated Peptides

The major synthetic obstacle to the preparation of acylamido boronic acids is the instability of α-amino boronic esters, which first was circumvented by preparing silylated amino boronic esters and acylating them promptly after desilylation [23]. Numerous syntheses of boropeptides have been based on this initial discovery [19, 24]. The present syntheses followed the same general pattern. The stereocontrol of the L-amino boronic ester synthesis and the ease of conversion of boronic ester to boronic acid were improved in the present work by the use of boronic esters of the chiral director, (R)-DICHED [25].

The general synthesis (Fig. 2) adopted involved treatment of a mixture of (*R*)-DICHED 1-[bis(trimethylsilyl) aminol]-2-phenylboronate (**1**) and *N*-protected polypeptide (**2**) in THF with methanol in THF to desilylate **1**, followed after a few minutes by the addition of dicyclohexylcarbodiimide to effect coupling to form **3**. Deacylation/reacylation of **3** as needed was carried out according to conventional procedures. Because equilibrium does not favor hydrolysis of the boronic ester, conversion to boronic acids, **4**, was accomplished by the exchange of (*R*)-DICHED with phenylboronic acid in a two-phase system, a method that has been used previously [25]. The ^{1}H-NMR spectra of **4a** and **4b** showed proton signals expected of the peptide chains but also indicated that removal of (*R*)-DICHED was incomplete and that the samples also contained substantial amounts of phenylboronic acid. The less lipophilic and more water-soluble boropeptides (**4c**, **d**, and **e**) were obtained in satisfactory purity.

Inhibition of HIV-1 Protease by Boronated Peptides

The effect of Ac-Thr-Leu-Asn-PheB pinacole on the activity of wild-type HIV-1 protease is presented in Figs. 3–5. Figure 3 depicts the inhibition of HIV-1 protease activity by the Ac-Thr-Leu-Asn-PheB pinacole, when the concentration of the enzyme was higher than the reported (10^{-9} M to 3×10^{-8} M) associative constant of HIV-1 protease. Two different substrates were used in these experiments with similar results. The inhibition constants, K_i, were determined from these data, presented in terms of the Dixon plot, to be 5×10^{-5} M and 8×10^{-5} M for Ac-Ser-Gln-Asn-Try-Pro-Val-Val and Ser-Gln-Asn-Try-Pro-Ile-Val, respectively. The K_m values for these two heptapeptide substrates were determined to be about 1.2×10^{-3} M and 1.1×10^{-3} M (data not shown).

Figure 4 depicts similar data as presented in Fig. 3, when the HIV-1 protease concentration was similar to or lower than the enzyme associative constant. These data cannot be linearized using common methods of enzyme kinetic analysis. When reaction velocity (V) is plotted versus enzyme concentration (E) (Fig. 4), it becomes apparent that both enzyme activity and inhibition are related nonlinearly to the enzyme concentration. The inhibition curves differ from what one would expect to observe if the inhibition were competitive or noncompetitive. Figure 5 depicts the same data, but V is plotted versus the square of the enzyme concentration (E^2) instead of E, and this now leads to the linearization of V. These effects are in agreement with the enzyme activity being a function of the square power of the

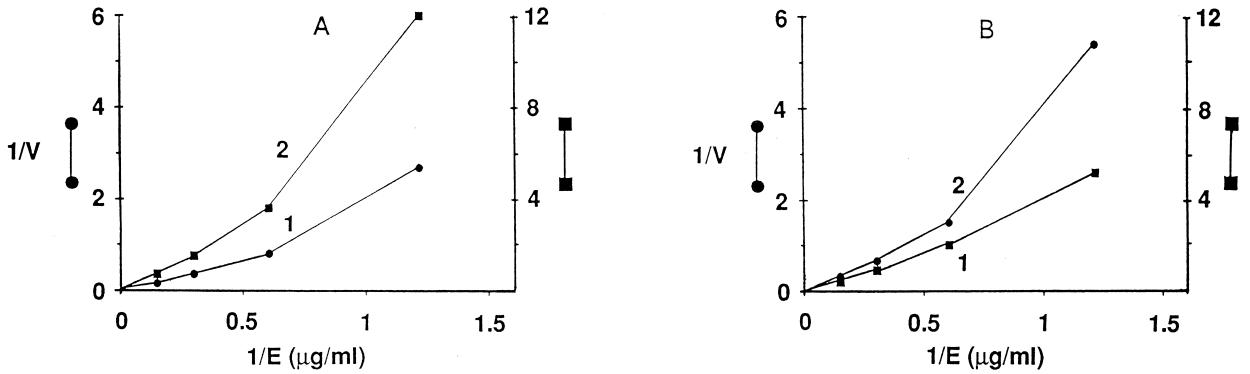


FIG. 4. Effect of enzyme concentration on the inhibition of HIV-1 protease activity by Ac-Thr-Leu-Asn-PheB. HIV-1 protease assay and HPLC analysis were performed as described in Materials and Methods. Panel A: Substrate 1, Ac-Ser-Gln-Asn-Try-Pro-Val-Val. Panel B: substrate 2: Ser-Gln-Asn-Try-Pro-Ile-Val. Line 1, no boropeptides; line 2, 2 μM borotetrapeptide. The results presented are from one experiment representative of a set of four experiments.

enzyme concentration and with associative-type inhibition. Similar data were obtained for various substrate concentrations in the range of 0.1 to 2.5 mM. Associative-type inhibition constants were calculated as described previously [26–28].

Other boronated tetrapeptides, with the exception of those modified with *t*-BOC, showed a similar inhibitory effect on HIV-1 protease, and the data are summarized in Table 1. *t*-BOC-modified boronated tetrapeptides were poorly soluble in the reaction mixture, and therefore were excluded from further analysis.

The potential inhibitory effect on HIV-1 protease by a non-boronated tetrapeptide with the same amino acid sequence, Ac-Thr-Leu-Asn-Phe, as that of the boronated analog was also evaluated. The non-boronated compound did not show any inhibitory activity up to a concentration of 10^{-4} M, which implies that the inhibition constant for this compound is at least two orders of magnitude higher than that of the boronated compound. This was also confirmed by analysis of product formation after various times of incubation when no inhibition of the reaction by the products formed was observed, even after accumulation of the products of substrate 1 (Ac-Ser-Gln-Asn-Tyr and Pro-Val-Val) and of substrate 2 (Ser-Gln-Asn-Tyr plus Pro-Ile-Val) up to 10^{-3} M (data not shown).

Boronated tetrapeptides did not inhibit three non-HIV-1 proteases (chymotrypsin, trypsin, and peptidase) even at concentrations of 10^{-4} M.

Activity against a Drug-Resistant HIV-1 Protease

Since Ac-Thr-Leu-Asn-PheB may interfere with the association of the HIV-1 protease subunits in addition to competing with the enzyme substrate, we next evaluated the activity of three boronated peptides against a drug-resistant HIV-1 protease. The protease variant used was derived from an RF strain of HIV-1 that was propagated in culture in the presence of ritonavir to select for resistant mutants. The ritonavir-resistant mutant virus recovered after selection contained the M461/V82F/I84V/L90M mutations in its protease and showed reduced sensitivities to ritonavir (71-fold), indinavir (29-fold), nelfinavir (22-fold), amprenavir (28-fold), and saquinavir (8-fold) (unpublished results). Consistent with these data, purified HIV-1 protease generated from a recombinant cDNA encoding M461/V82F/I84V/L90M mutant enzyme displayed elevated K_i

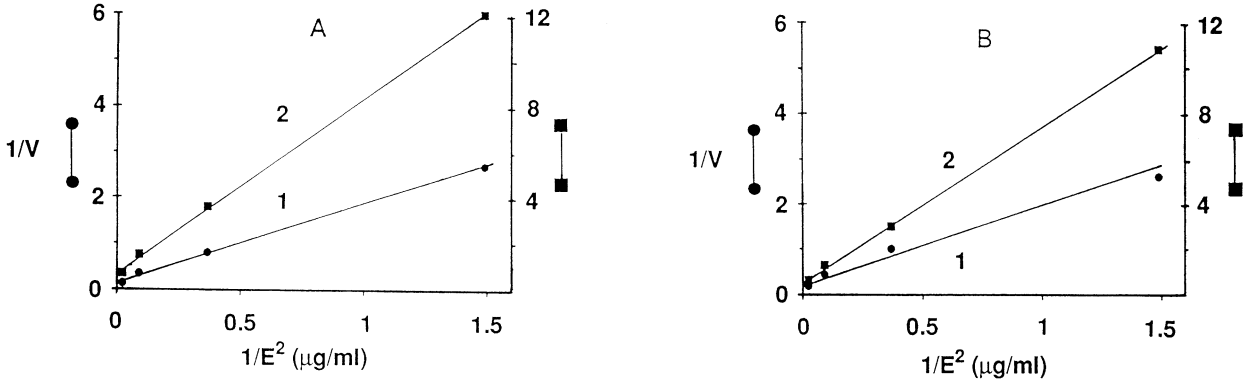


FIG. 5. Plot of the square of enzyme concentration on the inhibition of the HIV-1 protease. HIV-1 protease assay and HPLC analysis were performed as described in Materials and Methods. Panel A: Substrate 1, Ac-Ser-Gln-Asn-Try-Pro-Val-Val. Panel B: substrate 2, Ser-Gln-Asn-Try-Pro-Ile-Val. Line 1, no boropeptides; line 2, 2 μM borotetrapeptide. The results presented are from one experiment representative of a set of four experiments.

TABLE 1. Effects of boronated and non-boronated tetrapeptides on wild-type and mutant forms of HIV-1 protease

	K_i (μM)		
	Wild-type HIV-1 protease		Mutant 84-1A HIV-1 protease
Compound	Competitive-type inhibition	Associative-type inhibition	Total inhibition
Ac-Thr-Leu-Asn-Phe	$>400 \times 10^{-6}$ M	ND	ND
Thr-Leu-Asn-PheB	$18 \pm 3.5 \times 10^{-6}$ M	$4 \pm 1.5 \times 10^{-6}$ M	125×10^{-6} M
Ac-Thr-Leu-Asn-PheB	$5 \pm 1.5 \times 10^{-6}$ M	$3.5 \pm 2 \times 1\text{-}^{-7}$ M	ND
t-BOC-Thr-Leu-Asn-PheB*	$>10 \times 10^{-6}$ M		
Ser-Leu-Asn-PheB	$6 \pm 1.5 \times 10^{-6}$ M	$2 \pm 0.5 \times 10^{-6}$ M	46×10^{-6} M
Ac-Ser-Leu-Asn-PheB	$8 \pm 2 \times 10^{-6}$ M	$1 \pm 0.3 \times 10^{-6}$ M	150×10^{-6} M
t-BOC-Ser-Leu-Asn-PheB*	$>10 \times 10^{-6}$ M		

HIV-1 protease assay was performed as described in Materials and Methods. ND = not determined.
* Not soluble.

values when compared with the wild-type enzyme for the following inhibitors: ritonavir (95-fold), indinavir (153-fold), saquinavir (27-fold), and nelfinavir (78-fold) (Table 2).

When three boronated peptides were assayed against the multi-resistant protease, the results showed that K_i values for Thr-Leu-Asn-PheB, Ser-Leu-Asn-PheB, and Ac-Ser-Leu-Asn-PheB were 125, 46, and 150 μM, respectively. These values were only 7- to 19-fold higher than that observed for the wild-type protease (Table 1).

Anti-HIV Activity and Cytotoxicity

To determine whether any of the boronated peptides exhibited anti-HIV activity in cell culture, two of the more potent peptide inhibitors (Ser-Leu-Asn-PheB and Thr-Leu-Asn-PheB) were evaluated against HIV-1 RF infection of a T-cell line (CEM-SS) using an XTT cell protection assay [29]. Both compounds were inactive against HIV-1 replication up to their cytotoxic concentrations (102 and 82 μM, respectively).

Fluorescence Spectral Analysis of HIV-1 Protease

Ac-Thr-Leu-Asn-PheB pinacol produced a decrease in the fluorescence spectra of HIV-1 protease as shown in Fig. 6. This effect may be a consequence of the formation of the transition state complex and/or the dissociation of the dimer enzyme with formation of a protease subunit–boron tetrapeptide complex. The fluorescence emission spectrum of HIV-1 protease had a maximum at 360 nm when the excitation wavelength was 283 nm.

TABLE 2. Inhibition by known inhibitors of wild-type and mutant HIV-1 protease

	$K_{i_{wt}}$	$K_{i_{mut}}$	$K_{i_{mut}}/K_{i_{wt}}$
Ritonavir	1.01	95.95	95
Indinavir	0.73	111.7	153
Saquinavir	0.39	10.65	27.3
Nelfinavir	1.05	82.4	78.5

K_i values were determined using the equation $[K_i = IC_{50}/(1 + [S]/K_m]$ and are presented in nanomolar concentrations.

DISCUSSION

The replication of infectious HIV-1 is dependent on the proper formation and function of several viral proteins. One of the important viral proteins necessary for the formation of infectious HIV-1 is the virally encoded protease.

The development of HIV-1 protease inhibitors is under intensive investigation in many laboratories, and very potent inhibitors have been produced. Most of them are transition state peptide substrate analogs in which the scissile amide bond of the viral polyproteins has been replaced by nonhydrolyzable transition state isosteres such as hydroxyethylene, hydroxyethylamine, or phosphinate groups having a tetrahedral geometry [11]. Crystallographic and modeling studies also have found non-polypeptide compounds to have transition state inhibitory activities against HIV-1 protease [12, 13]. The inhibition constants for some of these inhibitors of HIV-1 protease are in the range of 10^{-9} to 10^{-10} M. Although strongly inhibitory, these compounds are not ideal, since they generally have poor aqueous solubility, poor oral bioavailability, rapid elimination, and brief duration of action. Furthermore, when the inhibitor is removed from the infected cells, infectious virions are produced. Thus, there is a need for protease inhibitors that do not have these undesirable characteristics.

HIV-1 protease is a dimeric enzyme that is active only in this form. Thus, besides regular inhibition via formation of an inactive enzyme–inhibitor complex, the prevention of association of HIV-1 protease may also serve as a pathway to prevent the activity of the enzyme. The kinetics of the association of the enzymes and their inhibition has been described earlier [28]. The inhibition of the association of the protease can lead to formation of inactive subunit–inhibitor complexes with formation of immature virions.

The synthesis of the associative type of inhibitors of HIV-1 protease has not been pursued rigorously. Several inhibitors that are analogs of the C-terminus of the scissile bond of the protease substrates have been reported to act as weak to very weak associative inhibitors with IC_{50} values in the range of 2×10^{-6} to 2×10^{-3} M [14, 26, 27].

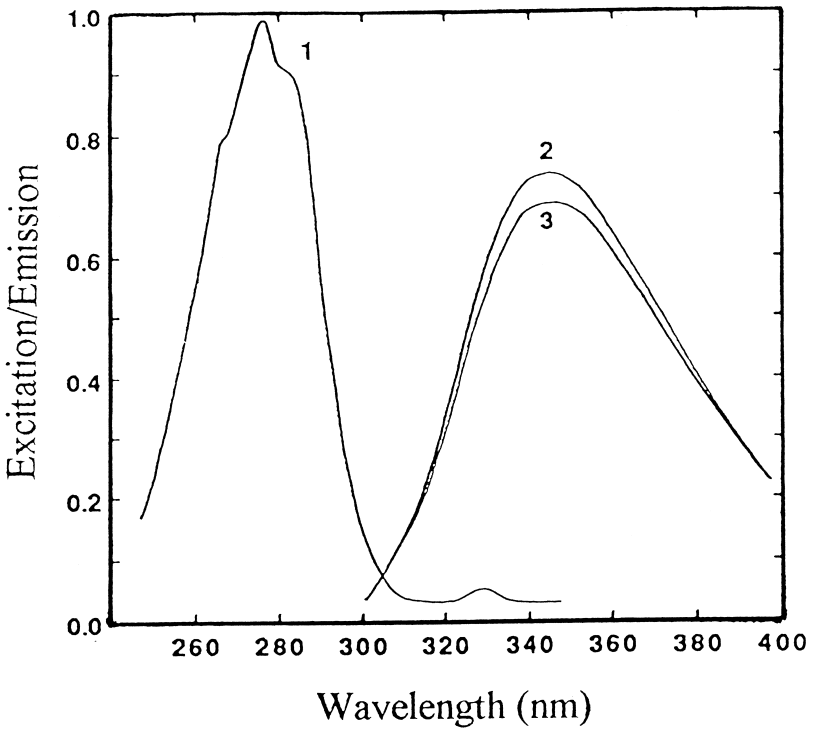


FIG. 6. Excitation and emission spectra of HIV-1 protease. The assay was performed as described in Materials and Methods. Line 1, excitation spectrum; line 2, emission spectrum of enzyme alone; line 3, emission spectrum of HIV-1 protease in the presence of 25 mM Ac-Thr-Leu-Asn-PheB. The results presented are from one experiment representative of a set of five experiments.

Peptide boronic acids have been reported to be effective inhibitors of mammalian and bacterial proteases with K_i values of 10^{-10} to 10^{-11} M [15–17]. The boron modification leads to an increase in the affinity of such compounds as a result of the formation of a strong transition state complex with the protease [15–17].

The boronated tetrapeptides Thr-Leu-Asn-PheB and Ser-Leu-Asn-PheB were conceived based on the known sequence of the scissile sites of the HIV-1 gag–pol polyprotein, and represent the phenylalanine-terminal part of the Phe–Pro scissile bond. We found that unprotected and acetylated forms of these tetrapeptides did inhibit HIV-1 protease. Since these boron tetrapeptides are analogs of the enzymatic reaction product, and are also analogs of the reported associative inhibitors of the HIV-1 protease [14, 26, 27], we investigated whether our boron tetrapeptides had dual inhibitory activity.

In our experiments the inhibition pattern of the HIV-1 protease by the boron tetrapeptides resembled competitive inhibition at high enzyme concentrations (Fig. 3) and had unusual inhibitory kinetics (Fig. 4) at low enzyme concentrations. The inhibition pattern of the HIV-1 protease was analyzed using associative inhibition kinetics. At very high concentrations of the enzyme (when the enzyme exists mainly as the dimer even in the presence of associative inhibitors), the reaction velocity will approach linear dependence on enzyme concentration and should resemble monodimeric enzyme kinetic behavior. We found that higher concentrations of enzyme resulted in a linear dependence of 1/V versus I (Fig. 3). At lower enzyme concentrations the reaction velocity was linearly related to the square power of the enzyme concentration (Fig. 5). Based on the equation, when the enzyme concentration is low (below the enzyme association constant) the increase of enzyme concentration will result in the square of the active enzyme form.

$$E_{subunit} + E_{subunit} = E_{active}$$

$$E_{active} = (E_{subunit} \times E_{subunit})/K_{ass} \text{ or}$$

$$E_{active} = E_{subunit}{}^2/K_{ass})$$

When an associative type of inhibitor is present in a reaction mixture with a low concentration of enzyme, the effective enzyme concentration will be affected. To determine the associative inhibition constant, we used the concentration of the enzyme producing 50% of the expected activity (thus 50% of the enzyme is in an active form) and further evaluated the inhibitors to find the concentration producing an additional 50% decrease of the enzyme activity. Because the boronated compounds were also competitive inhibitors of HIV-1 protease, we were able to calculate inhibition constants only for those compounds that exerted associative inhibition at concentrations much lower than that required to produce competitive inhibition, as described earlier [26, 28].

The association constant for HIV-1 protease was determined to be about 3.5×10^{-8} M, and the associative inhibition constant was equal to about 3.5×10^{-7} M and higher. At high enzyme concentrations, the inhibition patterns resembled competitive inhibition, and hence were analyzed by using Dixon plots. The inhibition constants were calculated to be 5×10^{-6} M and higher for the

substrates Ac-Ser-Gln-Asn-Try-Pro-Val-Val (substrate 1) and Ser-Gln-Asn-Try-Pro-Ile-Val (substrate 2), respectively.

HIV-1 protease inhibitors in clinical use are known to produce mutant forms of HIV-1 protease resistant to inhibition. Since associative inhibitors have a different site of inhibition, they may not be affected by these mutations. Indeed, boronated compounds were found to be inhibitory to the mutant form of HIV-1 protease, although they were less potent (Table 1). Boronated peptides are dual-mode inhibitors, and the relative role of each type of inhibition in their activity against mutant protease requires further study.

The non-boronated tetrapeptide Ac-Thr-Leu-Asn-Phe exerted no inhibition even at a concentration of 10^{-4} M. The specificity of the inhibitors of HIV-1 protease was indicated by the failure of the boronated tetrapeptides to inhibit other non-HIV-1 proteases such as chymotrypsin, trypsin, and peptidase at concentrations up to 10^{-5} M.

The binding of substrate analogs with an enzyme usually leads to the formation of a transition state complex; this may also include associative inhibitors. We found that binding of a boronated tetrapeptide with the HIV-1 protease decreased the fluorescence emission spectrum of the enzyme. This suggests that the binding of the boronated peptide with the HIV-1 protease produced a conformational change, with the probability that the binding had led to the formation of a transition state complex. It is also possible that the structural change observed in the HIV-1 protease is due to binding of the boronated peptide with the site of the protein responsible for formation of the dimer and/or dissociation of the enzyme.

Boronated compounds were not active against HIV-1-infected cells. This may be a result of proteolytic degradation, low permeability, compartmentalization, or short life span inside the cells. We tested the stability of the peptides in the medium and found that after 24 hr of incubation 35–45% of the boronated peptides was degraded. Therefore, instability in the medium is not the reason for the lack of anti-HIV-1 activity of the compounds.

Thus, the boronated peptides constitute a potential new class of inhibitors of HIV-1 protease, and with appropriate modification more potent boronated peptides may evolve.

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