characteristic reaction of phenols with 4-aminoantipyrine described previously (25). The order of addition of reagents was altered to achieve acceptable precision. With these samples, it was necessary to add the bicarbonate buffer first. Analyte II in an aqueous solution of the sample was quantitatively oxidized to benzaldehyde with alkaline periodate by the procedure described previously (20). The benzaldehyde was then extracted from the solution with chloroform and determined spectrophotometrically. Guaifenesin was extracted from the samples with chloroform and determined by its UV absorbance. As the data in Table III indicate, the spectrophotometric assays are in good agreement with the HPLC assay presented here.

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Kinetics and Mechanism of Degradation of Cefotaxime Sodium in Aqueous Solution

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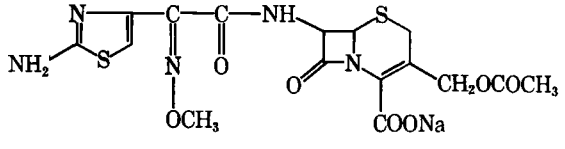
Received January 13, 1982, from the *Pharmaceutical Development Department, Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876.* Accepted for publication March 15, 1982.

Abstract □ The degradation kinetics and mechanism of a potent new cephalosporin, cefotaxime sodium, in aqueous solution were investigated at pH 0–10 at 25° and an ionic strength of 0.5. The degradation rates were determined by high-pressure liquid chromatography and were observed to follow pseudo first-order kinetics with respect to cefotaxime sodium concentration. The data suggested that the rate of degradation was influenced significantly by solvolytic, hydrogen ion, and hydroxide ion catalysis. No primary salt effects were observed in the acid or neutral regions; however, a positive salt effect was observed at pH 8.94. Buffer catalysis due to the buffer species employed was not seen during the kinetic studies. The pH–rate profile at 25° indicated that the maximum stability of cefotaxime sodium occurred in the pH 4.5–6.5 region. In aqueous solution, cefotaxime was shown to degrade by two parallel reactions: de-esterification at the C-3 position and β-lactam cleavage. Good agreement between the theoretical pH–rate profile and the experimental data support the proposed degradation process.

Keyphrases □ Kinetics—mechanism of degradation of cefotaxime sodium in aqueous solution □ Degradation—kinetics and mechanism, cefotaxime sodium in aqueous solution □ Cefotaxime sodium—kinetics and mechanism of degradation in aqueous solution □ Cephalosporins—cefotaxime sodium, kinetics and mechanism of degradation in aqueous solution

Cefotaxime sodium (I) is a potent new third generation cephalosporin possessing a broad spectrum of activity. Chemically, it is characterized by a 2-amino-4-thiazolyl ring which, in comparison to other cephalosporins, increases antibacterial activity against Gram-negative strains, and by an α-methoximino group which enhances



I

stability to β-lactamases (1–4). Compound I is active against Gram-positive and Gram-negative organisms, especially multiresistant strains, including many aminoglycoside-resistant strains. *In vitro*, its activity against Gram-negative organisms has been shown to be 10–200 times greater than that of the recently developed second generation cephalosporins (5).

The present report describes the stability kinetics of I in aqueous solution. The investigation was initiated to elucidate the mechanism by which I decomposes and to determine those kinetic parameters that will be of value in predicting the stability of the reconstituted antibiotic under a wide range of conditions.

EXPERIMENTAL

Materials—Cefotaxime sodium (I)[1] and desacetylcefotaxime (II)[2] were used without further purification. Desacetylcefotaxime lactone (III) was prepared using a modification of the method for cephalothin de-

[1] Claforan, Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.
[2] Hoechst AG, Frankfurt, West Germany.

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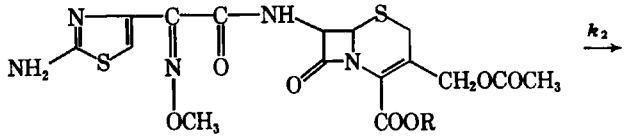
Cefotaxime

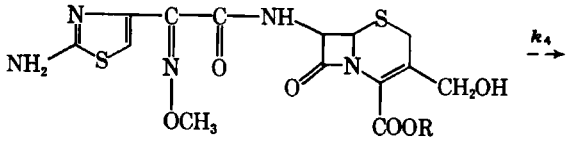


I

k_1

β-Lactam Ring Opened Products

Desacetylcefotaxime

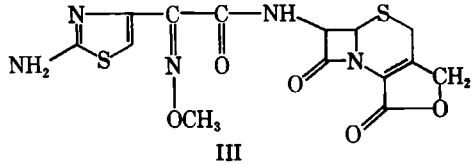


II

k_3

β-Lactam Ring Opened Products

Desacetylcefotaxime Lactone



III

k_5

β-Lactam Ring Opened Products

R = H, Na

Scheme I—Reaction pathways for the degradation of I in aqueous solution.

scribed previously (6). Buffers and all other chemicals were reagent grade.

Buffers—The buffers used in the kinetic studies were hydrochloric acid–potassium chloride (pH −0.10, 0.48, 1.15, and 2.23), citrate buffer (pH 2.83), acetate buffer (pH 3.93, 4.58, 5.07, and 5.52), phosphate buffer (pH 5.85, 6.51, 6.90, 7.57, and 7.93), borate buffer (pH 8.41, 8.93, and 9.06), and carbonate buffer (pH 9.89). The buffers were 0.1 *M* with respect to hydrochloric acid, citrate, acetate, phosphate, borate, and carbonate ions (except when a buffer effect was investigated), and they were adjusted to an ionic strength of 0.5 with potassium chloride, except when primary salt effects were studied. They were prepared by dissolving specific molar ratios of the buffer pair together with potassium chloride in water or by dissolving the acidic member of the buffer pair together with potassium chloride and adjusting the pH with 1.0 *N* NaOH. The pH of each solution was measured using a pH meter[3] equipped with a combination electrode[3].

Kinetic Procedure—Approximately 100 mg of I was accurately weighed and dissolved in 100 ml of the appropriate buffer to produce a solution having a concentration of 2.09×10^{-3} *M*. All buffers were preheated to the temperature of the study. The flasks were maintained at a constant temperature (±0.2°) in a thermostatically controlled water bath. Aliquots were withdrawn at desired time intervals, diluted, and immediately analyzed.

Analytical Procedure—Residual I was determined using a high-pressure liquid chromatographic (HPLC) method. The liquid chromatograph[4] was equipped with a variable wavelength UV detector[5] set at 235 nm; the separation was carried out using a 300 × 3.9-mm octadecylsilane column[6] with a methanol–phosphate buffer (pH 7.5) (12:88) mobile phase. The column pressure was 1700–1800 psi at a flow rate of 1.2–1.3 ml/min. Peak areas, which were calculated by a programmable integrator[7], were used to quantitate the amount of I remaining. Standard curves of the peak area of I *versus* concentration exhibited linear response in the working concentration range of 10–100 μg/ml. The sample peak areas were converted to concentrations by comparison with a standard curve which was obtained daily.

[3] Corning Glass Works.
[4] Waters Associates, Milford, Mass.
[5] Perkin-Elmer LC-75.
[6] μ Bondapak C_{18}, Waters Associates, Milford, Mass.

Table I—Buffer Systems, Observed Rate Constants, and $t_{90\%}$ for the Degradation of I at 25°

pH	Buffer System	k_{obs}, $hr^{-1} \times 10^3$	$t_{90\%}$, hr
−0.10	HCl	488.2	0.2
0.48	HCl	140.0	0.8
1.15	HCl–KCl	35.5	3.0
2.23	HCl–KCl	7.82	13.4
2.83	Citrate	4.96	21.2
3.93	Acetate	3.67	28.6
4.58	Acetate	2.72	38.6
5.07	Acetate	2.85	36.9
5.52	Acetate	3.24	32.4
5.85	Phosphate	3.08	34.2
6.51	Phosphate	3.23	32.5
6.90	Phosphate	3.44	30.5
7.57	Phosphate	4.23	24.8
7.93	Phosphate	5.73	18.3
8.41	Borate	8.27	12.7
8.94	Borate	25.5	4.1
9.06	Borate	27.0	3.9
9.89	Carbonate	177.0	0.6

RESULTS AND DISCUSSION

Degradation Reactions, Reaction Order, and Observed Rate Constants—Compound I, a 3-acetoxymethylcephalosporin, has three possible sites at which degradation can occur: the amide side chain, the β-lactam ring, and the acetoxy ester group. Under conditions of pharmaceutical interest, the β-lactam ring opening and acetoxy hydrolysis predominate. The degradation reactions for I have been proposed and are shown in Scheme I. Such a scheme has been shown to be valid for other 3-acetoxymethylcephalosporins (6–10) and is supported by the experimental results observed in this study. Compound I undergoes two parallel reactions simultaneously: β-lactam ring opening (k_1) and deesterification at the C-3 position (k_2). At pH values <4, II can undergo an internal ring closure (k_4) to form III. Both II and III are susceptible to β-lactam hydrolysis (k_3,k_5). Figure 1 shows typical chromatograms

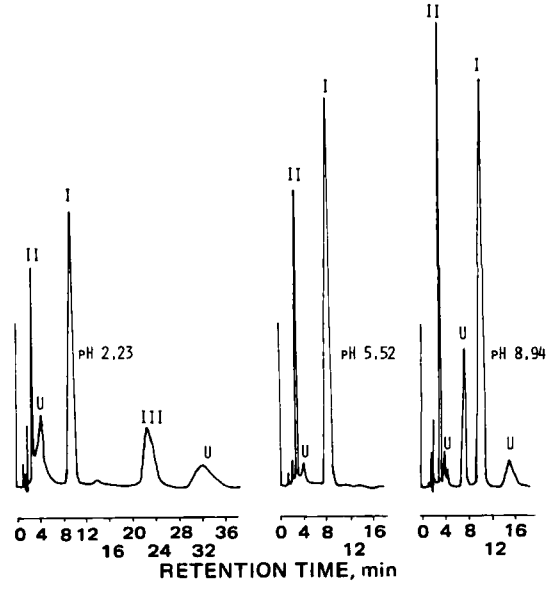


Figure 1—*High-pressure liquid chromatographs of 40–70% degraded I at 25°, μ = 0.5, and several pH values. Key: (I) cefotaxime; (II) desacetylcefotaxime; (III) desacetylcefotaxime lactone; (U) unknown peak.*

[7] Hewlett-Packard HP 3388.

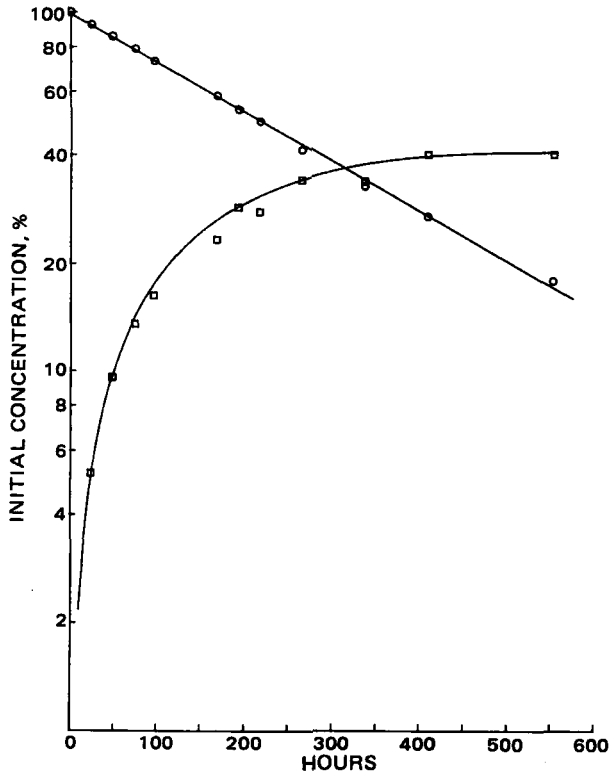


Figure 2—*Time courses for I (○) and II (□) during the degradation of I at pH 5.52, 25°, and μ = 0.5.*

of acidic (pH 2.23), neutral (pH 5.52), and basic (pH 8.94) reaction mixtures of I, which illustrate the formation of the degradation products described in Scheme I. The de-esterification reaction (k_2) occurs more rapidly, being ~1.8 times faster than the β-lactam ring opening (k_1) at pH 5.52. Figure 2 illustrates the kinetics of the disappearance of I and the formation of II at pH 5.52. The observed rate constants, k_{obs}, determined in this study are actually the sum of k_1 and k_2.

The degradation of I followed pseudo first-order kinetics at constant pH, temperature, and ionic strength over the pH range studied. This first-order dependence on the concentration of I is illustrated in Fig. 3, which shows that at pH 5.52, the log concentration *versus* time plots are

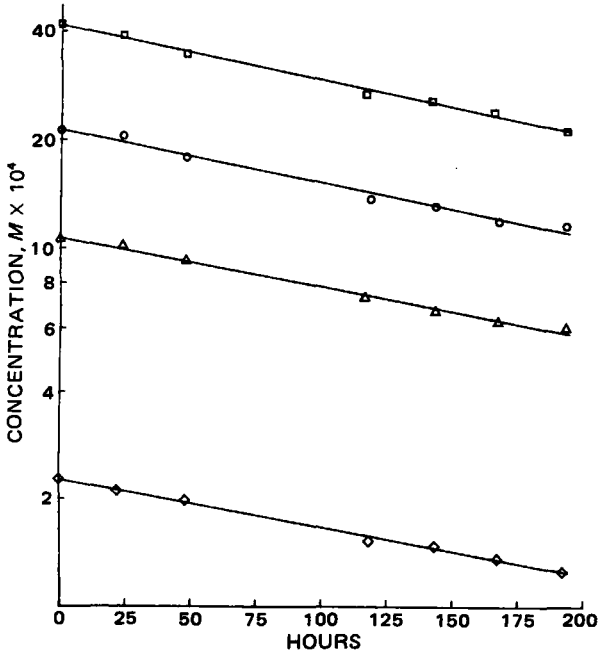


Figure 3—*Plots illustrating first-order dependence on the concentration of I at pH 5.52, 25°, and μ = 0.5. Key: (□) 2.0 mg/ml; (○) 1.0 mg/ml; (△) 0.5 mg/ml; (◇) 0.1 mg/ml.*

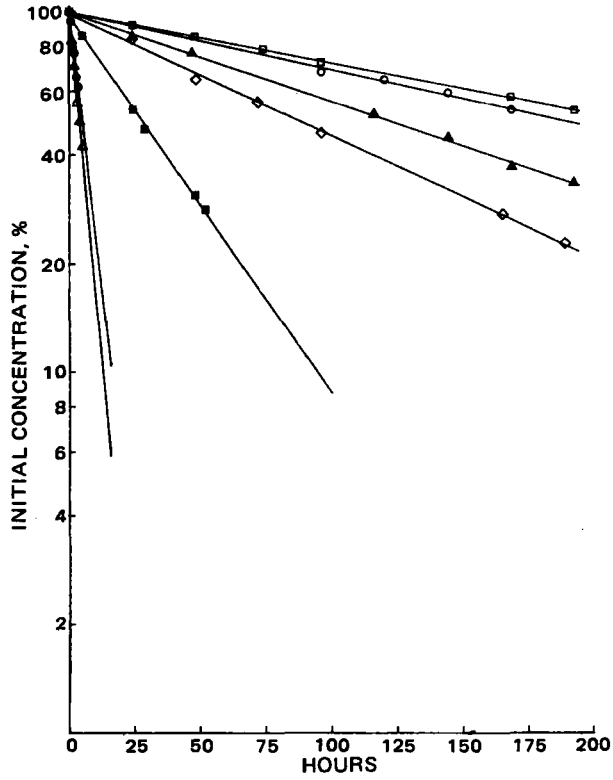


Figure 4—*Observed pseudo first-order plots for the degradation of I at various pH values, 25°, and μ = 0.5. Key: (□) pH 5.52; (○) pH 3.93; (▲) pH 7.93; (◇) pH 2.23; (■) pH 8.94; (●) pH 0.48; (△) pH 9.89.*

linear and parallel over a 20-fold concentration range (2.09×10^{-4}–4.19×10^{-3} *M*). Similar results were observed at pH 2.23 and 8.94. Further evidence of first-order kinetics is that semilogarithmic plots of the percent residual I *versus* time are linear over the pH range studied as shown in Fig. 4. The observed rate constants and the buffer systems employed are listed in Table I.

Catalytic Effect of Buffer Systems—The catalytic effect of the buffer systems used in the kinetic studies was determined at constant pH, temperature, ionic strength (μ = 0.5), and drug concentration, with only the buffer concentration varying (0.05–0.2 *M*). This experiment was done at several pH values within the effective range for each buffer employed. No appreciable effect on the degradation of I was observed for any of the buffer species used in the study.

Primary Salt Effect—The primary salt effect on the hydrolysis of

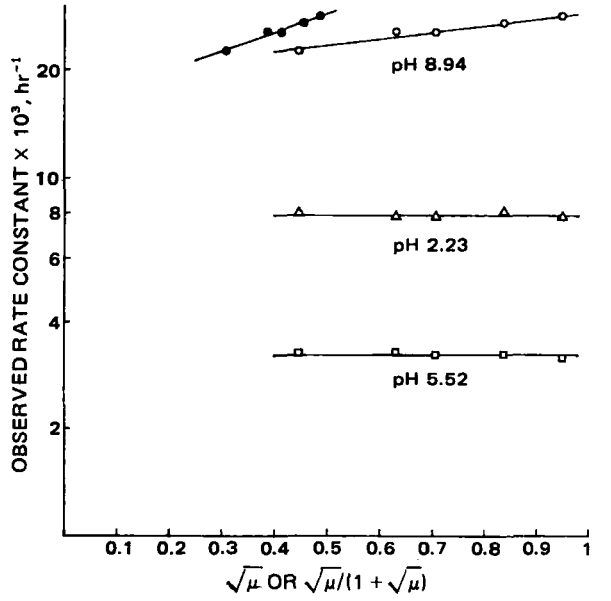


Figure 5—*Plots of log* k_{obs} versus $\sqrt{\mu}$ (○, △, and □) or $\sqrt{\mu}/(1+\sqrt{\mu})$ (●) *for the degradation of I at several pH values and 25°.*

Table II—Effect of Ionic Strength on Pseudo First-Order Rate Constants for the Degradation of I at pH 2.23, 5.52, and 8.94

Ionic Strength	k_{obs}, $hr^{-1} \times 10^3$		
	pH 2.23	pH 5.52	pH 8.94
0.2	7.99	3.28	22.6
0.4	7.82	3.30	25.6
0.5	7.82	3.24	25.5
0.7	8.07	3.25	27.1
0.9	7.79	3.17	28.3

I was studied at constant pH, temperature, and drug concentration, but the ionic strength was varied by potassium chloride addition. Studies were conducted at pH 2.23, 5.52, and 8.94 for ionic strength values ranging from 0.2 to 0.9. The data at these pH values and differing ionic strengths are given in Table II.

Within limits of the Debye-Huckel expressions, plots of k_{obs} *versus* $\sqrt{\mu}$ or $\sqrt{\mu}/(1+\sqrt{\mu})$ should yield slopes theoretically equal to $2AZ_AZ_B$, where A is a constant for the solvent at a given temperature ($A = 0.509$ at 25°, water) and Z_A and Z_B are the charges on reaction species A and B, respectively (11). Plots of k_{obs} *versus* $\sqrt{\mu}/(1+\sqrt{\mu})$ are perhaps preferred in that it has been pointed out that the use of $\sqrt{\mu}/(1+\sqrt{\mu})$ instead of $\sqrt{\mu}$ at higher ionic strengths more frequently produces values of $2AZ_AZ_B$ that are in agreement with the predicted values (12).

Figure 5 is a plot of the log k_{obs} *versus* $\sqrt{\mu}$ and $\sqrt{\mu}/(1+\sqrt{\mu})$ from the data in Table II. No kinetic salt effects were observed at pH 2.23 or 5.52, indicating that at least one of the reacting molecules is uncharged, while at pH 8.94 plots of $\sqrt{\mu}$ and $\sqrt{\mu}/(1+\sqrt{\mu})$ give positive slopes of +0.19 and +0.52, respectively. This result can be used as evidence that two negatively charged ions are reacting.

pH–Rate Profile—The pH dependence of the overall first-order rate constant for the degradation of I at 25° and an ionic strength of 0.5 is shown in Fig. 6. The pH–rate profile exhibits a U-shape which is typical of cephalosporins without an α-amino group in the C-7 side chain (7–9). This type of curve is characteristic of reactions that are susceptible to specific acid–base catalysis and that obey the general rate law:

$$k_{obs} = k_H a_{H^+} + k_w + k_{OH}[OH^-] \qquad \text{(Eq. 1)}$$

where k_H and k_{OH} are the second-order rate constants for the hydrogen ion-catalyzed degradation and hydroxide ion-catalyzed degradation, respectively; k_w is the first-order rate constant of spontaneous or water catalyzed degradation; a_{H^+} is the activity of hydrogen ion as measured by the glass electrode; and $[OH^-]$ is the hydroxide ion concentration.

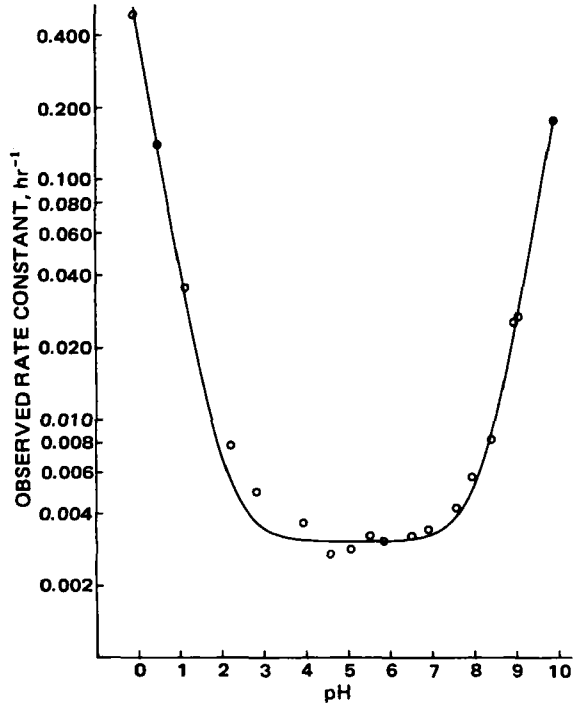


Figure 6—*Log k_{obs}–pH profile for the degradation of I in aqueous solution at 25° and $\mu = 0.5$. The points are experimental values, and the solid line is the theoretical curve calculated from Eq. 1.*

Table III—Effect of Temperature on the Degradation of I in Buffer Solutions at $\mu = 0.5$

pH	Temperature	k_{obs}, $hr^{-1} \times 10^3$	E_a, kcal/mole	log A, hr^{-1}	$k_{25°}$, $hr^{-1} \times 10^3$ (theoretical)	$k_{25°}$, $hr^{-1} \times 10^3$ (observed)
2.23	35	24.0	21.1	13.4	7.46	7.82
	45	71.2				
	55	196.7				
5.52	35	13.2	24.7	15.6	3.30	3.24
	45	44.7				
	55	154.2				
8.94	35	87.2	9.4[a]	15.2	24.50	25.50
	45	279.8				
	55	851.4				

[a] The calculated energy of activation E_a, was 22.9 kcal/mole, but this value includes the heat of ionization of water, which at 25° equals 13.5 kcal/mole (13).

Estimates of k_H, k_w, and k_{OH} were obtained from a simultaneous solution of k_{obs} (Eq. 1) at pH 0.48, 5.85, and 9.89. The hydroxide ion concentration was calculated according to:

$$[OH^-] = K_{w(exp)}/a_{H^+}\gamma\pm \qquad \text{(Eq. 2)}$$

where $K_{w(exp)} = 1.008 \times 10^{-14}$ and $\gamma\pm$ is the mean ionic activity coefficient product of water which is equal to 0.73, both at 25° and $\mu = 0.5$ (13). The resulting rate constants were $k_H = 0.4137\ M^{-1}\ hr^{-1}$, $k_w = 3.064 \times 10^{-3}\ hr^{-1}$ and $k_{OH} = 1616.5\ M^{-1}\ hr^{-1}$. The theoretical profile generated using these constants adequately describes the behavior of I and can be interpreted kinetically as follows:

Acid catalysis is important <pH 4 and base catalysis occurs >pH 8. The linear portions of the curve >pH 1.2 and 8.9 have slopes of −0.92 and +0.92, respectively, indicating that specific acid and specific base catalysis is occurring in these pH regions. The horizontal portion between pH 4 and 7 is pH independent, suggesting that the predominating reaction is the attack of water on the ionized species of I. The positive deviation in the pH 2–4 range can be postulated as being due to an intramolecular general acid catalysis of the carboxyl group on the deacetylation reaction. Such an explanation is logical in view of the facts that the pKa of I at 25° is 3.4[8] and that the drug concentration is significant in relation to the

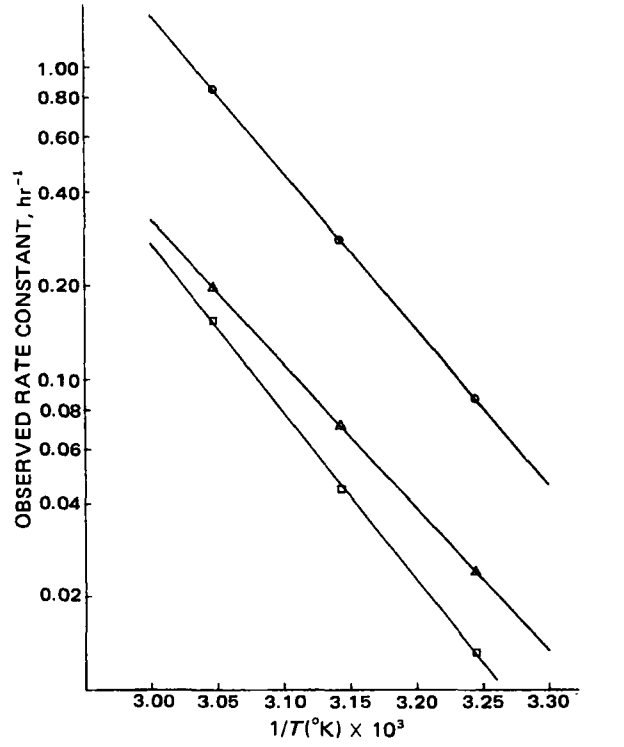


Figure 7—*Arrhenius plots for the degradation of I at several pH values and $\mu = 0.5$. Key: (△) pH 2.23; (□) pH 5.52; (○) pH 8.94.*

[8] Personal communication.

hydrogen ion concentration in this pH region. Ionic strength effects support this interpretation.

The theoretical pH minimum for the degradation of I obtained by taking the derivative of Eq. 1 and equating it to zero was pH 5.13.

Temperature Dependency—The dependence of degradation of I on temperature was determined by measuring the rate of decomposition at 35, 45, and 55° at pH 2.23, 5.52, and 8.94 and ionic strength is 0.5. The values of k_{obs}, E_a, and log A are given in Table III, and the corresponding Arrhenius plots are shown in Fig. 7. The theoretical $k_{25°}$ calculated using the energy of E_a, activation was in good agreement with the experimentally determined $k_{25°}$ at each of the three pH values, indicating that the data can be used to predict the stability of I over a wide range of pH and temperature conditions.

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Synthesis and Biological Activity of an Amino Analogue of a Tripeptide Inhibitor of Angiotensin-Converting Enzyme

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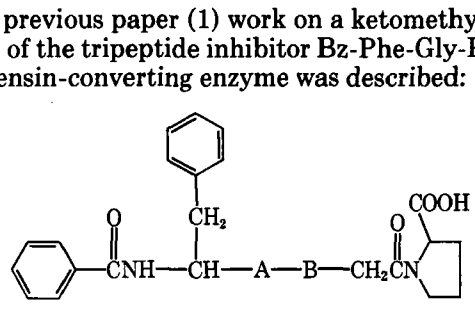
Received September 8, 1980, from the *Bio-Organic Chemistry Laboratory, SRI International, Menlo Park, CA 94025.* Accepted for publication February 25, 1982.

Abstract □ An amino analogue of *N*-benzoyl-phenylalanyl-glycyl-proline, a tripeptide inhibitor of angiotensin-converting enzyme, was synthesized. The analogue (III) has the phenylalanyl-glycine amide linkage of *N*-benzoyl-phenylalanyl-glycyl-proline reduced to a methylene amine. Compound III was tested as an inhibitor of porcine plasma angiotensin-converting enzyme and has an I_{50} of 620 μM compared with an I_{50} of 9.6 μM for its parent tripeptide. These results are explained in terms of a proposed model of the converting-enzyme active site.

Keyphrases □ Angiotensin-converting enzyme—synthesis and biological activity of an amino analogue of *N*-benzoyl-phenylalanyl-glycyl-proline, a tripeptide inhibitor □ *N*-Benzoyl-phenylalanyl-glycyl-proline—tripeptide inhibitor of angiotensin-converting enzyme, synthesis and biological activity of an amino analogue

In a previous paper (1) work on a ketomethylene analogue I of the tripeptide inhibitor Bz-Phe-Gly-Pro (II) of angiotensin-converting enzyme was described:



I: A = CO, B = CH_2
II: A = CO, B = NH
III: A = CH_2, B = NH

The replacement of the peptide amide linkage in II with a ketomethylene group was designed to stabilize this portion of the peptide molecule to peptidase cleavage. This substitution yielded a peptide analogue (I) with >100 times the inhibition activity against angiotensin-converting enzyme of the parent peptide.

A second approach to stabilizing this same peptide amide linkage in II to peptidase cleavage would be to reduce it to the corresponding methyleneamino compound (III). This compound has been synthesized and its inhibition activity against angiotensin-converting enzyme will be discussed.

BACKGROUND

A method for the synthesis of III was desired which would maintain the same stereochemistry around the optical centers as that found in the tripeptide (II). Initially, numerous attempts were made to selectively reduce the amide, but not the ester, group of *N*-benzyloxycarbonyl-phenylalanyl-glycine ethyl ester with borane, as reported previously (2), with *N*-benzyloxycarbonyl-glycyl-leucine methyl ester as the starting material. In contrast to those results the ester group of *N*-benzyloxycarbonyl-phenylalanyl-glycine ethyl ester was reduced more rapidly than the amide group by borane.

Because selective reduction of the desired amide functionality did not seem possible in this case, the dipeptide acid *N*-benzyloxycarbonyl-phenylalanyl-glycine (IV) was used as a starting material for the synthesis of III (Scheme I). The 9-fluorenyl-methoxycarbonyl amino blocking group (3) was used because of its stability to acidic conditions such as the Jones oxidation (4).

The low yield obtained for the transformation of VI to VIII was

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