Alkaline Hydrolysis of Cephaloridine: An ^{1}H—NMR Study. Temperature Dependence of the Rate Constants

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Abstract

A kinetic study on the basic hydrolysis of cephaloridine at $pD = 10.5$ was carried out by using the ^{1}H—NMR technique. Epimerization at H_7, a nucleophilic attack of hydroxyl ion on the β-lactam carbonyl group followed by the release of the pyridine molecule, and isomerization of the double bond at position 3 in the dihydrothiazine ring were the major reactions observed.

Based on the results obtained, it should be emphasized that the presence of a pyridine group at 3′ results in a slightly increased formation constant for the exo methylene compound relative to other cephalosporins with different substituents at that position.

The activation energy for the epimerization constant and the cleavage of the β-lactam ring at pD 10.5 was 21.2 kcal/mol. © 1993 John Wiley & Sons, Inc.

Introduction

The three-dimensional shape of an inhibitory molecule can be one of the important factors in determining how well it fits into the active site of each lethal target enzyme [1]. Cohen et al. [2] assumed that not only the antibiotic structure but also the chemical reactivity are important for antibacterial activity.

Hence, for a congenerous set of structures, where molecular shape is invariant in the sterically important regions, chemical reactivity becomes a dominant determinant of antibacterial activity [3]. This has fostered kinetic studies on the basic hydrolysis of cephalosporins [4–8] using the iodometric method, pH-stat titration, UV spectroscopy and HPLC, among others.

Cephaloridine belongs to the first generation of cephalosporins and is characterized by the presence of a thiopheneacetamido group at 7 and a pyridine group at 3′ (1 in Scheme I).

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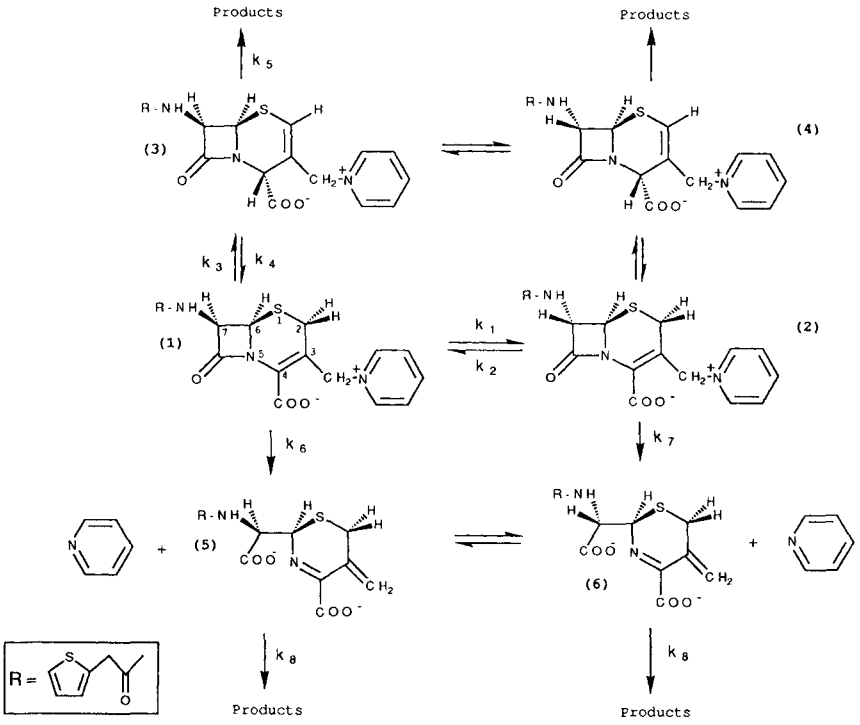

Scheme I

In the 1970s, Yamana and Tsuji [4] were the first to kinetically investigate the basic hydrolysis of this antibiotic; however, the kinetic constant they obtained only reflected the nucleophilic attack of the hydroxyl function on the β-lactam carbonyl as they considered no other potential reaction. Nishikawa et al. [8], who studied the basic hydrolysis of oxacephems by ^{1}H—NMR using a cephaloridine analogue, also concluded that the process only involved cleavage of the β-lactam ring and subsequent release of the pyridine group. However, HPLC experiments with cephaloridine [9] showed the occurrence of other degradation products in the basic hydrolysis reaction.

Recent research into cefotaxime conducted in our laboratory [10] showed the occurrence of another reaction in parallel with the hydrolysis of the β-lactam ring, viz. epimerization at H_7.

Since the occurrence of reactions other than the hydrolysis of the β-lactam ring in the basic hydrolysis of cephaloridine had never been considered to date, in this work we carried out a kinetic ^{1}H—NMR study on such a process, where the different reactions involved are described in terms of the degradation products obtained. In addition, we determined the influence of temperature on the kinetic constants for the process.

Experimental

Materials

Cephaloridine ($C_{19}H_{17}N_3O_4S_2$), obtained from Sigma was used without further purification. Deuterium oxide (99.8% D) was purchased from Sigma.

NMR Spectroscopy

The NMR spectra were obtained on Bruker AMX-300 spectrometer. The concentration of cephaloridine in D_2O was $8.41 \cdot 10^{-3}$ M.; the solution was stabilized at $pD = 10.5$ ($pD = -\log [D^+]$) by a carbonate buffer solution; ionic strength of 0.5 M. Sample tube diameter, 5 mm. 3-(trimethylsilyl)-1-propane-sulfonic acid (DSS) was used as internal reference. The resonances assignments were based on spectral characteristics previously described [6,7,11,12]. Chemical shift values (δ) are given in ppm.

Product Analysis

An amount of 35 mg of cephaloridine was dissolved in 2 mL of buffer at pH 10.5 and ionic strength $I = 2$ M. After 1 h at 37°C, the reaction mixture was frozen for subsequent isolation of the different compounds present by liquid chromatography on a Shimadzu LC-9A instrument furnished with a Rheodyne 7125 universal injector and a Spherisorb ODS-2 column (10 μm, 25 × 0.46 cm). A 12:88 mixture of acetonitrile and 0.01 M ammonium acetate at pH 6.50 was used as eluent. The solutions thus obtained were subsequently lyophilized. Finally, the residues were used to record their resonance spectra. Relevant spectroscopic data of the isolated compounds are given below.

Cephaloridine (1 in Scheme I). ^{1}H—NMR (300 MHz, DSS) δ: 3.18, 3.60 (2H, *AB*, J = 17.0 Hz, C_2—H_2), 3.90 (2H, *m*, thiophene—CH_2—CO), 5.16 (1H, *d*, J = 4.7 Hz, C_6—H), 5.35, 5.54 (2H, *AB*, J = 14.0 Hz, C_3—CH_2), 5.68 (1H, *d*, J = 4.7 Hz, C_7—H), 7.02 (2H, *m*, thiophene), 7.35 (1H, *m*, thiophene), 8.10 (2H, *m*, pyridine), 8.57 (1H, *m*, pyridine), and 8.94 (2H, *d*, J = 6.0 Hz, pyridine).

7-epimer of cephaloridine (2). ^{1}H—NMR (300 MHz, DSS) δ: 3.14, 3.59 (2H, *AB*, J = 18.7 Hz, C_2—H_2), 3.91 (2H, *m*, thiophene—CH_2—CO), 4.91 (1H, *d*, J = 4.3 Hz, C_6—H), 4.95 (1H, *d*, J = 4.3 Hz, C_7—H), 5.28, 5.41 (2H, *AB*, J = 14.0 Hz, C_3—CH_2), 7.04 (2H, *m*, thiophene), 7.39 (1H, *m*, thiophene), 8.10 (2H, *m*, pyridine), 8.57 (1H, *m*, pyridine), and 8.92 (2H, *d*, J = 5.3 Hz, pyridine).

Δ^2-cephaloridine (3). ^{1}H—NMR (300 MHz, DSS) δ: 3.92 (2H, *m*, thiophene—CH_2—CO), 4.57 (1H, *s*, C_4—H), 5.31 (2H, *s*, C_6—H and C_7—H), 5.34, 5.51 (2H, *AB*, J = 14.9 Hz, C_3—CH_2), 6.81 (1H, *s*, C_2—H), 7.05 (2H, *m*, thiophene), 7.38 (1H, *m*, thiophene), 8.08 (2H, *m*, pyridine), 8.58 (1H, *m*, pyridine), and 8.86 (2H, *d*, J = 6.2 Hz, pyridine).

Exo methylene compound (5). ^{1}H—NMR (300 MHz, DSS) δ: 3.38, 3.67 (2H, *AB*, J = 14.0 Hz, C_2—H_2), 3.83, 3.93 (2H, *m*, thiophene—CH_2—CO), 4.62 (1H, *d*, J = 2.7 Hz, C_6—H), 5.42 (1H, *d*, J = 2.7 Hz, C_7—H) 5.60,

5.65 (2H, 2*s*, J = 0 Hz, C_3=CH_2), 7.01 (2H, *m*, thiophene), and 7.33 (1H, *m*, thiophene).

Results and Discussion

Kinetic Constants

The basic hydrolysis of cephaloridine was studied by using the ^{1}H—NMR technique at 37°C, pD = 10.5, and I = 0.5 M. It should be noted that the use of D_2O resulted in slightly decreased kinetic constants relative to those obtained in H_2O owing to the isotopic effect of deuterium.

Figure 1 shows the resonance spectrum obtained over the range 3.0–4.0 ppm. As can be seen, it reflects the presence of an AB system corresponding to the protons in the C_2—H_2 of cephaloridine. These signals change according to a first-order kinetics with an apparent constant k_I = 2.0 ± 0.2 h^{-1}. Two other signals appear at 3.11 and 3.17 ppm in the course of the reaction which correspond to one proton in the C_2—H_2 group of the 7-epimer of cephaloridine. The two signals yielded by the other proton in the 3.60 ppm region are concealed. Four other *AB* signals at 3.69, 3.65, 3.41, and 3.46 ppm corresponding to the C_2—H_2 to the exo methylene compound are also observed. The figure shows a multiplet centred at 3.9 ppm which corresponds to the thiophene-CH_2—CO sequence of cephaloridine; as the reaction develops, other signals corresponding to the same protons, though in an open β-lactam ring, are observed.

The region between 4.6 and 5.8 ppm (Fig. 2) initially shows two doublets corresponding to protons 6 and 7 in cephaloridine. Their areas decrease (k_{II} = 1.8 ± 0.18 h^{-1}) on epimerization of H_7, which shifts the doublets upfield and decreases their coupling constant [12], the cleavage of the β-lactam ring and the isomerization of the double bond. A singlet corre-

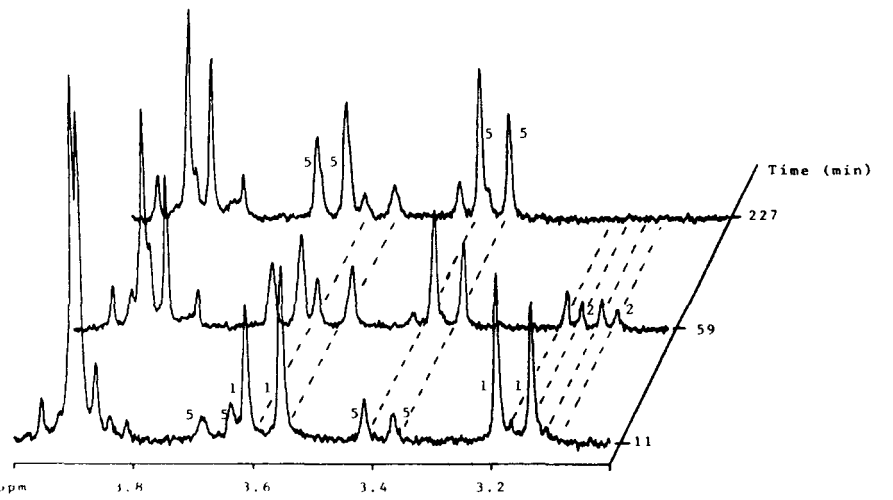

Figure 1. Change in intensities of ^{1}H—NMR signals during degradation of cephaloridine in deuterated carbonate buffer solution (pD 10.5, 37°C). Peak labels coincide with compounds numbers.

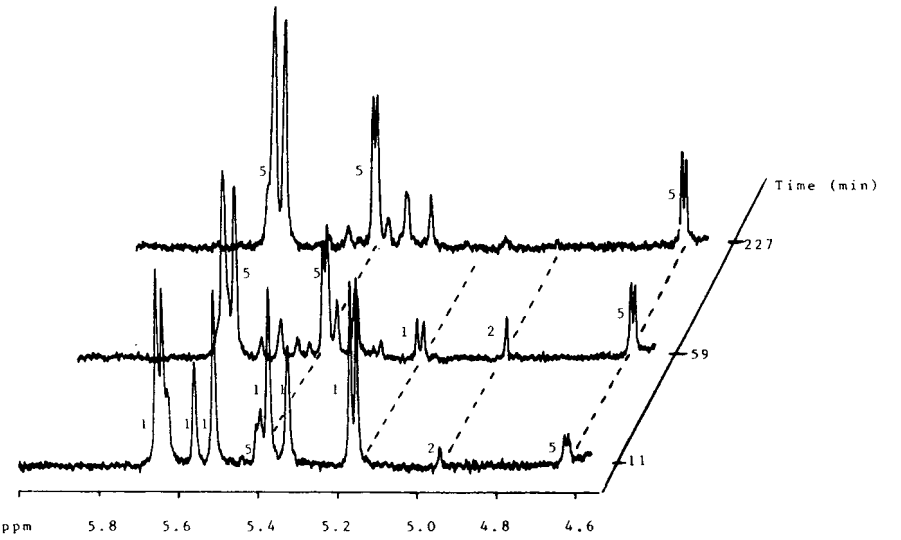

Figure 2. Change in intensities of ^1H—NMR signals during degradation of cephaloridine in deuterated carbonate buffer solution (pD 10.5, 37°C). Peak labels coincide with compounds numbers.

sponding to H_6 in the 7-epimer of cephaloridine also appears at 4.9 ppm. It does not occur as a doublet because the proton at 7 is converted into a deuterium atom in the course of the epimerization reaction in D_2O, so no coupling between D_7 and H_6 takes place. This region also includes an AB system corresponding to the C_3—CH_2 of the antibiotic. The signal intensity decreases as a result of the isomerization of the double bond, the epimerization reaction and the formation of the exo methylene group. The doublets corresponding to H_6 and H_7 in the exo methylene compound appear at ca. 4.65 and 5.4 ppm. The former doublet is smaller than the latter as a result of the signal transmitter being placed at 4.60 ppm in order to eliminate the H_2O peak.

The signals for the C_3—CH_2 protons in the exo methylene group appear at 5.60 and 5.65 ppm, i.e., in the olefin proton region. These signals are always observed in the resonance spectrum of the reaction mixture in the basic hydrolysis of cephalosporins with a suitable leaving group at 3′ [8].

The region between 7.4 and 6.6 ppm (Fig. 3) initially shows the signals corresponding to the three protons in the thiophene group at 7. As the reaction proceeds, a singlet corresponding to the C_2—H in the Δ^2-cephaloridine formed appears at 6.8 ppm.

Finally, the signals for the pyridine protons appear in the region from 9 to 7.4 ppm (Fig. 4). The initial signals include two triplets at 8.10 and 8.52 and a doublet at 8.94 ppm corresponding to the protons in pyridine bound to the dihydrothiazine ring. The signal at 8.10 evolves with an apparent constant $k_{III} = 0.85 \pm 0.15\ h^{-1}$. As the reaction develops, the signals corresponding to free pyridine appear at 8.58 (*d*), 7.87 (*m*), and 7.46 ppm (*m*) since the formation of the exo methylene group involves the release of the pyridine group from the dihydrothiazine ring. In addition, a doublet corresponding to the Δ^2-isomer of cephaloridine appears at 8.86 ppm. It should be noted that

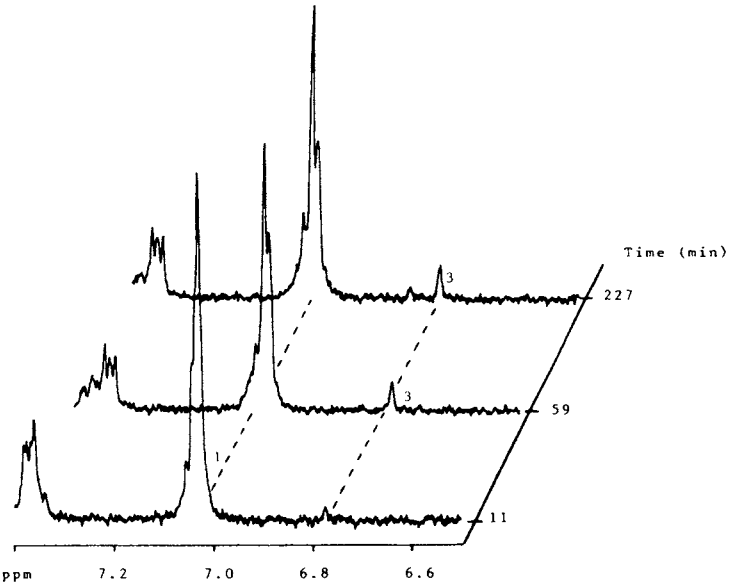

Figure 3. Change in intensities of ^1H—NMR signals during degradation of cephaloridine in deuterated carbonate buffer solution (pD 10.5, 37°C). Peak labels coincide with compounds numbers.

the epimerization reaction does not alter the chemical shift of the pyridine protons; also, the formation of the Δ^2-isomer only affects the two protons adjacent to the nitrogen atom in the pyridine ring.

Based on the changes in the resonance spectrum in the course of the reaction and on the degradation products isolated, we put forward the kinetic sequence depicted in Scheme I.

According to the scheme, cephaloridine can react via three pathways in an alkaline medium, namely: (a) with reversible epimerization at H_7 to yield its 7-epimer (2); (b) through nucleophilic attack of the hydroxyl ion on the carbonyl group of the β-lactam ring to give rise to the exo compound (5) and release pyridine; and (c) via the reversible, base-catalyzed isomerization of the Δ^3 double bond to yield the corresponding Δ^2-cephaloridine (3). The isomerization of the Δ^3- to the Δ^2-isomer introduces a new asymmetric centre at C_4. However, only a single stable isomer is reportedly obtained [13], viz. compound 3, whose acid group lies below the dihydrothiazine ring.

The nucleophilic attack on the carbonyl group in the β-lactam ring of the 7-epimer of cephaloridine gives rise to an exomethylene compound (6) that differs from 5 in its stereochemistry at C_7. However, compound 6 has not been detected due to its low concentration. Therefore we considered $k_6 = k_7$ and did not calculate the rate constants of epimerization at C_7—H of 5 and 6. Chemically, one would expect the Δ^2-isomer of the 7-epimer to be formed (4) by epimerization of H_7 in compound 3 or isomerization of the double bond in compound 2. Since compounds 2 and 3 occur at fairly low concentrations, then 4 must be produced in very small amounts. This precluded calculation of the constants involved in its formation.

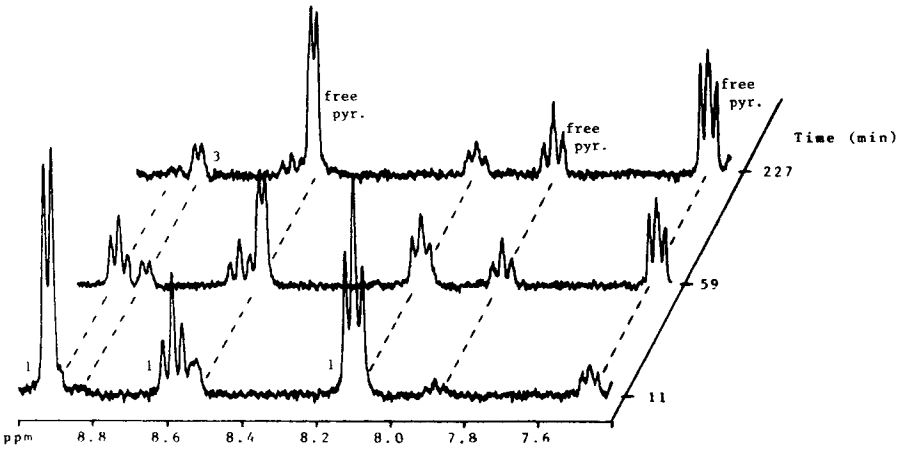

Figure 4. Change in intensities of ^{1}H—NMR signals during degradation of cephaloridine in deuterated carbonate buffer solution (pD 10.5, 37°C). Peak labels coincide with compounds numbers.

Compound 3 may undergo a nucleophilic attack on the carbonyl group of its β-lactam ring to yield products with an open ring. Finally, compounds 5 and 6 undergo subsequent degradation reactions.

The signals used in the kinetic study were the doublet at 5.60 ppm (compound 1), the doublets at 8.85 ppm (compound 3) and 3.7–3.60 ppm (compounds 5) and the singlet at 4.90 ppm (compound 2).

The rate constants were calculated by using the GIT kinetic data processing software package [14,15], which performs a numerical integration of the differential equations associated with a given kinetic scheme.

The different rate constants obtained were as follows (h^{-1}):

$$k_1 = 0.33 \pm 0.05 \quad k_4 = 0.12 \pm 0.03 \quad k_7 = 1.15 \pm 0.15$$
$$k_2 = 0.29 \pm 0.05 \quad k_5 = 0 \quad k_8 = 0.03 \pm 0.01$$
$$k_3 = 0.25 \pm 0.05 \quad k_6 = 1.15 \pm 0.15$$

Figure 5 shows the fitting of the experimental data to the kinetic scheme on the basis of the above rate constant values.

Taking into account that the concentrations of 2 and 3 are both very low, k_{I} and k_{II} must roughly correspond to the sum of constants k_1, k_3, and k_6. Also, k_{III} must correspond to k_6 in the kinetic scheme since the triplet at 8.10 ppm provides the overall concentration of compounds 1, 2, and 3.

On comparing the above results with those recently obtained for cefotaxime [9], a cephalosporin antibiotic bearing an acetyl group at 3′ and an oxime group at 7, namely $k_1 = 0.27\ \mathrm{h}^{-1}$, $k_2 = 0.24\ \mathrm{h}^{-1}$, $k_6 = 0.18\ \mathrm{h}^{-1}$, and $k_7 = 0.15\ \mathrm{h}^{-1}$ (all determined by ^{1}H—NMR at $pD = 10.5$, $I = 0.5$, and $T = 37$°C), it is seen that the epimerization constant increases only slightly as a result of the presence of a pyridine group at 3′, whereas that for the nucleophilic attack of the hydroxyl group on the β-lactam carbonyl increases to a greater extent for the same reason. This indicates that the epimerization reaction is minimally influenced by the electron-withdrawing character of the substituent at 3′.

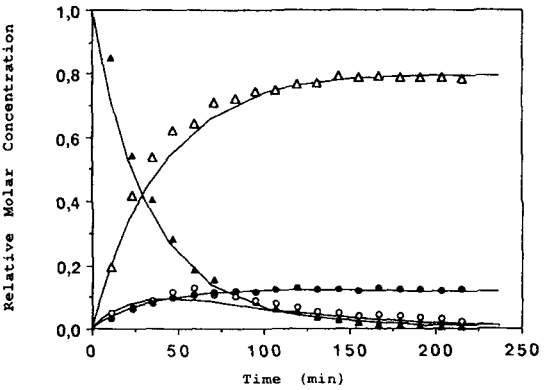

Figure 5. Time course of the alkaline degradation of cephaloridine in deuterated carbonate buffer solution. Points are the ^{1}H—NMR experimental values and continuous lines are the best theoretical fitting. (▲)— Compound 1, (○)—Compound 2, (•)—Compound 3, and (△)—Compound 4.

In a kinetic study on the hydrolysis of oxacephems by ^{1}H—NMR at pH 10.4, $\mu = 0.5$, and 35°C, Nishikawa et al. [8] obtained a constant of 1.26 h^{-1} which must be the sum of k_1, k_3, and k_6 in Scheme I. The small difference with our value can be ascribed to the different pH and temperature used.

Yamana et al. [4] obtained a value of 1.10 h^{-1} which corresponds essentially to k_6 in the kinetic scheme since their study was performed by UV spectroscopy, where the sole reaction that can decrease the band at 260 nm is the formation of the exo methylene compound.

We should emphasize that the resonance technique allows the formation of the 7-epimer to be clearly observed by monitoring the singlet at 4.9 ppm, and that of the Δ^2-isomer of cephaloridine by monitoring the singlet at 6.8 ppm and the typical double obtained in the aromatic proton region (8.86 ppm).

Influence of Temperature on the Reaction Rates

The effect of temperature on the degradation of cephaloridine was determined by measuring the degradation rates at different temperatures (between 23 and 41°C), in a carbonate buffer solution ($pD = 10.5$) and ionic strength of 0.5 M. In optimizing the kinetic constants at each temperature assayed, three approximations were adopted in order to decrease the number of parameters to be optimized and thus minimize any errors in the kinetic constants for products occurring at very low concentrations. Such approximations involved assuming (a) $k_1 = k_2$, (b) $k_3/k_4 = 2$, and (c) $k_6 = k_7$. The first approximation relies on the values for the cephaloridine constants at 37°C and those obtained for cefotaxime by ^{1}H—MNR. This is consistent with Figure 5, where the concentrations of 2 and 3 converge on the same value after a given reaction time.

The second approximation relies on the k_3 and k_4 values reported by Saab et al. [16] for the ester of cefazolin and its Δ^2-isomer at pH 7.4, $I = 0.3$ M, and $T = 40$°C, which are all in a 2:1 ratio. The electronic

environment offered by the uncharged cephalosporin esters and the free acids are different and the equilibration is more rapidly established in esters. However the ratio between the two rate constants (k_3/k_4) is similar in both antibiotics [9].

The third approximation is quite consistent with the chemical expectations since the epimerization of H_7 should not affect the nucleophilic attack of the hydroxyl ion on the β-lactam carbonyl. The values obtained for cefotaxime [10] and cephaloridine [10] at 37°C support this assumption.

The pseudo-first-order rate constants were fitted to the Arrhenius equation, $k = Ae^{-Ea/RT}$, by least-squares regression [17]. The Arrhenius plots for the reactions are shown in Figure 6 and the values of E_a and the enthalpies and entropies of activation are given in Table I.

However, the heat of ionization of water was included in the energies of activation. By assuming such a heat to be 13.0 kcal/mol [18], the actual energy of activation for each constant was calculated. The corrected values are given in brackets in Table I.

At the pH value used, $k_{obs} = k_i[OH^-]$ and $\Delta S^{\#}$ is given by

$$\Delta S_i^{\#} = 1.9872\ (\ln k_i + \ln[OH^-] - \ln(ekT/h) + E_a/RT) \quad (1)$$

where $ek/h = 5.664 \cdot 10^{10}\ \text{deg}^{-1} \cdot \text{s}^{-1}$.

The enthalpy of activation was calculated from the following equation

$$\Delta H^{\#} = E_a - RT \quad (2)$$

where E_a is the corrected energy of activation.

As can be seen in Table I, k_6 has a high energy of activation and a negative entropy of activation. Cefotaxime [19], cefazolin [20], and cefatrizine [21] have E_a values from 25 to 29 kcal/mol for their k_6 constants. On the other hand, k_3 has an E_a value that is 11 kcal/mol higher than those for the other two constants; hence, the reaction concerned is energetically unfavored, which is consistent with the experimental fact that the isomerization of the double bond has never been observed in cephalosporins with a free acid group at C_4 and no pyridine group at 3′. The presence of a free acid group at C_4 hinders the reaction, which primarily involves cephalosporins with an ester function at that position [16,22].

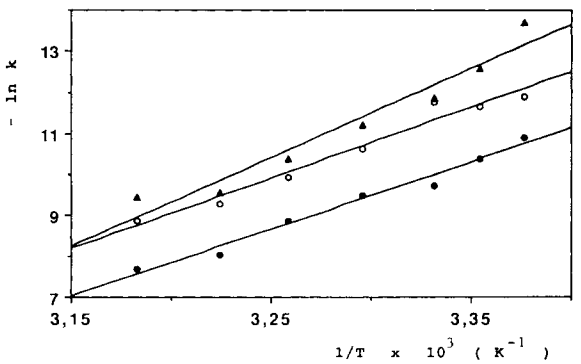

Figure 6. Arrhenius plots for the constants: k_1 (○), k_3 (▲), and k_6 (•) at $pD = 10.5$.

TABLE I. Arrhenius and activation parameters at 310 K.

Rate Constant	E_a (kcal/mol)	$\Delta H^{\#}$ (kcal/mol)	$\Delta S^{\#}$ (cal/deg mol)
k_1	34.2 (21.2)	20.5	−11.0
k_3	45.3 (32.3)	31.6	24.3
k_6	34.2 (21.2)	20.5	−8.4

In summary, the presence of a pyridine group at 3′ increases the hydrolysis constant for the β-lactam ring and also increases, though only slightly, that for the formation of the 7-epimer and the Δ^2-isomer of cephaloridine.

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