Degradation Kinetics of DMP 777, an Elastase Inhibitor

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Purpose. The objective was to evaluate the degradation profile of the elastase inhibitor DMP 777 and lay the foundation for formulation development.

Methods. The pK_a was determined by potentiometric titration in mixed-aqueous solvents. The degradation kinetics were studied as a function of pH, buffer concentration, ionic strength, methanol concentration and temperature using a stability-indicating HPLC assay. The degradation products were identified by LC-MS, NMR, and by comparison with authentic samples.

Results. The pK_a for the protonated piperazine nitrogen was estimated to be 7.04. The pH-rate profile is described by specific acid-, water-, and specific base-catalyzed pathways. The pH of maximum stability is in the range of 4 to 4.5 where water is the principal catalyst in the reaction. Buffer catalysis, primary salt effects and medium effects were observed. The proposed mechanism for acid catalyzed degradation is the rarely observed AAL1 which involves alkyl-nitrogen heterolysis. The driving force for the reaction appears to lie in the stability of the benzylic carbocation. The proposed mechanism for base catalyzed degradation is $B_{AC}2$ which involves β-lactam ring opening. The β-lactam ring of DMP 777, a monolactam, appears to be as reactive as that in benzylpenicillin in the k_{OH} controlled region where a similar mechanism of hydrolysis should be operative. A contributing factor to this increased reactivity may lie in the reduced basicity of the β-lactam nitrogen making it a good leaving group.

Conclusions. The degradation profile indicates that development of a solution dosage form of DMP 777 with adequate shelf-life stability at room temperature is feasible.

KEY WORDS: elastase inhibitor; monocyclic β-lactam; NMR; stability.

INTRODUCTION

Accumulation of polymorphonuclear leukocytes (PMN, neutrophils) at sites of inflammation is a prominent feature of a large number of acute inflammatory diseases such as cystic fibrosis, bronchitis, ulcerative colitis, rheumatoid arthritis, shock syndromes (1). There is much evidence to indicate that the release of proteinases such as elastase, a serine protease, oxidants and lipid mediators from these cells contributes to the erythema, edema and tissue destruction that occurs in these diseases (2). DMP 777, [S-(R*,S*)]-2-[4-[[(4-methyl)piperazin-1-yl]carbonyl]phenoxy]-3,3-diethyl-N-[1-(3,4-methylenedioxyphenyl)butyl]-4-oxo-1-azetidinecarboxamide (Figure 4), a novel, monocyclic β-lactam has been shown to be a highly selective and potent inhibitor of human PMN elastase (3) and is currently in phase I clinical trials.

The focus of this study is the degradation kinetics of DMP 777 as a function of pH with an objective to lay the foundation for formulation development.

MATERIALS AND METHODS

Materials

DMP 777 was prepared by Merck Research Laboratories, Rahway, New Jersey, and was used as received. Authentic samples of the degradation products (V, VI and VII, Fig. 3) were prepared and characterized by the Chemical Processing Division of The DuPont Merck Pharmaceutical Company and the Merck Research Laboratories, Rahway, New Jersey. The water used was of high purity with a specific resistance of not less than 18 megaohms-cm. All solvents were of HPLC grade. All other reagents were of analytical grade.

Ionization Constant

The pK_a value of the protonated piperazine nitrogen in DMP 777 was determined, in triplicate, by potentiometric titration (4a) of a 10 mM solution of DMP 777 in 60, 70 and 80% (v/v) aqueous methanol with 0.1 N hydrochloric acid at 22°C.

Solution Stability

The following aqueous solutions (μ = 0.5 M with sodium chloride except when stated) were used: hydrochloric acid (0.003–0.1 M, pH 1–2.5), acetate buffer (0.025–0.1 M, pH 3.5–5.5) and phosphate buffer (0.025–0.1 M, pH 6.5–8.0). Ionic strength effects were studied in 0.1 M hydrochloric acid at pH 1 adjusted to ionic strength values of 0.1, 0.3, and 0.5 M with sodium chloride. The effects of buffers were examined by varying the concentration of buffers while maintaining a constant pH. Three concentrations of buffers were used to study the effect of buffers at a pH.

All stability studies were performed at 80 ± 0.1°C except when stated. Activation parameters were determined in 0.1 M hydrochloric acid at pH 1 adjusted to an ionic strength of 0.5 M with sodium chloride at 60, 70, and 80°C. The pH values of the buffers were measured at the temperature of the study. The initial concentration of DMP 777 ranged from 10 μg/mL at pH values 1–6.5 to 5 μg/mL at pH values 7.3–8.4. For studies at pH values 1–6.5, a stock solution of DMP 777 in methanol was diluted with the desired buffer at room temperature, transferred into glass vials, crimp sealed and maintained at 80°C in an oven. The methanol concentration in these samples was 1% (v/v). The samples were allowed to equilibrate for 15 minutes before the first sample was taken. The samples were removed from the oven at appropriate time intervals, quench cooled in an ice:salt bath, and analyzed. For studies at pH values 7.3–8.4, 20% (v/v) methanol was incorporated in the buffer solutions to overcome precipitation of the drug substance. At these pH values, a stock solution of DMP 777 in methanol was diluted

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with the desired buffer preheated at 80°C in a constant temperature water bath. Aliquots of the samples were removed at appropriate time intervals, quenched 1:1 with 0.1 N hydrochloric acid, and analyzed. The effect of methanol on the rate was studied in 0.025 M phosphate buffer containing 10, 20, and 30% methanol at pH 8.4 (μ = 0.5 M). All solutions were prepared in triplicate.

Chromatographic Conditions

The HPLC separations were conducted on a system consisting of a pump programmed by a system controller (Model 600E), an autoinjector (Model 717 Wisp), a UV-vis spectophotometric detector (Model 486) operated at 240 nm, and a column oven programmed by a temperature control module and operated at 35°C, all from Waters, USA. The separations were accomplished on a Zorbax Rx-C18 column (5 μm, 4.6 mm × 125 mm, Macmod) using a gradient mode. The mobile phase consisted of the following: Component A: phosphate buffer (0.05 M, pH 2.5); Component B: 70% acetonitrile in water. The gradient scheme involved a change in mobile phase component B from 30% to 80% over 14 minutes at a flow rate of 2 ml/min. The chromatographic data was acquired and analyzed on a computer equipped with a data acquisition and analysis software program (Multichrom software, VG Instruments).

Liquid Chromatography-Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was performed on a HP1090 LC system interfaced with a PE SCIEX API III mass spectrometer. The separations were accomplished on a Zorbax SB-C18 (2.1 × 150 mm, 5 μm) maintained at 40°C. The solvent system consisted of the following: component A = 0.1% (v/v) trifluoroacetic acid (TFA) in water for acid degradation products and 0.15% (v/v) TFA for base degradation products; component B = 70% (v/v) acetonitrile in water. The gradient scheme involved a change in component B from 20% to 80% over 30 minutes and holding at 80% B for 5 minutes at a flow rate of 0.5 mL/min for acid degradation products, and from 0% B to 100% B over 15 minutes and holding at 100% B for 5 minutes at a flow rate of 0.25 mL/min for base degradation products. The detection was by UV at 240 nm. The mass spectrometric method consisted of atmospheric pressure chemical ionization with a nebulizer temperature of 400°C for acid degradation products and 440°C for base degradation products, a discharge current of 3.0 μA, and an orifice potential of 60 V.

NMR Analysis

The amine pK_a value for parabanic acid piperazineamide (VI, Fig. 3) was titrated at 80°C using ^{1}H NMR. The following buffers were used: pH 4.21–5.85 (0.1 M acetate), pH 6.58–7.98 (0.01 M phosphate), pH 8.25–9.20 (0.01 M borate). All buffers contained 10% (v/v) D_2O. A constant ionic strength of 0.1 M was maintained for each buffer by adding the appropriate amount of sodium chloride. The ^{1}H NMR spectra were acquired at 400 MHz on a Varian Unity-400 spectrometer. All chemical shifts were referenced to D_2O. Spectra were acquired with a spectral width of 8000 Hz, an acquisition time of 4.07 s, a relaxation delay of 2 s and a pulse angle of 30°.

The ^{1}H NMR spectrum of the degradation product I was recorded in DMSO-d_6 at 30°C. All chemical shifts were referenced to residual DMSO at 2.50 ppm.

Isolation of Degradation Product I

A solution of DMP 777 (1 mg/mL, 30 mL) in 0.1 N hydrochloric acid was heated at 80°C for about 16 h. After this time period, the solution was concentrated to a small volume, and chromatographed on a C18 preparative column (Rainin Dynamax-60A, 21.4 × 250 mm, 8 μm) maintained at 35°C. The mobile phase consisted of the following: Component A: 0.03% trifluoroacetic acid in water; Component B: acetonitrile. The gradient scheme involved a change in mobile phase component B from 30% to 95% over 75 minutes at a flow rate of 10 ml/min. Fractions were collected, pooled, and evaporated to dryness to obtain the degradation product I.

Data Analysis

The rate constants were generated by obtaining the best fit of the experimental data using a linear regression program (Cricket Graph, Computer Associates). The pH-NMR chemical shift and pH-rate profiles were analyzed by using a non-linear least squares regression program (PCNONLIN, SCI).

RESULTS AND DISCUSSION

Ionization Constant

The apparent dissociation constant of the protonated piperazine nitrogen in DMP 777 was determined at 22°C by potentiometric titration in a mixture of water and methanol, and extrapolating the pKa values so obtained to 100% water. The pKa of the protonated piperazine nitrogen in DMP 777 was estimated to be 7.04 ± 0.02.

Solution Stability

The degradation of DMP 777 followed apparent first-order kinetics in the pH systems studied over a range of 1–8. Buffer catalysis was observed, although minimally in some instances, and depended on the buffer system, and pH. For the instances where the rate of degradation exhibited buffer dependence, linear plots were obtained when the observed pseudo first-order rate constants were plotted against the total buffer concentration at constant pH. The criterion used to indicate if the data exhibited buffer dependence was whether the slope was significantly different from zero. The values for the rate constants in the absence of buffer are listed in Table 1.

The pH dependence of the buffer-independent rate constants, k', at 80°C and an ionic strength of 0.5 M is shown in Figure 1. The rate data generated at pH values 7.77 and 8.42 were not used in constructing the pH-rate profile as the solutions used in these studies contained 20% (v/v) methanol. The effect of methanol on the rate is discussed later under medium effects. The pH-rate profile shows that DMP 777 is most stable in the pH range of 4 to 4.5. The shape of the curve suggests an acid-catalyzed degradation pathway below pH 2.5, an uncatalyzed region between 4.0 to 4.5, and a base-catalyzed degradation pathway above pH 5. These reactions can be written as below, representing BH^+ as the protonated form of DMP 777:

Table I. The Buffer-Independent Rate Constants at Various pH Values for the Degradation of DMP 777 at 80°C and an Ionic Strength of 0.5 M

pH	Buffer (M)	k', day^{-1}
0.95	HCl	3.77
1.43	HCl	1.36
1.97	HCl	4.42×10^{-1}
2.50	HCl	1.33×10^{-1}
3.55	Acetate	2.30×10^{-2}[a]
4.54	Acetate	1.98×10^{-2}[a]
5.57	Acetate	4.35×10^{-2}[a]
6.49	Phosphate	1.68×10^{-1}[a]
7.77[c]	Phosphate[b]	1.15[a]
8.42[c]	Phosphate[b]	4.62[a]

[a] Obtained from intercept of plot of k_{obs} versus buffer concentration;
[b] Buffers contain 20% (v/v) methanol;
[c] Apparent pH of buffers containing 20% (v/v) methanol.

$$BH^+ + H^+ \xrightarrow{k_H} \text{Products}$$
$$BH^+ \xrightarrow{k_O} \text{Products}$$
$$BH^+ + OH^- \xrightarrow{k_{OH}} \text{Products}$$

The rate equation for this kinetic scheme is

$$k' = k_H[BH^+][H^+] + k_O[BH^+] + k_{OH}[BH^+][OH^-] \quad (2)$$

where k_H and k_{OH} are the second-order rate constants for specific acid- and specific base-catalyzed reaction, respectively, of BH^+, and k_O is the first-order rate constant for reaction of BH^+ with water. Substituting for $BH^+ = [H^+]/([H^+] + K_a)$ and $[OH^-] = K_W/[H^+]$, where K_a is the ionization constant of DMP 777 and K_W is the ionic product of water, results in

$$k' = \left(k_H[H^+] + k_O + k_{OH}\frac{K_W}{[H^+]}\right)\left(\frac{[H^+]}{[H^+] + K_a}\right) \quad (3)$$

The theoretical profile for k', shown in Figure 1, was generated with a k_H of 36.33 ± 1.41 M^{-1} day^{-1}, a k_O of 0.014 ± 0.001 day^{-1}, a k_{OH} of 373530 ± 6.1 × 10^4 M^{-1} day^{-1}, a K_a of 2.57 × 10^{-7} or a pK_a of 6.59, and a K_W of 2.44 × 10^{-13} or a pK_W of 12.613 at 80°C (5). Since the kinetic experiments were performed at 80°C and it is known that nitrogenous bases are highly sensitive to temperature and become weaker as the temperature is increased (4b), it was deemed important to evaluate the amine pK_a value of DMP 777 at this temperature. ^{1}H-NMR (6) was chosen as the method of choice over other methods because of the ease of operation at this temperature.

However, the aqueous solubility constraints posed by DMP 777 over the pH range of the experiment dictated the choice of parabanic acid piperazineamide (VI, Fig. 3) to verify the pK_a generated by Eq. 3 at 80°C. The parabanic acid piperazineamide is the most relevant choice since it forms an integral part of DMP 777. The amine and the phenol are well separated from each other in the molecule, and are not expected to influence each other's ionization constant.

The methyl protons on the N-methylpiperazine was used to follow the ionization of the amine pK_a. The relationship between the chemical shift and the ionization of the amine can be described by Eq. 4:

$$\delta_{obs} = (f^+_{BH} \times \delta^+_{BH}) + (f_B \times \delta_B) \quad (4)$$

where δ_{obs} is the observed chemical shift, δ^+_{BH} and δ_B are the intrinsic chemical shifts for the protonated and unprotonated species, respectively, and f^+_{BH} and f_B are fractions of the protonated and unprotonated species, respectively. Substituting for $f^+_{BH} = [H^+]/([H^+] + K_a)$ and $f_B = K_a/([H^+] + K_a)$ results in

$$\delta_{obs} = \frac{([H^+] \times \delta^+_{BH}) + (K_a \times \delta_B)}{[H^+] + K_a} \quad (5)$$

The observed chemical shift values (δ_{obs}) were plotted as a function of pH (Figure 2), and the data were fitted to Eq. 5 using a nonlinear curve fitting program (PCNONLIN) to generate a pK_a value of 6.54 ± 0.01 at 80°C. This result corroborates the kinetically generated pK_a value for DMP 777 at 80°C.

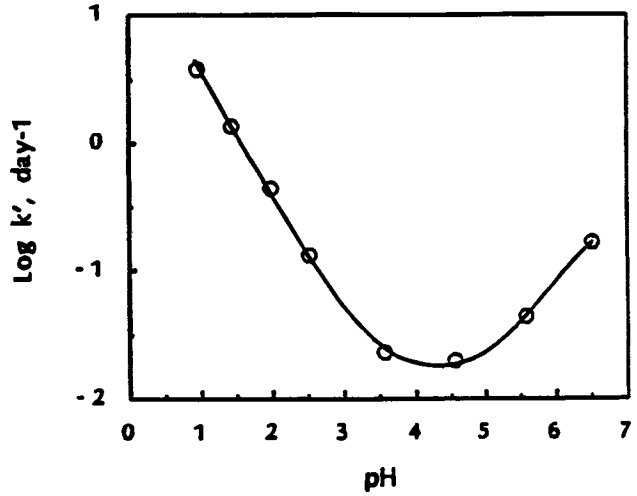

Fig. 1. The pH dependence of the buffer-independent rate constants for the degradation of DMP 777 at 80°C and an ionic strength of 0.5 M. The theoretical profile was generated using eq. 3 and the constants shown in the text.

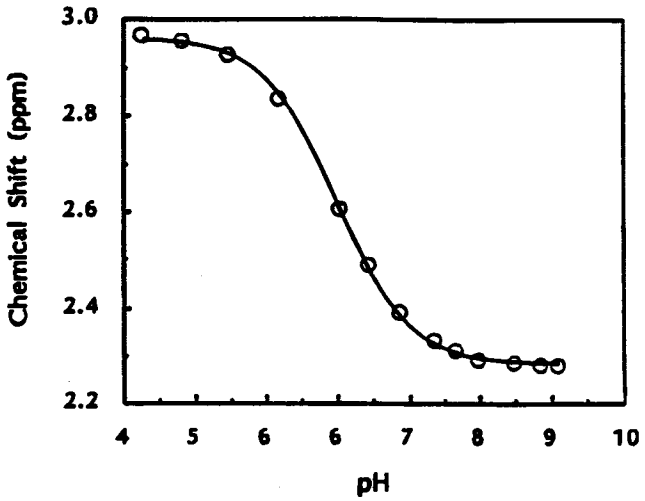

Fig. 2. The chemical shift changes for the methyl protons in parabanic acid-N-methylpiperazineamide as a function of pH at 80°C. The theoretical curve was generated by fitting the observed data to eq. 5.

Effect of Buffers

The rates of degradation of DMP 777 were affected by acetate and phosphate buffers. The reaction appeared to be subject to both general-acid and -base catalysis in acetate buffers. The buffer-dependent rate constants in acetate buffers were 0.087, 0.113, and 0.163 M^{-1} day^{-1} at pH 3.55, 4.55 and 5.57, respectively. The buffer species-dependent rate constants were $^{k}CH_3COOH$ = 0.08 M^{-1} day^{-1}, and $^{k}CH_3COO^-$ = 0.174 M^{-1} day^{-1}. In contrast, the reaction appeared to be predominantly subject to general-base catalysis in phosphate buffers. The buffer-dependent rate constants in phosphate buffers were 0.491, 6.56, and 31.97 M^{-1} day^{-1} at pH 6.5, 7.77, and 8.42, respectively. Since the reactions at pH 7.77 and 8.42 were studied in methanol-buffer mixtures, the catalytic rate constants in phosphate buffers must be interpreted cautiously for reasons explained later under medium effects.

Effect of Temperature

The activation parameters E_a (energy of activation), $\Delta S‡$ (entropy of activation), and $\Delta H‡$ (enthalpy of activation) were determined for the specific acid-catalyzed degradation of DMP 777 at pH 1 and an ionic strength of 0.5 M over a temperature range of 60 to 80°C. The values of $\Delta S‡$ and $\Delta H‡$ were determined from the zero intercept and the slope, respectively, of the Eyring plot, and were -11.6 ± 0.3 eu and 23.7 ± 0.1 kcal mol^{-1}, respectively. Arrhenius treatment of the same data gave an E_a value of 24.4 ± 0.1 kcal mol^{-1}. Using the E_a value, the rates of degradation in the specific acid-catalyzed region at 25°C were estimated. In the k_H controlled region (over a pH range of 1 to 3), the rate will be 600-fold slower at 25°C than at 80°C; the estimated t_{90} at 25°C varies from 18.4 days at pH 1 to 3.87 years at pH 3.

Effect of Ionic Strength

The influence of ionic strength (μ) on the rate constant for the specific acid-catalyzed degradation of DMP 777 was evaluated at pH 1 and 80°C. The result plotted according to the modified Debye-Hückel equation (7) indicated a positive primary salt effect with a slope of 0.94. This value is close to a theoretical value of 1.14 (7) and is consistent with a reaction between similarly charged ions (8a), in this case protonated DMP 777 and hydronium ion. The rate constant extrapolated to zero ionic strength at pH 1 and 80°C was 1.5 day^{-1}.

Effect of Medium

Methanol, which was added to keep the drug solubilized in buffers at high pH, had a significant effect on the reaction rate. The rate constants for the base-catalyzed degradation of DMP 777 at pH 8.4 increased with increasing methanol concentration in the medium (k_{obs} = 4.6, 5.3, 6.1 day^{-1} at 10, 20, and 30% methanol, respectively). This observation may seem consistent for a reaction between a neutral molecule (DMP 777 would be expected to be predominantly in its unionized form at this pH and temperature in this medium) and an ion (8b), where a decrease in solvent polarity would be expected to result in an increase in reaction rate. However, since the reaction is studied in methanol-water mixtures, not only would the solvent polarity change, but so would the ratio of hydroxide to methoxide, thereby changing the nature of the reaction from hydrolysis to methanolysis. A kinetic consequence of this is an increase in rate because of differences in nucleophilicity between OH^- and CH_3O^- (8b).

Degradation Products and Mechanism

The major degradation products of DMP 777 under acidic and basic conditions were identified by liquid chromatography-mass spectrometry, NMR, and by comparison with authentic samples of the degradation products. The structures of major degradation products and a selected mass spectral fragment ion are shown in Figure 3. The LC-MS analysis of a reaction mixture of DMP 777 in hydrochloric acid at pH 1 and 80°C exhibited mass peaks at *m/z* 389 and *m/z* 177. The mass peak at *m/z* 389 which corresponds to protonated molecular ion (M+H) was assigned structure I. The ^{1}H-NMR of a sample that was isolated by preparative LC was consistent with the assigned structure. The mass peak at *m/z* 177 was assigned structure II. This mass spectral fragment presumably has arisen from the corresponding chlorinated and/or the hydroxylated derivatives (structures III and IV).

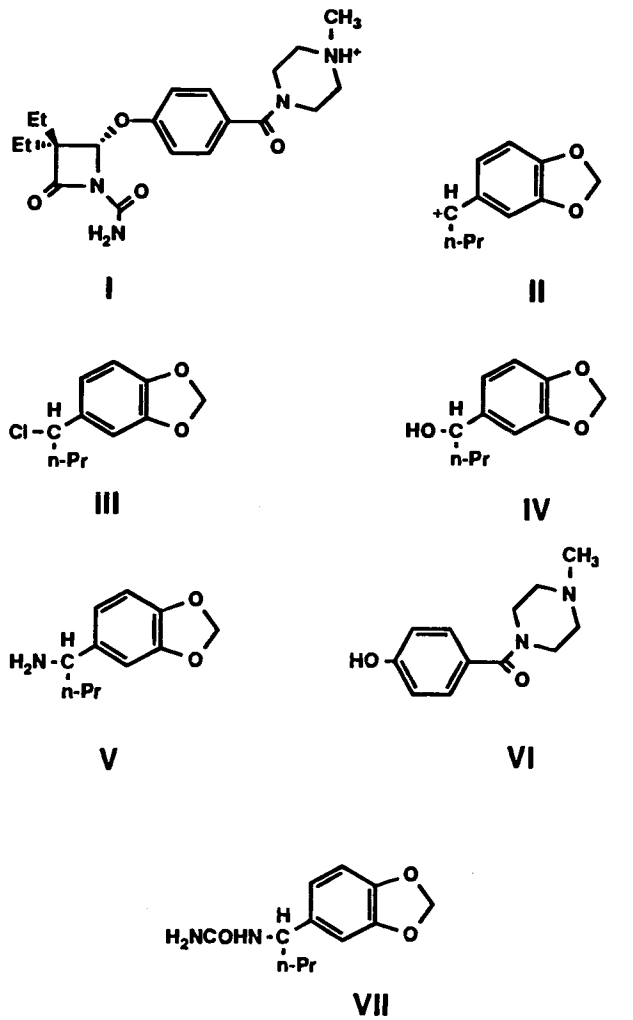

Fig. 3. The structures of major degradation products of DMP 777 and a selected mass spectral fragment ion.

The postulated mechanism for specific acid-catalyzed degradation of DMP 777 involves alkyl-N heterolysis. This reaction, while rare, has been observed for various N-t-butyl amides (9) and N-t-alkyl ureas (9) in mineral acids, where the mechanism was the AAL1 mechanism with the Ingold classification system (10). The two steps involved in the AAL1 heterolysis of an amide (R'CONHR) involve protonation of the carbonyl oxygen of the amide and unimolecular cleavage of the N-R bond to give the amide $R'CONH_2$ and R^+, the latter rapidly reacting with water or polymerizing. DMP 777 is postulated to degrade in acid by this mechanism leading to the urea derivative (I) and a stable benzylic carbocation (II). The main driving force behind the reaction must lie in the stability of the benzylic carbocation. The fate of this benzylic carbocation was not determined, although it was expected to rapidly react with chloride and/or water to form the corresponding chlorinated and/or hydroxylated derivatives (III and IV). Figure 4 shows the proposed mechanism for the specific acid-catalyzed degradation of DMP 777. The positive primary salt effect and entropy of activation support this mechanism. Unimolecular reactions of the A1 mechanism of acid catalysis usually have positive or small negative values of the entropy of activation (11). The value of ΔS‡ obtained for the specific acid-catalyzed reaction of DMP 777 is consistent with this observation and qualitatively supports the unimolecular mechanism.

The base-catalysed degradation products were identified by LC-MS, and by comparing with authentic samples for their LC characteristics. The LC-MS analysis of a reaction mixture

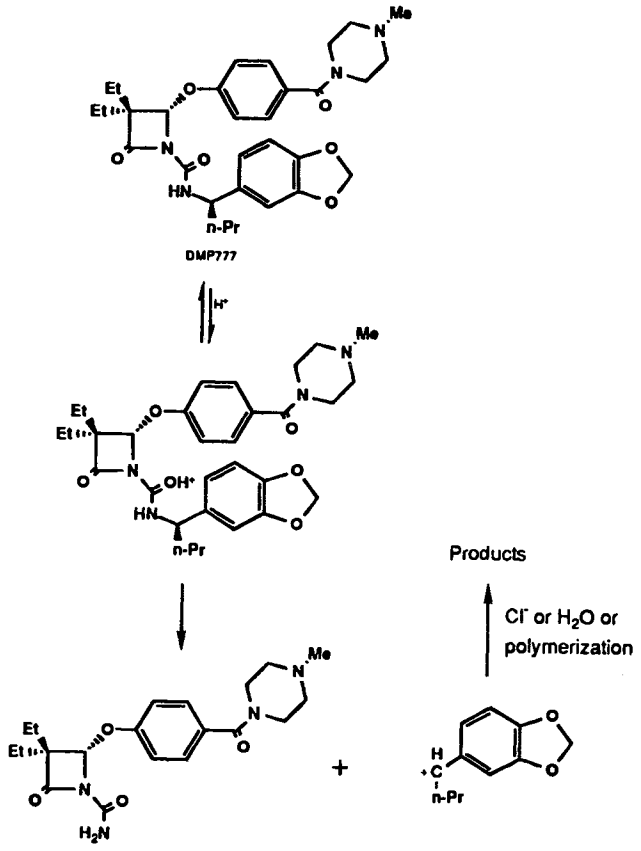

Fig. 4. The proposed mechanism for the specific acid-catalyzed degradation of DMP 777.

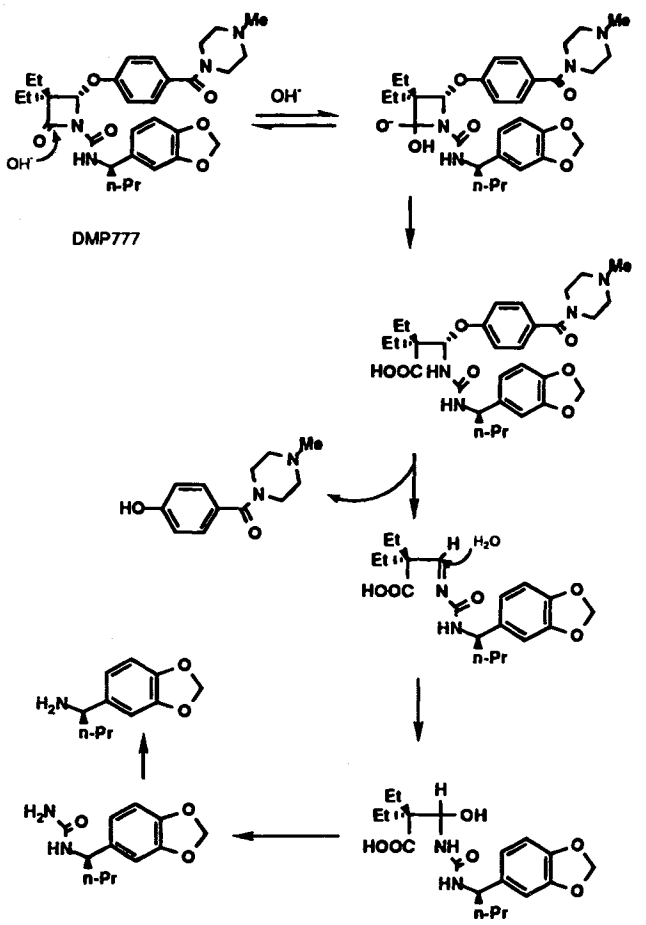

Fig. 5. The proposed mechanism for the specific base-catalyzed degradation of DMP 777.

of DMP 777 in phosphate buffer at pH 8.4 and 80°C exhibited mass peaks at *m/z* 194, *m/z* 221 and *m/z* 237, and were assigned structures V, VI and VII, respectively. The *m/z* 177 ion (II) is a stable fragment ion of *m/z* 194 and *m/z* 237.

The postulated mechanism for specific base-catalyzed β-lactam hydrolysis of DMP 777 involves nucleophilic participation by hydroxide ion, resulting in acyl cleavage via a tetrahedral intermediate, and the concomitant release of the parabanic acid piperazineamide; it has been classified as a BAC2 mechanism with the Ingold classification system (10). Figure 5 shows the proposed mechanism for the specific base-catalyzed degradation of DMP 777.

The bicyclic β-lactam ring of penicillins is readily susceptible to hydrolysis under alkaline conditions, and is ascribed to strain in the four-membered ring. In contrast, monocyclic β-lactams are resistant to alkaline hydrolysis (12). Woodward (13) suggested that this was due to resonance stabilization of the monocyclic lactam, which is not possible in the penicillins because of non-planarity of the system. However, the β-lactam ring of DMP 777, a monocyclic lactam, appears to be as reactive as that in benzylpenicillin in the k_{OH} controlled region where a similar mechanism of hydrolysis should be operative (k_{OH} = 152928 M^{-1} day^{-1} for benzyl penicillin at 60°C (14) *versus* 373530 M^{-1} day^{-1} for DMP 777 at 80°C). A contributing factor to this increased reactivity may lie in the reduced basicity of

the β-lactam nitrogen making it a good leaving group. The rates of hydroxide ion-catalyzed hydrolysis of amides and β-lactams have been shown to depend upon the basicity of the leaving group (15,16). Electron withdrawing substituents attached to nitrogen increase the rate of alkaline hydrolysis. Another factor contributing to the increased reactivity of DMP 777 may lie in the presence of the parabanic acid piperazineamide substituent that acts as a good leaving group concomitant to the carbon-β-lactam nitrogen bond fission, and facilitates further the collapse of the tetrahedral intermediate.

In conclusion, the degradation profile of DMP 777 indicates that it is feasible to formulate it as a solution dosage form with adequate shelf-life stability at room temperature.

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