Biochemistry

 Volume 32, Number 34 August 31, 1993

Articles

Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition†

William G. Gutheil and William W. Bachovchin*

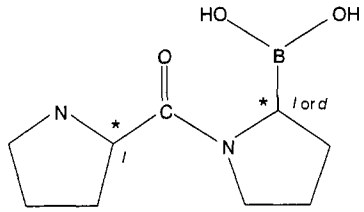
Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Received November 30, 1992; Revised Manuscript Received May 12, 1993

ABSTRACT: The potent dipeptidyl peptidase IV (DP IV) inhibitor [1-(2-pyrrolidinylcarbonyl)-2-pyrrolidinyl]boronic acid (L-Pro-DL-boroPro) [Flentke, G. R., Munoz, E., Huber, B. T., Plaut, A. G., Kettner, C. A., & Bachovchin, W. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1556–1559] was fractionated into its component L-L and L-D diastereomers by C18 HPLC, and the binding of the purified diastereomers to DP IV was analyzed. Inhibition kinetics confirms that the L-L diastereomer is a potent inhibitor of DP IV, having a K_i of 16 pM. The L-D isomer binds at least 1000-fold more weakly than the L-L, if it binds at all, as the ~200-fold weaker inhibition observed for the purified L-D isomer is shown here to be due entirely to the presence of a small amount (0.59%) of the L-L diastereomer contaminating the L-D preparation. The instability of Pro-boroPro, together with its very high affinity for DP IV and the time dependence of the inhibition, makes a rigorous kinetic analysis of its binding to DP IV difficult. Here we have developed a method which takes advantage of the slow rate at which the inhibitor dissociates from the enzyme. The method involves preincubating the enzyme and the inhibitor without substrate and then assaying the free enzyme by the addition of substrate and following its hydrolysis for a period of time which is short relative to the dissociation rate of the inhibitor. Data from experiments in which the preincubation time was sufficient for enzyme and inhibitor to reach equilibrium were analyzed by fitting to an appropriate form of the quadratic equation and yielded a K_i value of 16 pM. Data from experiments in which the incubation time was insufficient to establish equilibrium, *i.e.*, within the slow-binding regime, were analyzed by fitting to an integrated rate equation. The appropriate integrated rate equation for an $A + B \rightleftarrows C$ system going to equilibrium does not appear to have been previously derived. The analysis of the slow-binding curves yielded a K_i value of 16 pM, in agreement with that of 16 pM determined in the equilibrium titrations, and a bimolecular rate constant of association, k_{on}, of 5.0×10^6 M^{-1} s^{-1}. The experimentally determined k_{on} and K_i indicate that the dissociation rate constant, k_{off}, is 78×10^{-6} s^{-1} ($t_{1/2}$ = 150 min). The slow-binding curves are shown here to fit a simple $E + I \rightleftarrows EI$ model, indicating that it is not necessary to invoke a two-step mechanism to explain the inhibition kinetics.

Dipeptidyl peptidase IV (DP IV) is a type II membrane-anchored serine exoprotease found on the proximal tubules of the kidney (Gossrau, 1985; Wolf *et al.*, 1978), in the intestinal epithelium (Svensson *et al.*, 1978; Corporale *et al.*, 1985), on the surface of certain subsets of T lymphocytes, particularly $CD4^+$ helper cells (Ansorge & Ekkehard, 1987; Scholz *et al.*, 1985; Mentlein *et al.*, 1984), and in a number of other tissues. This protease has been implicated in a variety of physiological functions, including the salvage of amino acids (Miyamoto *et al.*, 1987) fibronectin-mediated cell movement and adhesion (Hanski *et al.*, 1985), and regulation of the immune system

† Supported by NIH Grant AI31866.
* Author to whom correspondence should be addressed.



trans Pro-boroPro

FIGURE 1: Structure of *trans*-Pro-boroPro showing chiral centers. The coupling of L-Pro with racemic LD-boroPro is expected to yield a mixture of two diastereomers: L-Pro-L-boroPro and L-Pro-D-boroPro.

(Schön *et al.*, 1985; Flentke *et al.*, 1991). Specific inhibitors of DP IV are therefore of some interest, both as tools to help elucidate the biological role or roles of DP IV and as potential therapeutic agents.

We have previously reported the synthesis and a preliminary kinetic characterization of two potent inhibitors of DP IV, Ala-boroPro and Pro-boroPro (boroPro refers to an analog of proline in which the carboxylate group is replaced by a boronyl group) (Bachovchin *et al.*, 1990; Flentke *et al.*, 1991). These inhibitors have an immunosuppressant activity, suppressing antigen-induced T-cell proliferation in T-cell culture systems (Flentke *et al.*, 1991) and antibody production in mice (Kubota *et al.*, 1992). These findings lend support to the hypothesis that DP IV plays a role in T-cell proliferation and suggest that DP IV inhibitors may be of therapeutic value.

Ala-boroPro and Pro-boroPro belong to a class of serine protease inhibitors known as peptide boronic acids (Kettner & Shenvi, 1984). Inhibitors of this class can have remarkably high affinities for their target enzymes. For example, MeO-Suc-Ala-Ala-Pro-boroPhe inhibits chymotrypsin with a K_i of 160 pM (Kettner & Shenvi, 1984), and Ac-D-Phe-Pro-boroArg inhibits thrombin with a K_i of 41 pM (Kettner *et al.*, 1990). The potency of these inhibitors is widely attributed to the ability of the boronyl group to form a tetrahedral adduct with the active site serine, which closely mimics the transition state of the enzyme-catalyzed reaction (Koehler & Lienhard, 1971; Lindquist & Terry, 1974; Rawn & Lienhard, 1974; Philipp & Maripuri, 1981; Bachovchin *et al.*, 1988). The peptide moiety, however, must also contribute importantly to the affinity, as simple alkyl- and arylboronic acids are many orders of magnitude less effective as inhibitors. X-ray crystallography and NMR spectroscopy have confirmed the presence of a boron–serine tetrahedral adduct in several serine protease–peptide boronic acid inhibitor complexes. However, NMR spectroscopy has also demonstrated that in certain cases tetrahedral boron–histidine adducts are formed (Bachovchin *et al.*, 1988; Tsilikounas *et al.*, 1992).

The more potent peptide boronic acid inhibitors usually inhibit their target enzymes in a time-dependent manner (Kettner & Shenvi, 1984; Shenvi, 1986; Kettner *et al.*, 1988, 1990), a phenomenon known as slow-binding inhibition [reviewed in Morrison and Walsh (1988)]. Both Ala-boroPro and Pro-boroPro are slow-binding inhibitors of DP IV. Morrison and Walsh (1988) have postulated that most, if not all, slow-binding inhibitors bind to their target enzymes in two steps, *i.e.*, the inhibitor first forms a relatively weak complex with the enzyme, which then undergoes slow conversion to a tighter complex. The molecular mechanism underlying the slow binding of peptide boronic acids to serine proteases is not yet clear, but is of considerable theoretical understanding the catalytic mechanism of serine proteases and for the rational design of inhibitors.

A major impediment to the study of slow-binding inhibition is that the kinetic analysis is not trivial. The high affinity these inhibitors typically have for their target enzymes means that kinetic experiments must often be carried out under conditions where $I \approx E$ and, thus, where the approximation that $I_{free} = I_{total}$, which greatly simplifies the kinetic analysis of weaker binding inhibitors, is no longer valid. The time dependence of the inhibition further complicates matters because it may prevent a steady-state rate from being reached until substrate depletion becomes significant. Such a system is described by a set of differential equations for which an integrated rate equation is not available, although expressions have been derived for the case where substrate depletion is not significant during the time course of inhibitor binding (Cha, 1975, 1976).

The kinetic analysis of Ala-boroPro and Pro-boroPro binding to DP IV is even more complicated because these inhibitors are unstable, having half-lives of about 5 and 30 min, respectively, at neutral pH. In the preliminary kinetic analysis we reported K_i values of 2 and 3 nM, respectively, for Ala-boroPro and Pro-boroPro, realizing that these values substantially overestimated the true K_i values owing to the simplified way in which K_i determinations were carried out and to the instability and slow binding of these inhibitors (Flentke *et al.*, 1991). The original analyses were also carried out with inhibitors which were diastereomeric mixtures, *i.e.*, L-Ala-DL-boroPro and L-Pro-DL-boroPro. The expectation is that only one of the isomers, presumably the L-L isomer, is the active inhibitor.

Because the potency of these inhibitors is unusually high for such small molecules, and because DP IV appears to have important biological functions, a more detailed analysis of how these small dipeptide boronic acids interact with DP IV should be of considerable interest. Here we report (i) the purification of L-Pro-L-boroPro and L-Pro-D-boroPro from the L-DL diastereomeric mixture and (ii) a more detailed kinetic analysis of each isomer's inhibition of DP IV. To circumvent the difficulties outlined above, we have developed a method which exploits the fact that dissociation of the inhibitor from the enzyme is a relatively slow process. The method involves incubating the enzyme with inhibitor in the absence of substrate. The amount of free enzyme at any time can then be determined by adding substrate and monitoring the time course of the enzyme-catalyzed reaction for a short period during which the inhibitor does not have time to measurably dissociate from the enzyme. These simplified experimental conditions allow the derivation of expressions which can be used to analyze inhibitor binding under both equilibrium and nonequilibrium conditions. Equilibrium conditions here refer to experiments in which the preincubation time was sufficient for enzyme and inhibitor to reach equilibrium prior to the addition of substrate and enzyme assay. Nonequilibrium conditions refer to experiments in which the preincubation time was insufficient for equilibrium to be reached, and thus the system is within the slow-binding time domain. The integrated rate equation needed to analyze the nonequilibrium data does not appear to have been previously derived and is therefore derived here for the first time. This approach and the derived equations should prove useful in the analysis of other slow-binding enzyme–inhibitor systems.

MATERIALS AND METHODS

Preparation of L-L *and* L-D *Pro-boroPro Diastereomers by*

previously (Bachovchin *et al.*, 1990). Analytical and semi-preparative C18 HPLC were performed on a 250 × 4.6 mm 5-μm Nucleosil C18 HPLC column (Alltech Associates Inc., Deerfield, IL) using a Hewlett-Packard 1050 quaternary pump HPLC equipped with a multiple wavelength detector (Hewlett-Packard, Rockville, MD). Several milligrams of the purified components could be prepared by repeatedly injecting 0.5 mg of the mixture on this column and then pooling and lyophilizing the appropriate fractions. The resulting material was redissolved in 0.01 N HCl. Analytical C18 HPLC chromatograms of the purified products are shown in Figure 2. The absolute configurations were assigned on the basis of a detailed NMR study (J. L. Sudmeier, W. G. Gutheil, and W. W. Bachovchin, unpublished results). An attempt to scale up this purification procedure on 200 × 10 mm and 400 × 10 mm Absorbosphere C18 HPLC columns (Alltech Associates) did not provide as pure a final product.

Quantitation of Pro-boroPro by Amino Acid Analysis. Amino acid analysis was performed by the PITC method (Bidlingmeyer *et al.*, 1984). Quantitation was based on proline. The boronylproline did not appear in this analysis.

Purification of Pig Kidney DP IV. Pig kidney DP IV was prepared as described previously (Wolf *et al.*, 1978). The concentration of DP IV active sites was assessed by stoichiometric titration with L-Pro-L-boroPro, as described further below.

Standard DP IV Enzyme Assays. Standard activity assays were performed in 50 mM sodium phosphate (pH 7.5) at 25 °C with the chromogenic substrate Ala-Pro-*p*-nitroanilide (APPNA) (Bachem Inc., Torrance, CA), monitoring the A_{410} on a Hewlett-Packard UV–vis spectrometer. The value $\Delta\epsilon$ = 8800 M^{-1} cm^{-1} upon hydrolysis of substrate was used to calculate rates and concentrations (Erlanger *et al.*, 1961). The hydrolysis time course was monitored for 2 min. The initial substrate concentration was 73.7 μM, 5 times the K_m (*vide infra*).

Equilibrium Titrations of DP IV with L-L *and* L-D *Pro-boroPro.* These experiments were performed by first preparing a DP IV stock in the assay buffer. The amount of DP IV used in each assay was the minimal amount necessary to obtain a sufficient absorbance change in 2 min with the substrate for accurate quantitation. A series of Pro-boroPro dilutions and a blank were prepared in 0.01 N HCl. To 0.980 mL of the stock-diluted DP IV was added 10 μL of diluted Pro-boroPro, the mixture was incubated for 30 min at 25 °C, and the free enzyme was assayed by the addition of APPNA in 10 μL of DMF.

Kinetics of DP IV and L-*Pro*-L-*boroPro Association.* For the association kinetics a fluorometric assay with Ala-Pro-7-amino-4-(trifluoromethyl)coumarin (APAFC) (Enzyme Systems Products, Livermore, CA) was used. Fluorescence was monitored on a Perkin-Elmer LS-5 fluorescence spectrometer (Oak Brook, IL) with an excitation wavelength of 400 nm and a detection wavelength of 505 nm. The response was calibrated with 1 μM 7-amino-4-(trifluoromethyl)-coumarin. The experiments were performed by incubating DP IV with inhibitor in the absence of substrate. After an appropriate time interval, APAFC was added to assay for free DP IV. The slow apparent rate of DP IV–inhibitor association under these dilute conditions gave a time course for the association reaction (Figure 5). Specifically, experiments were performed by diluting stock DP IV into 10 mL of the assay buffer to give a concentration one-fiftieth that used in the equilibrium binding experiments. A blank assay of the diluted enzyme with 10 μL of 1 mM APAFC in DMF in a cuvette (10 μM final concentration) and monitoring the fluorescence change for 2 min at 25 °C. This value was considered to be $t = 0$ for the binding time course. An aliquot of L-Pro-L-boroPro was then added to the remaining 9.010 mL of diluted DP IV, and 0.990-mL aliquots were removed and assayed at intervals with APAFC as above.

Stability of L-L *and* L-D *Pro-boroPro.* In 0.01 N HCl these compounds appeared stable for at least 1 month at room temperature. Both the L-L and L-D Pro-boroPro preparations lose their DP IV inhibitory activity in the assay buffer at pH 7.5. To partially characterize this behavior, the time course of inactivation was monitored at three different concentrations: two at relatively low inhibitor concentrations, using DP IV inhibition as an indicator of residual inhibitor concentration, and one at relatively high inhibitor concentration, using C18 HPLC to determine residual inhibitor concentration. For inactivation in the range of inhibitor used in the assays above, the inhibitor was diluted into assay buffer at a concentration sufficient to give roughly 90% inhibition initially (approximately 1 nM for the L-L and 100 nM for the L-D). At various time intervals DP IV was added, and after a 15-min incubation, APPNA was added to assay for the free enzyme analogous to the procedure used in the equilibrium titrations. In a second experiment the inhibitor concentration in the pH 7.5 buffer was 100 times the assay concentration. At time intervals between 1 and 150 min, 10 μL of the Pro-boroPro solution was added to 0.980 μL of assay buffer containing DP IV, and this mixture was allowed to incubate for 15 min. Substrate was then added to assay for free enzyme. The inhibition observed in these experiments was converted to the amount of active inhibitor using the inverse to the equilibrium relationships as derived in the Theory section. The inactivation of these compounds was also observed directly at higher concentrations (250 μM) by analytical C18 HPLC. Half-lives ($t_{1/2}$) for degradation were determined empirically from plotted degradation time courses as the time at which one-half of the inhibitor remained.

Progress Curves for Enzyme + Substrate + Inhibitor Assays. These experiments were performed in two ways. One was for the enzyme to be added to an assay mixture containing a known amount of both the substrate and the inhibitor. The other was to incubate the enzyme with inhibitor for 15 min to establish equilibrium and then add substrate. The control for this experiment was to monitor a complete time course for the hydrolysis of substrate by enzyme.

Numerical Inetegration of Rate Equations. Numerical integrations of rate equations were performed with the GEAR software package (Stabler & Chesick, 1978; McKinney & Weigert, 1986).

Data Analysis. Data were analyzed by fitting to the appropriate equation by derivative-free nonlinear regression using the IBM PC based version of the BMDP program AR (BMDP Statistical Software, Los Angeles, CA). The equations used are derived in the Theory section.

THEORY

Derivation of Equations Describing Simple $A + B \rightleftarrows C$ Equilibrium. In the enzymatically monitored equilibrium titrations, we are titrating DP IV with Pro-boroPro. The concentration of the stock Pro-boroPro is accurately known from amino acid analysis. The observed binding of the L-L Pro-boroPro was very tight, and this in principle allows the concentration of DP IV to be accurately determined by

exactly using the quadratic equation. Using E_T to represent the total DP IV concentration and I_T to represent the total inhibitor concentration, the following equations can be derived:

$$(EI) = \frac{(E_T + I_T + K_i) - \sqrt{((E_T + I_T + K_i)^2 - 4E_TI_T)}}{2} \quad (1)$$

$$E = E_T - (EI) \quad (2)$$

$$I = I_T - (EI) \quad (3)$$

The observable is the rate of APPNA hydrolysis, which is proportional to E:

$$\text{rate} = E(\text{SA}) \quad (4)$$

where SA is the specific activity in units of ΔOD_{410}/min/pM DP IV active sites at 73.7 μM APPNA. The experimentally variable parameter is I_T. The adjustable parameters to be fit are K_i, E_T (in terms of active sites), and SA. In the case of L-Pro-D-boroPro, the less potent inhibitor, the observed binding appeared to be due to contamination with the L-L diastereomer. This situation was analyzed by including another adjustable parameter, %L-L, in these equations. The actual amount of L-L present therefore was

$$I_T(\text{L-L}) = I_T(\text{L-D})\%\text{L-L}/100 \quad (5)$$

where I_T(L-L) is the true total L-L concentration, I_T(L-D) is the total inhibitor concentration (based upon amino acid analysis), and %L-L is an adjustable parameter describing the % contamination of L-L in the L-D preparation.

Inversion of the Equilibrium Equation To Measure the Rate of Inactivation of L-*Pro*-L-*boroPro*. In the preceding section, an equation was derived describing the observed rate of DP IV catalyzed substrate turnover as a function of the independent variable I_T(L-L) and the parameters K_i, E_T, and SA, which are to be fit to experimental data. Once these parameters have been fit, it is possible to find the inverse relationship to this equation and, with the fitted parameters, to calculate I_T(L-L) from an experimentally measured rate as required for analysis of the DP IV monitored inactivation of Pro-boroPro described above. The following equation is easily derived:

$$I_T(\text{L-L}) = E_T - K_i - \text{rate}/\text{SA} + E_TK_i(\text{SA})/\text{rate} \quad (6)$$

where the parameters are as defined above.

Derivation of an Integrated Rate Equation for the A + B ⇄ C System. Surprisingly, the appropriate form of the integrated rate equation for this system was not found in a number of standard sources. The system

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} (EI) \quad (7)$$

is described by the following set of differential equations

$$dE/dt = -k_{on}EI + k_{off}(EI) \quad (8a)$$

$$dI/dt = -k_{on}EI + k_{off}(EI) \quad (8b)$$

$$d(EI)/dt = k_{on}EI - k_{off}(EI) \quad (8c)$$

Substitution for E, I, and k_{off} of

$$E = E_T - (EI) \quad (9)$$

$$k_{off} = k_{on}K_i \quad (11)$$

into the expression for $d(EI)/dt$ and then expansion and rearrangement gives

$$d(EI)/dt = k_{on}(EI)^2 - (k_{on}E_T + k_{on}I_T + k_{on}K_i)(EI) + k_{on}E_TI_T \quad (12)$$

This can be rearranged for integration as

$$dt = \frac{d(EI)}{k_{on}(EI)^2 - (k_{on}E_T + k_{on}I_T + k_{on}K_i)(EI) + k_{on}E_TI_T} \quad (13)$$

The left side of this differential equation is trivial to integrate. The right side is given in standard math tables [*CRC Handbook of Chemistry and Physics*, Vol. 67, p A-26, eq 110, second equation). (Note that we use q for $-q$ and that q in our nomenclature can be shown to always be greater than or equal to 0, a prerequisite for using this equation.)

$$\int dx/X = \frac{1}{\sqrt{q}} \ln\left[\frac{2cx + b - \sqrt{q}}{2cx + b + \sqrt{q}}\right] \quad (14)$$

where $x = (EI)$, $X = a + bx + cx^2$, $a = k_{on}E_TI_T$, $b = -(k_{on}E_T + k_{on}I_T + k_{on}K_i)$, $c = k_{on}$, and $q = b^2 - 4ac$. The appropriate integrated expression is therefore (with an initial condition at $t = 0$ of $x = 0$ (i.e., $(EI) = 0$))

$$t = \frac{1}{\sqrt{q}} \ln\left[\frac{(2cx + b - \sqrt{q})(b + \sqrt{q})}{(2cx + b + \sqrt{q})(b - \sqrt{q})}\right] \quad (15)$$

Rearrangement to solve for x in terms of t gives

$$x = \frac{(1 - e^{t(q)^{1/2}})(b + \sqrt{q})}{2c(e^{t(q)^{1/2}} - ((b + \sqrt{q})/(b - \sqrt{q})))} \quad (16)$$

To check this result, note that at $t = 0$, $x = 0$ as expected. Also note that

$$\text{as } t \to \infty, \quad x \to \frac{(-\infty)(b + \sqrt{q})}{2c(\infty)} \quad (17)$$

$$x = \frac{-b - \sqrt{q}}{2c} \quad (18)$$

which is equivalent to the equilibrium expression derived above (eq 1), also as expected.

Analysis of Enzyme + Substrate Progress Curves. The data were collected at 5-s intervals over the time course of these experiments, up to 1.5 h. Several approaches have been described for the analysis of data of this type. Direct fitting to the integrated Michaelis–Menten equation (Kellershohn & Larent, 1985; Cox & Boeker, 1987) is complicated by the fact that this equation is a mixture of linear and transcendental functions in the dependent variable, and therefore t (time) must be fit as a function of P (product concentration). A more direct approach is to determine the rate (dP/dt) from the data and to fit this directly to the Michaelis–Menten equation (Canela & Franco, 1986). We use this approach here, but have not found it necessary to use a complicated weighting scheme nor to fit the time course data to a polynomial equation to extract derivatives. Instead, the data in terms of (OD,t) data pairs were converted into (P,t) data pairs and then into (S,dP/dt) data pairs in a Lotus 123 spreadsheet (Lotus Development Corporation, Cambridge, MA). The $dP/$

differences using the formula

$$(dP/dt)_i = (P_{i+1} - P_{i-1})/(t_{i+1} - t_{i-1}) \quad (19)$$

Parameters (K_m and k_{cat}) were then determined by fitting to the Michaelis–Menten equation

$$(dP/dt)_i = k_{cat}S_iE_T/(S_i + K_m) \quad (20)$$

The predicted time course was calculated from the fit parameters by summation using the following equations (Δt = 5s):

$$P_0 = 0 \quad (21)$$

$$S_0 = S_T \quad (22)$$

$$P_i = \sum_{n=1}^{i} (E_T S_{i-1} k_{cat}/(K_m + S_{i-1}))\Delta t \quad (23)$$

$$S_i = S_0 - P_i \quad (24)$$

which can also be performed easily in a Lotus 123 spreadsheet.

Alteratively, the predicted time course can be obtained by numerical integration. The system

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (ES) \overset{k_{cat}}{\rightarrow} E + P \quad (25)$$

is described by the following set of differential equations:

$$dE/dt = -ESk_1 + (ES)(k_{-1} + k_{cat}) \quad (26a)$$

$$dS/dt = -ESk_1 + (ES)k_{-1} \quad (26b)$$

$$d(ES)/dt = ESk_1 - (ES)(k_{-1} + k_{cat}) \quad (26c)$$

$$dP/dt = (ES)k_{cat} \quad (26d)$$

Given the initial values for the concentrations of the components in this system and values for the rate constants, the GEAR program will provide a simulated time course for comparison with the experimentally determined time course.

Numerical Simulation of the Model Shown in Figure 7A. The model shown in Figure 7A is described by the following set of differential equations:

$$dE/dt = -ESk_1 + (ES)(k_{-1} + k_{cat}) - k_{on}EI + k_{off}(EI) \quad (27a)$$

$$dS/dt = -ESk_1 + (ES)k_{-1} \quad (27b)$$

$$d(ES)/dt = ESk_1 - (ES)(k_{-1} + k_{cat}) \quad (27c)$$

$$dP/dt = (ES)k_{cat} \quad (27d)$$

$$dI/dt = -k_{on}EI + k_{off}(EI) \quad (27e)$$

$$d(EI)/dt = k_{on}EI - k_{off}(EI) \quad (27f)$$

Note that eqs 27 = eqs 8 + eqs 26, i.e., model(Figure 7A) = model($E + I \rightleftarrows EI$) + model($E + S \rightleftarrows ES \rightarrow E + P$). Also note that for the one species common to both models, E, the differential eq 27a is obtained as

$$dE/dt_{\text{model(Figure 7A)}} = dE/dt_{\text{model}(E+I\rightleftarrows EI)} + dE/dt_{\text{model}(E+S\rightleftarrows ES\rightarrow E+P)} \quad (28)$$

Given values for all of the initial concentration and the rate constants describing Figure 7A, it is possible to simulate experimental results for this system using the GEAR program.

RESULTS

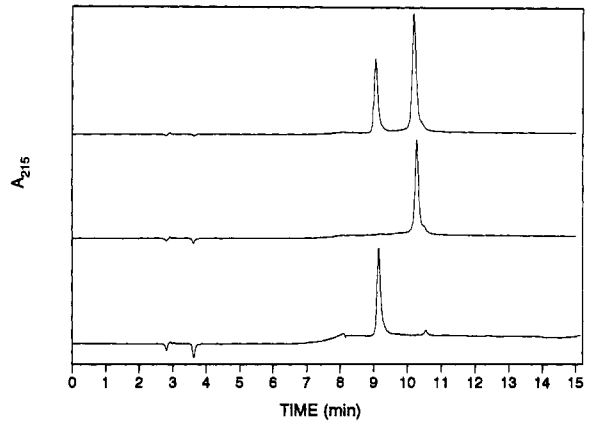


FIGURE 2: C18 HPLC chromatograms showing resolution of Pro-boroPro diastereomers. The upper chromatogram is of the starting mixture, the middle chromatogram is of the purified L-D diastereomer, and the lower chromatogram is of the purified L-L diastereomer. HPLC conditions: solvent A 0.1% trifluoroacetic acid (TFA) in H_2O; solvent B 70% acetonitrile/30% H_2O/0.086% TFA; gradient 0–2 min 0% B, 2–32 min 0–100% B. Only the first 15 min of each chromatogram are shown. The base-line disturbance at 8.15 min is the gradient entering the detector.

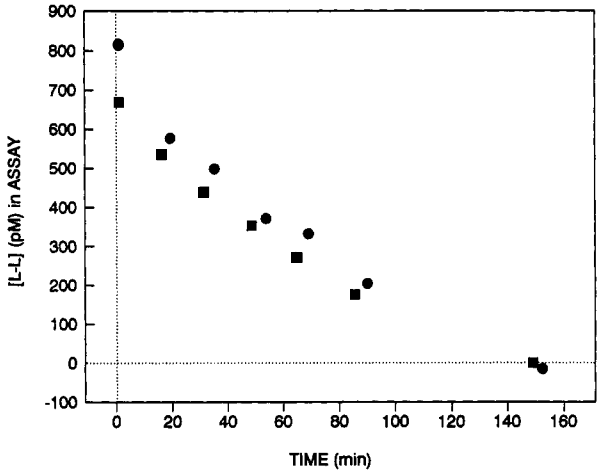


FIGURE 3: DP IV monitored inactivation kinetics of L-L (■) and L-D (●) Pro-boroPro at the very low concentrations (~1 nM of L-L) and pH (7.5) used in the equilibrium and kinetic experiments. The L-L concentration was calculated from the raw data using the inverse of the equilibrium equation derived in the Theory section (eq 6).

2. Attempts to scale up this separation were unsuccessful owing to the decreased resolution with larger column diameters, which could not be overcome by extending the length of the column. For separation on analytical columns, about 0.5 mg of the mixture was loaded for each run. A purity of >98% for the purified products was indicated by analytical HPLC (Figure 2).

Stability of Pro-boroPro Diastereomers. The concentration dependence of the inactivation of Pro-boroPro was examined at very low (~1 nM) and low (~100 nM) concentrations of Pro-boroPro, at pH 7.5, using DP IV inhibition to monitor the residual active inhibitor concentration, and at a relatively high (~250 μM) concentration of the inhibitors using C18 HPLC. The time courses of the degradation kinetics had the same shapes as the curves shown in Figure 3 in all cases, but with somewhat different half-lives. The inactivation reaction appears to follow a mixture of zero- and first-order kinetics, with zero-order dominating. The measured half-lives at pH 7.5 for the L-L isomer were 55 min at ~1 nM, 35 min at ~100

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.