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INHIBITION OF DIPEPTIDYL PEPTIDASE IV (CD26) BY PEPTIDE BORONIC ACID DIPEPTIDES

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Peptide boronic acid dipeptide compounds were analyzed for their ability to inhibit recombinant human dipeptidylpeptidase IV (CD26, DPPIV). Rate constants for the peptide boronates are difficult to obtain because the active boronic acid dipeptide exists in equilibrium with a cyclic inactive species in aqueous solution. Rate constants were determined for the inhibition of DPPIV using several peptide boronates at different pH values. Val-boroPro forms the most tightly bound complex with DPPIV; the first order half life for dissociation of the inactive enzyme/inhibitor complex at 23°C is approximately 27 days.

Keywords: Dipeptidyl Peptidase IV; DPPIV; peptide boronates; Val-boroPro; CD26.

INTRODUCTION

Dipeptidylpeptidase IV (DPPIV) [EC 3.4.14.5] is an ectoenzyme expressed on a variety of cell surfaces. It is a dimeric, type II transmembrane glycoprotein and possesses a serine protease activity with homology to other serine proteases.[1] The enzyme cleaves dipeptides from the N-terminus of a variety of bioactive peptides.

DPPIV (CD26) is found in human thymocytes and T lymphocytes[2] and has been shown to be involved in T cell activation.[3] Though only present at low levels on resting T cells, DPPIV is expressed strongly on activated T cells.[4] The enzyme activity of DPPIV has been shown to be required for T cell activation in a transfected human Jurkat system,[5] although other data suggests it may not be necessary.[6] Boronic acid analogues have been shown to result in immunosuppressive activity, probably through the inhibition of the enzymatic activity of DPPIV.[7,8]

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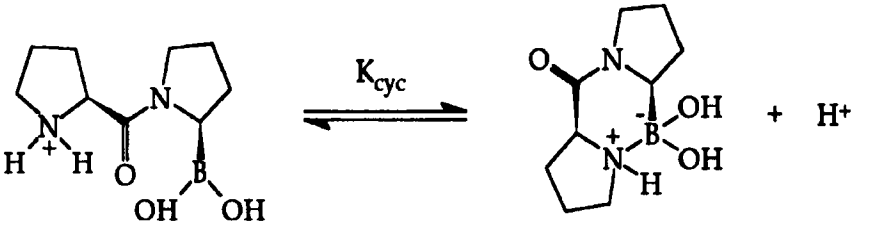
Some of these compounds have been demonstrated to be slow, tight binding inhibitors. The observed association and dissociation rate constants for the binding of Pro-boroPro to porcine DPPIV have been reported: $k_{on} = 5.0 \times 10^{+6}$ M^{-1} s^{-1} and $k_{off} = 7.8 \times 10^{-5}$ s^{-1}, respectively. This yields an equilibrium constant (K_i) of 1.6×10^{-11} M.[9] These compounds have association rate constants close to the slow binding limit of about 10^{+6} M^{-1} s^{-1};[10] the diffusion controlled rate for small molecule association to a protein is usually given as 10^{+7} to 10^{+8} M^{-1} s^{-1}.[11]

The use of tight binding inhibitors may enhance immunosuppression since published data indicates that DPPIV is relatively stable on the cell surface. Long-term pulse chase experiments estimate a half-life of 8 to 10 days on the cell surface of Caco-2 cells.[12] The average half-life for recombinant DPPIV expressed on the surface of CHO cells is about 40 hours.[13]

The peptide boronic acid compounds have also been shown to lose their inhibitory activity in aqueous buffer,[7] calling into question the previously reported rate constants. The molecular mechanism for the inactivation process has recently been shown to involve the conversion of the active species to an inactive, cyclic compound analogous to a diketopiperazine[14] (see Scheme 1). Cyclization of peptide boronate compounds has been shown to result in the loss of inhibitory activity.[14] Furthermore, the open form (I in Scheme 1) binds to the enzyme but the closed form (I^*) does not.[15] This cyclization reaction is accompanied by the loss of a hydrogen ion; therefore, the equilibrium concentrations of the active and inactive species can be manipulated by varying buffer pH. The process is relatively slow; the half life for cyclization of Pro-boroPro (Scheme 1) is approximately 160 minutes. Cyclization is also freely reversible; closed form peptide boronates undergo a slow time based ring opening upon transfer into low pH buffer.[14]



active Pro-boroPro **inactive Pro-boroPro**

I **I***

SCHEME 1

The most widely cited thermodynamic parameter used in the analysis of enzyme inhibitors is the inhibition constant K_i. Much less frequently, the association and dissociation rate constants (k_{on} and k_{off}) respectively are reported. The dissociation rate constant can be the most critical value for the selection of a drug candidate, since lower k_{off} values correspond to longer half lives for the inactive enzyme/inhibitor (EI) complex. Since K_i is equal to k_{off}/k_{on} many values of k_{off} and k_{on} can give rise to the same K_i. Hence, inhibitor selection based on K_i values alone is inadequate because it would overlook those inhibitors exhibiting the desirable property of being both slow binding and slow dissociating.[16]

k_{off} and k_{on} can be measured by following either the onset of inhibition or the dissociation of preformed EI complex. The computational analysis consists of fitting these nonlinear data to rate equations describing the conversion of substrate to product. In a previous report, the paired progress curve method of analysis was presented, in which a single step nonlinear regression analysis was applied to both association and dissociation data simultaneously for the determination of kinetic rate constants for inhibitors of HIV-1 protease.[17] There are several advantages to this method: (1) parameter bias is minimized, since both association and dissociation data sets are analyzed simultaneously. Analyses of single progress curves are often unsuccessful because the association data set is primarily dependent on k_{on} while the dissociation data set is primarily dependent on k_{off}. Hence, the less critical constant is subject to greater error. (2) Propagation of error is not a concern since parameters are not fixed to values determined in an earlier step. (3) The experimental method is rapid, because progress curves can be generated from a single concentration of inhibitor, substrate and enzyme. This method, adapted to account for the inactivation of peptide boronate inhibitors, can be used to determine: k_{on}, k_{off} and the catalytic rate constant (k_{cat}) from a pair of progress curves.

EXPERIMENTAL PROCEDURES

^{1}H NMR Quantitation of Active and Inactive Peptide Boronates

Peptide boronic acid dipeptides were prepared as reported previously.[14] ^{1}H NMR spectra were obtained with a Bruker Instruments, Inc. AM-500 spectrometer at ambient temperature (approximately 23°C). Samples were prepared by dissolving 5.0 mg of Pro-boroPro, Val-boroPro or Ala-boroPro in 0.6 mL of 0.5 M phosphate buffer prepared with D_2O. Measured pD values ranged from approximately 3.0 to 6.7 (pD = pH meter reading + 0.4). Deuterated-TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d4) was added to each sample as a chemical shift reference. The pD and the NMR spectra of the samples were monitored until

equilibrium was reached (usually within 24 h) between the active and inactive species. The equilibrium ratio of the active and inactive forms was measured by integration.

Enzyme Expression and Preparation

Recombinant DPPIV was produced in SF9 cells using a baculovirus expression system. A soluble DPPIV construct was used in which the transmembrane domain has been truncated (obtained from Bachovchin, Tufts University, Boston, MA). Previous work (data not shown) has demonstrated that this truncated DPPIV construct is kinetically indistinguishable from the full length DPPIV purified from YT cells discussed previously.[14] Media containing the recombinant DPPIV was concentrated ten-fold by ultrafiltration and the following protease inhibitors were added: 1.0 μM pepstatin A (Sigma), 10 μM leupeptin (Boehringer Mannheim), 0.2 mM phenylmethanesulphonyl fluoride (Boehringer Mannheim) and 1.0 mM disodium EDTA (Sigma). The concentrate was diluted with five volumes of 50 mM Hepes, 50 mM NaCl, 0.2% PEG 1000 (Sigma), 2.5% (v/v) glycerol, final pH 7.8 (4°C) and loaded onto a 3.2 × 17 cm column of Q Sepharose Fast Flow (Pharmacia). Enzyme present in the flow-through fraction was concentrated and re-equilibrated in a stirred cell with a YM30 membrane (Amicon) in 50 mM Na acetate pH 4.9 (4°C), 25 mM NaCl, 0.2% PEG 1000, 2.5% (v/v) glycerol. The concentrate was then loaded onto a 2.2 × 25 cm column of S Sepharose (Pharmacia) and eluted with a linear gradient of 50–300 mM NaCl. Enzyme eluting at about 90–130 mM NaCl was pooled, concentrated and equilibrated in 20 mM Tris-HCl pH 8.5 (4°C), 0.2% PEG 1000, 2.5% (v/v) glycerol. The pool containing recombinant DPPIV was then loaded on a 1 × 11 cm column of Arginine-Sepharose (Pharmacia) and eluted with a linear gradient of 0–200 mM NaCl. DPPIV eluting at 65–80 mM NaCl was pooled, concentrated as above, and stored after the addition of an equal volume of glycerol at –20°C. Purity was assessed at >90% by SDS-PAGE. Enzyme activity during fractionation was located by monitoring cleavage of the substrate L-alanyl-L-prolinyl-2-(4-methoxy)napthylamide (Bachem) using a colorimetric assay.[18] Typical recoveries of 600–700 μg of purified enzyme were obtained per liter of initial SF9 cell culture. The final enzyme concentration in the activity assays was determined by the BCA assay (Pierce) to be 1.93×10^{-11} M.

Enzyme Activity Assay

Enzyme activity was determined by monitoring the fluorescence change associated with the cleavage of the fluorogenic substrate L-alanyl-L-prolinyl-2-(4-

methoxy)napthylamide. Reactions were conducted at 23°C in a final volume of 2.0 mL containing: 0.025% sodium azide, 0.5% DMF, 0.5% DMSO and buffer. Two different buffer systems were used: 100 mM Tris-HCl at a pH of 7.97 or 100 mM Bis Tris-propane at a pH of 7.01. Except where noted, DPPIV was added to a final concentration of 1.93×10^{-11} M and substrate was added to a final concentration of 2.5×10^{-4} M. The K_m was determined to be: 1.00×10^{-5} M at a pH of 7.97 and 1.17×10^{-5} M at a pH of 7.01 (data not shown).

Fluorescence Detection of Product

The time course of the reaction was monitored using an SLM Aminco Bowman series 2 Model SQ-340 fluorescence detector equipped with a high speed 2-position magnetic stirrer. The excitation maximum of the product, 2-(4-methoxy)napthylamine, was found at 342 nm with an emission maximum at 421 nm. An excitation bandpass of 1 nm and an emission bandpass of 16 nm was used. The high voltage setting of the photomultiplier tube was set to 800 V. The data were collected as the ratio of emission to an internal diode standard (E_m/Rf) for varying periods of time. These data were subsequently converted to concentration of product.[19]

Determination of K_i by Equilibrium Methods

The dissociation of EI was conducted at several different total inhibitor concentrations ($[I]_T$). Inhibitor was preincubated in buffer for at least 16 h at 23°C in order to equilibrate active and inactive forms (see Scheme 1). DPPIV was added at a concentration of 1.93×10^{-11} M for a further 24 h. For Val-boroPro this period was extended to 216 h (data from Table II) so that equilibrium could be reached. Data were collected immediately after the addition of substrate, and subsequent points were acquired at 1 s intervals for 200 s. No significant EI dissociation was observed during this acquisition period for any of the three inhibitors. A linear regression was applied to the data to obtain the velocity at $t = 0$ (v_z). A control reaction, using DMSO vehicle, was performed to determine the uninhibited initial velocity (v_o). Pairs of v_z/v_o and associated $[I]_T$ values were fitted to Equation (4) using the value of ϕ calculated from Equation (7). The value for K_{cyc} was obtained from Table I, while $[E]_T$ and K_m were fixed to values shown above.

Determination of Rate Constants

The inhibitor was preincubated in buffer for 24 h at 23°C to allow active and inactive forms of the inhibitor to come to equilibrium. The initiation of inhibition

was obtained by treatment with substrate for 5 min followed by the addition of enzyme to start the reaction.

The dissociation of EI was obtained by adding enzyme to pre-equilibrated inhibitor solutions for a further 24 h period (216 h for Val-boroPro) followed by the addition of substrate to start the reaction. Data acquisition was initiated within 5 s and proceeded for various times as shown in the relevant figures.

Statistical Nonlinear Regression Analysis

The fluorescence intensity from an association and a dissociation data set (fluorescence *vs* time) was background corrected and converted to product concentration. The concentration of product at $t = 0$ was set equal to 0 and the data sets were combined to allow simultaneous analysis of both progress curves by nonlinear regression techniques. The values for the parameters k_{cat}, k_{on} and k_{off} were determined from the regression analysis, while $[E]_T$ and K_m were fixed to values shown above. Convergence of the regression to the model described by Equation (3) (ϕ defined appropriately by Equations (6), and (7)) occurs quickly with good initial parameter estimates.

Data analysis was performed by applying ordinary nonlinear least-squares regression techniques to the selected model using the Marquardt-Levenberg minimization method. All of the data were analyzed using the SAS® statistical software system (version 6.07, SAS Institute Inc., Cary, NC).

THEORY

pH Titration of Peptide Boronates

Peptide boronates, such as Pro-boroPro, have been shown to cyclize to an inactive form in aqueous solution.[14] This reversible cyclization requires the loss of a hydrogen ion and is therefore pH dependent (Scheme 1). It is possible to describe this process using acid-base equilibria.

NMR spectroscopy was used to determine the relative amounts of active and inactive species in buffered solutions at various pD values (Figure 4). Data were analyzed using the Henderson-Hasselbach expression for acid-base equilibria.

$$\text{pD} = pK_D + \log\left(\frac{100 - \%\text{ active form}}{\%\text{ active form}}\right) \quad (1)$$

A correction factor of 0.4 was required to convert the pH meter readings to pD values[20] to take into account the fact that the NMR quantitation was done in D_2O

and the enzyme assay was done in H_2O. Since the extent of ionization of weak acids decreases in D_2O, the resultant pK_D values were converted into pK_H values using the following expression.[21]

$$pK_H = (0.9804 \cdot pK_D) - 0.4020 \tag{2}$$

The pKa values determined by NMR were used to predict the equilibrium concentrations of active and inactive compound present in the enzyme assay.

Competitive Inhibition

The kinetic model assumed in the analysis of the peptide boronate inhibition of DPPIV is shown in Scheme 2. The active inhibitor is shown as I (see designation in Scheme 1) and the inactive form of the inhibitor is designated as I^*. The value for K_{cyc} is obtained from the corrected pK_H and the hydrogen ion equivalent to Equation (1).

$$E + S \overset{K_m}{\rightleftharpoons} ES \overset{k_{cat}}{\rightarrow} E + P$$

$$+$$

$$I^* \overset{K_{cyc}}{\rightleftharpoons} I \underset{k_{off}}{\overset{k_{on}}{\leftrightharpoons}} EI$$

SCHEME 2

Experimentally, association reactions are initiated by the addition of enzyme into a mixture of substrate and inhibitor; conversely, dissociation reactions are initiated by the addition of substrate to preincubated solutions containing preformed EI complexes. These two reactions, taken together and at a single $[I]_T$, constitute one pair of progress curves. Both curves are analyzed simultaneously in the determination of kinetic rate constants as described previously.[17] The derivation of the equation for the rate of change of the Michaelis complex to give product (Equation (3)) has been described elsewhere.[22,23]

$$[P]_t = [P]_0 + v_s t - \frac{(v_s - v_z)(1 - \gamma')}{\lambda \gamma'} \ln\left(\frac{1 - \gamma' e^{-\lambda t}}{1 - \gamma'}\right) \tag{3}$$

where $[P]_t$ = product concentration at time t, and $[P]_0$ = product concentration at $t = 0$, this has been set equal to 0 (see Methods), v_z is the velocity at $t = 0$ given by,

$$v_z = \phi \cdot v_o \tag{4}$$

with v_o as the velocity in the absence of inhibitor given by,

$$v_o = \frac{k_{\text{cat}} \cdot [E]_T}{\left(1 + \frac{K_m}{[S]}\right)} \tag{5}$$

k_{cat} = catalytic rate constant, $[E]_T$ = total enzyme concentration, $[S]$ = substrate concentration, and K_m = Michaelis-Menten constant.

The symbol ϕ, shown in Equation (4), is the fraction of active enzyme (free of inhibitor) at time zero. The value for ϕ will depend upon the presence or absence of an enzyme/inhibitor preincubation step. This preincubation occurs without substrate and proceeds to equilibrium. The paired progress curve method fits data simultaneously to Equation (3) with ϕ defined appropriately for both sets of data. When the reaction is started by the addition of enzyme into a mixture of substrate and inhibitor then all of the enzyme is available for binding to substrate at $t = 0$ and,

$$\phi = 1 \tag{6}$$

When enzyme is preincubated with inhibitor then ϕ will be given by,

$$\phi = \frac{1}{2[E]_T}\Big[[E]_T - K_{i(\text{app})} - [I]_T + \sqrt{\left(-[E]_T + K_{i(\text{app})} + [I]_T\right)^2 + 4[E]_T K_{i(\text{app})}}\Big] \tag{7}$$

$[I]_T$ = total inhibitor concentration and the apparent K_i is given by,

$$K_{i(\text{app})} = K_i[1 + K_{\text{cyc}}]. \tag{8}$$

K_{cyc} is the equilibrium constant for the active and inactive forms of the inhibitor (I and I^* respectively) defined as,

$$K_{\text{cyc}} = \frac{[I^*]}{[I]}. \tag{9}$$

v_s is the steady-state velocity given by,

$$v_s = \frac{k_{\text{cat}}}{2\left(1+\frac{K_m}{[S]}\right)}\left[[E]_T - K_{i(\text{app})}\left(1+\frac{[S]}{K_m}\right) - [I]_T + \sqrt{\left(-[E]_T + K_{i(\text{app})}\left(1+\frac{[S]}{K_m}\right) + [I]_T\right)^2 + 4[E]_T K_{i(\text{app})}\left(1+\frac{[S]}{K_m}\right)}\right]. \tag{10}$$

The value for λ is,

$$\lambda = \frac{q}{\left(1+\frac{[S]}{K_m}\right)} \tag{11}$$

with q defined as,

$$q = \sqrt{\left[\frac{k_{\text{on}}[I]_T}{1+K_{\text{cyc}}} - \frac{k_{\text{on}}[E]_T}{1+K_{\text{cyc}}} + k_{\text{off}}\left(1+\frac{[S]}{K_m}\right)\right]^2 + 4\left[k_{\text{off}}\left(\frac{[S]}{K_m}\right)[E]_T\right]\cdot\left[k_{\text{on}}\frac{1+\frac{K_m}{[S]}}{1+K_{\text{cyc}}}\right]} \tag{12}$$

where k_{off} = dissociation rate constant and k_{on} = association rate constant, and the value for γ' is given by,

$$\gamma' = \frac{[E]_T\frac{(2\phi-1)}{1+K_{\text{cyc}}} + \frac{[I]_T}{1+K_{\text{cyc}}} + K_i\left(1+\frac{[S]}{K_m}\right) - \frac{q}{k_{\text{on}}}}{[E]_T\frac{(2\phi-1)}{1+K_{\text{cyc}}} + \frac{[I]_T}{1+K_{\text{cyc}}} + K_i\left(1+\frac{[S]}{K_m}\right) + \frac{q}{k_{\text{on}}}} \tag{13}$$

The assumptions of this analysis are: (1) the active and inactive forms of the inhibitors are at equilibrium throughout the course of the kinetic experiment, (2) E and EI are at equilibrium prior to reaction initiation (addition of substrate) for the EI dissociation curves, and (3) the mechanism of inhibition indicated in Scheme 2 is valid. These assumptions are discussed below (see Discussion).

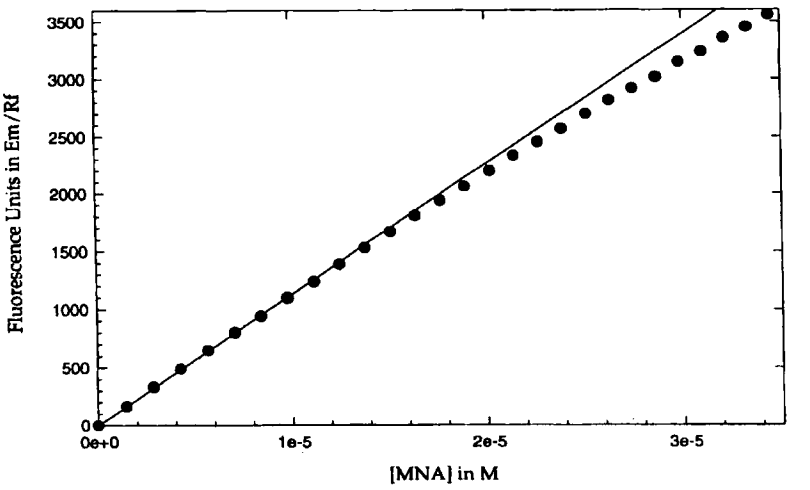


FIGURE 1 Fluorescence response of the DPPIV cleavage product. Emission is shown for the fluorescent product 2-(4-methoxy)napthylamine (MNA). The regression line to the raw data ([MNA] < 15μM) indicates the extent of the linear portion of the data.

RESULTS

The fluorescent amine, 2-(4-methoxy)napthylamine, provides a useful leaving group for monitoring the progress of enzyme catalyzed hydrolysis of substrate amides. It is very stable in aqueous buffer and the fluorescent response range is large. The linear range (Figure 1) extends to about 15 μM. In addition, DPPIV is an extremely stable enzyme giving rise to linear substrate conversion for long periods of time. Very little downward curvature is observed after 17 h (Figure 2) at either pH 7 or pH 8, making this system ideal for the analysis of tight binding inhibitors.

Peptide boronates are known to lose activity in aqueous buffer *vida supra*. Inactivation involves a reversible, time dependent cyclization process (Scheme 1). Reactions initiated by the addition of DPPIV into aqueous solutions of peptide boronates exhibit (t < 1000 seconds) concave downward curves typical of tight binding inhibitors (Figure 3). The degree of inhibition, indicated by reduced product formation at each instant of time, depends strongly upon the duration of the aqueous preincubation period. The peptide boronates, prepared from DMSO stock solutions, undergo a rapid, reversible cyclization following dilution into aqueous buffer and hence the degree of inhibition declines with increasing preincubation time. The $t_{1/2}$ for the cyclization process has previously been determined in D_2O to be: Ala-boroPro

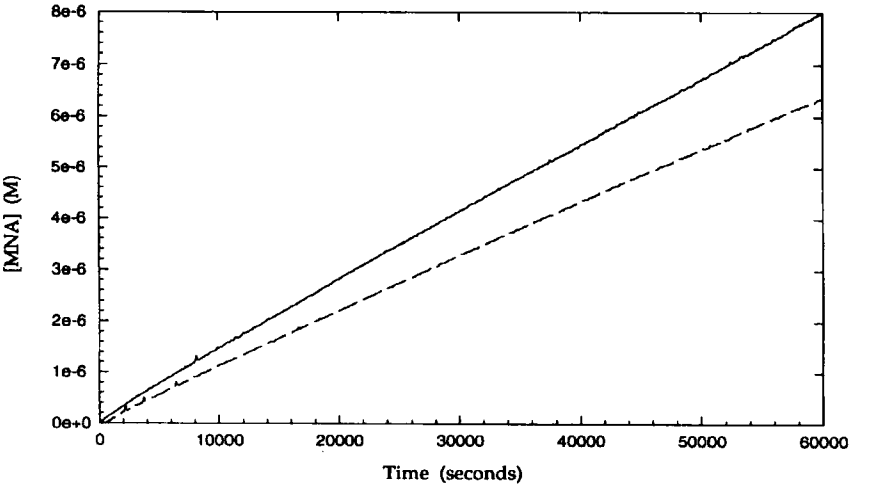


FIGURE 2 Linearity of control reactions. Control reactions were conducted for the longest reaction duration used in this work (see Figure 6, panels e and f). DPPIV is added to a final concentration of 1.93×10^{-11} M into reaction cuvettes containing 250 mM substrate and DMSO vehicle at 23°C. Data are shown at a pH of 7.97 (solid line) and at a pH of 7.01 (dashed line). The total substrate conversion, obtained at 16.7 h, lies within the linear range and does not exceed 5%.

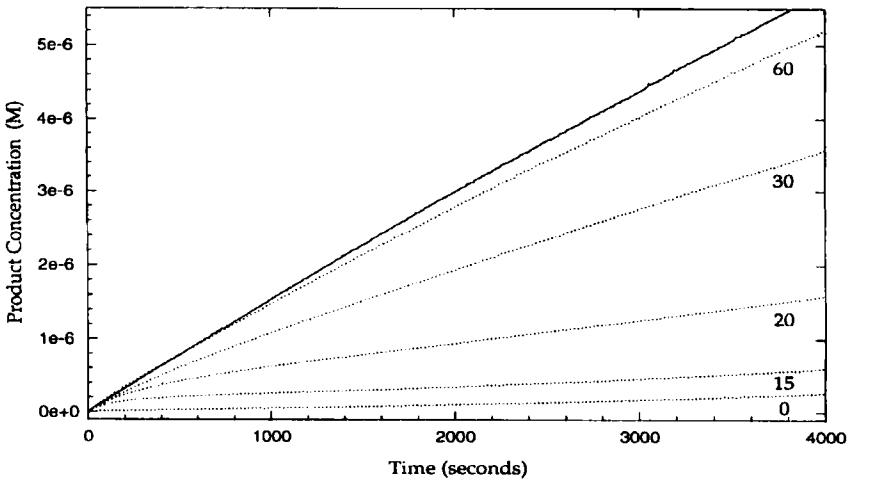


FIGURE 3 Time course for the inactivation of Ala-boroPro. Data are shown for the inhibition of DPPIV by both a control reaction containing DMSO (solid line) and by 2.25×10^{-6} M Ala-boroPro (dotted lines). The inhibitor is preincubated for various times (numbers indicate time in min) in buffered solution at a pH of 7.97 followed by the sequential addition of 250 mM substrate and DPPIV.

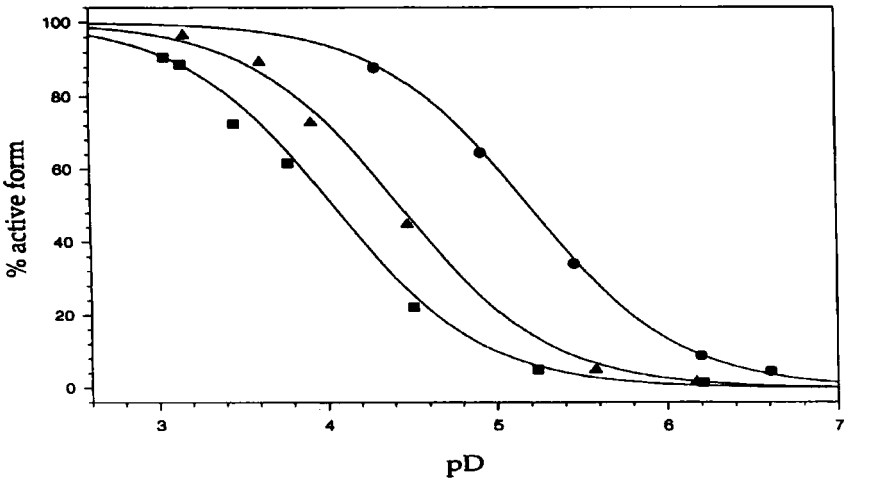


FIGURE 4 pH titration of peptide boronates. Peptide boronates were incubated in 0.5 M phosphate buffer until equilibrium was reached (usually less than 24 h) prior to the quantitation of the active and inactive forms (see Scheme 1) by NMR methods. Data are shown for: • Val-boroPro, ▲ Ala-boroPro and ■ Pro-boroPro. Values for pK_a (see Table I) were determined by nonlinear regression fit (solid lines) of the raw data to Equation (1).

= 44 min, Pro-boroPro = 160 min and Val-boroPro = 190 min.[14] These rates are significantly faster in H_2O, presumably due to a primary isotope effect.[15] The time course of inhibition for the enzyme reaction is similar to the time course of inhibitor inactivation. A reaction initiated by the addition of substrate and inhibitor will contain very high concentrations of active inhibitor at the beginning of the association phase and relatively low concentrations of active inhibitor at the end of the association phase 60 min later. Hence, it is not possible to determine kinetic rate constants from this data due to the rapid decrease in the concentration of active inhibitor throughout the time course of the reaction. Additional information about the inhibitor inactivation process is required before a successful analysis can occur, as the precise concentration of active inhibitor is required for the determination of kinetic parameters.

The equilibrium ratios of the active and inactive peptidyl boronates were determined by quantitative ^1H NMR over a range of deuterium ion concentrations. The percentage of active isomer was plotted against pD (Figure 4) and analyzed using Equation (1). The nonlinear regression analysis yielded a value for the parameter pK_D, which was then converted to pK_H using an appropriate conversion factor (Equation (2)); the values for K_{cyc} were calculated from the pK_H at each

TABLE I pH titration of inhibitors.

Compound	pK_H †	pH	K_{cyc} ‡
Pro-boroPro	3.56 (±0.02)	7.01	2,790
		7.97	25,500
Ala-boroPro	3.95 (±0.05)	7.01	1,150
		7.97	10,500
Val-boroPro	4.69 (±0.07)	7.01	207
		7.97	1,890

† The pK_H (standard error shown) was determined by nonlinear regression analysis to the data in Figure 4. ‡ K_{cyc}, defined in Equation (9), was determined from the pK_H using the protium equivalent to Equation (1).

pH value (Table I). The relative concentrations of the active and the inactive forms can then be calculated for any equilibrium solution of peptide boronate inhibitor using the calculated value for K_{cyc}. During the ^{1}H NMR quantitation a small amount (< 5%) of an unidentified isomer was observed. This isomer, observed for all three peptide boronates, is believed to correspond to a cis, open form in equilibrium with the active and inactive forms. The contribution of this unidentified molecule was ignored when determining the fraction of the active form.

An equilibrium method was used to determine K_i for several peptide boronates at two different pH values. Inhibitors were incubated overnight in aqueous buffer prior to the addition of enzyme for a further 24 h period. Reactions were started by the addition of substrate. The initial velocities (v_z) were determined by linear regression of the data derived from the first 200 s of reactions. For the control reaction lacking inhibitor, the value of v_z is equal to v_o. The data were fitted to Equation (4) (Figure 5, ϕ defined by Equation (7)) to determine the values of K_i presented in Table III.

The 36 h preincubation period, sufficient for the equilibration of both Ala-boroPro and Pro-boroPro with DPPIV, was insufficient for the equilibration of Val-boroPro with DPPIV. In this case an incubation time of 168 h is necessary to reach a constant K_i value (Table II).

Progress curves for both EI association and dissociation were obtained for each inhibitor at two different pH values (Figure 6). The addition of DPPIV into a mixture of pre-equilibrated inhibitor and substrate yields a characteristic association curve (concave downward) and the addition of substrate into pre-equilibrated mixture of DPPIV and inhibitor result in a dissociation curve (concave upward). The 24 h preincubation period used for the enzyme with pre-equilibrated Ala-boroPro or Pro-boroPro (stated above) was not sufficient for equilibration with

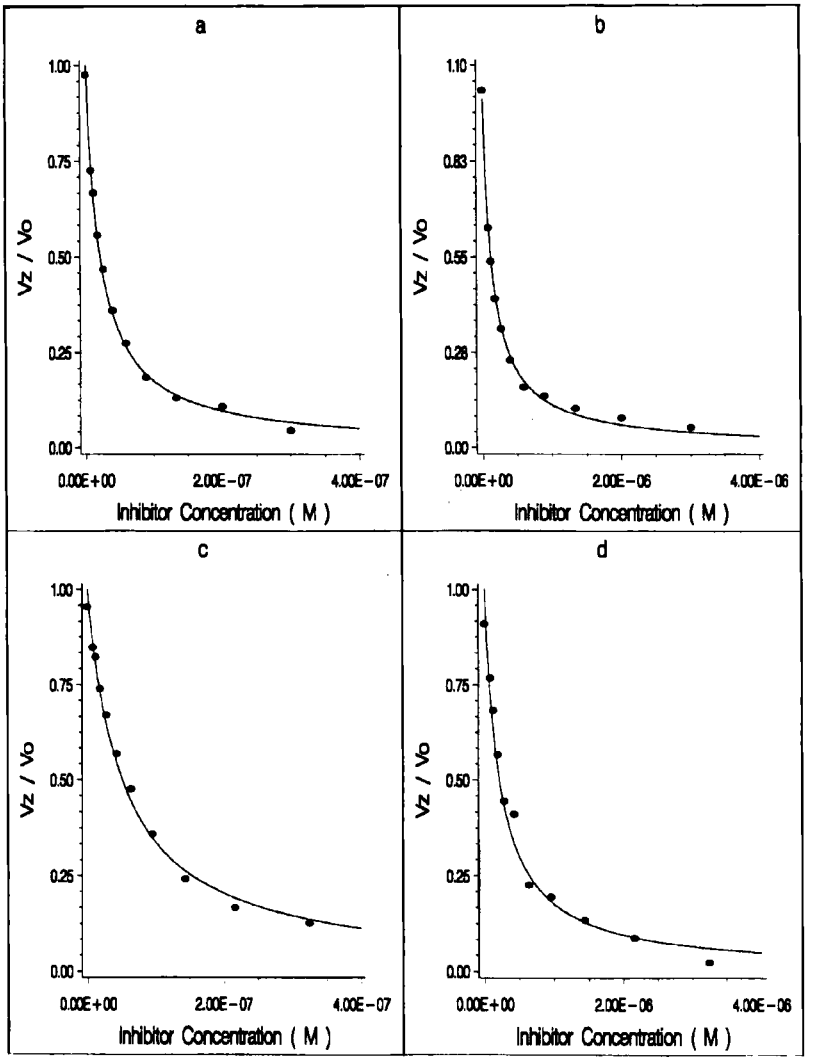


FIGURE 5 Determination of K_i by equilibrium binding. Titration data are shown for the inhibition of DPPIV by Ala-boroPro (panels a and b) and by Pro-boroPro (panels c and d) at each of two pH values: pH = 7.01 (panels a and c) and pH = 7.97 (panels b and d). Inhibitor was preincubated at 23°C overnight in buffer in order to obtain an equilibrium mixture of active and inactive forms prior to the addition of 1.93×10^{-11} M DPPIV. The reaction was initiated by the addition of 250 mM substrate after a further 24 h period. The initial velocity (v_z) was determined by a linear regression through the first 200 s of data. A control reaction was carried out using DMSO vehicle yielding a value for the initial velocity of the uninhibited reaction (v_o). Data were then fitted to Equation (4) with ϕ defined by Equation (7) (solid lines).

TABLE II Variation in the apparent K_i (M) for Val-boroPro.[a]

Time (h)	pH = 7.01	pH = 7.97
5	5.83×10^{-11}	1.57×10^{-11}
24	6.40×10^{-12}	3.47×10^{-12}
74	3.58×10^{-12}	1.53×10^{-12}
168	8.56×10^{-13}	8.26×10^{-13}
216	1.34×10^{-12}	9.54×10^{-13}

[a] Varying concentrations of Val-boroPro were incubated in buffer for 24 h at 23°C. DPPIV was added to a final concentration of 1.93×10^{-11} M for various periods of time as indicated followed by the addition of 250 mM substrate to start the reaction. Data were analyzed in the same way as shown for Figure 5.

TABLE III Inhibitory rate constants.

Compound	pH	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$K_i(k_{off}/k_{on})^a$ (M)	K_i (eq.)^b (M)
Pro-boroPro	7.01	$6.52 \times 10^{+5}$	7.93×10^{-6}	1.22×10^{-11}	1.84×10^{-11}
	7.97	$1.14 \times 10^{+6}$	9.13×10^{-6}	8.01×10^{-12}	8.48×10^{-12}
Ala-boroPro	7.01	$1.16 \times 10^{+6}$	2.68×10^{-5}	2.31×10^{-11}	1.85×10^{-11}
	7.97	$2.66 \times 10^{+6}$	3.05×10^{-5}	1.15×10^{-11}	1.35×10^{-11}
Val-boroPro	7.01	$4.08 \times 10^{+5}$	2.92×10^{-7}	7.16×10^{-13}	8.56×10^{-13}
	7.97	$1.15 \times 10^{+6}$	3.89×10^{-7}	3.38×10^{-13}	8.26×10^{-13}

[a] K_i determined as the ratio of k_{off}/k_{on} obtained from the paired progress curve analysis. [b] K_i determined using the equilibrium method described for Figure 5 (Ala-boroPro and Pro-boroPro) and from the limiting value for K_i obtained in Table II (Val-boroPro).

Val-boroPro. A preincubation period of 216 h (9 days) was selected for this inhibitor since this was previously determined to be sufficient for the establishment of equilibrium (Table II).

It was also necessary to vary the concentrations of Val-boroPro used for the two sets of curves, due to the tight binding of this compound. $[I]_T$ was 961 times greater for the association curve than for the dissociation curve at both pH values. It is necessary therefore, to set $[I]_T$ differently for the association and dissociation curves in addition to assigning the appropriate value of ϕ. Hence, the two pairs of curves for Val-boroPro (Figure 6, panels e and f) do not converge to identical steady-state velocities (v_s). In contrast, the pairs of curves for both Ala-boroPro and Pro-boroPro (Figure 6, panels a–d) will ideally converge since $[I]_T$ is the same within each pair. Rate constants (Table III) were determined as described above. This analysis yields, in all cases, values with low standard error (<1%) and with

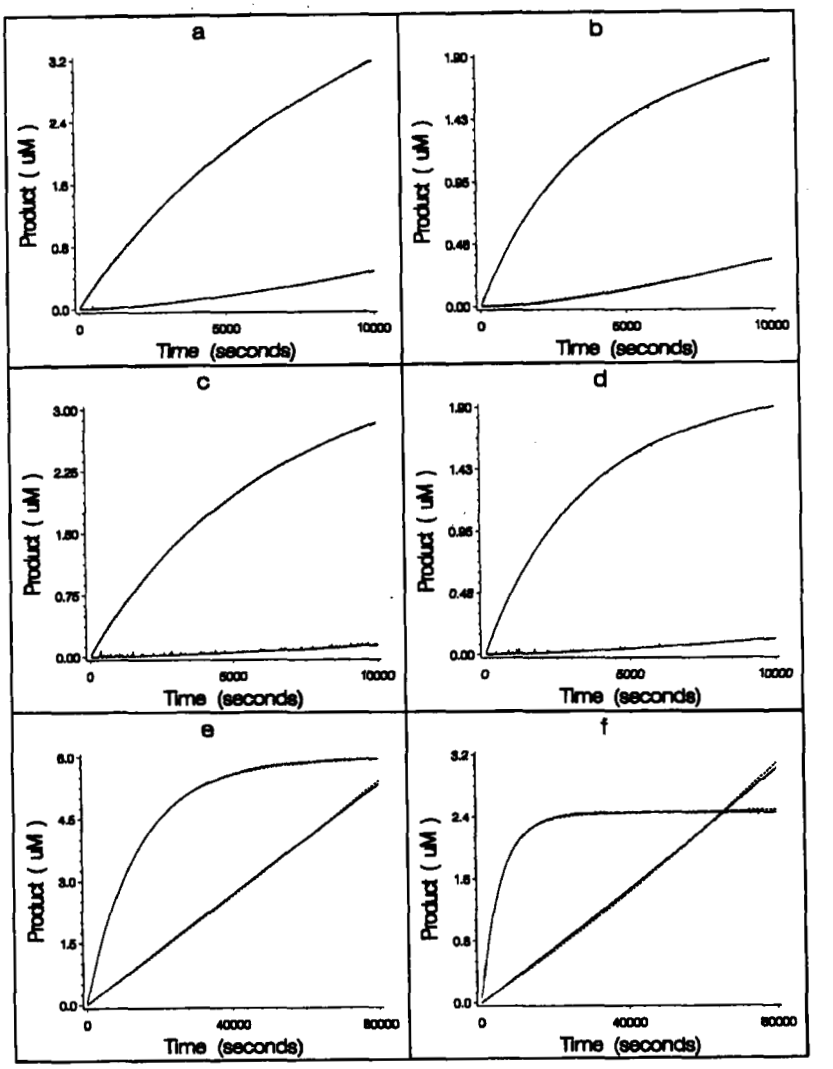


FIGURE 6 Paired progress curves for peptide boronates. Paired progress curves were determined (solid lines) at each of two pH values: pH = 7.01 (panels a, c, and e) and pH = 7.97 (panels b, d, and f). Peptide boronate concentrations were: Ala-boroPro, panel a ($[I]_T = 3.00 \times 10^{-6}$ M) and panel b ($[I]_T = 3.00 \times 10^{-5}$ M); Pro-boroPro, panel c ($[I]_T = 1.62 \times 10^{-5}$ M) and panel d ($[I]_T = 1.62 \times 10^{-4}$ M); and Val-boroPro, panel e ($[I]_T = 3.23 \times 10^{-9}$ M for EI dissociation and $[I]_T = 8.08 \times 10^{-7}$ M for EI association), and panel f ($[I]_T = 3.23 \times 10^{-8}$ M for EI dissociation and $[I]_T = 8.08 \times 10^{-6}$ M for EI association). Nonlinear regression analyses were performed (dashed lines) on both association curves (concave downward) and dissociation curves (concave upward) simultaneously, to generate values for k_{on} and k_{off} shown in Table III. $[E]_T$ was constant at 1.93×10^{-11} M and K_m was fixed to values presented earlier (see Methods).

low value ($\leq$0.0001). Val-boroPro forms the tightest EI complex observed with a dissociation half-life ($k_{off} = 3 \times 10^{-7}$ s^{-1}) of approximately 27 days.

DISCUSSION

The results presented in the previous section are dependent upon the validity of several assumptions. The first assumption is that I and I^* are at equilibrium throughout the time course of the kinetic experiment. For the association experiments (see Figure 6), the concentration of active I is about 250 times greater than $[E]_T$, and for the dissociation experiments, the amount of free enzyme available for binding active inhibitor is very small. In both cases, any perturbation to the $I \Leftrightarrow I^*$ equilibrium can be safely ignored. The second assumption is that E and EI are at equilibrium prior to the initiation of the reaction from which the EI dissociation curves were derived. The data presented in Table II supports this assumption because K_i is not observed to change significantly after 168 h. A third assumption concerns the mechanism of inhibition. Earlier kinetic studies, such as the determination of K_i [7] and the determination of rate constants,[9] indicate a competitive mechanism of inhibition. This is further supported by the observation in the crystal structure of a related protein, human alpha-thrombin, that the active site is blocked by a peptide boronate inhibitor.[24] A fourth assumption is that inhibition of DPPIV takes place via a single-step binding mechanism (see Scheme 2). Many tight binding EI complexes have been shown to arise via a two-step binding process which, for kinetic studies such as those undertaken here, can be simplified to a single-step mechanism.[25] Previous kinetic studies with DPPIV have assumed a single-step mechanism,[9] and other work[7] supports this assumption because no variation in initial velocity was observed with varying concentrations of inhibitor for a series of EI association curves.[26] In this work, the experimental conditions used for the equilibrium determination of K_i (9 day incubation period) can be assumed to result in the tightest possible EI complex. The close agreement between the equilibrium K_i and the K_i determined from the ratio of k_{off} to k_{on} implies that no rapidly forming EI complex exists. If such an EI complex was formed, then the paired progress curve analysis would have resulted in a different K_i.

The only published data which allows a discrimination between peptide boronate inhibitors for human DPPIV are IC_{50} and K_i values. Rate constants have only been reported for the inhibition of porcine DPPIV by Pro-boroPro.[9] The IC_{50} values for a number of peptide boronates which were published earlier[14] were determined through the use of an endpoint assay applied after a 60 min incubation. Since significant inhibitor inactivation has occurred during this period

(see Figure 3), determinations of relative inhibitor potency by this method are unreliable. Binding affinities in the low nM range for both Pro-boroPro and Ala-boroPro have been reported with the porcine enzyme.[7] Inhibitor inactivation during the course of the experiment was identified by the authors as a significant source of error. Therefore, we consider the inhibitory potencies for the peptide boronates with human DPPIV to be currently unavailable from published literature.

The kinetic analysis presented in this study overcomes the difficulty imposed by inhibitor inactivation. The equilibrium concentrations of active and inactive inhibitor were obtained by NMR and can be applied to the determination of true inhibitor binding affinities. Our results using the equilibrium binding method indicate that Val-boroPro is approximately 10 times more potent than either Pro-boroPro or Ala-boroPro. The K_i values that we obtained for the latter two compounds indicate a very similar degree of potency which is in agreement with previously published data.[7]

The equilibrium binding method was extended to the determination of kinetic rate constants in the presence of equilibrated inhibitor. The k_{off} values vary considerably, while little difference is seen for k_{on}. Val-boroPro possesses a k_{off} approximately 20 fold lower than either Ala-boroPro or Pro-boroPro. Fairly good agreement is observed between the K_i value calculated from the ratio of the kinetic constants and the K_i value determined directly from the EI equilibrium. These values vary by less than one quarter for Ala-boroPro and Pro-boroPro. The variation for Val-boroPro (about 2 fold at pH 8) is greater and may reflect the difficulty in attaining equilibrium for both active inhibitor and EI complex under the experimental conditions used.

Val-boroPro has been shown to have more immunosuppressive activity *in vivo* and to cyclize to a lesser extent than either Ala-boroPro or Pro-boroPro. It has been proposed[14] that the lower degree of cyclization for Val-boroPro might be expected to lead to increased immunosuppressive activity due to the increased plasma concentrations of active inhibitor. Previously, the actual binding affinities of the peptide boronate inhibitors were thought to play a minor role since all three compounds yield approximately equivalent IC_{50} values.[14]

The results of this work clarify the correlation between compound potency and compound instability. pK values were determined by NMR for three peptide boronic acid compounds: Ala-boroPro, Pro-boroPro, and Val-boroPro. Methods were developed for the determination of K_i, k_{off} and k_{on} in the presence of fully equilibrated inhibitor. Our results indicate that the inherently greater binding affinity of Val-boroPro may contribute to the increased immunosuppressive nature of this compound. In addition, the very low k_{off} may also affect immunosuppression due to the very slow dissociation of the EI complex ($t_{1/2} \approx 27$ days).

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References

[1] David, F., Bernard, A.-M., Pierres, M. and Marguet, D. (1993). *J. Biol. Chem.*, **268**, 17247–17252.
[2] Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Trotot, P., Barbat, A. and Trugnan, G. (1992). *J. Biol. Chem.*, **267**, 4824–4833.
[3] Dang, N.H., Torimoto, Y., Shimamura, K., Tanaka, T., Daley, J.F., Schlossman, S.F. and Morimoto, C. (1991). *J. Immunol.*, **147**, 2825–2832.
[4] Hegen, M., Neidobitek, G., Klein, C.E., Stein, H. and Fleischer, B. (1990). *J. Immunol.*, **144**, 2908–2914.
[5] Tanaka, T., Kameoka, J., Yaron, A., Schlossman, S.F. and Morimoto, C. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 4586–4590.
[6] Hegen, M., Mittrücker, H.-W., Hug, R., Demuth, H.-U., Neubert, K., Barth, A. and Fleischer, B. (1993). *Immunobiol.*, **189**, 483–493.
[7] Flentke, G.R., Munoz, E., Huber, B.T., Plaut, A.G., Kettner, C.A. and Bachovchin, W.W. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 1556–1559.
[8] Kubota, T., Flentke, G.R., Bachovchin, W.W. and Stollar, B.D. (1992). *Clin. Exp. Immunol.*, **89**, 192–197.
[9] Gutheil, W.G. and Bachovchin, W.W. (1993). *Biochemistry*, **32**, 8723–8731.
[10] Bartlett, P.A., Hanson, J.E. and Giannousis, P.P. (1990). *J. Org. Chem.*, **55**, 6268–6274.
[11] Hanson, J.E., Kaplan, A.P. and Bartlett, P.A. (1989). *Biochemistry*, **28**, 6294–6305.
[12] Yoshioka, M., Erickson, R.H., Matsumoto, H., Gum, E. and Kim, Y.S. (1991). *Int. J. Cancer*, **47**, 916–921.
[13] Hong, W., Piaza, G.A., Hixson, D.C. and Doyle, D. (1989). *Biochemistry*, **28**, 8474–8479.
[14] Snow, R.J., Bachovchin, W.W., Barton, R.W., Campbell, S.J., Coutts, S.J., Freeman, D.M., Kelly, T.A., Kennedy, C.A., Krolikowski, D.A., Leonard, S.F., Pargellis, C.A., Tong, L. and Adams, J. (1994). *J. Am. Chem. Soc.*, **116**, 10860–10869.
[15] Kelly, T.A., Adams, J., Bachovchin, W.W., Barton, R.W., Campbell, S.J., Coutts, S.J., Kennedy, C.A. and Snow, R.J. (1993). *J. Am. Chem. Soc.*, **115**, 12637–12638.
[16] Frieden, C., Kurz, L.C. and Gilbert, H.R. (1980). *Biochemistry*, **19**, 5303–5309.
[17] Pargellis, C.A., Morelock, M.M., Graham, E.T., Kinkade, P., Pav, S., Lubbe, K., Lamarre, D. and Anderson, P.C. (1994). *Biochemistry*, **33**, 12527–12534.
[18] Smith, R.E. and Van Frank, R.M. (1975). In *Frontiers of Biology Volume 43: Lysosomes in Biology and Pathology* (Neuberger, A., Tatum, E.L. (Eds.)) pp. 193–249, Amsterdam: North Holland.
[19] Matayoshi, E.D., Wang, G.T., Krafft, G.A. and Erickson, J. (1990). *Science*, **247**, 954–958.
[20] Glasoe, P.K. and Long, F.A. (1960). *J. Phys. Chem.*, **64**, 188–191.
[21] Rule, C.K. and LaMer, V.K. (1938). *J. Am. Chem. Soc.*, **60**, 1974–1981.
[22] Cha, S. (1980). *Biochem. Pharmacol.*, **29**, 1779–1789.
[23] Williams, J.W. and Morrison, J.F. (1979). *Meth. Enzymol.*, **63**, 437–467.
[24] Weber, P.C., Lee, S.L., Lewandowski, F.A., Schadt, M.C., Chang, C.W. and Kettner, C.A. (1995). *Biochemistry*, **34**, 3750–3757.
[25] Morrison, J.F. and Walsh, C.T. (1988). *Adv. Enzymol.*, **61**, 201–301.
[26] Williams, J.W., Morrison, J.F. and Duggleby, R.G. (1979). *Biochemistry*, **18**, 2567–2573.