Degradation Pathways of a Peptide Boronic Acid Derivative, 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$

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ABSTRACT: The peptide boronic acid derivative 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ is a potent inhibitor of 20S proteasome and a proposed anticancer agent. During preformulation studies, the compound presented erratic stability behavior. Efforts were made to isolate and identify the degradation products, thereby helping to identify possible mechanisms for the degradation. The reaction of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ with hydrogen peroxide not only provided a convenient way to isolate the initial degradation products seen from hydrolysis in aqueous buffers but also showed that the major, initial degradation pathway was probably oxidative in nature. The isolated degradation products were characterized by nuclear magnetic resonance spectroscopy, mass spectrometry, and optical rotation dispersion. In the presence of hydrogen peroxide, the boronic acid group was cleaved from 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ to give an alcohol with an apparent retention of the original stereochemistry. Subsequent isomerization and further hydrolysis were then seen. Surprisingly, added ascorbate and EDTA accelerated rather than inhibited degradation. Degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ under acidic and basic conditions seemed to be mediated by an initial oxidative degradation pathway similar to that seen with the peroxide. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 89: 758–765, 2000

Keywords: stability; peptide boronic acid; oxidation; peroxides

INTRODUCTION

Peptide boronic acid derivatives have been designed as potent inhibitors for serine proteases to achieve various potential therapeutic applications. For example, dipeptides of proline boronic acids were designed as inhibitors of dipeptidyl peptidase IV[1] and hence possessed immunosuppressant activity; Ac-(D)Phe-Pro-Arg-B(OH)$_2$ was synthesized as an inhibitor of thrombin to achieve anticoagulant activity.[2] The mechanism of inhibition of serine proteases by peptide boronic acid derivatives is formation of a tetrahedral intermediate between the boronic acid derivative and the catalytic serine group after the compounds bind to the enzymes.[3]

The compound, 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ (shown in Scheme 1), which was the focus of the present study, was synthesized as a potent inhibitor of 20S proteasome (K_i, 0.6 nM).[4] The 20S proteasome is a multicatalytic complex with chymotrypsin, trypsin-like, and peptidylglutamyl hydrolyzing activities. One of the potential therapeutic applications of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ is as an anticancer agent.

The chemical stability of peptide boronic acid derivatives, from a formulation perspective, has not been extensively reported in the literature to our knowledge.[5] During an effort to formulate 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ for parenteral administration, the compound showed erratic stability behavior and was quite unstable in certain solvents. For example, when the equilibrated

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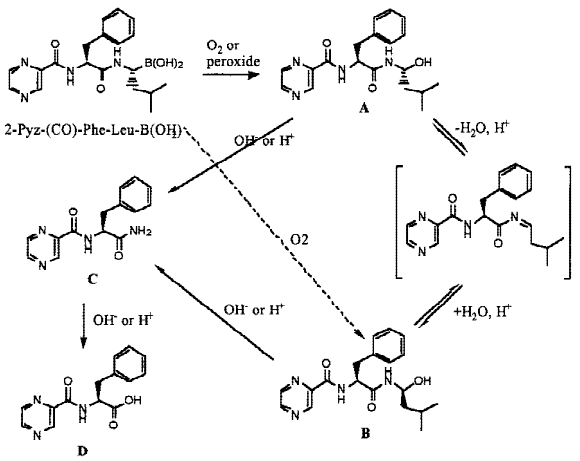


Scheme 1. Proposed mechanism for the degradation of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ under oxidative, acidic, and alkaline conditions.

solubility at 25 °C in 24 h was determined in a polyethylene glycol 300 (PEG300):EtOH:H_2O (40:10:50) solvent, significant quantities of two degradation products **A** and **B** were observed, with **A** as the major degradant (Figure 1a). The two degradants combined accounted for as much as 20% degradation. In an ethanol:normal saline solution (2:98, pH 2.8), 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (0.5 mg/mL) degraded 20% at 25 °C in 1 month, with trace amounts of degradants **A** and **B** and two additional degradants, **C** and **D**, observed (Figure 1b). In propylene glycol:ethanol:water (50:10:40), the stability of the compound improved but still degraded 20% in 8 months when stored at 25 °C; **A**, **B**, **C**, and **D** were observed as degradants. The stability of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ in aqueous acidic and basic pH conditions was also determined. Under acidic and basic conditions, **D** was observed as the major degradation product. It appeared, therefore, that the degradation of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ was quite complicated.

To understand the degradation pathways and possible mechanisms under various conditions, degradants **A**, **B**, **C**, and **D** were isolated and identified. Some observations on the effects of ascorbic acid and EDTA on the stability of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ were also observed.

EXPERIMENTAL SECTION

Materials and Instruments

The compound 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (NSC-681239) was supplied by the National Cancer Institute(NCI), Bethesda, MD. NSC-681239 was supplied to NCI by Proscript, Inc., Cambridge, MA. HPLC-grade acetonitrile was obtained from Fisher Scientific. Deionized distilled water was used to prepare aqueous solutions and mobile phase. Octadecyl spe C-18 extraction columns (3 mL) were purchased from J. T. Baker. All other reagents were ACS certified or reagent grade.

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with either a Bruker DRX400 or a Bruker AM500 spectrometer. Fast-atom bombardment (FAB) or exact mass spectrometry was obtained on a ZAB HS mass spectrometer (VG Analytical Ltd, Manchester, UK) equipped with a 11/250 data system. Optical rotation dispersion spectra were obtained on a Perkin-Elmer 241 polarimeter.

HPLC Assay Method

An HPLC assay method for 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ and its degradants was developed with a

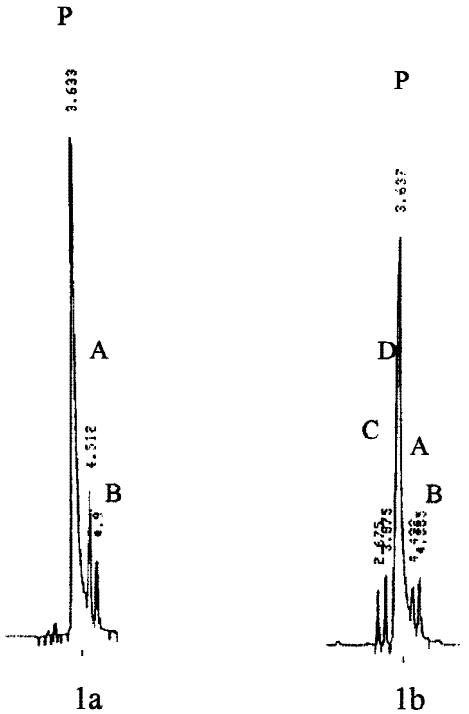


Figure 1. HPLC chromatograms illustrating degradation of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ in solvents of (a) PEG300:EtOH:H_2O (40:10:50) and (b) EtOH:normal saline (2:98) (pH 2.83); **P**, **A**, **B**, **C**, and **D** represent the parent compound and degradants **A**, **B**, **C**, and **D** respectively.

Shimadzu LC-6A pump, a Rheodyne 7125 injection valve fitted with a 20-μL loop, and a Shimadzu SPD-6A ultraviolet (UV) detector. A YMC-C8 column (150 × 4.6 mm, 5 μm) was employed. The isocratic mobile phase consisted of 40% (v/v) acetonitrile and 60% (v//v) 30 mM $KH_2PO_4H_3PO_4$ (pH 3.0). The flow rate was 1.0 mL/min and the detection wavelength was 280 nm. HPLC analyses were performed at room temperature (~20 °C). Under these conditions, 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ eluted at 3.6 min; and degradants **A**, **B**, **C**, and **D** eluted at 4.5, 5.0, 2.7, and 3.0 min, respectively.

Degradation in the Presence of Hydrogen Peroxide

Because alkyl borane compounds are known to be susceptible to oxidation by peroxides,[6] it was speculated that the degradation of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ observed in the PEG300:EtOH:H_2O (40:10:50) solvent was due to the presence of peroxides. PEG300 is known to undergo autooxidation and generate peroxide species.[7] To test this hypothesis, an aqueous solution of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (1.1 mM) was treated with hydrogen peroxide (15.7 mM) at 20 °C. The reaction was monitored by HPLC (Figure 2). After 1 h, 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ was transformed quantitatively to degradant **A**, the major degradation product observed in PEG300:EtOH:H_2O (40:10:50). At ambient temperature, **A** further degraded to **B** and **C**. Also, acidic conditions (pH 1–2) accelerated transformation of **A** to an equilibrium with **B** (Figure 3); in contrast, alkaline conditions (pH 11–12) accelerated the quantitative transformation from **A** to **C**.

Degradation under Acidic and Basic pH Conditions

To study degradation under aqueous acidic and basic pH conditions, a concentrated stock solution of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ in DMSO was diluted to 5 mL with 0.1 M HCl and 0.01 M NaOH to a final concentration of 0.19 mM. The solutions were then stored at 70 °C ; the degradation kinetics and products were monitored by HPLC (Figure 4). The major degradant observed at both pH 1.0 and 12.0 was **D**. A small amount of **C** was also

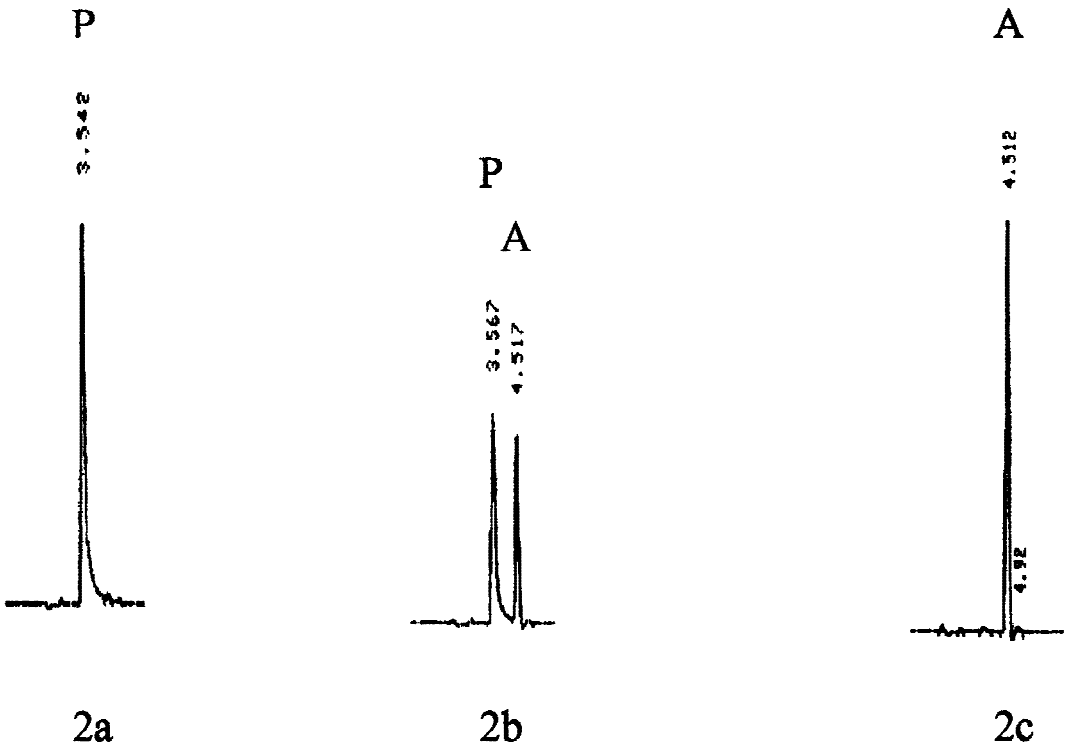


Figure 2. HPLC chromatograms of the reaction of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (1.1 mM) with H_2O_2 (15.7 mM) in H_2O at room temperature. Figures a, b, and c correspond to reaction times of 0, 15, and 60 min, respectively, and **P** and **A** represent the parent compound and degradant **A**, respectively.

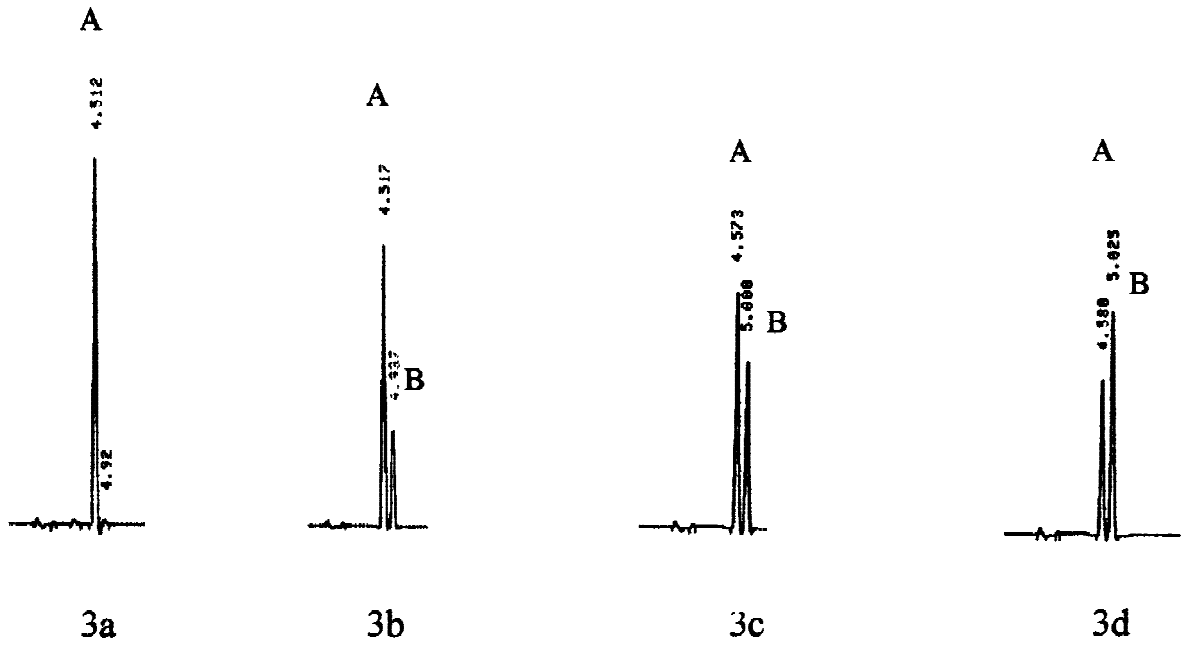


Figure 3. HPLC chromatogram of transformation of degradant **A** (1.1 mM) to **B** under acidic conditions. Figures a, b, c, and d correspond to times of transformation for 0, 20, 30, and 128 min, respectively, at pH 2.0 and room temperature.

observed during the process of alkaline-catalyzed degradation.

Isolation and Identification of Degradants A, B, C, and D

Degradant **A** was obtained by treating 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ with hydrogen peroxide.

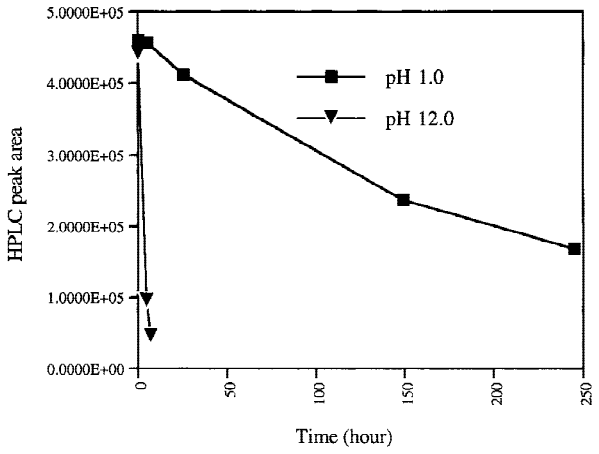


Figure 4. Degradation kinetics of 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (0.19 mM) at pH 1.0 (■) and 12.0 (▼), respectively, and 70 °C.

An aqueous solution (12.0 mL) containing 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ (1.1 mM) and hydrogen peroxide (24.1 mM) was incubated at ambient temperature (~20 °C). After 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ was completely transformed to degradant **A**, as monitored by HPLC, the reaction mixture was loaded onto a 3-mL C-18 extraction column. The column was washed with water to remove any remaining hydrogen peroxide. Degradant **A** was eluted with 50% ACN:water (v/v). The fractions that contained degradant **A** were collected and lyophilized. The ^1H and ^{13}C NMR and exact mass of degradant **A** were obtained. ^1H NMR (400 MHz, $CDCl_3$, δ): 0.87–0.90 (q, 6H), 1.26–1.52 (m, 2H), 1.62–1.73 (m, 1H), 3.16–3.30 (dddd, 2H), 3.31 (d, 1H, OH), 4.78–4.80 (dd, 1H), 5.31–5.38 (dd, 1H), 6.27–6.32 (d, 1H), 7.37–7.36 (m, 5H), 8.34–8.40 (d, 1H), 8.56–8.57 (dd, 1H), 8.78–8.79 (d, 1H), 9.38 (d, 1H). ^{13}C NMR (500 MHz, $CDCl_3$, δ): 22.1, 22.7, 24.2, 38.2, 43.7, 54.6, 73.5, 127.3, 128.8, 129.3, 136.1, 124.7, 143.7, 144.3, 147.6, 163.0, 171.1. MS (exact) *m/e*: 357.1913.

To obtain degradant **B**, an aqueous solution of **A** (10 mL, 1.1 mM) was adjusted to pH values between 1 and 2 by the addition of aqueous hydrochloric acid. When the transformation of **A** to **B** reached equilibrium, the mixture was separated by an isocratic HPLC method, with an ana-

lytical ODS-hypersil C-18 column and 26% acetonitrile:water (v/v) as the mobile phase (at room temperature). Under such conditions, **A** and **B** had retention times of 9.0 and 11.1 min, respectively. Fractions that contained degradant **B** were collected and lyophilized, and ^{1}H NMR and mass (FAB^+) spectra of **B** were obtained. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.84–0.87 (t, 6H), 1.12–1.46 (m, 2H), 1.49–1.53 (m, 1H), 3.10–3.28 (dddd, 2H), 3.41 (b, 1H, OH), 4.76–4.83 (dd, 2H), 5.28–5.33 (dd, 1H), 6.21–6.23 (d, 1H), 7.27–7.38 (m, 5H), 8.37–8.42 (d, 1H), 8.58–8.60 (t, 1H), 8.78–8.81 (d, 1H), 9.38–9.40 (d, 1H). MS (FAB^+): *M-1*/*e*356.3.

Degradant **C** was isolated by adjusting the pH of an aqueous solution of **A** (5.0 mL, 1.1 mM) to 12. After **A** was completely transformed to degradant **C**, the solution was loaded onto a 3-mL C-18 extraction column. After washing with water, **C** was eluted with 26% acetonitrile:water (v/v). The fractions that contained **C** were collected and lyophilized, and ^{1}H NMR and mass (FAB^+) spectra of **C** were obtained. ^{1}H NMR (400MHz, $CDCl_3$, δ): 3.18–3.32 (dddd, 2H), 4.84–4.90 (dd, 1H), 5.34 (b, 1H), 5.70 (b, 1H), 7.33–7.38 (m, 5H), 8.37–8.39 (d, 1H), 8.57 (s, 1H), 8.79 (d, 1H), 9.39 (s, 1H). MS (FAB^+): *M-1*/*e* 270.1.

To obtain **D**, 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$ was dissolved in 1.0 M HCl (1.5 mg/mL) and incubated at 70 °C for 5 days. The reaction mixture was loaded onto a 3-mL C-18 extraction column, and **D** was eluted with 35%acetonitrile:water. The fractions were collected and lyophilized, and ^{1}H NMR and mass spectra were obtained. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 3.22–3.38 (dddd, 2H), 5.04–5.09 (dd, 1H), 7.21–7.30 (m, 5H), 8.19–8.20 (d, 1H), 8.51 (s, 1H), 8.4 (d, 1H), 9.36 (s, 1H). MS (FAB^+):*M-1*/*e 271.*

Degradant **D** formed under alkaline conditions was also isolated and characterized to confirm that it was identical to **D** obtained under acidic conditions. A water solution of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$ (0.5 mg/mL) was adjusted to pH 12 by addition of a concentrated NaOH solution. The solution was then incubated at 70 °C for 48 h so that all the parent compound was degraded. The resultant solution was acidified to pH 2.5 and then loaded onto a 3-mL C-18 extraction column. Polar compounds were removed with 5% acetonitrile:water; **D** was eluted with 35% acetonitrile: water. The fractions that contained **D** were collected and lyophilized. The ^{1}H NMR and mass (FAB^+) spectra were obtained and were identical to the those of **D** obtained under acidic conditions.

The structures of **A**, **B**, **C**, and **D** identified according to NMR and mass spectra are illustrated in Scheme 1.

Optical Rotation Activities of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$ and Its Degradants

The optical rotations of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$, **A**, **B**, **C**, and **D** were measured at 25 °C in 95% ethanol at a wavelength of 365 nm. The cell length was 1.0 cm. The concentrations of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$, **A**, **B**, **C**, and **D** used for the measurement were 1.3, 0.76, 0.76, 0.15, and 1.3 mg/mL, respectively. The specific optical rotations of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$, **A**, **B**, **C**, and **D** were −64.2, −7.37, + 60.5, +130.0, and +70.4, respectively.

Effects of Ascorbic Acid and EDTA on the Stability of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$

To determine the effects of ascorbic acid on the stability of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$, the compound (1.3 mM) was dissolved in a mixed solvent of 2% EtOH and 98% normal saline (pH 2.8, adjusted with hydrochloric acid) with and without 5.7 mM ascorbic acid. The solutions were sealed in ampules and incubated at 25 °C. The samples were analyzed after 5 and 14 days, respectively. The results are given in Table 1.

To determine the effect of EDTA on the stability of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$, the compound (1.3 mM) was dissolved in a mixed solvent of 2% EtOH and 98% normal saline (pH 6.9) with and without 2.97 mM EDTA. The solutions were sealed in ampules and incubated at 25 °C. The samples were analyzed periodically over 8 months. The results are shown in Figure 5.

RESULTS AND DISCUSSION

The major degradation pathway of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$ seen in early attempts to formu-

Table 1. Effect of Ascorbic Acid on the Stability of 2-Pyz-(CO)-Phe-Leu-B$(OH)_2$ in 2% EtOH and 98% Normal Saline (pH 2.8 with HCl) at 25 °C

Time (day)	% Drug Remaining (without ascorbic acid)	% Drug Remaining (with 5.7 mM ascorbic acid)
0	100[a]	100[a]
5	96	83
14	94.1	78.2

[a] The initial drug concentration was 1.3 mM.

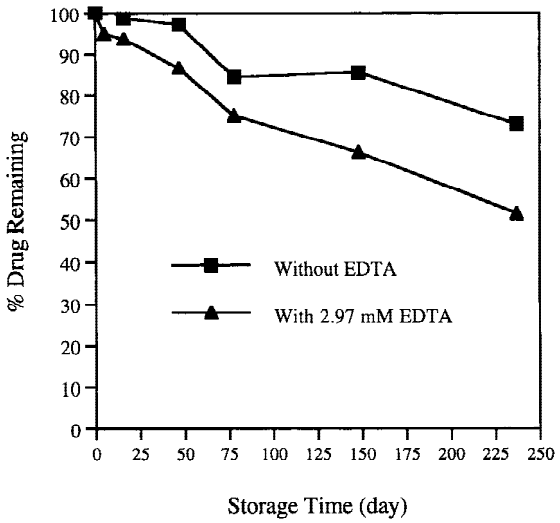


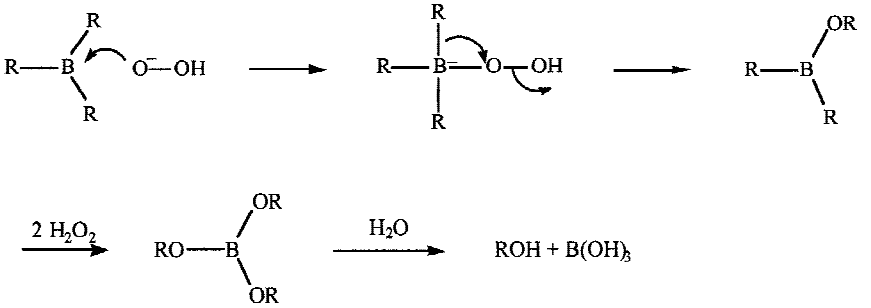
Figure 5. Plot showing the effect of EDTA on the stability of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ (1.3 mM) at 25 °C. The solvent was 2% EtOH and 98% normal saline (pH 6.9) with and without 2.97 mM EDTA.

late the drug for parenteral administration (as discussed earlier) seemed to be oxidative in nature. This pathway was suggested by the fact that treatment of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ with hydrogen peroxide generated **A**, the major degradation product seen in PEG300:EtOH:H_2O (40:10:50) and other solutions.

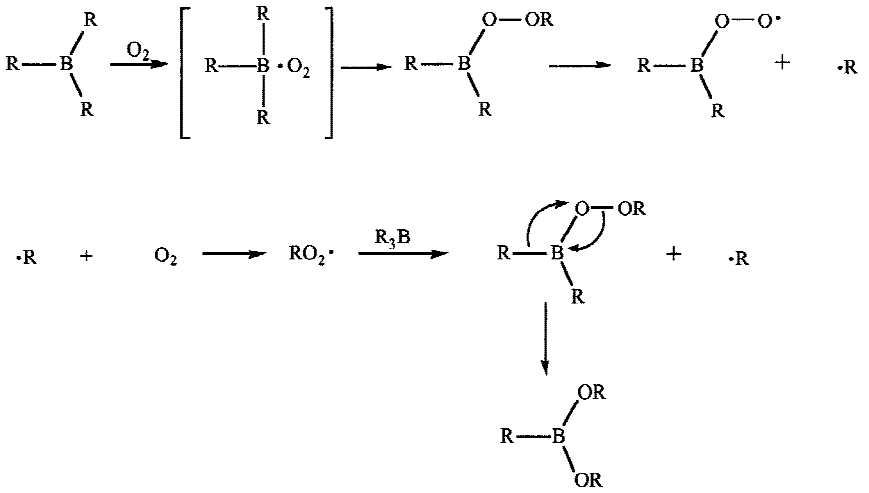
The identity of degradant **A**, characterized by ^{1}H NMR and exact mass information, was consistent with a known mechanism (Scheme 2) for the reaction of alkyl boranes with peroxides, including hydrogen peroxide.[8] Trialkyl boranes react with peroxides to give esters of boric acid. These esters then hydrolyze to alcohols and boric acid. This mechanism results in retention of configuration of the carbon to which the boron atom was attached. Therefore, the α-carbon of the pseudo leucine in **A** should have the same configuration as 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$. The α-carbon of phenylalanine in **A** also appeared to retain its configuration or experience little racemization during the oxidation because **C**, which was derived from **A**, was also optically active. Degradant **B** has a similar ^{1}H NMR spectrum and a molecular weight identical to that of degradant **A**, suggesting that **A** and **B** are diastereomers; it was probably the chiral center of the pseudo leucine that was converted to the opposite configuration when **A** isomerized to **B** under acidic conditions. A mechanism consistent with the isomerization is the reversible loss and addition of a water molecule, as illustrated in Scheme 1.

The oxidation of alkyl boranes, which gives the ester of boric acid, can also be due to reaction with alkyl peracids, alkyl peroxides, or oxygen radical species.[6] The mechanism of oxidation by oxygen involves a radical chain reaction, which is different from that seen in the reaction with peroxides. A possible mechanism for the radical chain reactions was summarized from the literature and is illustrated in Scheme 3.[9–11] Because the oxidation by oxygen involves radical reactions, the α-carbon to which boron is attached racemizes ~ 20%. Therefore, the degree of initial isomerization might allow one to differentiate the probable contributions from peroxides and oxygen.

Based on the known chemistry of boronic acids and the identity of the degradants, a degradation pathway of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ was proposed and is illustrated in Scheme 1. The initial oxidation can be attributed to peroxides or molecular oxygen and its radicals. Because light, metal ions, and alkaline conditions normally facilitate oxidation, these conditions should not be favorable to the stability of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ or any other alkyl boronic acid derivative. Consistent with this conclusion is the observation that light accelerated the degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$.[12]



Scheme 2. The mechanism for oxidation of alkyl boranes by hydrogen peroxide.[8]



Scheme 3. The mechanism for oxidation of alkyl boranes by oxygen.[9–11]

The degradation kinetics of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ at pH 1.0 and 12 and 70 °C are illustrated in Figure 4. The half-lives of the degradation at pH 1.0 and 12.0 were 10 days and 2.5 h, respectively. The major degradation product under both acidic and basic pH conditions was **D**. Compound **C** was also observed during the initial time points in the study, but eventually hydrolyzed to **D**. The presence of **C**, which was probably derived from the oxidative degradation products **A** and **B**, provides support that degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ under acidic or basic pH conditions is mediated through initial loss of the boronic acid group via oxidation. Presumably, peroxide species or dissolved oxygen caused the initial oxidative cleavage of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ to **A** and **B**, which were rapidly converted to **C**. This reaction was confirmed when authentic samples of **A** and **B** were exposed to conditions of pH 1 and they were immediately converted to **C**, which then began to form **D**. Degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ is more rapid at basic pH than at acidic pH, because of catalysis of the initial oxidative cleavage by hydroxide.[6] The stability of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ in acidified propylene glycol:ethanol:water (50:10:40) was much greater than in the corresponding non-acidified solvent.[12]

Compound **D** was optically active, suggesting that the chiral center of phenylalanine in 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ did not undergo any significant racemization even under acidic and basic conditions at 70 °C. Degradation under acidic and basic conditions mediated through oxidation was incorporated into the oxidative degradation and is illustrated in Scheme 1.

Because the major degradation pathway of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ was oxidative in nature, and the product appeared to be optimally stable under acidic conditions, ascorbic acid was added to the formulation mixtures. The results suggested that ascorbic acid actually accelerated the degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ (Table 1). After storage for 14 days, the sample containing 0.1% of ascorbic acid experienced 21.8% degradation, whereas the sample containing no ascorbic acid showed only 5.9% degradation. Degradants **A**, **B**, **C**, and **D** were observed in both samples based on both identical retention times and co-injection studies with standards. The apparent accelerated degradation of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ in the presence of ascorbic acid may have been due to production of hydrogen peroxide. Production of hydrogen peroxide from oxygen is accelerated when both ascorbic acid and transition metal ions are present (i.e., when ascorbic acid can act as a prooxidant).[13] Metal ions such as Cu^{2+} Fe^{3+}, or Zn^{2+} in the formulation mixtures could have come from the solvent or from tightly bound metal ions in the starting material, 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$; they may have also leached from the glass containers. EDTA was added to the formulation mixture of 2-Pyz-(CO)-Phe-Leu-B(OH)$_2$ at pH 6.9 to chelate the possible contaminant metal ions and see if its presence could reduce the oxidation caused by molecular oxygen. The results showed that the formulation containing 2.97 mM EDTA consistently degraded faster than the sample that did not contain EDTA over 8 months storage (see Figure 5); that is, the added EDTA appeared to accelerate the hydrolysis of 2-Pyz-(CO)-Phe-Leu-

$B(OH)_2$. EDTA has been reported to either stimulate or inhibit metal-catalyzed oxidation of amino acids and proteins, depending on the ratio of the concentration of EDTA to the transition metal ions.[14]

In summary, the peptide boronic acid derivative 2-Pyz-(CO)-Phe-Leu-$B(OH)_2$ appeared to be susceptible to oxidative degradation under a number of experimental conditions. The degradation was definitely accelerated by peroxide and perhaps molecular oxygen.

ACKNOWLEDGMENTS

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