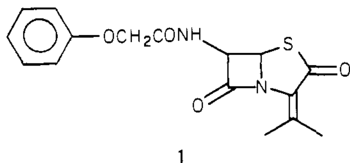
Products and Mechanism of the Oxyanion- and β-Lactamase-Catalyzed Hydrolyses of (Phenoxymethyl)anhydropenicillin

R. F. Pratt,* Yvonne S. Surh, and John J. Shaskus

Contribution from the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457. *Received June 28, 1982*

Abstract: The primary product of reaction of (phenoxymethyl)anhydropenicillin (**1**) in aqueous solution in the presence of phosphate or carbonate buffers, hydroxide ion, or class A β-lactamases arises from nucleophilic cleavage of the β-lactam ring. This conclusion is at variance with that previously reached by Bundgaard and Angelo,[7] whose results suggested that initial attack by oxygen nucleophiles occurred at the thiol ester carbonyl group. The present result arises from a closer examination of the properties of the primary reaction product and in particular of the ^1H and ^{13}C NMR spectra of it and of the analogous methanolysis product. Also important were the results of experiments with the thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid), and the elucidation of the details of the complete alkaline hydrolysis pathway (Scheme III). The β-lactam ring of **1** appears to be as susceptible to nucleophilic attack by hydroxide ion as would be expected in view of its highly strained structure. Some amine nucleophiles preferentially attack **1** at the thiol ester carbonyl group. The 4-alkylidenethiazolidin-5-one ring of the primary hydrolysis product was found to be stable in dilute neutral aqueous solution to ring-opening both by way of elimination through nitrogen and also through nucleophilic attack at the thiol ester carbonyl group; it was however labile to epimerization at C-2.

There has been considerable interest recently in the active sites of the β-lactamase enzymes. We, for example, have begun a survey of the potential chemical reactivity of these sites.[1] This and other interests have led us and others to investigate the interactions of these sites with a wide variety of nonclassical β-lactam structures. One fascinating example of such a structure is that of the anhydropenicillins, for example, (phenoxymethyl)-anhydropenicillin (*N*-[2-(1-methylethylidene)-3,7-dioxo-4-thia-1-azabicyclo[3.2.0]hept-6-yl]-2-phenoxyacetamide, **1**). Since this



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structure appeared to combine a high potential for chemical reactivity with the presence of a leaving group at the C-5 position (as found in several effective β-lactamase inhibitors[2-4]), we were interested in its mode of interaction with β-lactamases. As described below, we did in fact find that **1** is a substrate of typical class A β-lactamases, although not a covalent inhibitor. Of perhaps greater chemical interest however was the observation that the product of the enzyme-catalyzed reaction was the same as that obtained from the oxyanion buffer (phosphate and carbonate) and hydroxide catalyzed reactions. The significance of this point is made clear below.

Anhydropenicillins were first prepared and characterized by Wolfe and co-workers,[5] who were impressed with the stability of these compounds to boiling solvents such as alcohol, water, and toluene, despite indications from infrared spectra of high ring strain. Subsequently, Simon, Morin, and Dahl,[6] as part of a study of the relationship between the structure and activity of β-lactam antibiotics, confirmed through an X-ray structural determination the highly strained nature of this ring system and in particular of the β-lactam amide group. They also could not reconcile the reported stability with the structure. This early evidence of stability however did not involve any direct measurement of the reactivity of the β-lactam ring of anhydropenicillins or any comparison with that of other penicillins. Consequently, it was not surprising when Bundgaard and Angelo[7] demonstrated that the anhydropenicillin ring system of **1** was quite sensitive to base-catalyzed hydrolysis and considerably more so than (phenoxymethyl)penicillin itself. A striking further conclusion of this work, however, was that preferential nucleophilic attack occurred at the thiol ester carbonyl group, rather than the β-lactam, leading to a primary product derived from opening of the thiazolidin-5-one ring.

The dilemma that confronted us is now plain. If the conclusions of Bundgaard and Angelo are correct, and our results indicate that the spontaneous and enzyme-catalyzed hydrolyses yield the same product, then the enzyme, a β-lactamase, must have promoted attack at the non-β-lactam carbonyl group of **1**. Given the structural differences between **1** and normal penicillins, this is not a priori impossible, but given the pronounced specificity of the β-lactamase active site,[1] we felt it an important enough point to investigate further. The results of this investigation are the subject of this paper.

Experimental Section

Materials. (Phenoxymethyl)anhydropenicillin (**1**) was prepared by the method described by Wolfe et al.[5] for benzylanhydropenicillin and recrystallized, with one ethanol molecule of crystallization,[6] from ethanol. The product (mp 123–124 °C (lit.[8] mp 124 °C)) had IR[5] and NMR (see Results) spectra consonant with those expected of an anhydropenicillin. Acetonitrile was distilled from phosphorus pentoxide and stored over molecular sieves. The β-lactamase I of *Bacillus cereus* and the TEM β-lactamase were obtained as described previously.[1] 6-β-Bromopenicillanic acid was available from previous studies;[9] 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and (phenoxymethyl)penicillin were obtained from Sigma Chemical Co. and used without further purification. Potassium thioacetate was purchased from Fluka Chemical Corp.

General Methods. UV absorption spectra were obtained either from a Cary 14 or a Cary 219 recording spectrophotometer. Rapid kinetic measurements were made on a Durrum D-110 stopped-flow spectrophotometer. NMR spectra were obtained from a Varian XL-200 spectrometer, with either tetramethylsilane or sodium 3-(trimethylsilyl)-1-

(1) Pratt, R. F.; Anderson, E. G.; Odeh, I. *Biochem. Biophys. Res. Commun.* **1980**, *93*, 1266.
(2) Fisher, J.; Belasco, J. G.; Charnas, R. L.; Khosla, S.; Knowles, J. R. *Philos. Trans. R. Soc., London, Ser. B* **1980**, *289*, 309.
(3) Hill, H. A. O.; Sammes, P. G.; Waley, S. G. *Philos. Trans. R. Soc. London, Ser. B* **1980**, *289*, 333.
(4) Cohen, S. A.; Pratt, R. F. *Biochemistry* **1980**, *19*, 3996.
(5) Wolfe, S.; Godfrey, J. C.; Holdrege, C. T.; Perron, Y. G. *J. Am. Chem. Soc.* **1963**, *85*, 643. Wolfe, S.; Godfrey, J. C.; Holdrege, C. T.; Perron, Y. G. *Can. J. Chem.* **1968**, *46*, 2549.
(6) Simon, G. L.; Morin, R. B.; Dahl, L. F. *J. Am. Chem. Soc.* **1972**, *94*, 8557.
(7) Bundgaard, H.; Angelo, H. R. *Tetrahedron Lett.* **1974**, 3001.
(8) Thomas, R. *J. Chem. Soc., Chem. Commun.* **1972**, 478.
(9) Pratt, R. F.; Loosemore, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *76*, 4145.

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propanesulfonate as internal standard. A pulse recycle time of between 2 and 5 s was used during accumulation of the ^{13}C NMR spectra in order to detect the carbonyl resonances.

Reactions of **1** in aqueous buffer were followed spectrophotometrically after addition of a small aliquot of a solution of **1** in dry acetonitrile to the aqueous solution in a cuvette. The final concentration of **1** was around 0.05 mM except where noted. Some qualitative results were obtained at ambient temperature (20–25 °C), but all quantitative measurements were made at 30 ± 0.1 °C. Buffer solutions at higher pH (≥9) and potassium hydroxide solutions contained 0.1 mM EDTA in order to suppress (presumably metal-catalyzed oxidative) side reactions (see Results). The potassium hydroxide solutions for the kinetic studies at high pH were adjusted to an ionic strength of 1.0 by addition of potassium chloride. A stock 10 mM solution of Ellman's reagent was prepared in 0.1 M phosphate buffer,pH 7.5. The extinction coefficient of the dianion of 2-nitro-5-mercaptobenzoic acid (Ellman's anion) was assumed to be 13 700 M^{-1} cm^{-1}.[10]

Preparation of ^1H NMR Samples. 1. Enzyme-Catalyzed Hydrolysis Product (9). Solid TEM β-lactamase (0.5 mg) was added to 1 mL of a reaction mixture containing 1 mM of **1** and 10 mM of sodium bicarbonate in a solvent composed of 20% [$^2H_3$]acetonitrile in ^2H_2O (pD = 7.5–8.0). The reaction was complete in less than 30 min, at which time UV absorption and NMR spectra were recorded; the UV absorption spectrum of the product was shown not to change in the time necessary to obtain the NMR spectrum. A sample was also prepared by using [$^2H_8$]dioxane as cosolvent instead of acetonitrile. This yielded the same product NMR spectrum as the sample in acetonitrile and demonstrated that no further resonances had been hidden under the acetonitrile solvent peak that lay close to the product methyl resonances.

2. Spontaneous Hydrolysis Product (9). The reaction mixture was generated by addition of 0.8 mL of a 10 mM sodium carbonate solution in ^2H_2O at pD = 10.0 to 0.2 mL of a [$^2H_3$]acetonitrile solution containing sufficient **1** to give a final solution 1 mM in **1**. The NMR spectrum was recorded after 45 min at room temperature.

3. Methanolysis Product (10). An aliquot of triethylamine sufficient to give a final concentration of 6 mM was added to a solution containing 6 mM of **1** in 0.5 mL of [$^2H_4$]methanol. The NMR spectrum was then recorded as a function of time over a few hours, by which time the reaction was complete.

4. Hydroxide Product (12). To a solution of 5 mg of **1** in 0.6 mL of acetonitrile were added 20 mL of water and 4 drops of 2 M potassium hydroxide. An absorption spectrum of a diluted aliquot showed the expected 290-nm chromophore. The solution was then freeze–dried and the residue taken up into 0.5 mL of [$^2H_6$]dimethyl sulfoxide. Further absorption spectra confirmed the intact chromophore before and after NMR spectra were recorded.

Preparation of ^{13}C NMR Samples. 1. Methanolysis Product (10). Triethylamine (0.02 mL) was added to a solution of 65 mg of **1** in 200 mL of methanol. Afer 3 h at room temperature, the UV absorption spectrum indicated that the reaction was close to completion. The volatiles were removed by rotary evaporation at room temperature. The residual solid was taken up into 6 mL of [^2H]chloroform for the NMR spectrum (18-mm bore tube). The absorption spectrum was also checked after the NMR data were taken.

2. Hydroxide Product (12). This sample was prepared from 20 mg of **1**, 1.2 mL of acetonitrile, 2.0 mL of [^2H]water, 2.8 mL of water, and 6 drops of 2 M potassium hydroxide solution. For comparison, a spectrum of potassium thioacetate in the same solvent was obtained.

Other NMR spectra were obtained routinely in the designated solvents.

Results

The UV absorption spectrum of (phenoxymethyl)anhydropenicillin (**1**) in 0.1 M phosphate buffer at pH 7.5 is shown in Figure 1 along with the spectrum of the product obtained shortly (instantaneously at high enzyme concentration) after addition of either *B. cereus* β-lactamase I or the TEM β-lactamase (each at a final concentration of between 0.1 and 1 μM). A spectrum identical with the latter was obtained from the much slower spontaneous, i.e., not enzyme catalyzed (buffer catalyzed, according to Bundgaard and Angelo[7]) reaction whose half-time was about 2 h at 30 °C. The same product, as judged from the spectra, was obtained both in the presence and absence of enzyme in 0.1 M carbonate buffer, at pH between 9 and 10. At the higher pH, the identity of the initial product was less obvious because of the

(10) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332.

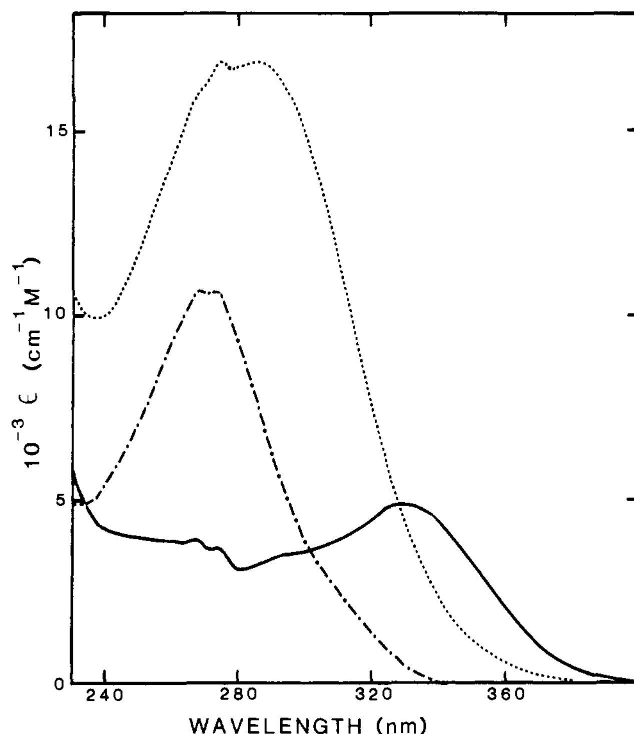


Figure 1. Absorption spectra of (phenoxymethyl)anhydropenicillin (·–·), the oxyanion- and β-lactamase-catalyzed hydrolysis product **9** (–), and the final alkaline hydrolysis product **12** (···) in 0.1 M phosphate buffer at pH 7.5.

presence of spectral changes accompanying subsequent reactions. The latter reactions could be suppressed by the presence of 0.1 mM EDTA (and thus presumably represent metal ion catalyzed oxidations) to such an extent that it was clear that the initial product, qualitatively and quantitatively, was the same as that observed at the lower pH. The subsequent reactions could also be retarded by the sparging of the solutions with nitrogen, although this procedure was less effective than inclusion of EDTA.

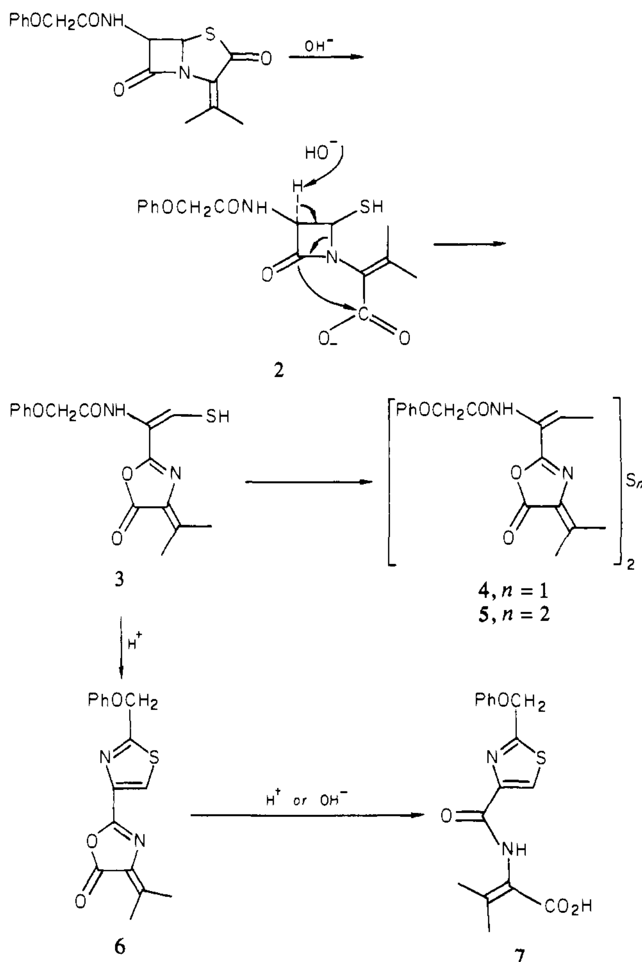
These observations suggested that **1** is a substrate of these typical β-lactamases and that the product of the enzyme-catalyzed reaction is the same as that of the spontaneous reaction in aqueous buffer. A stopped-flow experiment using high enzyme concentration (30 μM TEM β-lactamase) showed no induction period in the formation of the 330-nm chromophore. There was thus no evidence from this method that the product whose spectrum is shown in Figure 1 was of a secondary rather than primary nature. *B. cereus* β-lactamase I, which had been completely inactivated toward benzylpenicillin by treatment with 6-β-bromopenicillanic acid,[9] did not catalyze any reaction of **1**. This observation suggests that the reaction of **1** catalyzed by the β-lactamases does occur at the β-lactamase active site.

Measurement of the initial rates of the enzyme-catalyzed reaction as a function of **1** concentration resulted in a strictly linear relationship for both enzymes up to the solubility limit of **1** under these conditions (ca. 0.5 mM). This result indicates rather weak binding of **1** to these enzymes and on numerical analysis yielded k_{cat}/K_m values of 4.2×10^3 s^{-1} M^{-1} and 5.0×10^4 s^{-1} M^{-1} for the *B. cereus* and TEM enzymes, respectively. A value of $K_m \geq 0.5$ mM then yields $k_{cat} \geq 2.1$ s^{-1} and ≥ 25 s^{-1}, respectively. These numbers can be compared with those for *B. cereus* β-lactamase I and benzylpenicillin, a good substrate ($k_{cat}/K_m = 2.9 \times 10^7$ s^{-1} M^{-1}, $k_{cat} = 2.3 \times 10^3$ s^{-1}, $K_m = 0.08$ mM)[11] and suggest that **1** is a poor substrate of β-lactamases. Nor does it seem to be an irreversible inactivator: 0.5 mM of **1** did not affect the activity of 0.1 μM of *B. cereus* β-lactamase I over a 5-h period.

The absorption spectrum of the product of the above reactions does not resemble that of any product described by Bundgaard

(11) Waley, S. G. *Biochem. J.* **1975**, *149*, 547.

Scheme I



and Angelo.[7] In view of this and of the dilemma described in the introduction, it seemed important to characterize the product more fully. All attempts to isolate it failed however. Dilute solutions could not be evaporated to dryness, freeze-dried, or even concentrated to greater than 1 mM without drastic absorption spectral changes, which led to absorption maxima beyond 350 nm. Similar spectra were obtained, along with precipitation of products, when the reactions were attempted in more concentrated solutions, by using mixed aqueous/organic solvents, including the 50% aqueous acetonitrile employed by Bundgaard and Angelo.[7] Acidification of the dilute reaction solutions to pH 4 or lower also led to profound and irreversible spectral changes. It appeared that simple approaches to the isolation of the product(s) were not likely to be successful, presumably because of the reactivity of the primary product(s).

It seems likely that Bundgaard and Angelo[7] observed similar phenomena since they reported that the reaction of **1** in concentrated solution at pH 9–11 yielded a mixture of three products, from which they isolated and identified one after acidification and chromatography. Scheme I shows the reaction sequence proposed by Bundgaard and Angelo.[7] The major product (yield not reported), although it could not be obtained pure, was believed to be **3**, because of its conversion into compounds resembling **4–7** that had been earlier reported as products of penicillin rearrangements.[12] Beyond this, the only direct evidence presented as to the structure of **3** was a statement of its positive reactivity with thiol reagents. Hence, the characterized materials were far from the proposed primary product **2** and isolated under conditions that gave a series of complicated reactions. These considerations also suggested that a closer look at the primary product was

(12) Kukolja, S.; Cooper, R. D. G.; Morin, R. B. *Tetrahedron Lett.* **1969**, 3381.

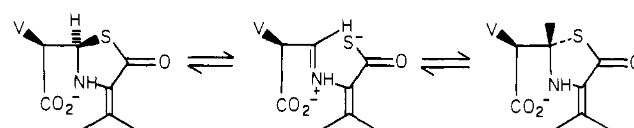
Table I. ^{1}H and ^{13}C NMR Spectra of (Phenoxymethyl)anhydropenicillin[a]

1

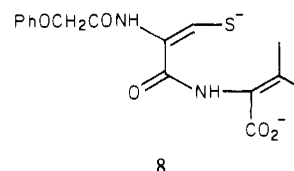
observed chemical shift	assignment
hydrogen	
2.09	{C-9 H_3, C-10 H_3
2.19	
4.58	C-13 H_2
5.61 (d, J = 4 Hz)[b]	C-5 H
6.00 (dd, J = 4, 9 Hz)[b]	C-6 H
6.9–7.4 (br m)	Ar H, NH
carbon	
18.7	{C-9, C-10
25.3	
61.0	C-5
62.4	C-6
67.5	C-13
116.3	C-15
124.2	C-17
127.7	C-3
131.6	C-16
145.1	C-8
158.9	C-14
170.6	{C-7, C-12
172.4	
194.8	C-2

[a] Solvent C^{2}HCl$_3$. [b] 5.80, 5.89 in 20% [^{1}H$_8$]dioxane/^{2}H$_2$O.

Scheme II



necessary. Note also that the primary product **2** of Bundgaard and Angelo would not have an absorption spectrum similar to ours (Figure 1).[13] Further ring-opening of **2**[14] could generate **8**, a product with an absorption spectrum similar to that of our product,[15] but the NMR spectra and further reactions (see below) of our product preclude this identification also.



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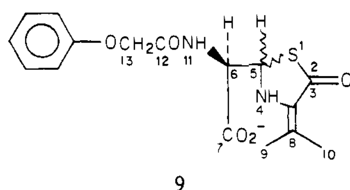
Spectral Studies. The ^{1}H and ^{13}C NMR spectra of **1** for comparison with subsequent spectra are given in Table I with structural assignments. The ^{1}H NMR spectrum of the product giving rise to the absorption spectrum of Figure 1 was obtained as described in the Experimental Section and is reported, with the proposed structure **9** and assignments in Table II. The initially striking feature of this spectrum was that two similar compounds were present in about a 60:40 ratio, as judged by the methyl resonances and the resonances between 5 and 6 ppm, which presumably derive from the β-lactam hydrogens of the starting material; the product is therefore represented as a mixture of the two C-5 epimers. Further evidence for these structures is presented

(13) Clayton, J. P.; Nayler, J. H. C.; Southgate, R.; Tolliday, P. *J. Chem. Soc. D* **1971**, 590.
(14) Baldwin, J. E.; Christie, M. A. *J. Chem. Soc., Chem. Commun.* **1978**, *239*. Baldwin, J. E.; Jung, M. *Ibid.* **1978**, 609.
(15) Leonard, N. J.; Ning, R. Y. *J. Org. Chem.* **1967**, *32*, 677.

Table II. ¹H NMR Spectra of the Enzyme-Catalyzed and Oxyanion Buffer Catalyzed Reaction Product[a,b]



9

observed chemical shift	assignment
1.71, 1.89[c]	C-9 H_3, C-10 H_3
1.78, 1.89	C-9 H_3, C-10 H_3
5.00 (d, J = 3.5 Hz)[c]	C-6 H }
5.68 (d, J = 3.5 Hz)[c]	C-5 H }
5.73 (d, J = 3.0 Hz)	C-5 H
7-7.6 (br m)	Ar H

[a] Other hydrogen resonances were not observed either because of exchange (N-H) or obscuration by the solvent ²HOH peak. [b] See methods section for details of sample preparation. [c] Major product.

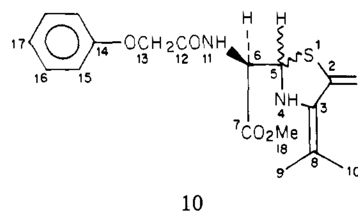
below, but the observed ¹H NMR spectrum is in accord with them. In order to explain the two products, it is assumed that epimerization at C-5 is rapid in the product via the mechanism of Scheme II. Epimerization at C-6 could only occur in ^2H_2O with exchange of the C-6 hydrogen. Since two doublets, at 5.68 and 5.73 ppm, one from each product, are present in the spectrum, no exchange at C-6 can have occurred, irrespective of which resonances, (C-5)-H (C-6)-H, are actually the ones observed. Further confirmation of this is seen in the presence of a third doublet, corresponding to the major epimer, upfield at 5.00 ppm; a decoupling experiment also showed that the 5.00 and 5.68 ppm doublets are coupled and hence in the same molecule. The 5.00 ppm resonance is assigned to C-6 since this one would probably move further upfield on opening the β-lactam ring; the corresponding peak of the minor epimer must be further upfield and obscured by the solvent peak.

An NMR spectrum of the spontaneous hydrolysis product at pH 7.5 could not be obtained because, even at an initial concentration of **1** of 1 mM, reactions leading to absorptions above 350 nm and precipitation of amorphous material occurred during the time required for the reaction to occur. It was possible however to obtain a spectrum of the product of reaction **1** in 0.1 M carbonate buffer at pH 10, where the half-time of reaction is much smaller, around 2 min. This product was shown to be the same as that from the enzyme-catalyzed reaction by absorption spectra (see above) and by thin-layer chromatography on cellulose, eluted with 4:1 methanol/0.1 M carbonate buffer at pH 10.0, where the R_f of each was 0.77. The ¹H NMR spectrum of this product was identical with that of the enzyme-catalyzed reaction reported in Table II, as anticipated on the basis of their very similar UV absorption spectra. The thin-layer chromatogram of the pH 10 reaction mixture also showed the presence of a little of the product obtained by hydroxide treatment of **1** (see below), R_f 0.41, but none of this material was seen in the NMR spectrum and so must account for less than 5% of the total product under these conditions.

Treatment of 0.04 mM **1** in methanol with triethylamine (5 mM) gives a product that absorbs maximally at 314 nm. This reaction could also be followed by ¹H NMR as described in the Experimental Section. The final ¹H NMR spectrum (Table III) looked very similar to that of the hydrolysis reaction; in particular, two very similar products were clearly present, again in a 60:40 ratio. Consequently, the products have been assigned the structures of the two C-5 epimers of **10**. Equilibration of the NMR sample solution with ^2H_2O led to collapse of the double doublets to doublets split by the smaller coupling constant and sharpening of the C-5 doublets that were perhaps broadened in the initial spectrum by N-H exchange at the 4-position.

Because of the greater stability of the methanolysis product in concentrated (millimolar) solutions, it was possible to also obtain a ¹³C NMR spectrum of it (Table III). This spectrum also showed the presence of two compounds, since here also most peaks appeared in duplicate. A comparison of the assignments of Table III with those of Table I suggests the former to be reasonable. It was not possible in these spectra to determine which peak of any pair belonged to the major isomer since the experiment was not carried out under conditions that allowed integration of the peaks to provide relative amounts. The C-5 and C-6 resonances cannot be separately assigned with the available data; nor can the carbonyl resonances of the amide and ester be distinguished from each other. The most important feature of this spectrum is the appearance of a pair of typical thiol ester resonances[16] in a position very similar to that of **1**. This observation points very strongly to the fact that in the first step of the base-catalyzed methanolysis of **1**, the thiol ester is *not* cleaved.

Table III. ¹H and ¹³C NMR Spectra of the Methanolysis Product

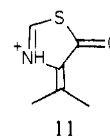


10

observed chemical shift	assignment
hydrogen[a]	
1.76, 2.01[d]	C-9 H_3, C-10 H_3
1.79, 2.01	C-9 H_3, C-10 H_3
3.79,[c,d] 3.82[c]	C-18 H_3
4.6 (br)	C-13 H_2
4.96 (dd, J = 3.2, 9.4 Hz)	C-6 H
5.61 (d, J = 3-4 Hz)[e]	C-5 H
5.25 (dd, J = 4.0, 9.0 Hz)[d]	C-6 H
5.59 (d, J = 3-4 Hz)[e]	C-5 H
7.0-7.5 (br m)	Ar H, N-11 H
carbon[b]	
10.3, 18.4	C-9, C-10
23.0, 23.4	C-9, C-10
56.9, 57.2, 62.3, 64.0	C-5, C-6
67.8	C-13
115.4, 115.6	C-15
123.1, 123.2	C-17
123.3, 126.1	C-3
130.7	C-16
133.6, 133.7	C-8
158.0, 158.1	C-14
169.4, 170.1, 170.5, 170.9	C-7, C-12
192.8, 193.3	C-2

[a] Solvent $C^2H_3O^2H$. [b] Solvent C^2HCl_3. [c] These chemical shifts are taken from a spectrum of the product prepared in CH_3OH. [d] Major product. [e] Overlapping and difficult to distinguish.

A mass spectrum of the methanolysis product contained a parent peak at m/e 364 (100%) that corresponds to a methanol adduct of **1**. Other prominent peaks were m/e 336 (9%, M − CO), 305 (9%, M − CO_2Me) and, significantly, 222 (31%, $PhOCH_2CONH^+{=}CHCO_2Me$) and 223 (19%, $PhOCH_2CON^+HCH_2CO_2Me$). In contrast, the mass spectrum of **1** contained only a small parent peak m/e 332 (3.6%) but had dominant peaks at m/e 142 (100%, **11**) and m/e 191 (17%,



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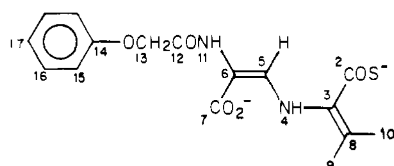
$PhOCH_2CONHCH{=}C{=}O$), these latter corresponding to the expected fragments of the principal cleavage of a β-lactam. The absence of a m/e 191 peak in the methanol adduct makes it seem

(16) Hall, C. M.; Wemple, J. *J. Org. Chem.* **1977**, *42*, 2118.

Table IV. ^{1}H and ^{13}C NMR Spectra of the Alkaline Hydrolysis Product



12

observed chemical shift	assignment
hydrogen[a]	
1.46	{C-9 H_3, C-10 H_3
1.56	
4.58	C-13 H_2
6.98 (d, J = 12 Hz)	C-5 H[b]
7.0-7.4 (br m)	Ar H
8.20 (d, J = 12 Hz)	N-4 H[c]
9.65	N-11 H[c]
carbon[d]	
15.6, 16.4	C-9, C-10
65.6	C-13
100.4	C-6
114.0	C-15
116.4	C-3
121.2	C-17
129.0	C-16
136.0	C-5
137.6	C-8
156.4	C-14
168.0	C-12
172.0	C-7
213.6	C-2

[a] Solvent Me_2SO-d_6. [b] Collapsed to singlet on addition of $^{2}H_2O$. [c] Disappeared on addition of $^{2}H_2O$. [d] Sample prepared as described in the methods section.

unlikely that it could be a β-lactam,[17] in further confirmation of the UV and NMR data presented above.

Reactions with Hydroxide Ion. Addition of **1** (final concentrations 0.01–0.1 mM) to potassium hydroxide solutions (0.01–1 M; ionic strength 1.0, adjusted with potassium chloride) yielded the absorption spectrum also shown in Figure 1. The nature of this product was investigated by NMR. ^{1}H and ^{13}C spectra were obtained as described in the Experimental Section and are given with a structural assignment, **12**, in Table IV. The most striking part of the ^{1}H spectrum was the olefin-like coupled (J = 12 Hz) doublets at 6.98 and 8.20 ppm. On addition of a drop of $^{2}H_2O$ to the NMR tube, the downfield doublet disappeared (as did the singlet at 9.65 ppm) while the upfield one collapsed to a singlet. Most significant in the ^{13}C spectrum was the resonance at 213.6 ppm. This cannot arise from a carboxylate carbon[18] but is very well situated to be from a thiocarboxylate. A spectrum of potassium thiolacetate under the same conditions contained a carboxyl carbon resonance at 222.7 ppm. The upfield shift from this point to that observed in **12** is typical of that found in carboxylic acid derivatives on incorporation of α,β unsaturation.[19]

The reaction pathway of **1** to **12** in hydroxide solutions is complex and difficult to study in detail quantitatively, but it is possible to prove that **9** is an intermediate. In solutions of low hydroxide ion concentration (0.001–0.01 M), the reaction apparently occurs in two phases, a fast phase consisting of a decrease in absorption at 270 nm (disappearance of **1**) and a concomitant increase in the 320–340-nm region (as would be expected if **9** were generated) followed by a slower phase involving an increase in absorption at essentially all wavelengths leading to the final product **12**. The first phase was first-order in hydroxide ion concentration (determined by measurement of pseudo-first-order rate constants at several hydroxide ion concentrations; data not shown) with a second-order rate constant of 31 s^{-1} M^{-1}. This rate constant can be compared with the value of 53 s^{-1} M^{-1} obtained by Bundgaard and Angelo[7] at lower pH (7–10) and 37 °C. Presumably, this first phase represents hydroxide ion attack on **1**. The slower phase of reaction although pseudo first order at a particular hydroxide ion concentration was apparently of higher order than one in hydroxide ion when examined at several hydroxide ion concentrations in the 0.001–0.01 M range. This phase was not analyzed numerically because of the complications leading to non-first-order behavior and arising even at 0.01 M as will be described below; the apparent higher order behavior will also be seen as in accord with the final scheme.

The addition of **9**, generated either by β-lactamase at pH 7–9 or spontaneously in phosphate or carbonate buffers at pH 7–10, to hydroxide solutions yielded **12** quantitatively, as judged by absorption spectra. Solutions of **9** (0.01–0.1 mM) in 1 M potassium chloride, adjusted to pH 7, could be obtained by addition of β-lactamase to solutions of **1**. The addition of small aliquots of strong potassium hydroxide solution then allowed the reactions of **9** in base to be studied. With manual mixing techniques (following the reaction on the Cary 219 spectrophotometer) and final hydroxide ion concentrations between 0.001 and 0.01 M, a single first-order reaction was observed where the amplitude at all wavelengths, the final spectrum, and the rate constants were the same as that observed in the slow phase of the reaction of **1** in the solutions of low hydroxide concentration. These results show clearly that **9** is not only the product of the enzyme-catalyzed and spontaneous reaction of **1** at neutral pH but also an intermediate in the reaction yielding **12** from **1** in alkaline solution.

At higher hydroxide ion concentrations, the reactions appeared to become more complex. The amplitude of the first phase of reaction of **1** decreased to such an extent that it became unobservable at any wavelength in the 0.05–1 M hydroxide ion concentration range, and concomitantly the absorption increase above 320 nm, ascribed to the formation of **9**, also vanished. Under these conditions, only the slow second phase involving appearance of **12** seemed to occur, but the absorbance changes with time were not strictly first order. These observations suggested that an intermediate other than **9** must also be involved. This problem was solved by stopped-flow studies of the reaction of **9** (enzyme generated at neutral pH as described above) in 0.01–0.3 M potassium hydroxide solutions. Under these conditions, two reaction phases were seen in the conversion of **9** into **12**, proving the existence of another intermediate. The slower phase was similar in rate and amplitude to the reaction of **1** under these conditions, described above, involving increasing absorbance at all wavelengths between 260 and 330 nm. It was however strictly pseudo first order at a given hydroxide ion concentration and first order in hydroxide ion concentration with a second-order rate constant of 5.2 s^{-1} M^{-1} (data not shown).

The faster phase of reaction of **9** with hydroxide ion involved loss of the 330-nm absorption band of **9** and an increase in absorption between 270 and 300 nm. Both the amplitude and rate of this phase were functions of hydroxide ion concentration as shown in Figures 2 and 3. These data are interpreted to mean that a hydroxide ion concentration dependent equilibrium is set up (eq 1) between **9** and the subsequent intermediate (**13**), with

$$\mathbf{9} \underset{k_{-2}}{\overset{k_2[\mathrm{OH}^-]}{\rightleftharpoons}} \mathbf{13} \qquad (1)$$

an apparent pK_a of 12.1 (from Figure 2). The observed pseudo-first-order rate constant, k_{obsd}, for establishment of this equilibrium is given by

$$k_{obsd} = k_{-2} + k_2[\mathrm{OH}^-]$$

A plot of k_{obsd} vs. $[\mathrm{OH}^-]$ yielded $k_2 = 352 \pm 4$ s^{-1} M^{-1} and $k_{-2} = 3.16 \pm 0.57$ s^{-1} from a least-squares line (Figure 3), and hence $K_a = k_2K_w/k_{-2} = 11.8 \pm 0.1$, in acceptable agreement with the value from Figure 2.

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(18) Stothers, J. B. "Carbon-13 NMR Spectroscopy"; Academic Press: New York, 1972; Chapter 8.
(19) Gensmantel, N. P.; Gowling, E. W.; Page, M. I. J. Chem. Soc., Perkin Trans. 2 1978, 335.

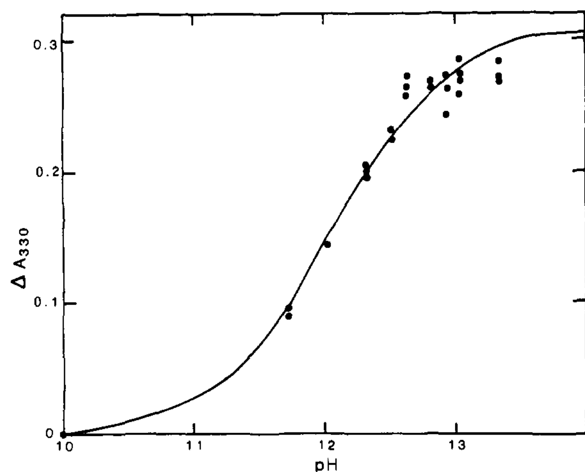


Figure 2. Decrease in absorbance at 330 nm, ΔA_{330}, during the first phase of reaction of **9** as a function of pH.

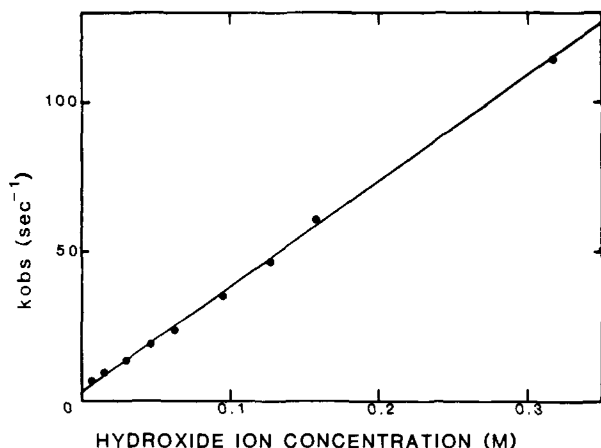
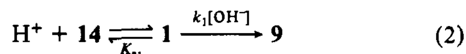


Figure 3. Pseudo-first-order rate constants k_{obsd} for reaction of **9** in alkaline solution as a function of hydroxide ion concentration. The reaction was followed spectrophotometrically at 330 nm.

On reaction of **1** at higher hydroxide ion concentrations, therefore, **9** is not seen as an intermediate, since it is rapidly and almost completely converted into **13**, the breakdown of which to **12** is largely rate determining in 0.1 M potassium hydroxide. The conversion of **1** to **13** is not observed directly, presumably because of the coincidental close similarity between the spectra of these compounds.

The proposed structure of **13** is shown in Scheme III, where the overall alkaline hydrolysis pathway is given. There is no direct evidence for the structure, but it is a logical intermediate in the pathway between **9** and **12**, being derived from **9** and converted into **12** in hydroxide-dependent steps.

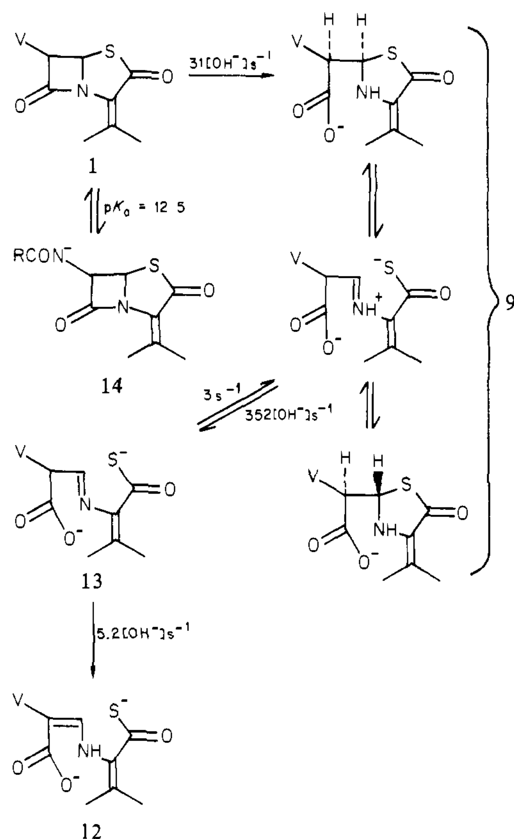
The remaining difficulty in interpreting the alkaline hydrolysis data lay in the observation that at the highest hydroxide ion concentration employed (1 M), the conversion of **1** to **12** again appeared to proceed with good first-order kinetics and without any appearance of intermediates. The rate constant observed in 1 M hydroxide ion was not 5.2 s^{-1}, as would be expected from the above discussion, for the rate-determining conversion of **13** into **12** but was only 1.5 ± 0.1 s^{-1}. Since this diminished rate was *not* seen when **9** was examined under the same conditions, the cause must lie in the conversion of **1** to **9** Since there was no sign of an intermediate between **1** and **9** under any conditions, a likely explanation would be that the ionization of the amide proton of **1** at high pH yields an inert anion **14** (eq 2). In this case the



$$H^+ + \mathbf{14} \underset{K_{a_1}}{\rightleftharpoons} \mathbf{1} \xrightarrow{k_1[OH^-]} \mathbf{9} \quad (2)$$

pseudo-first-order rate constant, k_{obsd}, would be given by eq 3. At low pH, $k_{obsd} = k_1[OH^-]$, and hence from above, $k_1 = 31\ s^{-1}$

Scheme III



M^{-1}. If $k_{obsd} = 1.5\ s^{-1}$ in 1 M hydroxide ion, then from eq 3, $pK_{a_1} = 12.5$.

$$k_{obsd} = k_1 K_w / (K_{a_1} + a_H) \quad (3)$$

The observations at high pH then could be explained by the ionization of **1**, with a pK_a of 12.5, to an inert anion. This would represent a further change in rate-determining step in the hydrolysis of **1** from **9** to **12** at low hydroxide ion concentrations (0.001–0.01 M) to **13** to **12** at intermediate concentrations (0.01–0.05 M) and finally to **1** to **9** at high concentrations. This complexity would explain the difficulty of observing good first-order kinetics at the intermediate concentrations.

If the above explanation for the behavior of **1** in strong base is correct, one might suppose that the same effect should be seen with all penicillins having 6-amido side chains. We do not know of any report of this phenomenon. Page et al. in recent comprehensive work on penicillin hydrolysis and aminolysis make no reference to it; their own data indicate that the rate of hydrolysis of benzylpenicillin is linear with hydroxide ion concentration to 0.1 M.[19] Consequently, we examined the hydrolysis of (phenoxymethyl)penicillin at high hydroxide ion concentration, with the results shown in Figure 4. These experiments were carried out at 25 °C. Clearly a phenomenon analogous to that deduced for **1** is occurring here. Application of eq 2 and 3 to the data of Figure 4 yields a second-order rate constant for hydroxide ion attack on (phenoxymethyl)penicillin of 0.20 s^{-1} M^{-1} (Page et al.[20] report 0.154 s^{-1} M^{-1} for benzylpenicillin at 30 °C) and a pK_a of 13.3. The latter figure is certainly consistent with a value of 12.5 for **1**, which should be more acidic. As a final check, it was noted that rate of hydrolysis of 6-aminopenicillanic acid, which does not have the potentially ionizable side chain, was linear with hydroxide ion concentration over the same range (Figure 4); the second-order rate constant obtained here was 0.037 s^{-1} M^{-1} (Page et al.[20] report 0.064 at 30 °C).

There seems, therefore, good indirect evidence for including eq 2 in the final scheme for the alkaline hydrolysis of **1** (Scheme

(20) Gensmantel, N. P.; Proctor, P.; Page, M. I. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1725.

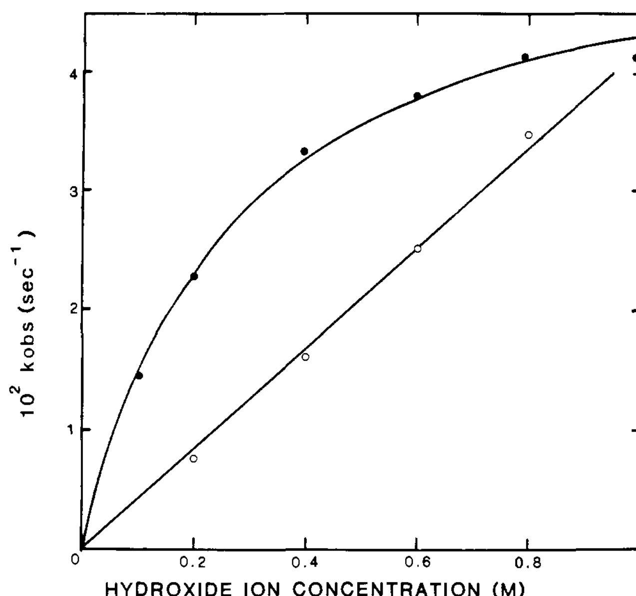
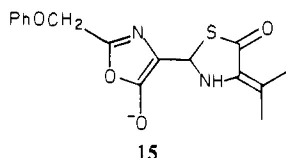


Figure 4. Pseudo-first-order rate constants k_{obsd} for hydrolysis of (phenxoymethyl)penicillin (●) and 6-aminopenicllanic acid (○) in alkaline solution as a function of hydroxide ion concentration. The reactions were followed spectrophotometrically at 232 nm.

III). It might also be noted here that although the suggestion has been made,[21] it does not seem to be commonly believed that there is intramolecular nucleophilic catalysis by the ionized 6-amido side chain in the alkaline hydrolysis of natural penicillins;[22,23] certainly the rate constants quoted above for benzylpenicillin and 6-aminopenicillanic acid do not suggest the need for any such catalysis. Thus although formation of an inert oxazolin-5-one anion **15** could also explain the results with **1** at high pH, there

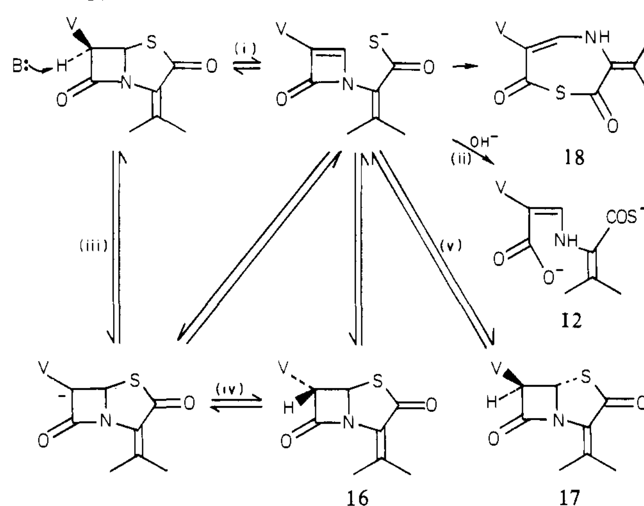


is no spectral evidence for such a species nor precedent from the penicillins. A further strong argument against such participation is that subsequent hydrolysis of **15** at high pH would be expected to yield 4-hydroxymethyleneoxazolin-5-one rather than **12**.[24]

Evidence from a Thiol Reagent. Ellman's reagent was used to detect the appearance of thiols during reactions of **1**. In one experiment, an aliquot of Ellman's reagent, sufficient to give a final concentration of 0.5 mM, was added to a cuvette containing the product of reaction of 0.04 mM of **1** in 0.1 M phosphate buffer at pH 7.5 in the presence of the TEM β-lactamase (i.e., containing 0.04 mM of **9**). No absorption increase was observed at 412 nm beyond the reagent background. Addition of Ellman's reagent (final concentration 0.5 mM) to 0.04 mM of **1** followed by β-lactamase addition also yielded no evidence of thiol production. These experiments seem to strongly suggest that the primary product does not contain a free thiol group, which would of course be much more in accord with initial β-lactam cleavage to **9** rather than the thiol ester cleavage of Bundgaard and Angelo.[7]

The addition of an aliquot of the alkaline hydrolysis product **12** to 0.5 mM of Ellman's reagent in 0.1 M phosphate buffer, pH 7.5 at 30 °C, led to an immediate increase in absorption at 412 nm. Since further aliquots yielded successively smaller absorption

Scheme IV



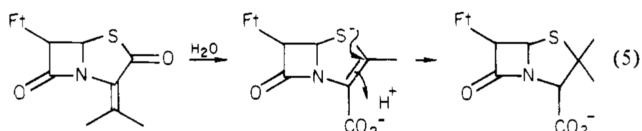
increases, it was clear that the reaction did not go to completion but that an equilibrium situation obtained (eq 4), where RS^-

$$RS^- + ArSSAr \overset{K}{\rightleftharpoons} ArS^- + ArSSR \qquad (4)$$

represents the thiolate released from **1** and ArSSAr is Ellman's reagent. Calculations using the measured absorbances at 412 nm as a function of volume of RS^- solution added yielded values of K (0.1 M phosphate buffer, pH 7.5, 30 °C) of 0.49 and a thiol content of 0.95 thiol groups per **1** reacted.

Not only does this result indicate that the reaction of hydroxide ion does, in the final product **12**, release a thiol from **1** but also it suggests, from the equilibrium constant of eq 4, that the thiol is of low pK_a, comparable to that of the Ellman's thiol (pK_a = 4.75[25]). The latter observation would be expected for a thio acid such as **12**; with higher pK_a thiols, the disulfide exchange would go to completion.[10]

Attempted Reversal of the Anhydropenicillin Rearrangement. Prior to the work of Bundgaard and Angelo,[7] there was already a suggestion in the literature that nucleophilic cleavage of anhydropenicillins occurred initially at the thiol ester center. Wolfe et al.[26] reported conditions under which anhydrophthalimidopenicillin apparently reverted to the penicillin. They interpreted this observation in terms of eq 5.



Employing exactly the conditions that Wolfe et al.[26] report, we were unable to observe any formation of (phenoxymethyl)penicillin from **1** over a period of several days. The reaction was followed by NMR so that the upper level of detection would be a few percent. (Wolfe et al. report an 86% reversal[26].) Baldwin et al.[27] have also reported being unable to effect this reconversion. Hence there seems no evidence here either for significant thiol ester cleavage.

Anhydropenicillin and Strong Base. The reaction of **1** with a strong base under aprotic conditions was examined in order to assess the facility of a direct base-catalyzed elimination (Scheme IV, (i)), which in penicillins is thought to lead to their rearrangement to thiazepinones,[28] but which here might represent

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another route of hydrolysis to **12** (Scheme IV, (ii)). Such an elimination might be more facile in anhydropenicillins because of the better leaving group at C-5. It should be noted however that penicillin sulfone esters do not appear to undergo this elimination—apparently epimerization at C-6 via the carbanion (analogous to reactions iii and iv, Scheme IV) and elimination of RSO_2^- from C-2 are more facile.[29]

Also of interest was the possibility of base-catalyzed intramolecular nucleophilic cleavage of the β-lactam of **1** to yield the oxazolin-5-one corresponding to the anion **15**. This type of rearrangement is known to occur both for penicillins[30] and anhydropenicillins,[31] although under conditions of acid catalysis.

In an NMR experiment, **1** (1 mg) was treated with 2.5 equiv of 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU) in 0.5 mL of C^2HCl_3 and the ^1H spectrum monitored at 22 °C as a function of time. An immediate observation was the disappearance of the side-chain amide N–H resonance and of its coupling to the hydrogen at C-6. Since the chemical shift of (C-6)-H did not change, the observation presumably reflects rapid exchange of the N–H, catalyzed by DBU, rather than complete anion formation. Despite the labilization of this proton, no sign of oxazolin-5-one formation was observed over several hours.

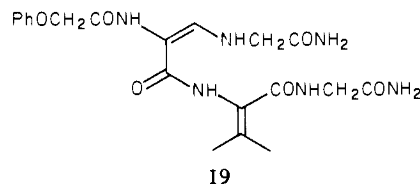
One reaction did occur, however, complete after about 10 min and characterized by the formation of a product with the following ^1H NMR spectrum: δ 2.21 (s, 3 H, CH_3), 2.22 (s, 3 H, CH_3), 4.60 (s, 2 H, CH_2), 5.20 (dd, J = 1.9, 8.0 Hz, 1 H, (C-6)-H), 5.42 (d, J = 1.9 Hz, 1 H, (C-6)-H), 7.0–7.5 (m, 6 H, Ar H, NH). This reaction did not go to completion but to a position of equilibrium with about 60% of the original **1** still present; further addition of DBU led to no further spectral changes in another 30 min. These observations are interpreted in terms of epimerization at C-6 via (iii) and (iv), Scheme IV, to yield the 6-epianhydropenicillin **16**. Another possible interpretation would be of equilibration of **1** via (i) and (v) with the 5-epianhydropenicillin **17**, whose spectrum would also contain the observed trans coupling constant between (C-5)-H and (C-6)-H. The latter interpretation seems less likely, however, because the absence of other products would imply that neither **16**, the 5,6-diepianhydropenicillin[29] nor the thiazepinone **18** formed along with **17** by the elimination route—this could be fortuitously possible through a combination of unfavorable kinetic and thermodynamic parameters but seems unlikely. A base-catalyzed reaction of phthalimidoanhydropenicillin was also interpreted by Wolfe et al.[32] in terms of epimerization at C-6. Under the same conditions as above, we noted that benzylpenicillin methyl ester revealed neither amide NH lability nor epimerization in 1 h.

These experiments give no indication of any particularly facile base-catalyzed rearrangements of **1** that needed to be considered in relation to the reactions in water.

The epimers **1** and **16** were separated from the equilibrium mixture as follows. DBU was extracted by washing the chloroform solution with 0.1 M phosphate buffer, pH 7.5, and the dried organic layer was evaporated to dryness. The residual mixture was separated by preparative thin-layer chromotography (silica gel, 50% ethyl acetate/cyclohexane; **1** and **16** have R_f values of 0.56 and 0.64, respectively). The isolated 6-epianhydropenicillin had a ^1H NMR spectrum as above, a mass spectrum containing peaks at m/e 332 (M, 1.3%), 304 (3.5%), 191 (33%), 142 (100%), 141 (92%), 108 (69%) and an ultraviolet absorption spectrum nearly identical with that of **1**.

As would be expected,[33] **16** was not a substrate of either the *B. cereus* I or TEM β-lactamases. It was however hydrolyzed in dilute base in a two-phased reaction and with rates not significantly different from those observed with **1**. The last observation here is further evidence that the reactions of **1** in aqueous base are not initiated by the elimination reaction of Scheme IV, (i).

Reaction of 1 with Amines. Some preliminary observations were made on the reaction of **1** with some typical amine nucleophiles since these are known to have affinity for both bicyclic β-lactams[34] and thiol esters,[35] leading to aminolysis. Hydroxylamine (10 mM) reacted immediately with 0.04 mM of **1** in 0.1 M phosphate buffer, pH 7.5, to give a product with absorption maximum at 327 nm (ε 4500 M^{-1} cm^{-1}). Ellman's reagent indicated no thiol release; so attack of this nucleophile at the β-lactam carbonyl seems likely. In contrast, 0.1 M glycinamide at pH 7.5 reacted more slowly to yield a product with absorption maximum at 283 nm (ε 16000 M^{-1} cm^{-1}). Addition of glycinamide to **9** did not give this chromophore. The glycinamide reaction with **1** did release thiol quantitatively. These observations suggested preferential nucleophilic attack of glycinamide at the thiol ester to yield **19**



perhaps.[36] Reaction with ammonia (0.1 M, pH 9.6) however gave a product spectrum similar to that from hydroxylamine. Results with imidazole and hydrazine indicated that even more complex reactivity was possible; the former appeared to yield different products at different imidazole concentrations (0.1–1 M, pH 7.0) while the latter (10 mM, pH 7.5) led to concerted loss of all absorption at wavelengths above 250 nm. These observations were not pursued further.

Discussion

The results indicate that (phenoxymethyl)anhydropenicillin, **1**, is a poor substrate of the *B. cereus* I and TEM β-lactamases. Although the binding of **1** to the β-lactamases is weak, it is apparently sufficiently strong in a specific fashion so as to lead, in combination with the presumed high reactivity of **1**, to significant turnover. Furthermore, as indicated by absorption spectra, by NMR spectra, and by thin-layer chromatography, the product of the enzyme-catalyzed reaction is the same as that of the spontaneous hydrolysis in phosphate and carbonate buffers at pH 7–10. The dilemma created by this observation is described in the introduction and could only be escaped by a closer examination of the primary products of the reaction than had been achieved by Bundgaard and Angelo.[7]

It became clear almost immediately that unless the hydrolysis reaction was studied at concentrations of **1** less than about 1 mM, the primary product was rapidly consumed by other reactions, presumably bimolecular, which led to a complex mixture of products. This was particularly true at higher pH in the absence of EDTA, and thus trace heavy metal ion catalyzed oxidations were to some extent involved. With care, however, at low concentrations, the nature of the primary product could be investigated.

The primary product of both the enzyme-catalyzed and the spontaneous hydrolyses in neutral and alkaline solutions is identified in this work as the β-lactam cleavage product **9**. It appears to exist as a mixture of isomers (Scheme III) where the cyclic epimers dominate in solution. The evidence for this identification rests on NMR spectra of the product, on NMR and mass spectra

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(36) Cheney, J.; Moores, C. J.; Raleigh, J. A.; Scott, A. I.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1974**, 986. Gunda, E. T.; Jaszberenyi, J. C.; Bognar, R. *Tetrahedron Lett.* **1976**, 2911.

of the analogous methanolysis product, on the absence of reactivity of the product with Ellman's reagent, and on the elucidation of the path of hydrolysis of **1** in alkaline solution.

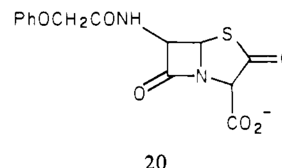
Particularly strong is the evidence from the Ellman's reagent and the spectra of the methanolysis product. Base-catalyzed methanolysis also produces two epimers, **10**, whose ^{13}C NMR spectrum shows clearly the continued existence of a thiol ester carbonyl group, and mass spectrum the absence of a β-lactam. Identification of the ultimate alkaline hydrolysis product as **12** is also important. The existence of a thiocarboxylate group in **12**, as indicated by the ^{13}C NMR spectrum and by the Ellman's reaction, demonstrates that cleavage of the thiol ester could not have occurred on conversion of **1** to **9** since **9** is shown to be an intermediate in the formation of **12**. In order to prove this point, we studied the rather complicated alkaline hydrolysis of **1** in some detail. Elucidation of the pathway was simplified considerably by the fact that intermediate **9** could be prepared in solution by hydrolysis at lower pH. The hydrolysis pathway of **1** in alkaline solution is given in Scheme III with the relevant rate and equilibrium constants.

The property of **9** whose observation originally led to most of this work, the absorption spectrum (Figure 1), was not a useful factor in the structure analysis because we were unable to find other examples in the literature of a 4-alkylidenethiazolidin-5-one ring system for comparison.

This work indicates that the 4-alkylidenethiazolidin-5-one ring is both stable and labile in aqueous solution; stable enough to not provide a concentration of the ring-opened isomer sufficient to react with Ellman's reagent at a significant rate but labile enough to allow rapid epimerization at C-5 of **9**—the half-time of epimerization can be estimated to be less than 5 min at pH 7–8.

We conclude, contrary to Bundgaard and Angelo,[7] that the initial site of nucleophilic attack of hydroxide ion and water (and phosphate and carbonate ions if they participate[7] as nucleophilic rather than general-base catalysts[37]) is at the β-lactam rather than the thiol ester carbonyl group, leading to β-lactam cleavage. The β-lactamases then do remain true to their calling.

The apparent failure of **1** to significantly inhibit β-lactamases may well be a result of the stability of the 4-alkylidenethiazolidin-5-one ring; **20**, containing a thiazolidin-5-one ring that is probably less stable to ring-opening after β-lactam hydrolysis, is reported to inhibit β-lactamases.[38]



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The effect of the structure of **1** vs. that of natural penicillins on the susceptibility of the β-lactam carbonyl group to nucleophilic attack can now be directly assessed by comparison of the second-order rate constants for hydroxide ion attack, 53 s^{-1} M^{-1} and 0.65 s^{-1} M^{-1}, respectively.[7] This considerable difference seems well in accord with the high β-lactam carbonyl stretching frequency and highly pyramidal β-lactam nitrogen atom in **1**.[6] Other nucleophiles such as amine nucleophiles may preferentially attack the thiol ester carbonyl group. It is likely that amine attack at these centers requires general-acid or general-base catalysis[35,36] and that the site of attack may vary with the mode of catalysis.[39] The thiol ester carbonyl group of the 4-alkylidenethiazolidin-5-one ring system does not seem readily susceptible to nucleophilic attack, presumably because of the conjugated double bond, such that in **9** the ring opens via an elimination pathway (**9** to **12**) rather than through thiol ester cleavage.

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Registry No. 1, 47295-33-0; **9** (isomer 1), 84303-62-8; **9** (isomer 2), 84303-68-4; **10** (isomer 1), 84303-63-9; **10** (isomer 2), 84303-69-5; **12**, 84303-64-0; (*S*)-**13**, 84303-65-1; **16**, 84303-66-2; **19**, 84303-67-3; β-lactamase I, 9001-74-5; carbonate, 3812-32-6; hydroxide, 14280-30-9; DBU, 6674-22-2; hydroxylamine, 7803-49-8; Ellman's reagent, 69-78-3; glycinamide, 598-41-4.

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Hydride vs. Electron Transfer in the Reduction of Flavin and Flavin Radical by 1,4-Dihydropyridines

Michael F. Powell and Thomas C. Bruice*

Contribution from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106. *Received July 9, 1982*

Abstract: Literature dealing with the existing controversy concerning the mechanism of hydride equivalent (H^- or e^-, H^+, e^-) transfer from *N*-alkyl-1,4-dihydronicotinamides is presented. Included are references to the dihydronicotinamide reduction of flavins. As a means of assessing the H^- and e^-, H^+, e^- reduction mechanism of flavins, we have studied the reaction of the *N*-alkyl[4,4-$^1H_2$]- and -[4,4-$^2H_2$]pyridines **3a–f** with the flavin **1** and flavin cation radical **2**. By electrochemical calculations, the 1e^- reductions of both **1** and **2** by **3a–f** are exothermic. The second-order rate constants and kinetic isotope effect for reactions of **3a–f** with **1** are reported. The disappearance of **2** in the presence of excess **3a–f** is zero order in [**2**]. Various arguments and experimental evidence have been combined to show that **2** does not react with the dihydropyridines **3a–f**. The reduction of **2** takes place by dihydronicotinamide reduction of flavin to yield dihydroflavin, which then reduces the flavin radical **2** by 1e^- transfer. It is concluded that since dihydronicotinamide does not reduce flavin radical cation by 1e^- transfer, it is unlikely to undergo stepwise 1e^-, H^+, 1e^- reduction of flavin. These results are best interpreted to indicate that flavin reduction by dihydronicotinamides occurs by a hydride-transfer mechanism.

Despite the importance of the 1,4-dihydronicotinamides NADH and NADPH in biochemical redox reactions, the mechanisms for these reactions continue to be debated. It is yet to be settled as to whether the transfer of a hydride equivalent from dihydronicotinamide to substrate involves (i) transfer of a hydride ion in a single step; (ii) two-step transfer of an electron and a hydrogen atom; or (iii) overall transfer of two electrons and a proton in three separate steps. Abeles, Hutton, and Westheimer,[1] in a pioneering

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