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α-Aminoboronic Acid Derivatives: Effective Inhibitors of Aminopeptidases†

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ABSTRACT: α-Aminoboronic acids and their derivatives have been synthesized as stable white solids. These compounds are effective inhibitors of human enkephalin degrading aminopeptidase, microsomal leucine aminopeptidase (EC 3.4.11.2), and cytosolic leucine aminopeptidase (EC 3.4.11.1) at micro- to nanomolar concentrations. The inhibition of cytosolic leucine aminopeptidase has been studied in some detail. Kinetic data correspond to the mechanism for biphasic slow-binding inhibition: $E + I \rightleftarrows E{\cdot}I \rightleftarrows E{\cdot}I^*$, in which a rapid initial binding is followed by a slow transformation to a stable enzyme inhibitor complex. The initial and final binding constants are dependent on the nature of the side chain at the α-carbon atom but are independent of the protecting group on the boronic acid moiety and follow the trend for the hydrolysis of the corresponding amino acid amides. The first-order rate constant for the transformation of E·I to E·I* is similar for all four compounds studied. These data suggest that the slow-binding step represents the formation of tetrahedral boronate species from trigonal boronic acid.

Aminopeptidases are a group of metalloenzymes that catalyze the hydrolysis of the NH_2-terminal peptide bonds in polypeptides. Aminopeptidases with similar or identical properties have been found in many tissues, including the lens, kidney, pancreas, muscle, and liver [for reviews, see Smith & Hill (1960) and Delang & Smith (1971)]. Although their mechanism of action is unclear, the primary amino acid sequence of leucine aminopeptidase from bovine eye has been reported (Cuypers et al., 1982). Studies on the mechanism of action of aminopeptidases have been reported (Lin & Van Wart, 1982; Makinen et al., 1982; Taylor et al., 1982; Carson & Carpenter, 1983; Baker & Prescott, 1980; Allen et al., 1983; Baker et al., 1983). Effective inhibitors reported for aminopeptidases include amino ketones and derivatives (Birch et al., 1972; Kettner et al., 1974), small peptide analogues from microbial sources (Umezawa et al., 1976; Aoyagi et al., 1978; Rich et al., 1984), aliphatic and aromatic boronic acids (Baker et al., 1983), amino acid hydroxamates (Chan et al., 1982; Baker et al., 1983; Wilkes & Prescott, 1983), α-aminoaldehydes (Andersson et al., 1982), and amino acid thiols (Chan, 1983). Of these, aliphatic boronic acids and α-aminoaldehydes are thought to act as "transition-state analogues".

In the course of synthesis of enzyme inhibitors, we prepared a number of α-aminoboronic acids and their derivatives, **1** (Figure 1), using a synthesis scheme similar to the one reported by Matteson et al. (1981). These compounds, in which the boron atom has trigonal geometry, can form a tetrahedral boronate ion and are expected to act as transition-state analogues for proteases (Koehler & Leinhard, 1971; Philipp & Bender, 1971). Indeed, [2-(*N*-acetylamidophenyl)ethyl]boronic acid (Matteson et al., 1981), as well as peptide boronic acids (Kettner & Shenvi, 1984), have been shown to be effective inhibitors of chymotrypsin (a serine protease). Intrigued by the possibility that α-aminoboronic acids may act as transition-state analogues for aminopeptidases, we synthesized several of these compounds and tested them as inhibitors of human enkephalin degrading aminopeptidase (Coletti-Previero et al., 1982), microsomal leucine amino-

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peptidase (EC 3.4.11.2) and cytosolic leucine aminopeptidase (EC 3.4.11.1).

Materials and Methods

Materials. α-Aminoboronic acid derivatives were synthesized by a method similar to that of Matteson et al. (1981). All intermediates were isolated and characterized by nuclear magnetic resonance spectroscopy (NMR),[1] MS, and elemental analysis. Spectral and physical properties of these compounds and intermediates are given in the supplementary material (see paragraph at end of paper regarding supplementary material). All other chemicals were purchased in highest available purity.

Enzymes. Cytosolic leucine aminopeptidase from porcine kidney, 100 units mg^{-1}, was purchased from Sigma (type III-cp, lot 111F-8015) and was dialyzed for 24 h at 5 °C against 5 mM Tris buffer, pH 8.5, containing 3.0 mM $(NH_4)_2SO_4$ and 5.0 mM $MgCl_2$ at 5 °C. This material (0.30 mL, 0.70 mg of protein) was then activated by incubating at 37 °C for 2 h after adding 0.10 mL of 0.25 mM $MnCl_2$ and 0.10 mL of 0.50 M Tris buffer, pH 8.5, and adjusting the final volume to 2.50 mL with H_2O. This enzyme preparation was stored at 5 °C for not longer than 1 week. Microsomal leucine aminopeptidase from porcine kidney, 6 units mg^{-1}, purchased from Sigma as lyophilized powder (type IV, Lot 33F-0842), diluted with 50 mM PO_4^{3-} buffer,[2] pH 7.2, was used directly. Stock solutions of human enkephalin degrading aminopeptidase, kindly supplied by M.-A. Coletti-Previero, INSERM, France, as lyophilized powder, were prepared in 2.0 mM PO_4^{3-} buffer, pH 7.8. The specific activity of this preparation of enzyme was 0.15 μmol min^{-1} mg^{-1} (Coletti-Previero et al., 1981). For the purposes of preliminary experiments samples of human enkephalin degrading aminopeptidase were prepared from human blood plasma by a method similar to that reported by Coletti-Previero et al. (1981).

Kinetic Determination. Human enkephalin degrading aminopeptidase was assayed by using a method similar to that described by Lee and Snyder (1982) for brain dipeptidyl-aminopeptidase. Thus, 135.0 μL of 2.0 mM PO_4^{3-} buffer, pH 7.8, containing 0.45 milliunit of the enzyme and varying concentrations of the inhibitor were incubated, in duplicate, for 2 h at 37 °C. Following the addition of 15 μL of a 10.0 mM solution of the acetate salt of Leu-enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH, in 2.0 mM PO_4^{3-} buffer, pH 7.8, the incubation was continued at 37 °C. The hydrolysis of Leu-enkephalin was monitored by removing aliquots, 25.0 μL, of the assay mixture and adding to 125.0 μL of 50 mM PO_4^{3-} buffer, pH 2.5. Samples, 25.0 μL, were subjected to chromatographic analysis on a Hewlett-Packard 1084B HPLC equipped with a Zorbax ODS column (0.45 × 25 cm); the column elution was run isocratically at 2.0 mL min^{-1} by using 75% acetonitrile–25% 50 mM PO_4^{3-} buffer, pH 2.5, and was monitored at 215 nm. The progress of the enzymatic reaction was determined from the ratio of the areas under the peak corresponding to (des-Tyr)-enkephalin, H-Gly-Gly-Phe-Leu-OH, at 2.9 min and to that by Leu-enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH, at 3.9 min. No other significant peaks were seen, except for a peak at the solvent front, presumably H-Tyr-OH. The ratio of (des-Tyr)-enkephalin to Leu-enkephalin was plotted vs. time, and a straight line through the points was drawn by using a least-squares fit. The slope of this line was taken as the rate of hydrolysis of Leu-enkephalin. The maximum amount of (des-Tyr)-enkephalin produced in the control experiment without any added inhibitor was 13% of Leu-enkephalin as measured by the area under the peaks obtained in HPLC. The concentration of the inhibitor which reduced the enzyme activity by 50% was taken as the IC_{50} value.

Leucine aminopeptidases were assayed in a Cary 219 spectrophotometer using cells with 1-cm light path, thermostated at 25 ± 0.5 °C, and the final volume of all the assay mixture was 2 mL. For microsomal leucine aminopeptidase the IC_{50} values were determined by adding a solution of the enzyme to a 0.50 mM solution of substrate, L-leucine-*p*-nitroanilide, in 50 mM PO_4^{3-} buffer, pH 7.2, containing five to six different concentrations of the inhibitor. Substrate hydrolysis was monitored by measuring the increase in absorbance at 405 nm after 20 s from the time of addition of enzyme up to 10 min. The rate of the reaction was determined by drawing tangents to the progress curve at the desired point. The concentration of the inhibitor necessary for 50% inhibition was used as the IC_{50} value for the inhibitor. The concentration of the binding site in the enzyme solution for the inhibitor was estimated by a method described by Williams and Morrison (1979). Samples of the enzyme stock solutions were incubated with various concentrations of the inhibitor for 15 min, and an aliquot was assayed for residual activity. The intercept of the plot of the residual activity vs. corresponding inhibitor concentration on the inhibitor axis gave the concentration of of the binding sites for the inhibitor. In all cases the IC_{50} values were determined at an inhibitor concentration of at least 10-fold greater than the binding sites in the assay mixture.

For cytosolic leucine aminopeptidase the assays were carried out in 50 mM Tris buffer, pH 8.5, containing 5.0 mM $MgCl_2$. Because of the limited solubility of L-leucine-*p*-nitroanilide, the maximum concentration of this substrate used for the determination of kinetic parameters was 2.1 mM, which was determined by measuring the absorbance at 320 nm and using $\epsilon_{320} = 1.39 \times 10^4$ M^{-1} cm^{-1} (Lin & Van Wart, 1982). Solutions of the substrate in the buffer were prepared by adding a solution of L-leucine-*p*-nitroanilide in Me_2SO to the buffer and filtering the resulting solution to remove any insoluble material. These solutions contained a final concentration of 2.5% Me_2SO. The concentration of enzyme binding sites for the inhibitor was determined as described for microsomal leucine aminopeptidase. The initial inhibition constant, K_i(initial), was determined by estimating the initial rate of hydrolysis of the substrate, in duplicate, in the presence of inhibitor at various concentrations of the substrate. This rate was determined by drawing a tangent at the starting point of the progress curve generated by adding the inhibitor to a mixture of enzyme and substrate. The assay conditions were chosen with care to minimize the effect of the slow binding step on the initial rate. Average values of duplicate determinations of initial velocities were used for the determination of K_i(initial) (as will be discussed). The final inhibition constant K_i(final) for H-boroAla-(pinacol)[3] was determined by measuring the rate of hydrolysis of the substrate after equilibrating the enzyme, the substrate, and the inhibitor in

[1] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; MS, mass spectrum; Tris, tris(hydroxymethyl)aminomethane.

[2] PO_4^{3-} buffer refers to phosphate buffer prepared from monobasic sodium phosphate and the pH adjusted with sodium hydroxide.

[3] For convenience the α-aminoboronic acids have been abbreviated by the prefix "boro" and the name of the corresponding amino acids. The protecting group, if present, follows the abbreviated name. All α-aminoboronic acids are "DL" mixture unless otherwise specified. The designation "L" for the configuration of α-aminoboronic acids is that of naturally occurring L-amino acids.

the assay buffer for 2 h. In order to determine the rate constant for the onset of inhibition, k_{on}, different concentrations of the inhibitor were added to the assay mixture containing the enzyme and 2.0 mM substrate. The absorbance values at various time intervals were fit to integrated equation 2. In all cases the concentration of the enzyme binding sites was 3 orders of magnitude less than inhibitor concentration, and less than 5% of the substrate was hydrolyzed. The rate of dissociation of the enzyme–inhibitor complex, k_{off}, was estimated by diluting an equilibrated mixture of the enzyme and the inhibitor with the assay mixture. The assay mixture contained 2.2 mM substrate, 50 mM Tris buffer, pH 8.5, and 5.0 mM $MgCl_2$. The absorbance values at different time intervals were then fit to eq 2. In a typical experiment the enzyme binding site concentration was at least 5 times smaller than the concentration of the inhibitor. Less than 10% of the substrate was hydrolyzed, and the assay was conducted for at least 3 times the half-life of the enzyme–inhibitor complex. In all cases a control experiment with no inhibitor was used to monitor the stability of the enzyme and the instrument. Values of K_m and k_{cat} for the hydrolysis of L-alaninamide, L-leucinamide, and L-phenylalaninamide were determined by measuring the rate of decrease of absorbance at 238 nm at various concentrations of these compounds in the presence of the enzyme (Wagner et al., 1972). The change in the extinction coefficients for these compounds upon hydrolysis was determined by measuring the absorbance of the standard solutions of these amides and corresponding amino acids in the assay buffer. The rate of hydrolysis of the substrate reached at 3–5 min after starting the assay was used for the calculation of kinetic constants.

Data Processing. Kinetic data were fitted by using the MLAB computer program (Knott, 1979) to eq 1 and 2. v is the experimentally determined velocity, V is the maximum velocity, S is the concentration of the substrate, K_i is the initial or final steady-state inhibition constant, K_m is the Michaelis constant, and I is the inhibitor concentration. P is the product produced at time t, v_i is the initial velocity, v_f is the final velocity, k_{app} is the first-order rate constant, and P_0 is the product at zero time (Schloss et al., 1980).

$$1/v = K_m(1 + I/K_i)/(SV) + 1/V \quad (1)$$

$$P = P_0 + (v_i - v_f)/k_{app} + v_f t + (v_f - v_i)(1/k_{app})e^{-k_{app}t} \quad (2)$$

The constants k_{app}, k_{on}, k_{off}, K_i(initial), and K_i(final) are related as defined in eq 3 and 4.

$$k_{app} = k_{off} + k_{on}[I/K_i(\text{initial})/(1 + I/K_i(\text{initial}) + S/K_m)] \quad (3)$$

$$K_i(\text{final}) = K_i(\text{initial})k_{off}/(k_{on} + k_{off}) \quad (4)$$

Stability of the Inhibitor. The stability of H-boroPhe-(pinacol) was monitored by TLC on silica gel plates using a 7:3:0.5 (v/v) chloroform–methanol–acetic acid solvent system. A 10.0 mM solution in 100 mM Tris buffer, pH 8.5, as well as in 100 mM PO_4^{3-} buffer, pH 7.2, was stored at ambient temperature (18–25 °C) and analyzed at different time intervals. After being developed, the TLC plates were visualized by dipping in a 0.5% ninhydrin solution in ethanol and heating to 150 °C for 1–3 min. In addition, the inhibitory activity of a 2.0 μM solution of H-boroPhe-(pinacol) in 50 mM Tris buffer, pH 8.5, and in 50 mM PO_4^{3-} buffer, pH 7.2, stored at ambient temperature, toward microsomal leucine aminopeptidase was monitored at various intervals (1–6 days).

RESULTS

α-Aminoboronic acids and their derivatives (Figure 1), synthesized as stable white solids, were found to be strong inhibitors of aminopeptidases. Moreover, time-dependent inhibition was observed for microsomal and cytosolic leucine aminopeptidases. Table I lists IC_{50} values for human enkephalin degrading aminopeptidase and microsomal leucine aminopeptidase. The IC_{50} values for microsomal leucine aminopeptidase are reported at 20 and 400 s after initiating the assay. The observed first-order rate constant, k_{obsd}, and the second-order rate constant for the onset of inhibition, k_{obsd}/I, were calculated from the half-life ($t_{1/2}$). In order to ensure that the concentration of the inhibitor is not substantially altered by binding to the enzyme, the concentration of the binding sites were kept several orders of magnitude smaller than the concentration of the inhibitor.

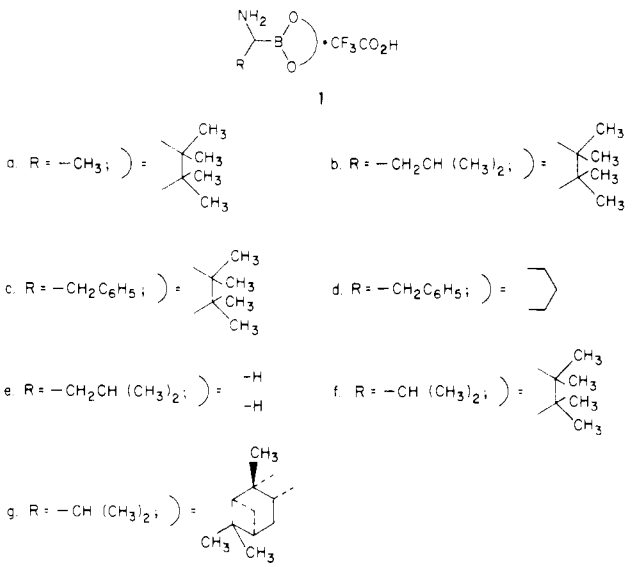

FIGURE 1: α-Aminoboronic acids and derivatives.

Table I: Inhibition of Human Enkephalin Degrading Aminopeptidase (HEDA) and Microsomal Leucine Aminopeptidase (LAPM) by α-Aminoboronic Acids and Their Derivatives, **1**

inhibitor	IC50 HEDA[a] (nM)	IC50 LAPM 20 s[b] (nM)	IC50 LAPM 400 s[b] (nM)	LAPM k_{obsd}/I[c] (M^{-1} s^{-1})
H-boroAla-(pinacol) (**1a**)	70	350	40	4.3×10^4
H-boroLeu-(pinacol) (**1b**)	74	250	25	6.9×10^4
H-boroPhe-(pinacol) (**1c**)	50	200	20	8.6×10^4
H-boroPhe-(propanediol) (**1d**)		200	25	6.9×10^4
H-boroLeu-OH (**1e**)		250	20	8.6×10^4

[a] Concentration of the inhibitor to reduce the rate of hydrolysis of Leu-enkephalin (1.0 mM) by 50% in PO_4^{3-} buffer, pH 7.8. [b] Concentration of the inhibitor needed to reduce the rate of hydrolysis of L-leucine-*p*-nitroanilide (0.5 mM) by 50% in PO_4^{3-} buffer, pH 7.2, 20 or 400 s after initiating the assay. [c] k_{obsd}, the observed first-order rate constant, was calculated by using the equation $t_{1/2} = 0.69/k_{obsd}$.

The binding site concentration for the inhibitor in the enzyme stock solution was determined by using a titration procedure similar to the one reported by Williams and Morrison (1979). As shown in Figure 2, a straight line was obtained for the plot of percent activity of microsomal leucine aminopeptidase vs. concentration of the inhibitor H-boroPhe-(pinacol). An x intercept of 2.55 μM was obtained after extrapolation of this line to zero activity. A similar result was obtained for H-boroVal-(pinacol) (I = 2.71 μM) while for L-H-boroVal-(pinanediol) the x intercept was 1.34 μM. These

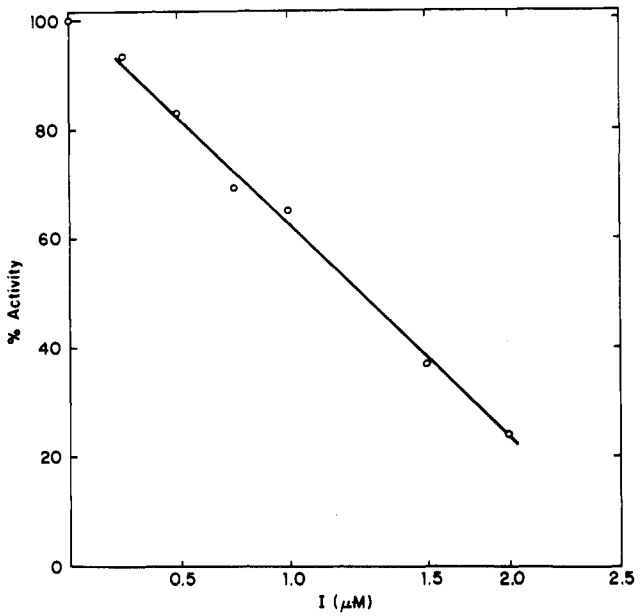

FIGURE 2: Titration of microsomal leucine aminopeptidase with H-boroPhe-(pinacol) (**1c**). The enzyme solution, 100 μL, containing 6 units mL^{-1} enzyme, was treated with the inhibitor solution, 100 μL. After 15 min, 10 μL of this solution was added to the assay mixture, and the rate of increase of absorbance at 405 nm due to the hydrolysis of L-leucine-*p*-nitroanilide was measured. Extrapolation of the least-squares fit to the plot of percent activity vs. inhibitor concentration gave the active site concentration in the enzyme solution.

results suggest that only the L-enantiomer of these compounds binds to the enzyme. Because of the lower affinity of cytosolic leucine aminopeptidase for these compounds, the titration of the enzyme binding site was more cumbersome. In particular, considerable deviation from linearity in the plot of percent activity vs. inhibitor concentration was observed at higher than 50% inhibition. However, the concentration of the binding sites for this enzyme could be estimated by using the linear portion of the plot.

In an attempt to understand the nature of the slow-binding behavior exhibited by the α-aminoboronic acid derivatives, the inhibition of cytosolic leucine aminopeptidase by H-boro-Ala-(pinacol), H-boroLeu-(pinacol), H-boroPhe-(pinacol), and H-boroPhe-(propanediol) was studied in some detail. For H-boroAla-(pinacol), the steady-state reaction rate obtained by adding the inhibitor to the mixture of the enzyme and the substrate was similar to the steady-state reaction rate obtained after diluting a mixture of the inhibitor and the enzyme into a solution of the substrate. Similarly, when a preincubated mixture of cytosolic leucine aminopeptidase and the inhibitor H-boroPhe-(pinacol) was diluted in a large volume of the assay mixture containing the substrate, a recovery of activity was seen. These results indicate that the inhibition of cytosolic leucine aminopeptidase by these compounds is a reversible process (Schloss et al., 1980). Since the initial as well as the final rate is decreased, the initial formation of an enzyme–inhibitor complex, E·I, appears to be followed by a slow process leading to a more tightly bound complex, E·I*. In determining various kinetic constants for the inhibition (Scheme I) certain problems associated with the assay of cytosolic leucine aminopeptidase had to be circumvented. The enzyme was remarkably stable during the assay for up to 33 h with less than 10% variation in the enzymatic hydrolysis of L-leucine-*p*-nitroanilide. However, the reaction rate fluctuated during the first 30 s of the assay. This effect could be reduced considerably by adding 1 mg mL^{-1} BSA to the assay buffer and therefore is attributed to the adsorption of the enzyme to the glass surface of the spectrophotometer cells. The influence of this effect on the estimation of the initial velocity was circumvented by carrying out the inhibition experiment by first adding the enzyme to the substrate solution in the assay buffer, waiting for 30 s, and then adding the inhibitor to this mixture. In addition, because of the relatively fast onset of inhibition, care had to be taken to keep the influence of the second transition, E·I to E·I* (see Scheme I), on the initial rate minimal. The values of K_i(initial) are given in Table II. In all cases the inhibition was competitive as indicated by Lineweaver–Burk double-reciprocal plots.

Table II: Kinetic Constants for Binding of α-Aminoboronic Acid Derivatives to Cytosolic Leucine Aminopeptidase[a]

compound	K_i(initial) (mM)	k_{on} (× 10^{-2} s^{-1})	k_{off} (s^{-1})	K_i(final) (μM)
H-boroAla-(pinacol) (**1a**)	0.40	1.2[b]	1.6×10^{-3}	90[c] (53[d])
H-boroLeu-(pinacol) (**1b**)	0.30	4 ± 1[e]	1.7×10^{-5}	0.13[d]
H-boroPhe-(pinacol) (**1c**)	0.06	5 ± 1[e]	4.2×10^{-5}	0.05[d]
H-boroPhe-(propanediol) (**1d**)	0.03	3 ± 1[e]	2.9×10^{-5}	0.03[d]

[a] Inhibition constants K_i(initial) and K_i(final) and first-order rate constants k_{on} and k_{off} are defined in eq 3 and 4 and were determined in Tris buffer, pH 8.5 at 25 °C. [b] Rate constants were determined by using graphical solution of eq 3. [c] This value was determined by the method of Lineweaver–Burk. [d] These values were calculated by using eq 4. [e] These values are the averages of five determinations at different concentrations of the inhibitor.

Scheme I

$$E + I \underset{}{\overset{K_i(\text{initial})}{\rightleftharpoons}} E{\cdot}I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}I^*$$

The first-order rate constant for the transformation of E·I to E·I* (see Scheme I), k_{on}, was determined from the progress curves obtained by adding the inhibitor to a mixture of the enzyme and the substrate in the assay buffer. For the inhibitors H-boroLeu-(pinacol), H-boroPhe-(pinacol), and H-boroPhe-(propanediol), the inhibitor concentration was comparable to the initial inhibition constant, K_i (initial). In addition, k_{on} is very large compared to k_{off}. Therefore, from eq 3 it can be deduced that under these conditions k_{app} is very similar to k_{on}. However, for H-boroAla-(pinacol), k_{on} and k_{off} are comparable in magnitude, and therefore, the values of these rate constants were obtained by plotting the term in the brackets of eq 3 vs. k_{app}. While the y intercept of this plot gave the value of k_{off}, the slope gave the value of k_{on}.

The first-order rate constant, k_{off}, for the dissociation of E·I* was determined from the progress curves generated by diluting into the assay mixture containing the substrate and equilibrated mixture of the enzyme and the inhibitor. The inhibitor concentration in this mixture was at least 5 times the concentration of the enzyme's inhibitor-binding sites. The progress curves were recorded for at least 3 times the half-life of E·I* and fitted to eq 2. In all cases, control experiments with no inhibitor were run to obtain progress curves with only about 10% change in activity in about 33 h. Because the final inhibitor concentration is very low compared to K_i (initial), the second term in eq 3 is very small relative to k_{off}. Under these conditions k_{app} is approximately equal to k_{off}. The values of k_{off} are listed in Table II.

In order to demonstrate the competitive nature of the final inhibition, the enzyme, the inhibitor, and the substrate should reach a steady state. As described above, at low concentrations of the inhibitor, the first-order rate constant for the inhibitor to reach a steady state is determined by k_{off}. As k_{off} for

Table III: Kinetic Constants for Hydrolysis of Amide Substrates by Cytosolic Leucine Aminopeptidase[a]

compound	K_m (M)	k_{cat}[b] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
L-alaninamide[c]	1.2	1800	1 500
L-leucinamide[d]	0.009	710	78 000
L-phenylalaninamide[e]	0.04	296	74 000

[a] Values of K_m and k_{cat} were determined in Tris buffer, pH 8.5, at 25 °C by using the method by Lineweaver–Burk. [b] Values of k_{cat} are based on the active site concentration determined by a titrimetric method described by Williams and Morrison (1979). [c] The concentrations of the substrate used for the determination of K_m and k_{cat} were between 10 and 100 mM. [d] The concentrations of the substrate used for the determination of K_m and k_{cat} were between 1.5 and 80 mM. [e] The concentrations of the substrate used for the determination of K_m and k_{cat} were between 1.5 and 20 mM.

inhibitors H-boroLeu-(pinacol), H-boroPhe-(pinacol), and H-boroPhe-(propanediol) is relatively small, it would take several days to reach a steady state. However, for H-boro-Ala-(pinacol), this steady state can be reached in a few hours, rendering the determination of the steady-state velocity practical. Therefore, a mixture of the inhibitor H-boro-Ala-(pinacol), the enzyme, and various concentrations of the substrate in the assay buffer was allowed to equilibrate for 2 h, and the rate of hydrolysis of the substrate was determined. Lineweaver–Burk plots indicate that the inhibition is competitive with an inhibition constant, K_i(final), of 90 μM.

A number of studies have been reported dealing with the hydrolysis of L-amino acid amides by aminopeptidases [see, for example, Smith & Spackman (1955), Glenner et al. (1962), Hopsu et al. (1966), Wachsmuth et al. (1966), Vogt (1970), Wagner et al. (1972), Taylor et al. (1981), Carson & Carpenter (1983), Allen et al. (1983), Baker & Prescott (1980), and Baker et al. (1983)]. In order to be able to correlate the binding of α-aminoboronic acids and their derivatives and the substrates to cytosolic leucine aminopeptidase, the determination of the K_m/k_{cat} values of various substrates under the assay conditions used in this study was necessary. The determination of these kinetic constants was achieved by spectrophotometric methods (Wagner et al., (1972), and the values obtained are listed in Table III.

Finally, in order to demonstrate that the recovery of enzyme activity upon dilution of the enzyme–inhibitor complex is not due to the slow decomposition of the inhibitor itself, the stability of H-boroPhe-(pinacol) was tested under the assay conditions. Indeed, less than 10% decomposition was observed in 3 days as monitored by TLC for the formation of phenylethylamine. In addition, a 2.0 μM solution of this compound in the assay buffers showed no significant loss of activity toward the inhibition of microsomal leucine aminopeptidase even after 6 days at ambient temperature.

Discussion

Boronic acids have been demonstrated to be effective inhibitors of proteases presumably because they act as "transition-state" analogues (Koehler & Lienhard, 1971; Philipp & Bender, 1971; Matteson et al., 1981; Kettner & Shenvi, 1984). Until recently this inhibition was restricted to serine proteases. However, Baker and Prescott (1980) reported that butylboronic acid inhibits *Aeromonas* aminopeptidase with a K_i value of 9.6 μM but did not report a time dependence for the inhibition.

A newly developed synthetic method (Matteson et al., (1981) has allowed us to prepare a number of α-aminoboronic acids and their derivatives. These compounds are clearly potent inhibitors of aminopeptidases. The inhibition seems to be general since these compounds inhibit human enkephalin degrading aminopeptidase, microsomal leucine aminopeptidase, and cytosolic leucine aminopeptidase. Interestingly, these compounds exhibit slow-binding kinetics with the last two enzymes. While additional studies are needed to elucidate in detail the mechanism of this slow-binding inhibition, at first approximation the inhibition of cytosolic leucine aminopeptidase can be represented as in Scheme I. Both initial and final inhibitions are competitive, indicating reversible binding of the inhibitor at the substrate binding site. Moreover, as can be seen from Table II, the final inhibition constant, K_i(final), for cytosolic leucine aminopeptidase reflects specificity for the substrate. For example, H-boroLeu-(pinacol) and H-boroPhe-(pinacol) are 2–3 orders of magnitude better inhibitors than H-boroAla-(pinacol). This observation is consistent with the fact that L-alaminamide is a relatively poor substrate (k_{cat}/K_m = 1500 M^{-1} s^{-1}) for this enzyme, compared to L-leucinamide and L-phenylalaninamide (k_{cat}/K_m = 78 000 and 74 000 M^{-1} s^{-1}, respectively; see Table III). However, the inhibition appears to be independent of the substituents on the oxygen atoms of the boronic acid. For example, the inhibition constants for H-boroPhe-(pinacol) and H-boroPhe-(propanediol) are similar (Table II), as are the IC_{50} values for H-boroLeu-(pinacol) and H-boroLeu-OH (Table I). This observation indicates that the slow-binding step does not represent the hydrolysis of the borate ester to the free boronic acid. In all cases studied, the first-order rate constants for the formation of E·I* from E·I, designated as k_{on}, are similar, and the difference in the final inhibition constant is mostly determined by the first-order rate constant for the dissociation of the final enzyme–inhibitor complex, E·I*. While the exact implication of this observation awaits further studies, it is tempting to speculate that the slow transition of E·I to E·I* involves transformation of the trigonal boronic acid species into the tetrahedral boronate ion at the active site of the enzyme. Further support for the formation of the tetrahedral boronate species at the active site of aminopeptidases can be derived from the pH profile of boronic acid inhibition of *Aeromanas* aminopeptidase (Baker & Prescott, 1980). In addition, two-phase binding of (phenylethyl)boronic acid to a serine protease, subtilisin BPN′, has been previously demonstrated (Nakatani et al., 1975). It should be noted that for subtilisin BPN′, the rate constants k_{on} and k_{off} are several orders of magnitude larger than those observed in the present study. Peptide boronic acids also show slow binding behavior, but the second step in these cases appears to involve a conformational change (Kettner & Shenvi, 1984). Recently, peptide inhibitors from a microbial source, bestatin and amastatin, have been shown to exhibit time-dependent inhibition with microsomal leucine aminopeptidase and cytosolic leucine aminopeptidase (Rich et al., 1984).

Even though an understanding of the mechanism of action of metalloproteases exemplified by carboxypeptidase and thermolysin has increased rapidly, much less is known about the mechanism of action of aminopeptidases. Evidence has been accumulated for the involvement of a tyrosyl residue in *Aeromonas* aminopeptidase (Mäkinen et al., 1982) and for the proximity of the essential metal ion to the active site (Baker et al., 1983). It is hoped that α-aminoboronic acids, because of their unique slow-binding inhibitory behavior, will play a role in the elucidation of the mechanism of action of this group of enzymes.

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Supplementary Material Available

Spectral and physical properties and synthetic scheme of α-aminoboronic acids and intermediates (3 pages). Ordering information is given on any current masthead page.

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