in the presence of an appropriate bridging ligand, these complexes exhibit a strong preference for inner-sphere electron transfer ($K_{i.s.}/K_{o.s.} > 1000$). Bridging μ-1,2 peroxy binuclear cobalt(II) coordination chemistry further indicates the importance of inner-sphere electron transfer to oxygen reduction, where, in all complexes studied,[51] peroxide further reduces via a μ-1,1 rearrangement to the hydroperoxide, which yields a pathway for inner-sphere electron transfer. However, blue copper centers are capable of rapid outer-sphere electron transfer,[52] and in reoxidation studies of fully reduced laccase, the type 1 and type 3 centers are reported[53] to rapidly reoxidize while the type 2 copper appears to remain reduced; these rates of reoxidation are, however, not consistent with turnover kinetics.[53] Nonetheless, a three-electron-reduced oxygen intermediate has been observed,[53,54] and an alternative role for the type 2–type 3 site could be in stabilizing this intermediate through a delocalized inner-sphere superexchange pathway. It is now important to determine the contribution of this type 2–type 3 cluster with respect to oxygen reactivity at the multicopper oxidase active site.

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Registry No. Cu, 7440-50-8; N_3^-, 14343-69-2; F^-, 16984-48-8; laccase, 80498-15-3.

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Direct Observation by ^{1}H NMR of Cephalosporoate Intermediates in Aqueous Solution during the Hydrazinolysis and β-Lactamase-Catalyzed Hydrolysis of Cephalosporins with 3′ Leaving Groups: Kinetics and Equilibria of the 3′ Elimination Reaction

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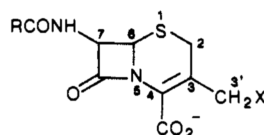
Abstract: The hydrolyses of 3,4-dimethylcephaloridine, thiophenoxycephalothin, and desacetylcephalothin, catalyzed by the β-lactamases of *Enterobacter cloacae* P99 and the TEM-2 plasmid, yield cephalosporoate intermediates in solution that retain the 3′ substituent. These intermediates have been characterized by their UV absorption and ^{1}H NMR spectra. The 3′ substituents, 3,4-lutidine, thiophenoxy, and hydroxyl, respectively, are then eliminated in a reaction, which is not enzyme-catalyzed, to give the well-known 5-*exo*-methylene-1,3-thiazine as final product. The elimination of 3,4-lutidine and thiophenoxide occurs rapidly and spontaneously but is also hydroxide ion catalyzed: phosphate buffer catalysis was not observed. On the other hand, the elimination of hydroxide ion from the other cephalosporoate is much slower and catalyzed by protons, hydroxide ions, and phosphate buffer. The elimination of 3,4-lutidine, pyridine (from cephaloridine), and thiophenoxide proceeds to a position of equilibrium, observable by ^{1}H NMR. Analogous intermediates are generated on hydrazinolysis of these cephalosporins. Thus, the elimination of 3′ leaving groups from cephalosporins is not, in general, concerted with nucleophilic β-lactam C–N bond cleavage.

The cephalosporins (**1**) are an important group of β-lactam antibiotics. Recently, we have provided evidence that, during the hydrolysis of cephalosporins with good 3′ leaving groups, X, catalyzed by the RTEM-2 β-lactamase, elimination of the leaving groups is not concerted with β-lactam cleavage.[1] An intermediate, whose absorption spectral characteristics were consistent with the structure **2**, was released by the enzyme, implying that the two-step reaction of Scheme I obtained. Preliminary observations of a similar nature have been reported by Page and co-workers.[2]

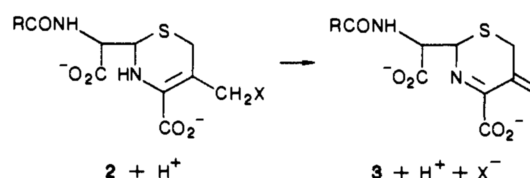
We have also shown that the elimination of the 3′ leaving group can occur at a β-lactamase active site at the acyl–enzyme stage.[3] Subsequently, Grabowski et al.[4] showed, by direct ^{13}C NMR observations, that intermediates analogous to **2** arise upon ammonolysis of cephamycins in liquid ammonia at −50 °C. Nevertheless, the structural identity of these intermediates has been questioned,[5] in our specific case and in general. This criticism

Scheme I



β-lactamase, H_2O

1a: R = 2′-thienylacetyl; X = 3,4-lutidinium
b: R = 2′-thienylacetyl; X = SPh
c: R = 2′-thienylacetyl; X = OH
d: R = 2′-thienylacetyl; X = H



2 + H^+ → **3** + H^+ + X^-

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stems from a broad base of earlier thought and experiment that suggested that elimination of 3'substituents was concerted with β-lactam C–N bond cleavage.[6-11] In this paper, therefore, we describe the results of ^1H NMR experiments that show clearly that the structure of the intermediates released by the RTEM-2 and *E. cloacae* P99 β-lactamases is **2**. Furthermore, we show that such intermediates can also be generated in aqueous solution by nonenzymic nucleophilic cleavage and that, in the presence of pyridine and thiol nucleophiles, **2** and **3** exist in equilibrium in solution, i.e., that the elimination reaction is reversible.

Experimental Section

Materials. Cephalothin, cephaloridine, desacetylcephalothin, and desacetoxycephalothin were generous gifts of Eli Lilly and Co. Thiophenol, which was purified by distillation under reduced pressure prior to use, 3,4-lutidine, and 99.8% ^2H_2O were purchased from Aldrich Chemical Co. Hydrazine hydrate and 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were procured from J. T. Baker Chemical Co. and Sigma Chemical Co., respectively. The *E. cloacae* P99 and RTEM-2 β-lactamases were obtained from the PHLS Centre for Applied Microbiology and Research, Porton Down, England, and used as received. Deionized water was used throughout, and all buffer materials were reagent grade.

3,4-Dimethylcephaloridine (1a). This material was synthesized according to the general procedure of Spencer et al.[12] Thus, 1.0 g of sodium cephalothin was dissolved in 1.0 mL of water, and to the resulting solution were added 4.55 g of potassium thiocyanate, 50 μL of 85% phosphoric acid, and 0.42 mL of 3,4-lutidine. The mixture was then stirred at 70 °C for 5 h. After cooling, it was diluted into 20 mL of water and extracted several times with chloroform. Acidification of the cooled (4 °C) aqueous layer yielded a tan precipitate that was collected by filtration, washed with water, and dried. This material was then stirred with Dowex 1-X2 anion-exchange resin (acetate form) until it had dissolved. After removal of the resin by filtration, the solution was freeze-dried, yielding 0.5 g of a pale yellow powder, whose ^1H NMR spectrum [(^2H_2O, HCO_3^-) δ 2.44 (s, 3, py CH_3), 2.56 (s, 3, py CH_3), 3.13, 3.55 (AB q, J = 18 Hz, 2, 2-H), 3.96 (s, 2, Th CH_2), 5.24, 5.40 (AB q, J = 15 Hz, 2, 3'-H), 5.16 (d, J = 4 Hz, 1, 6-H), 5.69 (d, J = 4 Hz, 1, 7-H), 7.05 (m, 2, Th 3', 5'), 7.38 (m, 1, Th 4'), 7.80 (d, J = 7 Hz, 1, Py 5-H), 8.62 (m, 2, py 2, 6-H)] showed it to be the required compound and devoid of cephalothin to the level discernible by NMR.

Thiophenoxycephalothin (1b). The procedure mentioned by Hatfield et al.[13] was employed in this case. Cephalothin free acid (500 mg, obtained by acidification of a solution of the sodium salt and carefully dried in vacuo) was dissolved in 50 mL of freshly distilled 1,2-dichloroethane under a nitrogen atmosphere. To this solution was added 200 μL of freshly distilled thiophenol and the mixture heated under reflux and nitrogen for 24 h. Extraction of cephalosporin from the cooled solution was effected by extraction with saturated aqueous sodium biocarbonate. The combined bicarbonate extracts were then acidified, and the cephalosporin was extracted into ethyl acetate. Subsequent evaporation under reduced pressure of the dried ethyl acetate solution yielded the crude product. This material was dissolved in water containing 1 equiv of sodium bicarbonate, applied to a Sephadex G25-40 column (18 × 1.5 cm), and eluted with water. The fractions with strong absorption at 260 nm were pooled and freeze-dried, yielding ca. 50 mg of the sodium salt of **1b** [^1H NMR (^2H_2O) δ 3.30, 3.70 (AB q, J = 18 Hz, 2, 2-H), 3.60, 4.43 (AB q, J = 14 Hz, 2, 3'-H), 3.85, 3.94 (AB q, J = 15 Hz, 2, Th CH_2), 4.90 (d, J = 4 Hz, 1, 6-H), 5.50 (d, J = 4 Hz, 1, 7-H), 7.04 (m, 2, Th 3', 5'), 7.32–7.44 (m, 6, Th 4', Ph)], also containing no discernible cephalothin. Alkaline hydrolysis of this material yielded, according to absorption spectra, 1 equiv of thiophenoxide; assay of the hydrolysis mixture with Ellman's reagent (see below) indicated the presence of 1 equiv of thiol.

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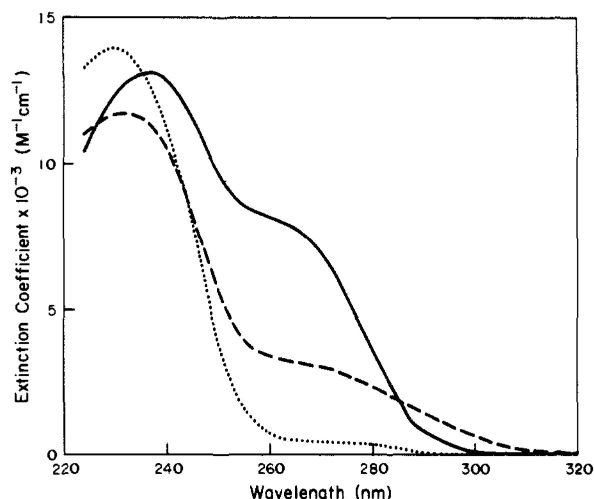

Figure 1. Absorption spectra of **1c** (—), **2c** (---), and **3** (···).

General Methods. ^1H NMR were obtained by means of a Varian XL-200 spectrometer. Absorption spectra and spectrophotometric reaction rates were measured with a Cary 219 spectrophotometer. Fast kinetic measurements were made with a Durrum D-110 stopped-flow spectrophotometer.

Preparation of ^1H NMR Samples. The intermediates **2** and their α-hydrazides **4** were generated for NMR observation as follows. The cephalosporin **1a** (6.0 mg) was dissolved in 0.50 mL of ^2H_2O at 5 °C containing 0.25 M sodium carbonate buffer at pD 10.2 and either 0.3 mg of solid P99 β-lactamase (to generate **2a**) or 5 μL of hydrazine hydrate (to generate **4a**) was added. ^1H NMR spectra at 5 °C were taken immediately and subsequently at appropriate intervals. Solutions of **1b–1d** were prepared similarly; the spectra of **1d** were also taken at 5 °C while those of **1b** and **1c** were taken at 22 °C.

Equilibrium between **2** and **3** in the presence of pyridine and sulfur nucleophiles was also achieved by addition of the nucleophiles to solutions of **3**. The latter were generated by addition of solid P99 β-lactamase (0.3 mg) to sodium cephalothin (5.0 mg) dissolved in 0.5 mL of the appropriate buffer in ^2H_2O. Addition of small aliquots of the neat pyridine or thiol then yielded the desired equilibrium mixture, suitable for NMR investigation.

The reference compound included in all solutions was sodium 3-(trimethylsilyl)-1-propanesulfonate. pD values were obtained by adding 0.40 to pH meter readings.[14]

Kinetics of the 3' Elimination Reaction. The elimination reactions of **2** could be followed spectrophotometrically at 260 nm, subsequent to the β-lactamase catalyzed β-lactam hydrolysis (Scheme I). The latter reaction is accompanied by a decrease in extinction coefficient at 260 nm of about 4000 M^{-1} cm^{-1}, as seen, for example, in the hydrolysis of **1d** to **2d**,[3] while the former leads to a further decrease of around 2600 M^{-1} cm^{-1}, as seen in the **2c** to **3c** elimination, where the 3' leaving group does not absorb at 260 nm. Figure 1 shows the spectral changes accompanying the conversion of **1c** to **2c** and then to **3c**.

Typically, 10 μL of a P99 β-lactamase solution (yielding a final concentration of 0.35 μM) was added to 150 μM solutions of **1a** or **1c** in 0.1 M buffer at 30 °C. Total volumes were 1.0 mL, and the buffers used were acetate, phosphate, and carbonate. The ionic strength of the solutions was maintained at a value of 1.0 M with potassium chloride. The absorbance at 260 nm was then followed until no further changes were observed. Buffer catalysis was assessed through rate measurements in solutions of total buffer concentration between 0.01 and 0.1 M. Some rates were also measured at 5 °C in order to complement the equilibrium measurements at that temperature. The rates of the elimination reactions in potassium hydroxide solutions were determined in solutions prepared by addition of 25-μL samples of solutions of **2** to 0.975-mL aliquots of appropriate hydroxide solutions (ionic strength also at 1.0 M by addition of potassium chloride). The former were generated by addition of β-lactamase solution (10 μL) to 15-μL aliquots of 6.0 mM **1a** or **1b** in dilute phosphate buffer. The potassium hydroxide was added immediately (10 s) after the β-lactamase. Control experiments showed the β-lactam hydrolysis to be complete prior to hydroxide addition.

Since, at neutral and slightly basic pH, the elimination of the 3' leaving group from **1b** did not go to completion, even at the low concentrations employed in the above experiments (see Results), the elimi-

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nation rate was best studied by trapping the released thiophenol and thus drawing the reaction forward to completion. Ellman's reagent was used both to trap the thiophenol and to readily enable the rates to be determined by following formation of the 2-nitro-5-mercaptobenzoate dianion, which absorbs strongly (extinction coefficient 13 600 M^{-1} cm^{-1}) at 412 nm.[15] Thus, to 0.90 mL of buffer solution containing **1b** (75 μM) and Ellman's reagent (300 μM) in a thermostated (30 °C) cuvette in the spectrophotometer was added 10 μL of a P99 β-lactamase solution, giving an enzyme concentration of 0.35 μM. The ensuing reaction was followed at 412 nm. The observed rates did not vary with the Ellman's reagent concentration or the enzyme concentration and were thus presumably rates of the elimination reaction.

In potassium hydroxide solutions, however, the dissociation of **1b** at low concentrations did go to completion and could be followed directly spectrophotometrically. In these experiments **2b** (200 μM) was generated (in equilibrium with **3** and thiophenol) in dilute buffer at neutral pH from **1b** and the P99 β-lactamase, as described above, and mixed with appropriate hydroxide ion solutions in the stopped-flow apparatus. The subsequent reaction was followed at 260 nm.

Under the conditions described herein, neither the addition of 0.1 mM ethylenediaminetetraacetic acid nor the degassing of solutions changed the observations qualitatively or quantitatively; thus, oxidation of the thiophenol was not a problem in the time frame of these experiments.

Kinetics of the Hydrazinolysis Reaction. Aliquots of hydrazine monohydrate, yielding final hydrazine concentrations of 0.10 or 0.20 M, were added to 0.1 M sodium carbonate buffer at pH 9.6 containing 160 μM **1a**, equilibrated at 5 °C in a thermostated cuvette; final volumes were 1.0 mL. The ensuing reaction was followed through absorbance changes at 260 nm. The same procedure was used for reaction of **1b** (110 μM) with hydrazine.

Determination of Equilibrium Constants. The equilibrium constant of dissociation of **2** to **3** and pyridine nucleophiles was determined from the NMR spectra described above, where the relative amounts of **2** and **3** at equilibrium were determined from measurement of appropriate peak areas.

Again because of the much smaller dissociation constants of **2b** to **3** and thiophenol, this constant could not be determined at the concentrations used in NMR experiments and was obtained from spectrophotometric results at lower concentration. Solutions containing concentrations of **1b** varying between 25 and 65 μM in 0.90 mL of 0.1 M phosphate buffer were incubated in a thermostated cuvette, 20 μL of a P99 β-lactamase solution (final concentration 0.35 μM) was added, and the system was allowed to reach equilibrium. Addition of a 25-μL aliquot of Ellman's reagent (to a final concentration of 300 μM) led to an immediate increase in absorbance at 412 nm, corresponding to the amount of free thiol in solution, which was followed by a time-dependent increase in absorbance corresponding to dissociation of **2b**. Measurement of the amplitudes of the two phases of thiol production allowed calculation of the amounts of **2** and **3** in equilibrium and hence of the equilibrium constant.

pK_a Determinations. The pK_a's of pyridine and 3,4-lutidine at 5 °C were determined by potentiometric titration of approximately 0.05 M solutions in ^2H_2O with concentrated deuteriohydrochloric acid in ^2H_2O. The pK_a of thiophenol (50 μM) was determined in H_2O spectrophotometrically in phosphate buffers of pH between 5.78 and 7.20; at 262 nm the extinction coefficient change on formation of thiophenoxide was 13 000 M^{-1} cm^{-1}.

Results and Discussion

Since the purpose of this work was to unequivocally demonstrate the two-step reaction of Scheme I, and in particular the structure of the intermediates **2**, 3' substitutents of poorer leaving group ability than those used in the initial study,[1] which led to the proposal of the reaction sequence of Scheme I, were employed, in order to yield longer lived and more stable intermediates. The cephalosporins used were 3,4-dimethylcephaloridine (**1a**), thiophenoxycephalothin (**1b**), desacetylcephalothin (**1c**), and desacetoxycephalothin (**1d**). Of these, the first is analogous to the compounds used previously,[1] but it has a higher pK_a leaving group and thus, in view of the trends we observed earlier,[1,3] was anticipated to yield a longer lived species, **2a**. Desacetylcephalothin has an even poorer leaving group while desacetoxycephalothin, for comparison, has no leaving group. Thiophenoxycephalothin was chosen to have a leaving group of comparable proton basicity to the pyridines but with the likelihood of forming a more stable intermediate because of the high alkyl carbon basicity of thiolate nucleophiles.[16]

The enzyme-catalyzed hydrolysis, alkaline hydrolysis, and ammonolysis of deacetylcephalosporin C, which contains the same 3' leaving group as **1c**, have previously been examined in some detail by Abraham and co-workers,[17] whose results suggested that the initial product of the enzyme-catalyzed reaction involved hydrolysis of the β-lactam ring accompanied by elimination of the leaving group, while the product of the alkaline hydrolysis and ammonolysis involved only the former reaction. The significance of this work appears to have been overlooked somewhat. Our results confirm and amplify these observations and extend them to leaving groups much better than the hydroxyl group.

When **1a**–**1c** were hydrolyzed in neutral buffered solution in the presence of sufficient amounts of either the RTEM-2 or the P99 β-lactamase, a two-step reaction is observed spectrophotometrically at 260 nm, the first of which is enzyme catalyzed, the second not. The first step involved a decrease in extinction coefficient of around 4000 cm^{-1} M^{-1} at 260 nm, typical of that expected on conversion of **1** into **2**, for example, as observed on hydrolysis of **1c** to **2c**,[3] on the hydrolysis of dansylcephalothin, where no loss of 3' leaving group occurs,[18] and as deduced to occur on β-lactam ring opening of PADAC [(pyridine-2-azo-4'-(*N'*,*N'*-dimethylaniline) substituted on cephalosporin)].[3] This spectral change is shown in Figure 1 for **1c** where the spectrum is uncomplicated by aromatic 3' substituents. The second step involved a further decrease in absorbance at 260 nm, yielding a final spectrum of **3** from **1c** (Figure 1) and of **3** plus 3,4-lutidine from **1a**; the case of **1b** will be discussed in detail below. These observations are interpreted, as before,[1] in terms of Scheme I, where the intermediate generated by β-lactamase-catalyzed reaction is **2**.

Observation and Characterization of Cephalosporoates by ^{1}H NMR. These intermediates could be observed and characterized by ^{1}H NMR spectroscopy. The results of these experiments are given in Table I. After treatment of **1c** with either the RTEM-2 or the P99 β-lactamase (essentially identical results were obtained with both these β-lactamases in all the experiments below so the enzymes will not henceforward be distinguished), the initial spectrum changed instantly (<1 min) to that of an intermediate and then, over a longer time period, changed into that of **3**.[3] The NMR spectrum of the intermediate, like the absorption spectrum, is consistent with that of the proposed structure **2c**, showing, in particular, upfield movements of the C-6 and C-7 protons, as expected on the β-lactam ring opening, and little change in the C-3' methylene chemical shift. This interpretation is supported by the spectrum of **1d** after β-lactamase-catalyzed hydrolysis (Table I) since, in the absence of a 3' leaving group, the structure of the product must be **2d**.

A similar experiment with **1a**, carried out at 5 °C rather than 20 °C, also gave the NMR spectrum of an intermediate (Table I), consistent with that of the structure **2a**. In this case, not only did the positions of the C-6, C-7, and C-3' proton resonances, as above, indicate the structure **2a** but the chemical shifts of the pyridine ring and accompanying methyl protons showed that the pyridine was still positively charged and thus attached to C-3' (at the pH of the experiment, free 3,4-lutidine would be neutral). There is no evidence for an (highly unlikely) intermediate structure between **1** and **2**.[5] This spectrum rapidly ($t_{1/2} \sim 2$ min) changed into that of **3** and neutral 3,4-lutidine.

At lower pH (6–8), a further discovery was made. The same intermediate, **2a**, was generated by β-lactamase-catalyzed hydrolysis of **1a**, but it did not totally convert into **3**; significant amounts of **2a** remained after the composition of the reaction mixture ceased to change (Figure 2). This observation suggested that, in the presence of 3,4-lutidine, an equilibrium between **2a**

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Table I. ¹H Chemical Shifts of Cephalosporins and Cephalosporoates

compd	chemical shift (δ), ppm			
	py CH_3	H-6, H-7	CH_2X	py
1a[a]	2.42, 2.54	5.14, 5.69	5.23, 5.40	7.82, 8.59, 8.61
2a[a]	2.36, 2.48	4.54, 4.72	5.28, 5.44	7.72, 8.50, 8.50
4a[b]	2.37, 2.49	4.54, 4.78	5.30, 5.48	7.72, 8.50, 8.50
3,4-lutidinium[c,d]	2.46, 2.58			7.83, 8.44, 8.48
3,4-lutidine[h]	2.22, 2.27	*e*	*e*	7.22, 8.22, 8.22
1b[f]		4.90, 5.50	3.61, 4.42	
2b[f]		4.55, 4.55	4.05, 4.17	
4b[g]		4.36, 4.58	4.14, 4.14	
1c[h]		5.10, 5.61	4.26, 4.26	
2c[h]		4.64, 4.64	4.26, 4.26	
4c[i]		4.56, 4.67	4.27, 4.27	
1d[j]		5.07, 5.59	1.90	
2d[j]		4.51, 4.59	1.84	
4d[g]		4.55, 4.55	1.87	

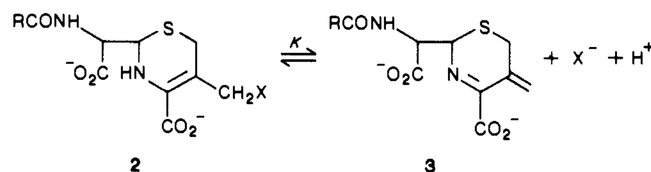
[a] 0.25 M carbonate, pD 10.2, 5 °C. [b] 0.25 M carbonate, 0.25 M hydrazine, pD 10.2, 5 °C. [c] 0.20 M formate, pD 4.9, 5 °C. [d] The pK_a of 3,4-lutidine was measured to be 7.12 under the relevant conditons. [e] Chemical shifts[g] in the final product, **3**, are 5.47 and 4.65 ppm for H-6 and H-7, respectively, and 5.62 and 5.68 ppm for the 3-*exo*-methylene protons. [f] 0.1 M carbonate, pD 8.4, 22 °C. [g] 0.25 M carbonate, 0.25 M hydrazine, pD 9.4, 5 °C. [h] 0.25 M carbonate, pD 9.4, 22 °C. [i] 0.25 M carbonate, 0.25 M hydrazine, pD 9.4, 22 °C. [j] 0.25 M carbonate, pD 9.4, 5 °C.

Table II. Rate and Equilibrium Parameters for the Dissociation of Cephalosporoates (Scheme II)

compd	K,[a] M^2	k_{fo},[a] s^{-1}	k_{ro},[a] s^{-1} M^{-2}	k_{fo},[b] s^{-1}	k_{fH},[b] s^{-1} M^{-1}	k_{fOH},[b] s^{-1} M^{-1}
2a	1.55×10^{-9} [c]	0.0050	3.2×10^6	0.057	$<5.7 \times 10^2$ [d]	50
2 (X = py)[e]	1.73×10^{-8} [c]	0.12	6.8×10^6	0.44[f]	ND	ND
2b	1.21×10^{-12}	0.014	1.2×10^{10}	0.21	$<2.1 \times 10^3$ [d]	4.7
2c	$>2 \times 10^{-13}$ [g]	ND	ND	$<10^{-5}$ [h]	7.1×10^4	0.03

[a] Determined at 5 °C. [b] Determined at 30 °C except where otherwise noted. [c] Determined in ^2H_2O. [d] No indication of acid catalysis at or above pH 5.0. [e] From cephaloridine. [f] Determined at 20 °C.[1] [g] No indication of **2c** at equilibrium at pH 8–9. [h] No indication of a spontaneous reaction with a rate constant above this value. ND, not determined.

Scheme II



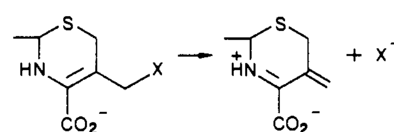
and **3** existed in solution and favored **2a** at lower pH. This was confirmed by attainment of the same position of equilibrium by addition of 3,4-lutidine to **3** (generated from cephalothin and the P99 β-lactamase). The equilibrium must then be that of Scheme II, which will clearly favor association at lower pH. Calculation of the equilibrium constant at 5 °C from the NMR data yielded for X = 3,4-lutidine: $K = [3][3,4\text{-Lu}][H^+]/[2] = [3][3,4\text{-Lu}]_T[H^+]/[2](1 + [H^+]/K_a) = 1.55 \times 10^{-9}$ M^2 (where [3,4-Lu] is the concentration of neutral 3,4-lutidine, $[3,4\text{-Lu}]_T$, the total concentration of 3,4-lutidine, and K_a, the acid dissociation constant of 3,4-lutidinium, measured under these conditions to be $10^{-7.12}$ M). Addition of the less basic ($pK_a = 5.84$ under these conditions) unsubstituted pyridine to **3** yielded, as expected, a less stable adduct, the cephalosporoate of cephaloridine, $K = 1.73 \times 10^{-8}$ M.[2]

Addition of β-lactamase to a solution of **1b** at any pH between 6 and 9 under the conditions described in the Experimental Section yielded an NMR spectrum (Table I) consonant with the structure **2b**. No indication of **3** was present, suggesting that **2b** was more stable than the above pyridinium adducts. This position of equilibrium was confirmed by the observation of complete formation of **2b** on adding thiophenol to equimolar **3**. The equilibrium constant for dissociation of **2b** was determined at lower concentration spectrophotometrically, as described in the Experimental Section; at 5 °C, in H_2O, $K = 1.21 \times 10^{-12}$ M^2 (the pK_a of thiophenol was determined to be 6.24).

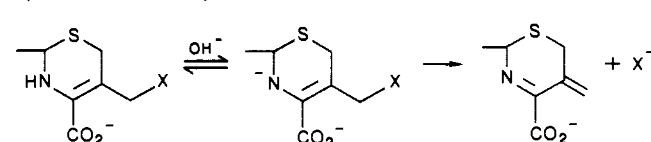
Kinetics and Mechanism of the Dissociation Reaction. The cephalosporoate intermediates, **2a–2c**, could be generated in a variety of buffered solutions as described in the Experimental Section. Their first-order rates of dissociation to **3** plus nucleophile could then be determined spectrophotometrically. Under the conditions employed in these kinetic studies, dissociation was complete, i.e., the observed pseudo-first-order rate constant was k_f ($K = k_f/k_r$). Buffer catalysis was observed only in the case of **2c** but was not pursued in detail; the solvent-catalyzed rate constants shown below for this compound were obtained from extrapolations to zero buffer concentration.

Scheme III

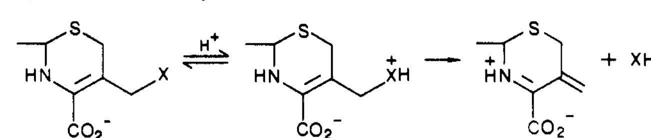
spontaneous elimination



specific-base catalyzed



specific-acid catalyzed



The pseudo-first-order rate constants of the elimination reaction of Scheme II for compounds **2a–2c** at pH values greater than 5 were given by eq 1, where the values of the various rate parameters are given in Table II.

$$k_f = k_{fo} + k_{fH}[H^+] + k_{fOH}[OH^-] \quad (1)$$

In general, therefore, the elimination reaction can be apparently specific-acid and -base catalyzed. There is also, in general, a significant apparently spontaneous reaction that, in the case of **2a** and **2b** at least, is the dominant route at neutral pH. Reasonable mechanisms for these reactions are given in Scheme III. The data of Table II shows that thiophenoxide has leaving-group ability comparable to that of a pyridine of similar basicity. The

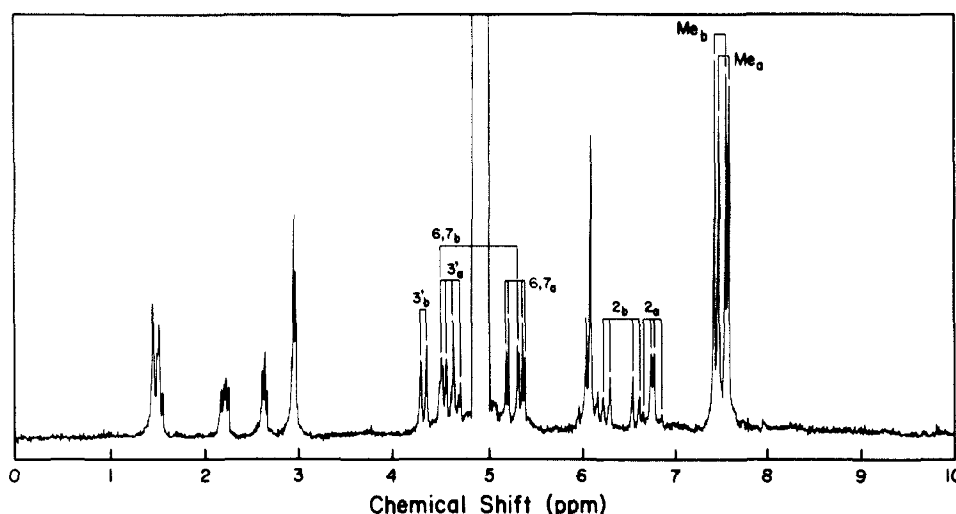


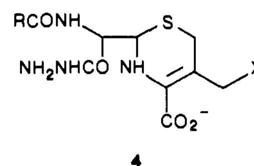
Figure 2. Final ^1H NMR spectrum (^2H_2O, 0.2 M phosphate, pD 6.4, 5 °C) of **1a** after β-lactamase addition. The methyl (Me), 2-H, 3-H, 6-H, and 7-H resonances are labeled, and the subscripts a and b refer to **2a** and **3**, respectively. Chemical shifts are given in Table I.

hydroxyl group is a much poorer leaving group, particularly in the spontaneous reaction; in the specific-base-catalyzed reaction the hydroxyl group performs relatively better, presumably because of the stronger driving force produced by the enamine anion. The greater stability of **2b** with respect to **2a** is more strongly reflected in the association rate than in the dissociation rate, where the thiolate is seen to exhibit a much greater nucleophilicity toward the C-3′ methylene than does the pyridine. The other striking feature of the data of Table II is the specific-acid-catalyzed mechanism involved in elimination of the hydroxyl group from **2c**, which is the dominant path of elimination at all pH below 10. The appearance of this feature presumably reflects both qualitative (oxygen) and quantitative (poor) aspects of the leaving group in this compound. It seems likely that the buffer catalysis also observed in this case would prove to be general-acid catalysis. The importance of acid catalysis in this elimination reaction probably explains why Abraham and co-workers thought that **3** [with the δ-[(D-α-aminoadipamido) side chain at C-7)] was the product of the P99 β-lactamase-catalyzed hydrolysis of deacetylcephalosporin C.[17] At lower enzyme concentrations and/or with a poorer β-lactamase substrate (as deacetylcephalosporin C is with respect to **1c**), and in neutral buffered solution, the analogue of **2** may well not accumulate in solution.

Comparison of the hydroxide-catalyzed elimination rate constant for **1a** with that of hydroxide-catalyzed β-lactam hydrolysis (0.124 s^{-1} M^{-1}) suggests why the intermediates **2** would not be seen,[1,5,8,19] if they occurred, during alkaline hydrolysis of cephalosporins with good 3′ leaving groups.

Cephalosporoate Intermediates during Cephalosporin Hydrazinolysis. Although, as shown above, cephalosporoate intermediates would not be expected to accumulate during the alkaline hydrolysis of cephalosporins with good 3′ leaving groups, it seemed possible that good nucleophiles at lower pH might cleave the β-lactam ring fast enough to achieve accumulation in cases such as **1a** and **1b** where the elimination reaction appeared to not be general-acid/-base catalyzed. Morris and Page have shown that hydrazine is a very effective nucleophile toward the β-lactam carbonyl group.[20] Consequently, we examined the reactions of hydrazine with **1a**–**1d** at pH 9 and at 5 °C. Absorption spectra showed a hydrazine-catalyzed, rapid (<10 s at 0.1 M hydrazine) absorbance decrease at 260 nm, of similar magnitude to that observed in the β-lactamase-catalyzed hydrolysis. This was followed, in the cases of **1a** and **1b**, by first-order reactions, not hydrazine catalyzed, with rate constants of 5.8×10^{-3} s^{-1} (cf. 5.0×10^{-3} s^{-1} for conversion of **2a** to **3**) and 4.0×10^{-3} s^{-1} (cf. 1.40×10^{-2} s^{-1} for conversion of **2b** to **3**), respectively.

No comparable second stage of reaction of **1c** was observed over the same time scale, presumably because of the stability of **4c** at this pH (cf. **2c**). These results are interpreted in terms of a reaction pathway analogous to that of Scheme I, where the accumulating intermediates generated on cephalosporin hydrazinolysis have the structure **4**. This interpretation was confirmed



by ^1H NMR experiments, which also revealed transient hydrazinolysis intermediates whose spectra (Table I) are consistent with structure **4a**–**4c** in the same way as described above for the hydrolysis intermediates. Hydrazinolysis of **1d** yielded **4d** whose H-6 and H-7 chemical shifts (Table I) support this structural assignment.

Concluding Discussion. The combination of absorption and NMR spectral evidence and the kinetic evidence presented here, along with the results of previously reported experiments, shows clearly that, in the cases investigated, 3′ eliminations from cephalosporins are not concerted, in any practical sense of the word,[5] and irrespective of the existence[21] or otherwise of a tetrahedral intermediate, with nucleophilic β-lactam C–N bond cleavage. Species of structure **2** are well-defined chemical compounds whose stability and lability depend on the nature of the substituent X. We have now demonstrated this result not only with β-lactamase-catalyzed hydrolysis but with a nonenzymic nucleophile, hydrazine; it thus may be general. The result has interesting and important implications, some of which we have already reported,[3,22] with respect to the interaction of cephalosporins with β-lactam-processing enzymes.

It may be, with better leaving groups than those examined here, that a concerted elimination can occur, but claims for this must now be very closely scrutinized; it is not easy, in general, to prove that a reaction is concerted. It may be relevant to note at this point that orbital alignment, following α-face attack on the β-lactam carbonyl, does not appear optimal for a concerted reaction; there are clear indications that such alignment is important in eliminations concerted with β-lactam ring cleavage.[23] If the

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reaction is in fact not concerted over a wide range of leaving groups, the system might be useful for the study of leaving-group abilities or nucleofugalities,[24] which still appear to be poorly defined.[24-26]

The other major finding of this work, which to our knowledge is novel, is that the elimination reaction is readily reversible with certain nucleophiles. This feature makes the system attractive for physical organic studies and also has implications, which we are pursuing, with respect to the β-lactam-processing enzymes.

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Nonplanar Amide Groups as Ligands

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Abstract: When the *trans*-osmium(IV) complexes $Os(\eta^4\text{-HBA-B})(PPh_3)_2$, **1** ($H_4$HBA-B = 1,2-bis(2-hydroxybenzamido)benzene), and $Os(\eta^4\text{-CHBA-DCB})(PPh_3)_2$, **2** ($H_4$CHBA-DCB = 1,2-bis(2-hydroxy-3,5-dichlorobenzamido)-4,5-dichlorobenzene), are treated with a strong π-acid ligand, carbon monoxide or *tert*-butyl isocyanide, a phosphine ligand is replaced and the substituted complexes are produced as the *cis*-α diastereomers $Os(\eta^4\text{-HBA-B})(CO)(PPh_3)$, **3**, and $Os(\eta^4\text{-CHBA-DCB})(t\text{-BuNC})(PPh_3)$, **4**. X-ray crystal structure analyses show that **3** and **4** contain nonplanar amido ligands. The twisting about the C–N bond and the pyramidal distortions at the carbonyl–carbon and nitrogen atoms of the nonplanar amido groups are compared with all other reported cases of structured RC(O)NR'M and RC(OM')NR'M groups (R and R' are general groups but do not include H). The twist angles about the C–N bonds are significantly larger for **3** and **4** than have been observed previously. The effects of these deformations upon the bonding properties of the *N*-amido ligands are discussed.

The organic amide functional group, one of the most important building blocks in biological systems, is almost invariably found in a near-to-planar form. Rotational processes around the amide C–N bond disrupt amide delocalization and consequently are subject to substantial activation barriers (10–35 kcal·mol^{-1}).[2] Nonplanar amides have been recognized in formamide[3] and in some constrained molecules such as certain lactams[4,17] (including penicillin and cephalosporin antibiotics[5]), polycyclic spirodilactams,[6] and anti-Bredt bridgehead nitrogen compounds.[7] As part of a program aimed at developing new polyanionic chelating (PAC) ligands for highly oxidized and highly oxidizing complexes,[8-12] we have discovered a series of remarkable isomerization reactions in which planar N-coordinated organic amido groups are converted to distinctly nonplanar forms. The angular parameters of these unusual molecules are discussed in the context of all structurally characterized species containing RC(O)NR'M and RC(OM')NR'M fragments (R- and R'- are general groups but do not include H).

Experimental Section

Materials. Benzene (thiophene free, Aldrich), ethanol (U.S.I.), and hexanes (Aldrich) were reagent grade and were used as received. Dichloromethane (Baker) was distilled from calcium hydride (Aldrich) prior to use. *tert*-Butyl isocyanide (Alfa) and carbon monoxide (Matheson) were all used as received. Silica gel used in column chromatography was 60-200 mesh (Davison).

Physical Measurements. ^{1}H NMR spectra were recorded at 90 MHz on a Varian EM-390 spectrometer, at 89.83 MHz on a JEOL FX90-Q spectrometer, or at 500.135 MHz on a Bruker WM-500 spectrometer. ^{1}H chemical shifts are reported in ppm (δ) vs. Me_4Si with the solvent

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