Specificity, Stability, and Potency of Monocyclic β-Lactam Inhibitors of Human Leucocyte Elastase

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ABSTRACT: Stable, potent, highly specific, time-dependent monocyclic β-lactam inhibitors of human leucocyte elastase (HLE) are described. The heavily substituted β-lactams are stable under physiological conditions including in the presence of enzymes of the digestive tract. The β-lactams were unstable in base. At pH 11.3 and 37 °C they were hydrolyzed with half-lives of 1.5–2 h. Hydrolysis produced characteristic products including the substituent originally at C-4 of the lactam ring, a substituted urea, and products resulting from decarboxylation of the acid after ring opening. The most potent β-lactam displayed only 2-fold less activity versus HLE than α_1PI, the natural proteinaceous inhibitor. The compounds were more potent against the human and primate PMN elastases than versus either the dog or rat enzymes. Differences in the structure–activity relationships of the human versus the rat enzymes suggest significant differences between these two functionally similar enzymes. The specificity of these compounds toward HLE versus porcine pancreatic elastase (PPE) is consistent with the differences in substrate specificity reported for these enzymes [Zimmerman & Ashe (1977) *Biochim. Biophys. Acta 480,* 241–245]. These differences suggest that the alkyl substitutions at C-3 of the lactam ring bind in the S_1 specificity pocket of these enzymes. The dependence of the stereochemistry at C-4 suggests additional differences between HLE and PPE. Most of the compounds do not inhibit other esterases or human proteases. Weak, time-dependent inhibition of human cathepsin G and α-chymotrypsin by one compound suggested a binding mode to these enzymes that places the N-1 substitution in the S_1 pocket.

Human leucocyte elastase (HLE, EC 3.4.21.37) is a glycoprotein found in the azurophilic granules of neutrophils (Dewald et al., 1975). The enzyme is released from neutrophils upon stimulation by inflammatory agents (Bonney et al., 1989). HLE is a potent serine protease with the ability to degrade a number of structural proteins as well as elastin. The degradation of connective tissues is evident in the pathogenesis of a number of disease states. Therefore, a role for elastase has been suggested for diseases such as emphysema (Kaplan et al., 1973; Powers, 1983) and atherosclerosis (Travis et al., 1980), and the selective inhibition of HLE could relieve the progression of these diseases.

A number of low molecular weight inhibitors of HLE have been reported [for a review, see Stein et al. (1985)]. Unfortunately, many of these are nonspecific inhibitors of other serine proteases as well [for example, see Hemmi et al. (1985)]. Peptide-based inhibitors such as the trifluoromethyl ketones are somewhat more specific but suffer from a lack of oral availability in animal models (Williams et al., 1991). A class of cephalosporin derivatives were found to be potent inhibitors of HLE (Doherty et al., 1986). Bonney et al. (1989) reported that L-659,286, a member of this class, displayed a favorable pharmacological profile when instilled intratracheally. From mechanistic studies, Knight et al. (1992b) concluded that this class of inhibitors could be classified as mechanism-based inhibitors of HLE. Unfortunately, the β-lactams in these studies suffered from stability problems (Knight et al., 1992b) and a lack of oral availability in animal models. Knight et al. (1992a) recently reported that simple monocyclic β-lactams were also mechanism-based inhibitors of HLE. But, these β-lactams were relatively nonspecific and displayed similar potency versus HLE and Cat-G.[1] Doherty et al. (unpublished data, 1992) described a potent, orally active monocyclic β-lactam inhibitor of HLE. We have examined the stability and specificity of a series of these monocyclic β-lactams.

EXPERIMENTAL PROCEDURES

Materials. MeOsucc-AAPV-pNA[2] was purchased from Calbiochemical Co. Succ-AAPA-pNA, FAGLA, N^{α}-*t*-Boc-A-*p*-nitrophenyl ester and MeOsucc-AIPM-pNA were pur-

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[1] Abbreviations: α_1PI, α_1-proteinase inhibitor; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Cat-G, cathepsin G; CTL, cytotoxic T-lymphocytes; DMSO, dimethyl sulfoxide; E-64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; Fut-175, 6-amidino-2-naphthyl-4-guanidinobenzoate dihydrochloride; HLE, human leucocyte elastase; PMN, polymorphonuclear neutrophils or leucocytes; PPE, porcine pancreatic elastase; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

[2] Peptide-based substrates and inhibitors are abbreviated using the standard one-letter representation of the amino acids. Additional functionalities present were abbreviated as follows; Boc, butoxycarbonyl; FAGLA, *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide; MeOsucc, methoxysuccinyl; pNA, *p*-nitroanilide; succ, succinyl; tosyl, *p*-toluenesulfonyl; Z, carbobenzoxy.

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chased from Chemical Dynamics Corp. *N*-Succ-AAPF-pNA was purchased from Bachem. Benzoyl-R-pNA and tosyl-GPR-pNA were obtained from Boehringer Mannheim. The synthesis of the compounds listed in Table I and [4-^{13}C]-L-680,833 will be described elsewhere.[3] All substrate stock solutions were prepared in DMSO. Peptide *p*-nitroanilide stock concentrations were either determined from the absorbance at 315 nm (ϵ = 15 400) or by complete hydrolysis to free *p*-nitroanilide (ϵ = 9350). Buffers (Sigma Chemical Co.) were titrated to the appropriate pH with either NaOH or HCl prior to use. All enzyme assays were conducted at 25 °C unless noted.

Human neutrophil elastase, cathepsin G, trypsin, α-chymotrypsin, pancreatic elastase 2, and α_1PI were purchased from Athens Research and Technology, Inc. In early experiments human Cat-G was prepared according to the method of Ashe et al. (1981). Human CTL chymase and granzyme A were gifts from M. Poe of these laboratories. Human stromelysin was obtained from Celltech Ltd. Human thrombin, plasmin, papain, thermolysin (*Bacillus thermoproteolyticus*), and acetylcholine esterase (*Electrophorus electricus*) were purchased from Boehringer Mannheim. Fut-175 and E-64 were obtained from Torii and Sigma Chemical Co., respectively. PPE was obtained from Serva Chemical Co. Bovine α-chymotrypsin and bovine trypsin were purchased from Worthington. The mixture of human pancreatic elastases (1 and 2) was prepared according to the method of Largmann et al. (1976). Human sputum elastase and bovine neck ligament elastin (catalog no. ES60, prepared by the neutral extraction method) were purchased from Elastin Products. The methods of Green et al. (1991) were used to pretreat the elastin and to prepare the isozymes of HLE.

PMN elastases from rat, dog, chimpanzee, and African green, cynomolgus, and rhesus monkeys were obtained in the form of granule extracts and used without further purification. The granule extracts were prepared from PMN's isolated from whole blood according to a modification (Green et al., 1991) of the procedure of Feinstein and Janoff (1975). The Michaelis constants for the hydrolysis of MeOsucc-AAPV-pNA by rhesus and chimpanzee granule extracts were determined by varying the substrate concentration from 0.03 to 2 mM in buffer A (45 mM Tes at pH 7.5, 450 mM NaCl in 10% DMSO).

Human pancreatic elastase 2 was assayed spectrophotometrically with 0.2 mM succ-AAPL-pNA in buffer B (45 mM TES at pH 7.5, 135 mM NaCl in 10% DMSO). The other enzymes were assayed in a 96-well plate format in 0.2 mL of buffer B except where noted with conditions as follows: Thrombin and trypsin were assayed with 0.2 mM tosyl-GPR-pNA, plasmin was assayed with tosyl-GPK-pNA, and cathepsin G and α-chymotrypsin were assayed with 0.2 mM succ-AAPF-pNA as substrates. Fut-175 (1, 5, and 20 μg/mL) was used as a positive control for inhibition of serine proteases (Fujii & Hitomi, 1981). Papain was assayed with 0.2 mM benzoyl-R-pNA in buffer B except 10 mM EDTA and 5 mM cysteine were added. E-64 (1, 5, and 20 μg/mL) was used as a positive control for inhibition of papain (Barrett et al., 1982). Acetylcholinesterase was assayed with 0.2 mM *p*-nitrophenyl ester in 0.2 mL of 90 mM potassium phosphate at pH 7.8. Acetylcholine chloride (3, 30, and 300 μg/mL) was used as a positive control for inhibition of the esterase. The 96-well plates were read every minute for 20 min at 405 nm. Stromelysin (Harrison et al., 1989; Teahan et al., 1989) and the human CTL proteases (Poe et al., 1992) were assayed according to published procedures. Thermolysin was assayed spectrophotometrically at 345 nm using 1.5 mM FAGLA in 5% DMF and 0.1 M HEPES at pH 7.2.

The concentration of HLE stock solutions was determined on the basis of the activity versus 1 mM MeOsucc-AAPV-pNA in 1 mL of buffer A at 25 °C. The turnover number under these conditions reported by Green et al. (1991) was 472 ± 30 min^{-1}. The activity (k_{inact}/K_i) of inhibitors versus HLE-catalyzed hydrolysis of either 0.2 mM succ-AAPA-pNA or 1 mM MeOsucc-AAPV-pNA in 1 mL of buffer A at 25 °C was determined at inhibitor concentrations that produced greater than 90% inhibition over either 15 or 7.5 min. The second-order rate constants for the inhibition of PPE (0.04 μg) were determined similarly with 0.2 mM succ-AAPA-pNA. The second-order rate constants for the inhibition of human Cat-G (5 μg) and α-chymotrypsin (0.18 μg) by L-683,845 were determined spectrophotometrically with 0.2 mM succ-AAPF-pNA in 1 mL of buffer A (Knight et al., 1992a). The concentrations of L-683,845 were 20 and 4.1 μM, respectively.

The elastase-catalyzed solubilization of elastin was determined by a modification (Green et al., 1991) of the procedure of Ardelt et al. (1970). In a typical experiment 7 mL of 1.5 mg/mL elastin in 50 mM TES at pH 7.5 and 42 μg/mL HLE were incubated at 37 °C for 60 min. The reactions were initiated with HLE. One-milliliter aliquots were removed every 10 min and filtered through Millipore Millex GV filters. The optical density at 276 nm was determined. The change in absorbance was linear with time over 60 min. The rate of solubilization of elastin was determined by a linear least-squares fit to the data. The effect of monobactams on this reaction was determined by varying their concentration in the incubation mixture. The percent inhibition is a reflection of the decrease in the slope in the presence of inhibitor divided by the control slope.

HPLC was conducted on either 250 × 4.6 or 150 × 4.6 mm C-18 columns eluted with either gradient A (80:10:10 to 10:80:10 H_2O–AcCN–CH_3OH in 0.1% acetic acid) or gradient B (45:45:10 to 10:80:10 in 0.1% acetic acid). The eluant was monitored at either 223 or 250 nm by on-line UV–visible detection. The stability of L-669,844, L-670,258, L-680,831, and L-680,833 was monitored at 37 °C in 10% DMSO and an ionic strength (controlled with NaCl) of 200 mM at pH 7.3 and 11.3 with 50 mM sodium phosphate by HPLC. In typical experiments 1.2 mM inhibitor was incubated, and 5–10-μL aliquots were removed over time. The half-lives for the disappearance of parent and liberation of the C-4 substituent were calculated by fitting the peak areas to eq 1. The UV–visible spectra of the base-catalyzed decomposition products were determined by HPLC with a Waters 990 diode array detector after quenching by lowering the pH to 7.7 with HCl at 25 °C. The buffer dependence of the stability of L-670,258 was examined in 50 mM TES and 50 mM imidazole at pH 7.4 and 50 mM CAPS at pH 11.3.

The new products observed during base-catalyzed hydrolysis of the β-lactams were purified by HPLC. In a typical synthesis the hydrolysis product from L-670,258 (U-1) was synthesized from 8 mg of L-670,258 by incubating at 37 °C in 4 mL of 100 mM Na_3PO_4 at pH 11.3. The progress of the reaction was monitored using gradient B. After 2 h the reaction which had become cloudy was cooled to 25 °C and the pH adjusted to 6.8. The reaction solution was centrifuged, and the supernatant which was enriched in L-670,258, *N*-(β-naphthylmethyl)urea, and *p*-hydroxybenzoic acid was decanted.

[3] For an example, see EPO O 337 549.

The precipitate enriched in U-1 was dissolved in 20% DMSO, 36% AcCN, 8% CH_3OH, and 36% H_2O and purified by HPLC on gradient B. The fractions containing U-1 were pooled and solvents removed by rotary evaporation under reduced pressure and lyophilization. The hydrolysis product (1.2 μmol) derived from L-680,833 (2.1 μmol) was prepared similarly except the incubation time was 6 h. A 0.7-μmol sample of ^{13}C-labeled hydrolysis product was produced from 1.1 μmol of [4-^{13}C]-L-680,833 in similar fashion.

The new compounds were analyzed by electron impact mass spectrometry recorded on a Finnigan-MAT TSQ70B or MAT212 instrument at 70 eV. Exact mass values were obtained using the peak matching method with perfluorokerosene as the internal standard. U-1 was examined by ^1H NMR data collected on a Bruker AM500. The solvent was CD_3CN with external TMS as a standard. The proton NMR spectrum of U-4 was obtained on a Varian XL400 and referenced to CH_3CN (1.93 ppm).

Two-dimensional $^1H\{^{13}C\}$ HMQC spectra (Bax et al., 1983) of U-4 were collected on a Bruker AM500 spectrometer operating at 125 MHz for ^{13}C and 500 MHz for ^1H. GARP (Shaka et al., 1985) decoupling during the acquisition time was achieved by using a BSV-3 X-nucleus decoupler equipped with a hardware modulator from Tschudin Associates. The decoupling field strength was 4000 Hz. The ^1H sweep width was 4854.37 Hz, and the data size was 4096 real data points (acquisition time of 0.421888 s); the ^{13}C sweep width was 20000 Hz, and the data size was 256 real data points (acquisition time of 640 μs). Quadrature detection in the ^{13}C dimension was obtained by the TPPI method (Marion & Wuthrich, 1983). The relaxation delay was 2 s, and the delay time in HMQC for the buildup of antiphase ^1H–^{13}C coherence was 2.87 ms. The digital resolution in the transformed spectra was 2.37 Hz/point in the ^1H dimension and was 78.125 Hz/point in the ^{13}C dimension. The temperature of the sample was 25 °C.

The acid stability of L-680,833 and L-680,831 was examined at 37 °C in 10% DMSO and an ionic strength of 200 mM at pH 2.0. After 18 h at pH 2.0 an aliquot of the L-680,833 solution was assayed for activity versus HLE. This was compared to fresh stock solutions prepared in pH 2 phosphate and DMSO.

The stability of L-680,833 and L-683,845 was examined under conditions expected in the GI tract (*U.S. Pharmacopeia*, 1989). The GI fluid mimic (*GIfm*) with a final pH of 1.2 contained 2 g of NaCl, 3.2 g of pepsin, and 7 mL of HCl in 1 L of H_2O. The intestinal fluid mimic (Ifm) contained 0.87 g of monobasic potassium phosphate, 0.038 N NaOH, and 10 g of pancreatin. The pH was adjusted to 7.5 with 0.2 N NaOH for a final volume of 1 L. Then 0.4 mM L-680,833 or L-683,845 was dissolved in 1 mL each of GIfm and Ifm, and the mixture was incubated for 3 days at 37 °C. Aliquots were removed over time and filtered through Millipore LGC filter cups (10 000 MWCO), and 10-μL aliquots were injected.

UV–visible spectroscopy was conducted on a Varian DMS-300 spectrophotometer or on a Waters 990 diode array detector for chromatographic separations. The hydrolysis of peptide *p*-nitroanilides was monitored at 410 nm. Kinetic data were collected either on Cary 210 and Varian DMS-300 spectrophotometers or on an AVIV-14DS spectrophotometer equipped with a Zymark robot for sample preparation and a Hewlett-Packard 900/320 series computer for data analysis and storage.

DATA ANALYSIS

Linear kinetic data were fit by nonlinear regression to eq 2 to obtain the initial rates. The initial rates as a function of substrate concentration were fit by nonlinear regression to eq 3 to determine the kinetic constants toward substrate for the rhesus and chimpanzee enzymes. The nonlinear progress curves observed with time-dependent inhibitors of HLE were fit to eq 4 (Morrison & Walsh, 1988) to obtain the first-order rate constant k_{obs}. The first-order rate constant for the inhibition of 2.65 mM HLE by 20 nM α_1PI was determined by fitting the nonlinear progress curve to eq 5 ($a = [E_t]/[I_t]$), which corrects for depletion of inhibitor upon binding to enzyme (Williams & Morrison, 1979). Second-order rate constants (k_{inact}/K_i) were estimated by calculating $k_{obs}/[I]$ and then correcting for the substrate concentration and Michaelis constant according to eq 6.

$$y = ae^{-kt} + c \tag{1}$$

$$Y = v_i X + B \tag{2}$$

$$Y = V_m[S]/(K_m + [S]) \tag{3}$$

$$Y = v_s t + ((v_0 - v_s)(1 - e^{-k_0 t})/k_0) + A_0 \tag{4}$$

$$Y = v_s t + ((1 - a)(v_0 - v_s)/k_0 a) \ln ((1 - ae^{-k_0 t})/(1 - a)) + A_0 \tag{5}$$

$$k_{obs}/[I] = k_{inact}/(K_i(1 + [S]/K_m)) \tag{6}$$

RESULTS

Stability of β-Lactams. In Figure 1 a representative chromatogram of L-670,258 after incubation in phosphate buffer at pH 11.3 and 37 °C is shown. L-670,258 disappeared with a $t_{1/2}$ of 118 ± 10 min, and *p*-hydroxybenzoate appeared with a half-life of 110 ± 5 min at pH 11.3 (see Figure 2). We observed the production of 98% and 36% of the theoretical amount of *p*-hydroxybenzoate and *N*-(β-naphthylmethyl)urea, respectively. In addition a new species (U-1) with a retention time intermediate between L-670,258 and the urea was observed. The UV spectra of L-670,258 displays maxima at 224 and 250 nm (not shown) while the urea and U-1 absorb maximally at 222 and 224 nm, respectively (see Figure 3). *p*-Hydroxybenzoic acid absorbs maximally at 250 nm (not shown). U-1 and the urea lack the 250-nm absorption band characteristic of the *p*-hydroxybenzoate. The amount of U-1 present was maximal at 83 min and decreased with time after 188 min. At 165 h 37% of the maximal amount was still present. Since the amount of urea did not continue to increase, it is unlikely that U-1 converts to the urea. The decrease in U-1 with time may have resulted from precipitation (see Experimental Procedures).

The mass spectrum of U-1 displayed a parent ion of 282 amu. The ^1H NMR spectrum of U-1 was similar to the L-670,258 spectrum except in the aromatic region (data not shown) due to the loss of the elements of benzoic acid and the region around 6 ppm (see Figure 4). The ^1H NMR spectrum of L-670,258 displayed an N–H proton resonance at 5.9 ppm, broadened due to the quadrupolar nitrogen (data not shown). This effect presumably masks the observation of coupling of this proton to the benzylic methylene hydrogens. The spectrum of U-1 in this region displayed two broad resonances at 5.8 and 6.8 ppm, consistent with quadrupolar broadening by urea nitrogens. The doublet at 6.5 ppm with a coupling constant of 9.8 Hz is consistent with a vinyl proton (H_a) coupled to the urea proton (H_b). In this case the large coupling constant between the vinyl proton and the urea proton is evident in the

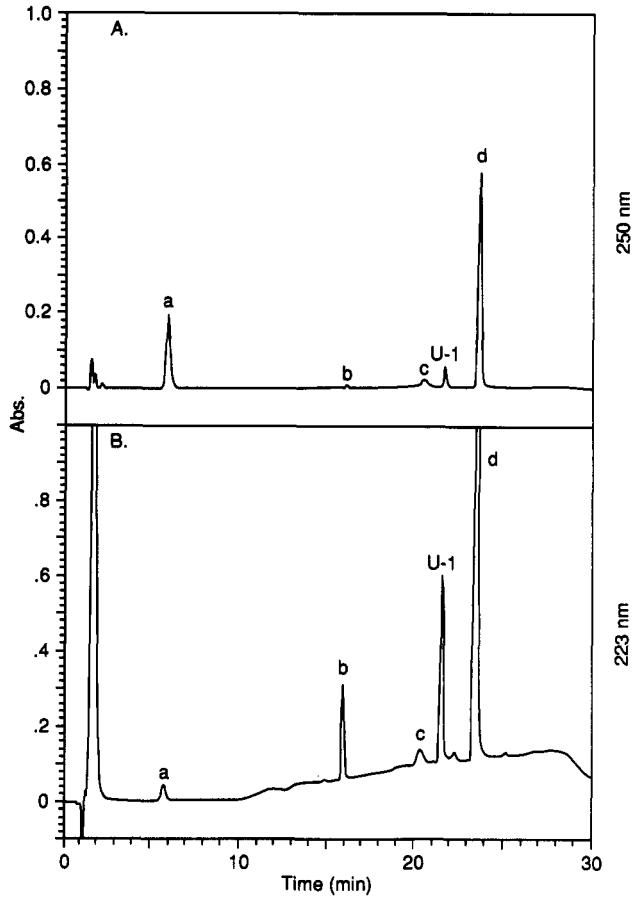


FIGURE 1: HPLC chromatogram of the base-catalyzed hydrolysis of the L-670,258 reaction after quenching: (A) detection at 250 nm; (B) detection at 223 nm. Peaks: a is *p*-hydroxybenzoic acid, b is *N*-(β-naphthylmethyl)urea, c is a gradient artifact as it is observed when only buffer is injected, d is L-670,258, and U-1 is a new species, not observed in control reactions.

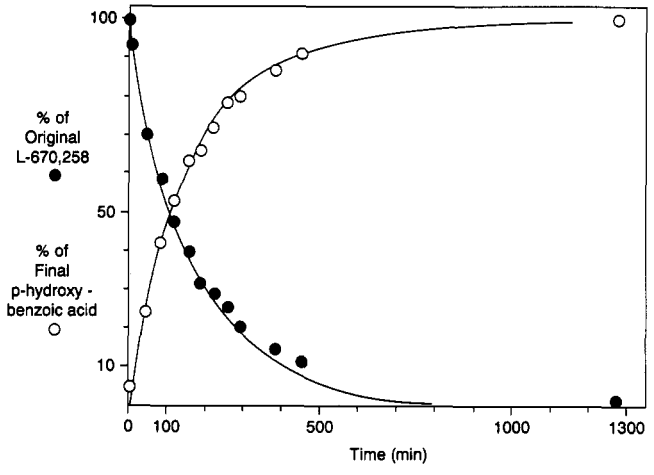


FIGURE 2: Loss of L-670,258 (●) and appearance of *p*-hydroxybenzoic acid (○) as a function of incubation time at 37 °C and pH 11.7.

line shape and line width of the resonance at 6.8 ppm. The spectral data are consistent with the structure shown in Figure 5 for U-1.

L-670,258 was stable at pH 7.3 and 37 °C for greater than 7 days. In phosphate buffer at pH 7.3, 2% of the theoretical amount of *p*-hydroxybenzoate and small amounts of both the *N*-(β-naphthylmethyl)urea (≤1% of the theoretical amount) and U-1 were produced. After 7 days in Tes or imidazole at pH 7.3 only small amounts of *p*-hydroxybenzoic acid (≃1% of theoretical) and the urea (≃1%) were evident.

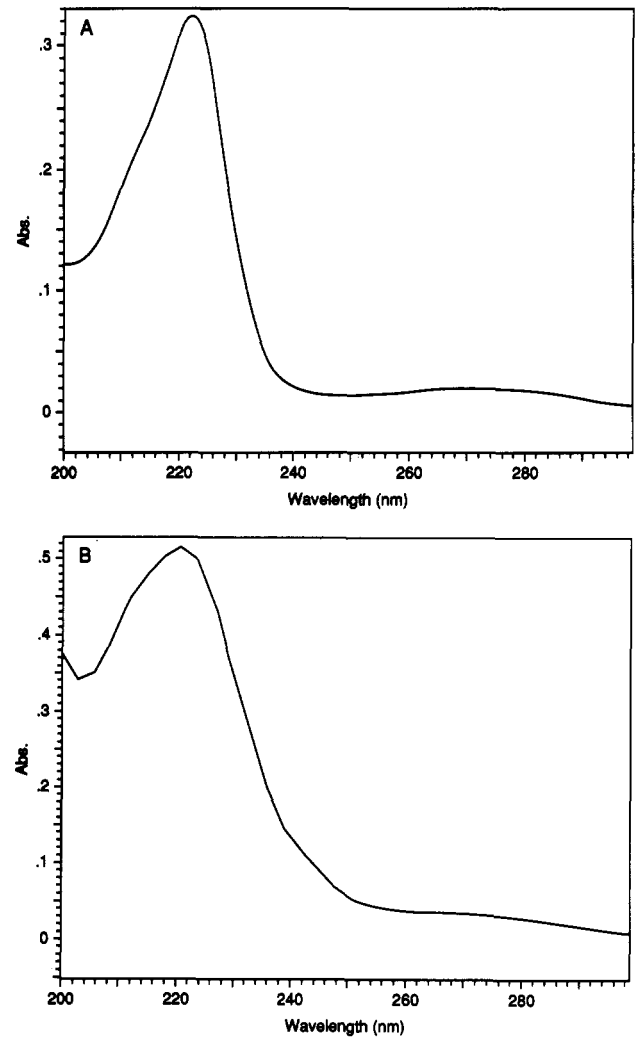


FIGURE 3: UV–visible spectra of (A) *N*-(β-naphthylmethyl)urea and (B) U-1.

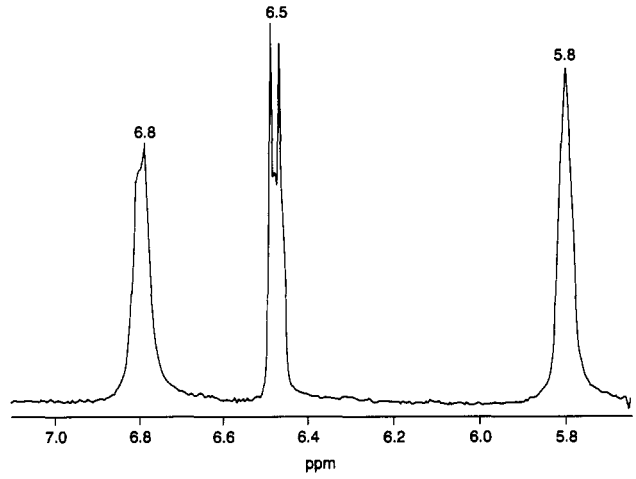


FIGURE 4: ^{1}H NMR spectrum of U-1 in the region of 6 ppm.

Base-catalyzed decomposition of L-669,844 also produced an unknown (U-2) with a retention time relative to the *N*-benzylurea and the parent compound similar to that observed for the L-670,258 reaction (data not shown). On the basis of the L-670,258 spectral data we predict the structure in Figure 5 as the identity of U-2. The half-life for the decomposition of L-669,844 based upon loss of compound was 126 ± 7 min at 37 °C and pH 11.3. There was no measurable decomposition of L-669,844 at pH 7.3 and 37 °C

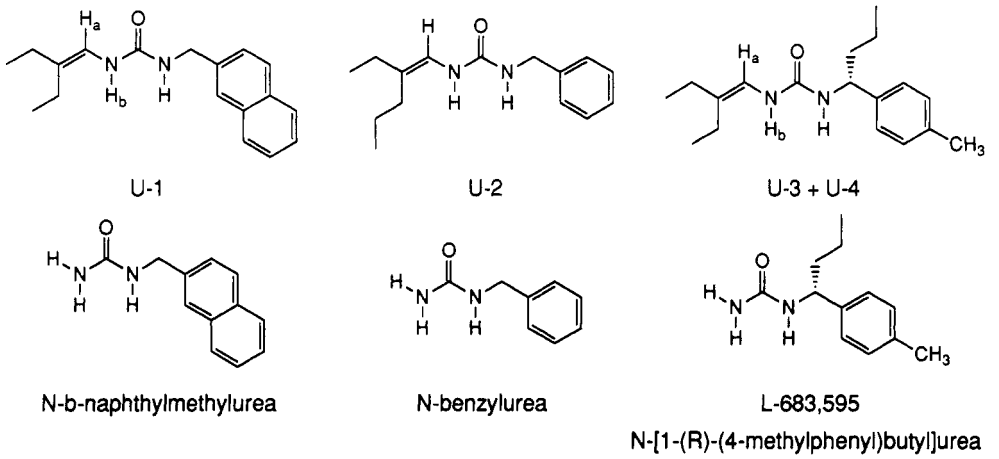


FIGURE 5: Structures of the substituted ureas and base-catalyzed decomposition products produced from L-669,844, L-670,258, L-680,831, and L-680,833.

after 15 h. After 13 days there was a small amount of U-2 (4.6%) present at this pH.

We observed no significant decomposition of L-680,833 at pH 7.5 after 23 days at 37 °C. This compares to a half-life of 76 ± 10 min based upon loss of compound under identical conditions at pH 11.3. Under these conditions *p*-hydroxyphenylacetic acid appeared with a half-life of 104 ± 18 min. L-680,831 decomposed with a half-life of 97 ± 12 min, producing *p*-hydroxybenzoate with a half-life of 107 ± 21 min. The decomposition of both L-680,831 and L-680,833 produced *N*-[1(*R*)-(4-methylphenyl)butyl]urea (L-683,595) and a new species with identical retention times (U-3 and U-4). On the basis of the extinction coefficient (area) at 223 nm for L-683,595 the maximal amount of U-3 and U-4 in each reaction was 2-fold greater than the urea produced. The UV spectra of these unknowns were similar to the urea spectrum (data not shown). The ^{1}H NMR spectrum of U-4 displayed broad resonances at 6.3 and 5.2 ppm and a doublet at 6.0 ppm with a coupling constant of 11.3 Hz.[4] The ^{13}C and proton chemical shifts of ^{13}C-labeled U-4 obtained from a proton-detected carbon spectra were 141 and 6 ppm, respectively. J_{C-H} and J_{H-H} were 173 and 12 Hz, respectively. The 2-D spectrum confirms the assignment of the doublet as the proton on the carbon originally at C-4 of the β-lactam ring. The mass spectra of U-4 and ^{13}C-labeled U-4 displayed parent ions of 288 and 289 amu, respectively. The spectral data are consistent with the structure presented in Figure 5 for U-4. L-680,831 and L-680,833 are equivalent except for the group at C-4; therefore, U-3 is likely the unknown produced from L-680,831.

We found both L-680,833 and L-680,831 to be stable for greater than 7 days (no measurable decomposition) at pH 2.0 in 50 mM phosphate (10% DMSO) at 37 °C. The second-order rate constant versus HLE obtained from stock solutions of L-680,833 incubated at 37 °C and at pH 2.0 for 18 h was identical to that obtained from a fresh solution (pH = 2.0) or a stock solution stored in DMSO alone. L-680,833 and L-683,845 were stable to the conditions and proteases expected in both the GI tract and the intestine for greater than 48 h.

β-Lactam Inhibition of HLE. A typical progress curve of the hydrolysis of MeOsucc-AAPV-pNA by 6 nM HLE in the presence of L-680,831 is shown in Figure 6. Control curves in the absence of inhibitor were linear. The inhibitor displayed time-dependent inhibition of HLE. From these data we obtain k_{obs} and could estimate k_{inact}/K_i. The rate constant did not vary with enzyme concentration (data not shown) or the identity of the HLE isozyme. The other compounds listed in Table I also displayed time-dependent inhibition of HLE. The second-order rate constants are reported in Tables II and III. α_1PI inhibited HLE with a second-order rate constant of 2.2 × 10^6 M^{-1} s^{-1} under these conditions.

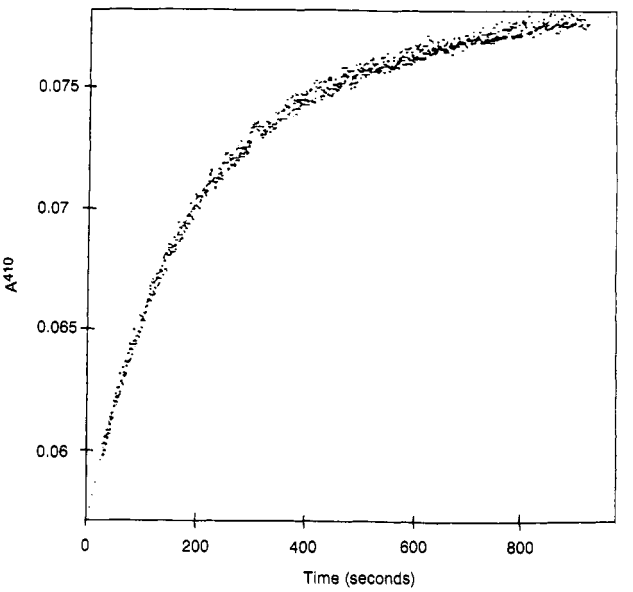


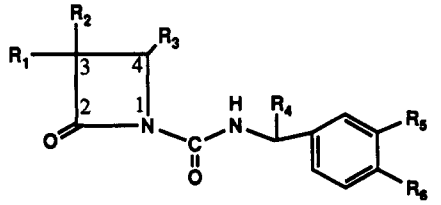
FIGURE 6: Progress curve of the hydrolysis of 1 mM MeOsucc-AAPV-pNA in the presence of 80 nM L-680,831. The HLE concentration was 6 nM.

Monolactam Inhibition of HLE-Catalyzed Elastinolysis. The hydrolysis of elastin in the presence and absence of L-680,833 is presented in Figure 7. Similar data were obtained with L-683,845, L-680,831, and α_1PI. In Table III the data are summarized. The β-lactams and α_1PI completely inhibit the elastinolysis at a ratio of inhibitor to enzyme of 3:1 to 1:1.

Specificity of β-Lactams. The β-lactams were tested as inhibitors versus PMN elastases from other species. The data are reported and compared to the activities versus HLE in Table II. Chimpanzee and rhesus monkeys displayed Michaelis constants of 0.09 ± 0.006 and 0.37 ± 0.05 mM, respectively, versus MeOsucc-AAPV-pNA. L-680,831 and L-680,833 and several analogs containing dimethyl substitutions at C-3

[4] These proton chemical shifts are relative to 1.93 for the protons of CH_3CN. The apparent difference of 0.5 ppm between the vinyl protons and urea nitrogen protons of U-1 and U-4 may be misleading due to differences in the assignment of the standards. In the spectrum shown in Figure 4, the CH_3CN protons occur at 2.2 ppm.

Table I: Structures of Compounds Used in This Study



L no.	R_1	R_2	R_3	R_4	R_5	R_6
668,651	(*R*)-ethyl	methyl	(*S*)-*p*-hydroxybenzoic acid	H	H	H
669,844	ethyl	(*S*)-propyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-ethyl	H	H
670,258	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	H	–CH(CH)$_2$CH–	
670,449	(*R*)-ethyl	methyl	*p*-hydroxybenzoic acid[a]	methyl	H	H
671,070	ethyl	(*S*)-propyl	(*S*)-*p*-hydroxybenzoic acid	H	–CH(CH)$_2$CH–	
674,085	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-methyl	H	H
674,194	(*R*)-ethyl	methyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-ethyl	H	H
679,723	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-ethyl	H	H
680,414	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-propyl	H	H
680,755	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-CH_2CHCH_2	H	CH_3
680,831	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-propyl	H	CH_3
680,833	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-propyl	H	CH_3
680,860	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-CH_2CHCH_2	–OCH$_2$O–	
680,861	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-propyl	–OCH$_2$O–	
683,845	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetate	(*R*)-propyl	–OCHCH–	
684,248	methyl	methyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-propyl	H	CH_3
684,249	methyl	methyl	(*R*)-*p*-hydroxybenzoic acid	(*R*)-propyl	H	CH_3

[a] The lower R_f, C-4 diasteromer of this compound, based upon the TLC separation of the *tert*-butyl esters of the two diastereomers on silica with 20:80 ethyl acetate–hexane. Similar separations with a number of analogous diastereomeric pairs and the activity of these two C-4 isomers versus HLE suggest that L-670,449 is likely an (*S*)-*p*-hydroxybenzoic acid (S. K. Shah, personal communcation).

Table II: Specificity of Monobactams toward PMN Elastases[a]

	k_{inact}/K_i (M^{-1} s^{-1}) for species						
compd	human	chimp	green monkey	rhesus monkey	cynomolgus monkey	dog	rat
L-669,844	29 000	nd	nd	nd	nd	nd	nd
L-679,723	26 000	nd	nd	nd	nd	nd	3 600
L-680,414	188 000	nd	nd	nd	nd	nd	6 500
L-680,755	664 000	nd	132 000	nd	nd	5 000	23 000
L-680,831	300 000	nd	102 000	nd	nd	4 000	13 000
L-680,860	605 000	nd	nd	nd	nd	nd	26 000
L-680,861	867 000	nd	nd	nd	nd	nd	34 000
L-683,845	848 000	802 000	nd	204 000	nd	7 700	6 400
α_1PI	2 200 000	nd	nd	nd	nd	nd	nd
L-680,833	622 000[b]	864 000	160 000	210 000	222 000	4 100	20 000
L-680,833 (I-2)[c]	622 000						
L-680,833 (I-3)	618 000						
L-680,833 (I-4)	621 000						

[a] k_{obs} was determined at 25 °C, and k_{obs}/I was calculated from the inhibitor concentration. This was used to calculate the reported values according to eq 6. The Michaelis constants for the rat, dog, and green monkey were reported by Green et al. (1991) to be 0.77, 1.0, and 0.26 mM, respectively. The K_m values for the rhesus and chimpanzee enzymes were determined in this study (see text). The standard deviations from two to three determinations were <15% of the reported inhibition constants. nd = not determined. [b] Doherty, unpublished data, 1992. [c] I-2, I-3, and I-4 refer to the HLE isozymes.

(instead of diethyl) of the β-lactam ring were tested as inhibitors of the PPE-catalyzed hydrolysis of succ-AAPA-pNA. In Table IV the results are compared to those obtained versus HLE. L-671,070, containing ethyl–propyl substitution at C-3 did not inhibit PPE at concentrations as high as 0.11 mM.

L-683,845, L-680,755, and L-680,831 were tested as inhibitors of a variety of enzymes. The data are reported in Table V. These three monobactams at 20 ng/mL yielded greater than 97% inhibition of HLE activity versus 1 mM MeOsucc-AAPV-pNA (7-fold greater than K_m). There was no significant inhibition of most of the enzymes tested at inhibitor concentrations as high as 20 μg/mL.[5] The apparent inhibition of thrombin by L-680,831 did not appear to be dose dependent. L-680,831 may be a weak inhibitor of papain although there was no evidence for time dependence in these studies. L-670,449, L-671,070, L-668,651, L-669,844, L-674,194, L-674,085, and L-679,723 did not display significant inhibition[5] of any of the enzymes tested (data not shown). These compounds displayed significant potencies (k_{inact}/K_i) versus HLE ranging from 5000 to 100,000 M^{-1} s^{-1} (Tables II and IV and Shah, Finke, Maycock, and Knight, unpublished data). There was slight inhibition of Cat-G by L-671,070 that appeared to be concentration dependent (3%, 10%, and 17% at 1, 5, and 20 μg/mL). The inhibition of Cat-G and α-chymotrypsin by L-683,845 was significant although weak at 20 μg/mL. The inhibition was time dependent. The second-order rate constants for the inhibition of Cat-G and α-chymotrypsin were 14 and 40 M^{-1} s^{-1}, respectively.

[5] Significant inhibition is defined as greater than 10% inhibition at 20 μg/mL and dose dependent.

DISCUSSION

The monobactams studied in this work are extremely stable at physiological pH and temperature. This is in contrast to

Table III: Effect of Inhibitors on the Solubilization of Elastin by HLE at 37 °C[a]

compd	% inhibition	inhibitor/HLE
L-680,831	94	2.6
	45	1.3
	23	0.65
L-680,833	99	5.2
	98	2.6
	59	1.84
	27	0.92
L-683,845	88	2.14
	62	1.08
	24	0.54
α_1PI	82	1.0[b]
	85	1.0
	59	0.75
	62	0.75
	33	0.5
	17	0.25

[a] The standard errors in the slopes of progress curves such as those in Figure 7 were from 2% to 8%. Therefore, the errors in the percent inhibitions are from 2.3% to 11% of the reported value. [b] Based on a molecular weight of 52 000.

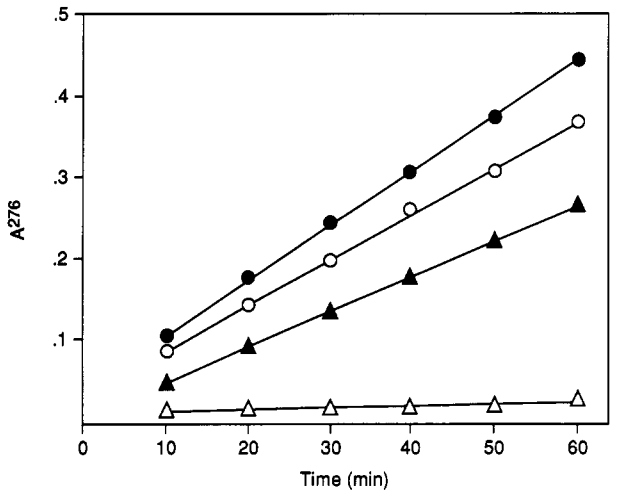


FIGURE 7: Inhibition of HLE-catalyzed solubilization of elastin by L-680,833: (●) no inhibitor; (○) 1.1 μM, (▲) 2.2 μM, and (△) 4.4 μM L-680,833.

the cephalosporin derivatives reported previously (Doherty et al., 1986; Knight et al., 1992b). The stability of these compounds is likely due to both the *N*-carbamoyl substitution and dialkyl substitution at C-2. L-680,833 and L-683,845 were also stable to the conditions and enzymes found in the GI tract. Thus the oral availability of these compounds should not be limited by compound stability.

The monobactams are labile in base. Unexpectedly, they decompose to decarboxylated adducts as well as the urea. The group at C-4 is liberated at a rate similar to the rate of loss of parent. In Figure 8, a plausible mechanism for this process is presented. Initial opening of the β-lactam ring by hydroxide produces the acid, possibly with concerted departure of the group at C-4. Considering the steric hindrance of the neopentyl C-4 carbon, this is an attractive possibility. As presented in Figure 8, the imine acts as the electron sink for decarboxylation. The intermediacy of the imine would explain the production of both the ureas and the vinyl adducts. The imine intermediate could partition between hydrolysis (a) to produce the urea and an aldehyde–acid (2,2-diethyl-3-oxopropanoic acid in the case of L-680,833) or decarboxylation (b) to produce the vinyl adducts. During the interaction of HLE with this class of inhibitors an intermediate along the reaction pathway partitions to produce either a relatively stable enzyme–inhibitor

Table IV: Comparison of the Activity of β-Lactams against PPE and HLE[a]

	k_{inact}/K_i (M^{-1} s^{-1})	
L no.	HLE	PPE
671,070	33 000	no inhibition[b]
680,833	622 000[c]	$K_i \simeq 0.2$ mM[d]
680,831	300 000	37
684,248	160 000	69 000
684,249	6 900	57 000

[a] The standard deviations from two to four determinations were <15% of the reported values. [b] The highest L-671,070 concentration tested was 110 μM. [c] Doherty and Knight, unpublished data, 1992. [d] Only a K_i could be estimated for this compound versus PPE. This was estimated by assuming competitive inhibition.

complex or the release of active enzyme and modified forms of the inhibitor (W. B. Knight, unpublished data). In those studies, approximately two-thirds of the modified inhibitor containing the urea portion of the molecule was released as either the urea or the decarboxylated product. These two species were produced in similar amounts. In addition, a species with both carbon and proton chemical shifts suggestive of the aldehyde of 2,2-diethyl-3-oxopropanoic acid (the aldehyde–acid in Figure 8) was also observed during the enzymatic reaction with [4-^{13}C]-L-680,833. These results suggest that hydrolysis of the L-680,833-derived enzyme–inhibitor complex releases the imine of Figure 8 which then partitions in an analogous fashion to the base-catalyzed nonenzymatic reaction.

As Doherty and Knight (unpublished data) reported for L-680,833, the compounds discussed in this work are potent inhibitors of HLE. The structure–activity relationships will be described in detail elsewhere. L-680,833 displayed identical potency versus the isozymes of HLE. This supports the conclusion by Green et al. (1991) that the isozymes of HLE are catalytically indistinguishable. The second-order rate constants obtained with these compounds versus HLE are in many cases several orders of magnitude greater than similar numbers obtained with either cephalosporin derivatives, peptide chloromethyl ketones (Green et al., 1991), or the simple monocyclic β-lactams reported by Knight et al. (1992a). The most potent β-lactams of this study displayed potency rivaling that of α_1PI, the natural proteinaceous inhibitor of HLE.

The monobactams were also effective inhibitors of the HLE interaction with macromolecular susbstrates. It required slightly more inhibitor to completely inhibit the HLE-catalyzed elastinolysis than was required to completely inhibit the HLE-catalyzed hydrolysis of MeOsucc-AAPV-pNA (Knight et al., unpublished data). While we do not know the significance of this observation, the apparent decrease in efficiency in the presence of elastin may be a result of the depletion of the β-lactams from solution by nonspecific binding to the insoluble elastin. In fact, Doherty et al. (unpublished data) report that L-680,833 has a high affinity for plasma proteins. Alternatively, the presence of the elastin substrate could effect the actual partition ratio between inhibitor hydrolysis and inactivation of the enzyme (Knight et al., unpublished data).

The monobactams are highly specific for PMN elastases and do not significantly inhibit most of the enzymes tested which utilize an active site serine and covalent catalysis such as human thrombin, plasmin, cathepsin G, human pancreatic elastase, bovine pancreatic trypsin, α-chymotrypsin, or acetylcholinesterase. In addition, the compounds did not significantly inhibit proteases from other classes such as the metalloproteases, stromelysin and thermolysin, or the thiol protease, papain. These compounds represent a significant

Table V: Selectivity of Four β-Lactams toward Other Proteases and Esterases[a]

	% inhibition											
	L-680,755 concn (mg/mL)			L-680,831 concn (mg/mL)			L-683,845 concn (mg/mL)			L-680,833 concn (mg/mL)		
enzyme	1	5	20	1	5	20	1	5	20	1	5	20
human												
plasmin	0	0	2	1	0	2	0	0	0	2	1	5
thrombin	8	6	12	13	15	17	0	0	0	9	8	3
Cat-G	0	4	1	8	12	11	13	15	27	0	0	7
pancreatic elastase	0	3	3	0	3	0	nd	nd	nd	3	3	5
pancreatic elastase 2	nd			nd			0	0	0	nd	nd	9
trypsin	nd			nd			0	0	6	0	0	0
α-chymotrypsin	nd			nd			2	22	61	0	3	4
stromelysin	nd			nd			0	0	0		nd	5
CTL granzyme A	nd			nd			0	0	0	0	0	2
CTL chymase	nd			nd			3	2	5	1	7	12
bovine												
α-chymotrypsin	0	0	7	9	7	3	nd			0	6	7
trypsin	0	0	0	0	2	0	nd			0	3	4
E. electricus acetylcholine esterase	0	2	0	0	0	0	0	0	0	1	0	1
B. thermoproteolyticus thermolysin	nd	nd	0	nd	nd	2	nd				nd	0
C. papaya papain	2	13	11	4	12	16	0	0	0	0	11	9

[a] The standard deviations were within 25% of the reported value. For example, 20 mg/mL L-683,845 inhibited α-chymotrypsin 61 ± 12% for a 20% standard deviation.

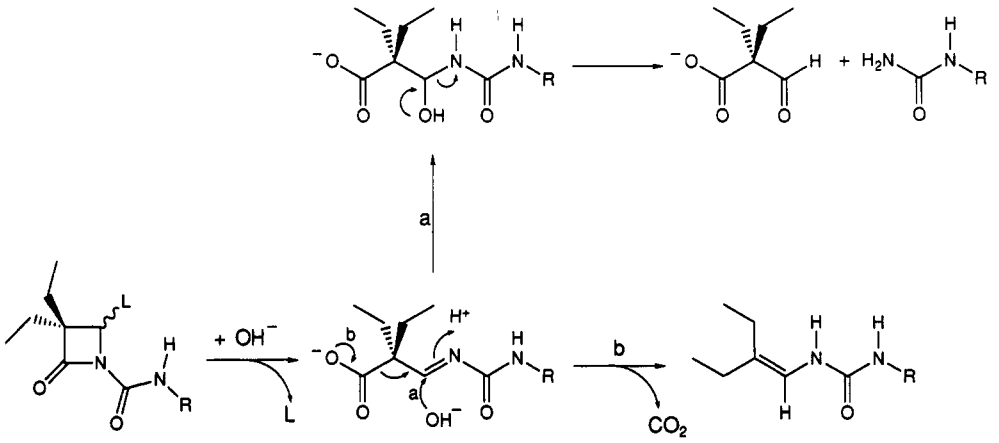


FIGURE 8: Possible mechanism for the base-catalyzed hydrolysis of the β-lactams.

increase in specificity over the monobactams reported by Knight et al. (1992a) and many of the low molecular weight inhibitors reported in the literature. The order of potency versus PMN elastases was human ≃ primate > rat ≃ dog. L-680,833 and L-683,845 were actually slightly more potent versus PMN elastase from chimpanzee.

Comparison of the activity of the β-lactams versus rat and human PMN elastases suggests significant differences between these two enzymes. For example, two benzodioxolanes, L-680,860 and L-680,861, displayed similar relative activities versus both enzymes. The vinyl analog of L680,860, L-680,861, is only slightly less active versus both enzymes. On the other hand, the benzofuran, L-683,845, is 5-fold less active versus the rat enzyme than is L-680,861. These two compounds displayed similar activity versus the human enzyme.

There was weak, time-dependent inhibition of both human Cat-G and α-chymotrypsin by L-683,845. L-680,833 did not inhibit these enzymes. As these compounds differ only in the identity of the urea portion of the molecule (benzofuran versus toluene), these observations could be explained by different binding modes. Both Cat-G and α-chymotrypsin prefer aromatic residues such phenylalanine at P_1[6] of their substrates. The binding of L-683,845 to these two enzymes could place the benzofuran into the specificity pocket (S_1)[6] rather than the diethyl functionality. It is evident from the model depicted in Figure 9 that there is sufficient room for flat side chains such as the benzofuran to fit into the S_1 pocket.[7] This proposal is also predicted by the substrate specificity of α-chymotrypsin. Furthermore, binding of L-683,845 in this mode leaves the γ-oxygen of Ser^{195} in position for nucleophilic attack on the lactam carbonyl. A similar binding mode was proposred by Knight et al. (1992a) for the inhibition of Cat-G by an N-substituted, *p*-nitrobenzenesulfonate β-lactam. This conclusion is also supported somewhat by the weak inhibition of human Cat-G by the naphthyl derivative L-671,070. The phenyl-substituted analog L-669,844 did not inhibit Cat-G. These data suggest that the naphthyl group might also be accommodated in the S_1 pocket. This binding mode is reminiscent of the "inverse" substrates that have been reported for serine proteases [for example, see Tanizawa et al. (1987)].

If L-683,845 bound to α-chymotrypsin in the same manner as that proposed for the binding of the monocyclic β-lactams to HLE, then the asymmetry of the benzofuran could result

[6] The enzyme subsites and the amino acid residues of substrates are numbered according to the nomenclature of Schecter and Berger (1967).

[7] L-683,845 has not been energy optimized in the active site of α-chymotrypsin. The compound was placed in a conformation that has been observed for this inhibitor type and was manually docked into the active site.

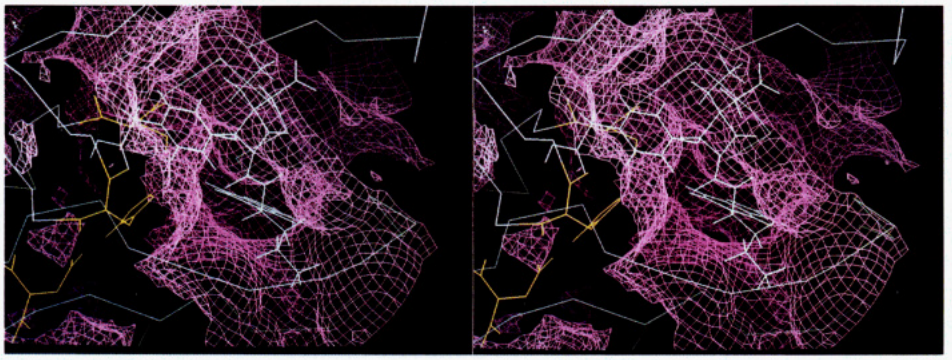


FIGURE 9: A stereoview of the active site region of bovine α-chymotrypsin with L-683,845 bound in an inverse mode. The van der Waals surface of the enzyme is shown. The orientation of the enzyme is the same as that of PPE in Figure 10. The model is based upon the structure of the enzyme bound to phenylethaneboronic acid (Tulinsky & Blevins, 1987).

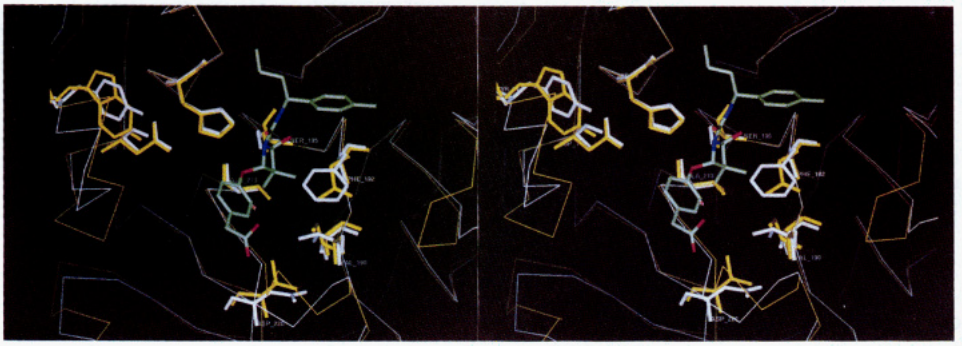


FIGURE 10: A stereoview of the active site region of PPE with a model of the Michaelis complex of L-684,248. For simplicity, much of PPE is represented by a C_α chain trace. PPE's "catalytic triad" (Ser^{195}, His^{57}, and Asp^{102}) is highlighted as well as residues around the S_1 pocket.

Table VI: Comparison of the Amino Acid Residues in the Active Site Regions of PPE and HLE[a]

PPE vs HLE	site	effect
Thr^{226} to Asp	S_1	bottom of pocket; Asp is held back, away from ligands in the S_1 pocket, by a tight H_2O network for HLE and deepens the HLE pocket in comparison to that of PPE; Thr^{226} occludes the PPE S_1 pocket
Thr^{213} to Ala	S_1	opening of the pocket for HLE
Gly^{190} to Val	S_1	"pinches" HLE's pocket
Ile^{138} to Ala	S_1	opening of the back of the HLE pocket
Gln^{192} to Phe	S_1, S_2	the "character" of the mouth of the S_1 pocket changes
Tyr^{94} to Trp	S_2	distal to the bound ligand

[a] The structural alignment of the residues between PPE and HLE is that of Navia et al. (1989). The selection of the significant residues was made on the basis of modeled structures of β-lactams in the active sites of the X-ray crystallographic structures of HLE and PPE (Navia et al., 1987, 1989).

in asymmetric binding modes in the S_2' subsite. The shape of this subsite is such that one "edge" of the furan would face the solvent. If L-683,845 bound to α-chymotrypsin with the furan oxygen facing the solvent, then the face presented to the S_2' site would not be significantly different from the face that L-680,833 would present to this site. If, on the other hand, the benzofuran was rotated such that the furan oxygen faced the enzyme, then differences in the specific interactions between α-chymotrypsin and the two compounds might be expected. Alignment of the active site residues of HLE and α-chymotrypsin [based upon bovine α-chymotrypsin complexed with phenylethaneboronic acid (Tulinsky & Blevins, 1987)] suggests significant differences between the two enzymes in the S_2' site. Significantly, HLE's Ile^{151}, which is part of the loop which defines the end of the S_2' region, is Thr^{151} in bovine α-chymotrypsin. This difference, along with others in this region, may yield an improved interaction with monocyclic β-lactams that contain a potential hydrogen bond acceptor in the urea portion of the inhibitor (benzofuran versus toluene). Thr^{151} is conserved in human pancreatic prechymotrypsin and also in human Cat-G but not in PPE (Salvesen et al., 1987). In addition, His^{40} is a conserved residue in this region which may also be involved in hydrogen bonding. Thus the weak, time-dependent inhibition of α-chymotrypsin and Cat-G by L-683,845 may be the result of interaction of the furan oxygen with the S_2' sites of these enzymes.

PPE has often been used as a model for HLE. While a number of groups have reported that PPE and HLE display similar specificity toward substrates, Zimmerman and Ashe (1977) argued that they are significantly different. These workers reported that PPE and HLE differ in their specificity for the P_1 residue of peptides. HLE prefers larger groups such as valine while PPE prefers alanine. This can be attributed to a number of amino acid substitutions that result in increasing the steric constraints of the S_1 pocket of PPE compared to HLE (see Table VI). The inhibition data reported in Table IV are consistent with these differences if the C-3 substituents of the β-lactams bind in the S_1 pocket of PPE and HLE. Diethyl substitutions at C-3 of the β-lactam ring result in very weak inhibitors of PPE, while dimethyl-substituted analogs (L-684,248 and L-684,249) are relatively potent inhibitors of PPE. In Figure 10, a stereoview of the model of the Michaelis complex between PPE and L-684,248 in the region of the active site is presented. The S_1 pocket extends away behind the inhibitor and is seen to contain the *gem*-dimethyl group at the C-3 position of the β-lactam ring. Those residues in the S_1 pocket which are critical to the difference in binding of inhibitors between HLE and PPE are

highlighted (refer to Table VI). On the left is the Thr^{213}, on the bottom is Thr^{226}, and on the right is Gly^{190}. The effect of reducing the size of the C-3 substituent decreases the potency versus HLE by 2-fold, which parallels the known substrate P_1 preference of HLE for hydrophobic residues with branched side chains (valine versus alanine). The larger ethyl–propyl substitution at C-4 (L-671,070) resulted in no inhibitiohn of PPE, although the complete interpretation of this result would rely on a dimethyl analog with the appropriate urea substitution. These data support the proposal by Doherty (unpublished data) that the C-3 substituent of L-680,833 binds in the S_1 subsite of HLE.

Unlike HLE, PPE is not specific for the stereochemistry at C-4 of the β-lactam ring. Doherty et al. (unpublished data) reported that the L-680,833 was 23-fold more potent than the diastereomer with *R* stereochemistry at C-4. We observed a similar trend with the dimethyl analogs in that the *S* isomer at this position, L-684,248, is a factor of 23-fold more active versus HLE than is the *R* isomer, L-684,249, while both isomers were equipotent versus PPE. A further difference between the two elastases is that the stability of the β-lactam-derived PPE–I complexes does not rival that of L-680,833-derived HLE–I complexes. The reactivation half-lives were 3–7 h at 25 °C compared to 40–50 h for the L-680,833-derived HLE–I complex (Knight, unpublished data). There are a number of differences in the region of the active sites of the two elastases which may account for these differences between the two enzymes. A key difference between PPE and HLE in the S_2 site is the presence in HLE of a hydrophobic Phe residue in place of Gln^{192}. The X-ray structure indicate that in both HLE and PPE the side chain of residue 192 is at the "mouth" of the S_1 pocket and that the side chain reaches into the S_2 site (Navia et al., 1987, 1989). The function of this residue in both enzymes may be to "pack" in around the ligand, especially the P_1 group, by means of hydrophobic interactions and thereby provide further stabilization to the enzyme–inhibitor or Michaelis complex; for HLE the entire side chain of Phe^{192} is hydrophobic whereas for PPE it is the methylenes of the side chain which are hydrophobic. It is clear from the models of the β-lactam bound to HLE and PPE that the presence of a 4(*R*)-phenoxy substituent can enter into hydrogen bonding with the amide side chain of Gln^{192} (the 4-O is 3.2 Å from Gln^{192} N^{ϵ}) but will encounter a hydrophobic aromatic side chain in the case of HLE's Phe^{192} (the 4-O is 2.1 Å from Phe^{192} C^{ϵ}) which is presumably a bad interaction.

The observations reported in this work support the proposal by Knight et al. (1992a) that β-lactams could be developed as a general class of serine protease inhibitors. The stability of these compounds in this study increases the likelihood that they will be orally available and useful as agents against serine proteases of therapeutic interest. The structure–activity relationships and specificity can be fine tuned by substitutions at either C-3 or N-1 and most likely C-4. That these compounds are likely mechanism-based inhibitors of serine proteases should also increase the specificity and reduce the chance of nonspecific toxicity (Knight et al., 1992a,b). The observation that these inhibitors display similar potency (although reduced) versus primate enzymes suggests that mechanism-based toxicity should be detectable in these species. In addition, the formation of stable enzyme–inhibitor complexes (Knight et al., unpublished data) should circumvent the necessity of maintaining high levels of drug in vivo.

CONCLUSION

The monobactams discussed in this work are stable, potent, specific, and time-dependent inhibitors of HLE. Work is underway to define their therapeutic utility and mechanism of action.

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REFERENCES

Ardelt, W., Ksiensky, S., & Niedzwiecka-Namyslowska, I. (1970) *Anal. Biochem. 34*, 180–187.

Ashe, B. M., Clark, R. L., Jones, H., & Zimmerman, M. (1981) *J. Biol. Chem. 256*, 11603.

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., & Hanada, K. (1982) *Biochem. J. 201*, 189–198.

Bax, A., Griffey, R. H., & Hawkins, B. L. (1983) *J. Am. Chem. Soc. 105*, 7188.

Bonney, R. J., Ashe, B., Maycock, A., Dellea, P., Hand, K., Osinga, D., Fletcher, D., Mumford, R., Davies, P., Frankenfield, D., Nolan, T., Schaffer, L., Hagmann, W., Finke, P., Shah, S., Dorn, C., & Doherty, J. (1989) *J. Cell. Biochem. 39*, 47–53.

Dewald, B., Rindler-Ludwig, R., Bretz, U., & Baggiolini, M. (1975) *J. Exp. Med. 141*, 709–723.

Doherty, J. B., Ashe, B. M., Argenbright, L. W., Barker, P. L., Bonney, R. J., Chandler, G. O., Dahlgren, M. E., Dorn, C. P., Finke, P. E., Firestone, R. A., Fletcher, D., Hagmann, W. K., Mumford, R., O'Grady, L., Maycock, A. L., Pisano, J. M., Shah, S. K., Thompson, K. R., & Zimmerman, M. (1986) *Nature 322*, 192–194.

European Patent Office (1989) Publication EPO O 337 549.

Feinstein, G., & Janoff, A. (1975a) *Biochim. Biophys. Acta 403*, 477–492.

Feinstein, G., & Janoff, A. (1975b) *Biochim. Biophys. Acta 403*, 493–505.

Fujii, S., & Hitomi, Y. (1981) *Biochim. Biophys. Acta 661*, 342–345.

Green, B. G., Weston, H., Ashe, B. M., Doherty, J., Finke, P., Hagmann, W., Lark, M., Mao, J., Maycock, A., Mumford, R., Walakovits, L., & Knight, W. B. (1991) *Arch. Biochem. Biophys. 286*, 284–292.

Harrison, R., Teahan, J., & Stein, R. L. (1989) *Anal. Biochem. 180*, 110–113.

Hemmi, K., Harper, J. W., & Powers, J. C. (1985) *Biochemistry 24*, 1841–1848.

Kaplan, P. O., Kuhn, C., & Pierce, J. A. (1973) *J. Lab. Clin. Med. 82*, 349.

Knight, W. B., Chabin, R., & Green, B. (1992a) *Arch. Biochem. Biophys.* (in press).

Knight, W. B., Maycock, A. L., Green, B. G., Ashe, B. M., Gale, P., Weston, H., Finke, P. E., Hagmann, W. K., Shah, S. K., & Doherty, J. B. (1992b) *Biochemistry 31*, 4980–4986.

Largmann, C., Broderick, R., & Geokas, M. (1976) *Biochemistry 15*, 2491–2497.

Marion, D., & Wuthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967.

Morrison, J. F., Walsh, C. T. (1983) *Adv. Enzymol. 61*, 201–301.

Navia, M. A., Springer, J. P., Lin, T.-Y., Williams, H. R., Firestone, R. A., Pisano, J. M., Doherty, J. B., Finke, P. E., & Hoogsteen, K. (1987) *Nature 327*, 79–82.

Navia, M. A., McKeever, B. M., Springer, J. P., Lin, T.-Y., Williams, H. R., Fluder, E. M., Dorn, C. P., & Hoogsteen, K. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7–11.

Poe, M., Bennett, C. D., Biddison, W. E., Blake, J. T., Norton, G. P., Rodkey, J. A., Sigal, N. H., Turner, R. V., Wu, J. K., & Zweerink, H. J. (1988) *J. Biol. Chem. 263*, 13215–13222.

Powers, J. C. (1983) *Am. Rev. Respir. Dis. 127*, 554–558.

Salvesen, G., Farley, D.; Shuman, J., Przybyla, A., Reilly, C., & Travis, J. (1987) *Biochemistry 26*, 2289–2293.

Schecter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Shaka, A. J., Barker, P. B., & Freeman, R. (1985) *J. Magn. Reson. 64*, 547.

Stein, R. L. (1983) *J. Am. Chem. Soc. 105*, 5111–5116.

Stein, R. L., Trainor, D. A., & Wildonger, R. A. (1985) in *Annual Reports in Medicinal Chemistry* (Egan, Ed.) Vol. 20, pp 237–246, Academic Press, London.

Tanizawa, K., Kanaoka, Y., & Lawson, W. B. (1987) *Acc. Chem. Res. 20*, 337–343.

Teahan, J., Harrison, R., Izquierdo, M., & Stein, R. L. (1989) *Biochemistry 28*, 8497–8501.

Travis, J., Giles, P. J., Porcelli, L., Reilly, C., Baugh, R., & Powers, J. (1980) in *Protein Degradation in Health and Diseases* (Evered, D., & Whelan, J., Eds.) Vol. 75, pp 51–68, Excerpta, Amsterdam.

Tulinsky, A., & Blevins, R. A. (1987) *J. Biol. Chem. 262*, 7737–7743.

U.S. Pharmacopeia (1989) Vol. 22, pp 1788–1789, USPC, Inc., Rockville, MD.

Williams, J. C., Stein, R. L., Giles, R., & Krell, R. D. (1991) *Ann. N.Y. Acad. Sci. 624*, 230–243.

Williams, J. W., & Morrison, J. F. (1979) *Methods in Enzymology* (Purich, D. L., Ed.) Vol. 63, pp 437–467, Academic Press, New York.

Zimmerman, M., & Ashe, B. M. (1977) *Biochim. Biophys. Acta 480*, 241–245.