8-Hydroxypenillic Acid from 6-Aminopenicillanic Acid: A New Reaction Catalyzed by a Class C β-Lactamase

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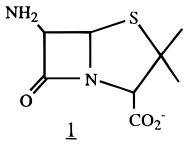
Abstract: The hydrolysis of 6-aminopenicillanic acid (APA) at neutral pH, catalyzed by the class C β-lactamase of *Enterobacter cloacae* P99, was observed from ^{1}H NMR spectra to yield an unexpected product, 8-hydroxypenillic acid, in bicarbonate-containing buffers. This product probably arises from turnover of the carbamate of APA by the enzyme. ^{1}H and ^{13}C NMR spectra of APA in bicarbonate solutions clearly demonstrate the presence of the carbamate. Turnover of APA at saturating concentrations by the enzyme is accelerated by bicarbonate and by methanol. These results suggest that deacylation of the enzyme is rate determining and that the presence of the carbamate affects this step. A mechanism involving rate-determining intramolecular nucleophilic attack by the carbamate on the acyl enzyme is proposed to rationalize these observations and lead to formation of 8-hydroxypenillate. This reaction can be seen as an example of substrate-assisted enzymic catalysis. Quantitative analysis of the data indicated a dissociation constant of the APA-carbamate to APA and bicarbonate of 7 mM at pH 7.5. The P99 β-lactamase also catalyzes the formation of an alternative product from 7-aminocephalosporanic acid in the presence of bicarbonate. Ampicillin and cephaloglycine which also possess an amine-bearing side chain [PhCH(NH_2)CO−] react with bicarbonate to form carbamates at neutral pH but these do not yield alternative products on turnover by the P99 β-lactamase. Typical class A β-lactamases do not catalyze 8-hydroxypenillate formation from APA and bicarbonate even when deacylation is rate determining. This difference is discussed in terms of β-lactamase active site structure.

Introduction

The interactions of substrates and mechanism-based inhibitors, particularly those that are β-lactams, with serine β-lactamases have produced an impressive range of chemical reactions and quite a number of surprises.[1] In most of these cases, the covalent acyl-enzyme intermediate has provided the staging point for these unexpected reactions. It is now clear therefore that product identification must remain an essential component of studies of these enzymes with their broad range of now-available mutants[2] and their even broader range of substrates. Even with simple and extensively studied substrates, surprises can still emerge. The present paper, for example, describes a new reaction of 6-aminopenicillanic acid (APA, **1**) and its cephalosporin analog, 7-aminocephalosporanic acid (ACA, **2**), catalyzed by the class C β-lactamase of *Enterobacter cloacae* P99. This enzyme, but not typical class A β-lactamases, was found to catalyze the reaction of APA with carbon dioxide to form 8-hydroxypenillic acid. This reaction represents an example of one type of substrate-assisted enzymic catalysis.[3]



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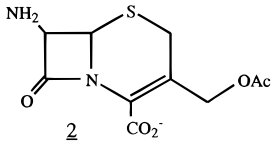

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Experimental Section

Materials. The β-lactamase of *E. cloacae* P99, the TEM-2 β-lactamase from *E. coli* W3310, and the PC1 β-lactamase of *S. aureus* were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. Samples of APA were purchased from Aldrich Chemical Co., Fluka Chemical Corp., and U. S. Biochemical. ACA and cephaloglycin were gifts of Eli Lilly and Co. ^{13}C-Labeled (98 atom %) sodium bicarbonate was purchased from Aldrich Chemical Co. and ampicillin from Sigma.

APA was purified by its dissolution in water (1 g/40 mL) containing the minimum necessary amount of sodium bicarbonate and reprecipitated by acidification to pH 4.5 with hydrochloric acid. After isolation by filtration, the APA was washed with water and dried at room temperature *in vacuo*. This procedure removed a small amount of an impurity which behaved as a transient inhibitor (a β-lactam?) of the P99 β-lactamase. APA from the three above-mentioned sources contained similar amounts of this material. It may be responsible for the complex kinetics reported for APA and the P99 β-lactamase by Matagne et al.[4] Certainly, after its removal, we observed clean Michaelis−Menten kinetics with this enzyme. The presence of impurities in commercial β-lactam preparations thus represents another source of unusual β-lactamase kinetics.

Kinetic Methods. The hydrolysis of APA was monitored spectrophotometrically at a wavelength of either 232 nm ($\Delta\epsilon = -830$ M^{-1} cm^{-1}) or 248 nm ($\Delta\epsilon = -235$ M^{-1} cm^{-1}). The hydrolysis of ACA was followed in two ways, by disappearance of starting material at 285 nm ($\Delta\epsilon = -1000$ M^{-1} cm^{-1}) and by appearance of product at 320 nm ($\Delta\epsilon = 290$ M^{-1} cm^{-1}). Steady-state kinetic parameters were obtained from spectrophotometrically determined initial rate measurements by the method of Wilkinson.[5] All kinetics studies, except where otherwise specified, were performed at 25 °C in 20 mM MOPS buffer at pH 7.50.

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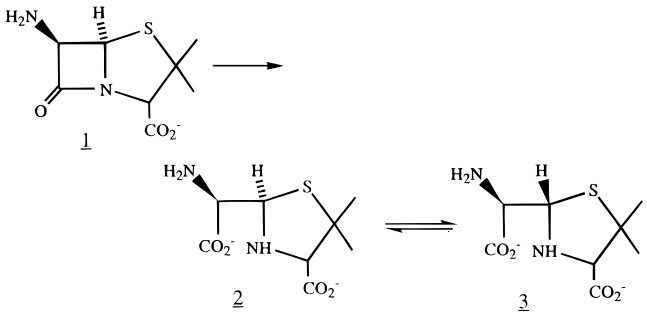
Scheme 1



Quantitative initial rate and product analysis data were fitted to Scheme 3 by the Kinsim program[6] in the Macintosh version.[7] Progress curves produced by this program were used to obtain initial rates and product composition at completion. Values of K_c, ($k_b + k_c$), and $k_b/(k_b + k_c)$ were varied by trial and error in order to obtain the best visual fits.

Synthesis of the Disodium Salt of 8-Hydroxypenillic Acid (3,3-Dimethyl-8-oxo-4-thia-1,7-diazabicyclo[3.3.0]octane-2,6-dicarboxylic Acid). This compound was prepared in a manner very similar to the original method of Johnson and Hardcastle.[8] Thus, 5 g of APA was dissolved in 23 mL of water containing 3.9 g (2 equiv) of sodium bicarbonate. Carbon dioxide was slowly bubbled through the stirred mixture at room temperature for 12 h. Addition of acetone and cooling then produced the disodium salt of the required product, mp 248−251 °C (lit.[6] mp 250−251 °C). The ^{1}H and ^{13}C NMR spectra are described below.

NMR Spectra and Product Analysis. ^{1}H and ^{13}C NMR spectra were obtained by means of a Varian Gemini-300 spectrometer. Chemical shifts are reported as ppm downfield of either tetramethylsilane or sodium 3-(trimethylsilyl)propanesulfonate. Product ratios in the APA experiments were obtained from ^{1}H NMR spectra of samples from the kinetics runs after the reaction had reached completion, as determined by the absorbance measurements. These solutions were removed from the cuvettes, freeze dried, and redissolved in ^2H_2O, and ^{1}H NMR spectra obtained. The ratio of **2** to **6** was obtained from integration of the methyl peaks at 1.23 and 1.50 ppm, respectively.

Results and Discussion

The hydrolysis of purified APA, catalyzed by the P99 β-lactamase, exhibited classical Michaelis−Menten kinetics under steady state conditions with $k_{cat} = (0.92 \pm 0.06)$ s^{-1} and $K_m = (0.29 \pm 0.05)$ mM. It is thus a rather poor substrate of this β-lactamase.[9] It was noticed, however, that the enzyme-catalyzed reaction yielded an unusual product, X, under conditions commonly used in this laboratory for NMR studies, *viz.*, 0.1 M sodium bicarbonate solution.

The alkaline hydrolysis of APA, as we and others[10] have observed, yields first the penicilloate **2**, which then epimerizes at C-5 to give **3** (Scheme 1), almost to completion at equilibrium. (The ^{1}H and ^{13}C NMR spectra of **1** and **2** are reported in Tables 1 and 2, respectively.) In neutral phosphate buffer, the P99 β-lactamase catalyzes the hydrolysis of APA to give **2** which, under these conditions, more slowly epimerizes. In neutral bicarbonate solutions however the P99 β-lactamase produces X. The addition of bicarbonate to **2** did not generate X. The ^{1}H and ^{13}C NMR spectra of X are reported in Tables 1 and 2, respectively.

Table 1. ^{1}H NMR Spectra of APA and Its Reaction Products

	chemical shift (ppm)			
resonance	**1**	**2**	**4**	**6**
$(CH_3)_2$[a]	1.53, 1.63	1.23,1.56	1.52,1.63	1.50,1.55
C_3-H[a]	4.16	3.48	4.17	4.22
C_6-H[b]	4.58 (4.0)	3.64 (8.0)	5.25 (4.0)	4.19 (2.1)
C_5-H[b]	5.49 (4.0)	4.86 (8.0)	5.51 (4.0)	5.49 (2.1)

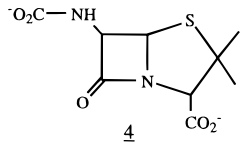
[a] Singlet. [b] Doublet, coupling constant (Hz) in parentheses.

Table 2. ^{13}C NMR Spectra of APA and Its Reaction Products

	chemical shift (ppm)			
resonance	**1**[a]	**2**	**4**[a]	**6**
$(CH_3)_2$	26.6, 30.8	26.2, 26.3	26.6, 30.4	26.1, 31.7
C-2	63.9	59.6[b]	64.2	57.9[b]
C-3	72.8		73.1	
C-5	68.5	59.8, 63.8, 74.6[c]	67.9	59.7, 69.6, 73.8[c]
C-6	60.4		60.3	
C-7, 3-CO_2^-	175.0, 179.9	172.4, 175.4	174.1, 178.3	176.1, 177.6
NH−CO			162.4	164.6

[a] Assignments following Chang and Hem.[32] [b] Assigned on the basis of its small intensity with respect to C-3, C-5, and C-6. [c] Assignment between C-5, C-6, and C-7 uncertain.

Closer inspection of the ^{1}H NMR spectrum of APA in bicarbonate showed the presence of two β-lactam species, one of which was presumably APA itself in view of the similarity of its spectrum to those taken in solutions of other buffers. The other species (^{1}H and ^{13}C spectra reported in Tables 1 and 2, respectively) is suggested to be carbamate **4**. There is considerable evidence for this assignment. First, the quantity of the second species relative to the first increased with total carbonate (carbonate plus bicarbonate) concentration. For example, at pH 9.7, the amount of **4**, determined from integration of ^{1}H NMR peaks, increased linearly (data not shown) with total carbonate (0−100 mM) from 0% to 25% of the total APA (10 mM). Essentially total conversion was achieved at 1 M total carbonate. Second, at constant total carbonate (100 mM), the proportion of **4** increased as the pH was decreased (<5% at pH 11.4, 22%



at pH 10.3, 33% at pH 8.9). The amount decreased again at pH <7, but carbon dioxide was also lost. The acidification of a solution of APA in 1 M bicarbonate to pH 3 followed by aspiration to remove carbon dioxide and finally rebuffering to pH 7 with phosphate yielded a solution whose ^{1}H NMR spectrum showed the presence of only APA. A bell-shaped equilibrium distribution with pH, with maximum around the pK_a of the amine, is typical of carbamate formation, for example from amino acids.[11] Third, the doublet corresponding to C_6-H moved downfield from 4.58 ppm in **1** to 5.25 ppm, closer to that in an N-acylated penicillin. A similar downfield shift of C_α protons has been observed on carbamate formation from amino acids.[11a,12]

Further, in the ^{1}H NMR spectrum of APA (0.1 M) in $NaH^{13}CO_3$ (0.67 M), the resonance peak for C_6-H at 5.25 ppm appears as a double doublet ($J = 3.4$, 3.9 Hz) rather than the

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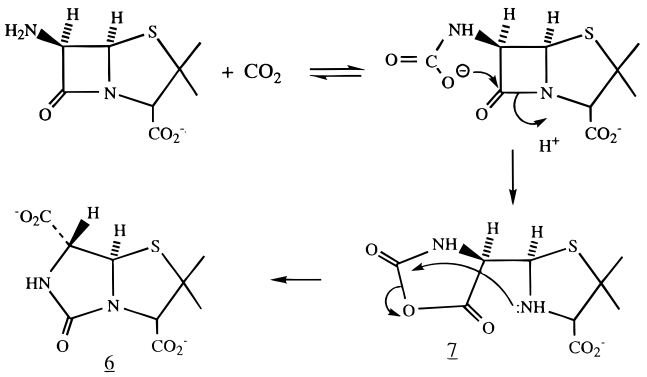
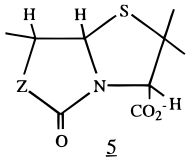
Scheme 2



doublet observed in $NaHCO_3$. In terms of structure **4**, this would of course arise through the coupling of both C_5-H and the carbamate ^{13}C to C_6-H. Finally, the ^{13}C spectrum of 0.667 M APA in 0.0667 M $NaH^{13}CO_3$ shows an enhanced peak at 162.4 ppm, just downfield of the $H^{13}CO_3^-$ peak. Similar ^{13}C chemical shifts for carbamates have been previously reported.[11b,12]

It is therefore very likely that the additional peaks observed in NMR spectra of APA in bicarbonate buffers derive from carbamate **4**. It further seemed reasonable that the novel product, X, obtained from APA in these buffers in the presence of the P99 β-lactamase, arose from interaction of the enzyme with **4**.

In order to test the hypothesis that X was derived from **4**, APA (0.1 M) in $NaH^{13}CO_3$ (0.2 M) was converted to X by addition of the P99 β-lactamase. The ^{13}C spectrum of X thus obtained showed an enhanced carbonyl resonance at 164.6 ppm. Further, the ^1H proton spectrum of X (Table 1) showed ^{13}C coupling to protons on C-5 (5.8 Hz), C-6 (4.2 Hz), and C-3 (3.7 Hz). These results suggest the skeleton structure **5**, where



Z presumably would be either O or NH. At this stage, perusal of the literature revealed a well-established reaction of APA in bicarbonate solutions (Scheme 2,)[8,13] producing 8-hydroxypenillic acid **6** (although 8-hydroxypenillic acid is the traditional trivial name for this compound it probably exists predominantly in the keto form,[14] as depicted in **6**); the mechanism proposed for the reaction is also shown in Scheme 2.

The disodium salt of **6** was prepared as described by Johnson and Hardcastle[8] and was found to exhibit identical ^1H and ^{13}C NMR spectra to those of X. The identification of X as **6** was thus made. A final point that might be mentioned here with respect to the nature of **6** concerns the stereochemistry at C-5 and C-6. The NMR spectra clearly indicate only one isomer is produced, and the same isomer, in both the spontaneous and β-lactamase-catalyzed reactions. Casy and Lipczynski[13] have suggested on indirect grounds that the correct assignment is *not* 5*R*6*R*, the original configuration in **1**. Such a result would imply epimerization at C_5, C_6, or both. The mechanisms of Scheme 2 and Scheme 5, however, do not require epimerization at either center. Epimerization at C_6 seems unlikely on the grounds that exchange with solvent deuterium was not observed in the product, and that at C-5 similarly because of the electron-withdrawing nature of the thiazolidine substituent in **7**.[15] The observed coupling constants also do not support either 5*R*6*S*, or 5*S*6*R*. Models suggest $H-C_5-C_6-H$ dihedral angles of around 22° in the latter copounds and around 110° in the 5*R*6*R* isomer. The small observed coupling constant ($\leq$2 Hz) is more in accord with the latter possibility than either one of the former pair. The evidence thus seems more in accord with the 5*R*6*R* stereochemsitry for **6**, as shown in Scheme 2, than with any of the alternatives.

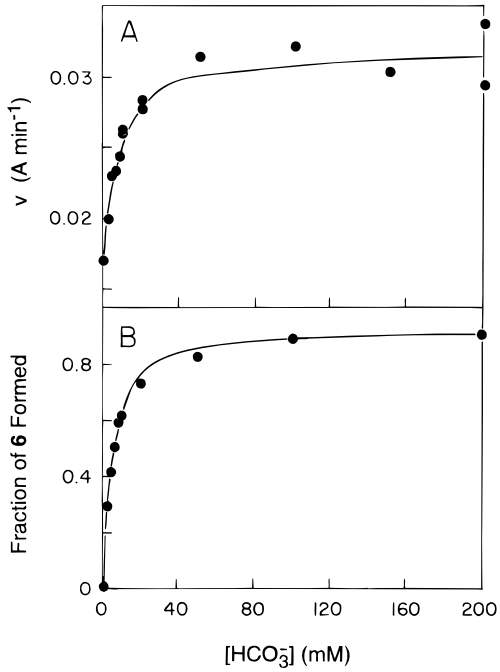
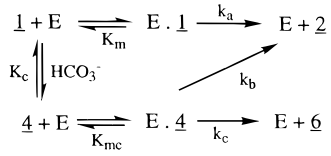


Figure 1. Effect of HCO_3^- concentration on the rate of disappearance of APA (3 mM) catalyzed by the P99 β-lactamase (1.3 μM) (A) and on the fraction of **6** among the products of the reaction (B).

Scheme 3



The remaining question now involves the question of how the P99 β-lactamase might catalyze formation of **6**.

Kinectis and Mechanism of the P99 β-Lactamase-Catalyzed Formation of 6. Under substrate saturation conditions (3.0 mM), HCO_3^- accelerates the disappearance of APA (Figure 1A); under the same conditions, HCO_3^- has little or no effect on benzylpenicillin (0.5 mM) turnover (not shown). In 0.2 M HCO_3^- and 0.02 M MOPS at pH 7.5 the steady state parameters for turnover of APA were $k_{cat} = 1.86 \pm 0.06$ s^{-1} and $K_m = 0.36 \pm 0.03$ mM. There has thus been a 2-fold increase in k_{cat}. Since K_m is little affected, k_{cat}/K_m is increased by a similar amount. The acceleration of APA turnover correlates with the change in product composition from **2** to **6**, as revealed by ^1H NMR measurements (Figure 1B). These data could be quantitatively fitted to Scheme 3, as described in the Experimental Section, yielding the solid lines of Figure 1. Values for K_m, K_{mc}, and k_a were fixed at the steady state values

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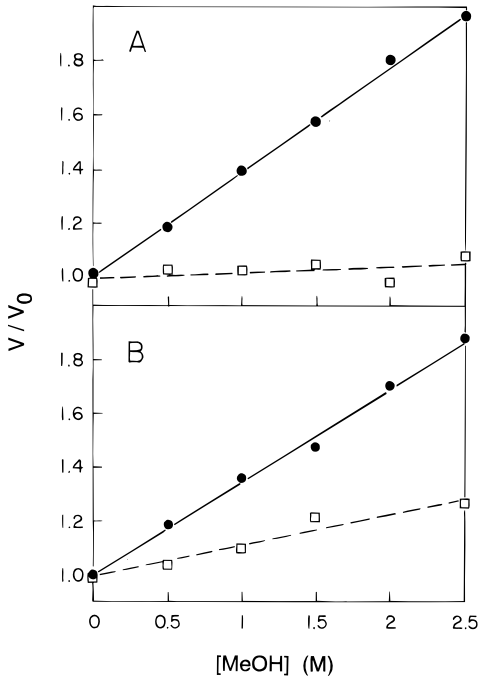
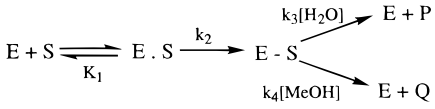


Figure 2. Effect of methanol concentration on the rate of disappearance of APA (3 mM) catalyzed by the P99 β-lactamase (1.3 μM), in the absence (○) and presence (□) of 0.17 M HCO_3^- (panel A). The lower panel (B) shows the results of the same experiment with ACA (0.75 mM).

Scheme 4



described above, 0.29 mM, 0.36 mM, and 0.92 s^{-1}, respectively. The solid lines of Figure 1 correspond to values of ($k_b + k_c$), $k_b/(k_b + k_c)$, and K_c of 1.72 s^{-1}, 0.93, and 7.0 mM, respectively. The value of ($k_b + k_c$) thus obtained at 3.0 mM APA would correspond, if $K_m = 0.36$ mM, to a k_{cat} value of 1.93 s^{-1} which is in good agreemnt with that obtained by direct experiment in the presence of 0.2 M HCO_3^- (1.86 ± 0.06 s^{-1}). Partitioning of the enzyme−carbamate complex, E.**4**, between rearrangement to **6** and hydrolysis to **2**, is required by the product analysis data of Figure 1B—approximately 7% of the turnovers yield the hydrolysis product **2** even at saturating HCO_3^- concentration.

Mechanistic analysis of these data is informed by an appreciation of the mechanism of P99 β-lactamase catalysis and would be further so by knowledge of the nature of the rate-determining step under saturating APA conditions, i.e. of the k_{cat} transition state. Figure 2 shows the effect of methanol on the rate of P99 β-lactamase-catalyzed disappearance of APA under close to saturating conditions (3.0 mM) in the absence and presence of 0.17 M HCO_3^-. In the absence of HCO_3^-, methanol accelerates the disappearance of APA in a linear fashion. This is similar to the effect of methanol on the reaction of benzylpenicillin[16] and other substrates[16,17] catalyzed by this enzyme, and is interpreted in terms of Scheme 4 where E-S represents the acyl-enzyme intermediate,[18] and P and Q the products of its hydrolysis and methanolysis respectively.

Methanol will obviously accelerate the steady state rate if deacylation is rate determining at saturation, i.e. $k_{cat} = k_3$. It seems likely from the data of Figure 2, that for APA, as for most substrates of the P99 β-lactamase, deacylation is rate determining. The slope of the least-squares line fitting these data in Figure 3 gives[16,17] a k_4/k_3 value of 19.3 which is very similar to the value of this ratio for benzylpenicillin, *viz*. 16.[16]

The acceleration of the enzyme-catalyzed reaction of APA at saturating concentration (Figure 1A) by bicarbonate probably then derives from the effect of HCO_3^- on the deacylation step. Given the above considerations and the mechanism of spontaneous formation of **6** from APA and carbon dioxide shown in Scheme 2, a rational reaction sequence by which the P99 β-lactamase would catalyze the formation of **6** (and it is greatly catalyzed since the spontaneous rate under the conditions of the enzyme-catalyzed reaction is very small) would be that of Scheme 5. After the normal acyl enzyme is achieved by attack of the active site serine hydroxyl group E-OH on the β-lactam ring, deacylation occurs, not by the usual enzyme-catalyzed hydrolysis, but by intramolecular nucleophilic participation by the carbamate (**8**). This would regenerate the free enzyme and the intermediate **7** which could rearrange to **6** while still at the enzyme surface or after diffusion into solution. The mechanism of turnover of the APA carbamate **4** would therefore represent an example of substrate assisted enzyme catalysis in the sense the deacylation of the enzyme, the slow step in turnover of APA, is accelerated, although only slightly in this case, by an intramolecular nucleophile incorporated in the substrate.

Related precedent for Scheme 5 is available. The hydrolysis of aryl esters of amino acids is catalyzed by carbon dioxide, and a mechanism analogous to that of Scheme 3, with an intermediate Leuchs anhydride analogous to **7**, has been proposed.[19] Similar intramolecular catalysis of hydrolysis of these esters is also observed in the presence of aldehydes by way of carbinolamine adducts.[19b,20] Although there have been many suggestions of intramolecular nucleophilic participation by amido side chains in the hydrolysis of penicillins and other β-lactams, the direct evidence for such interactions is slight at best.[21] In acylic systems however, with good leaving groups, as for example in aryl hippurates, such participation is well-documented.[22] The latter pair of observations may be reconciled in terms of the difficulty that the 6β-amido substituent of a penicillin has in approaching the β-lactam carbonyl group on a suitable trajectory on the β-face of the molecule because of steric interactions with the sulfur atom and 2β-methyl substituent of the thiazolidine ring. This same problem may well account for the slow spontaneous formation of 8-hydroxypenillate from APA and carbon dioxide. The β-lactamase is able to catalyze this reaction because, at the acyl-enzyme stage, after sterically unhindered attack by the active site serine on the α-face of the β-lactam ring, rotation about the C5−C6 bond of the penicilloate occurs[23,24] to relieve the eclipsing interaction between the β-substituents at C5 and C6, and which, at the same time, permits more facile access of the carbamate to the acyl group.

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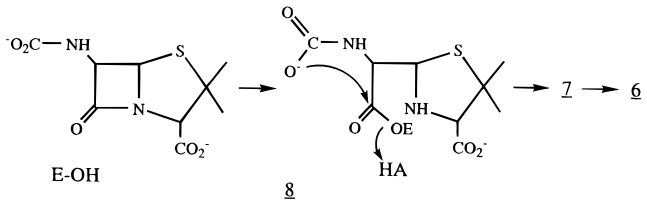
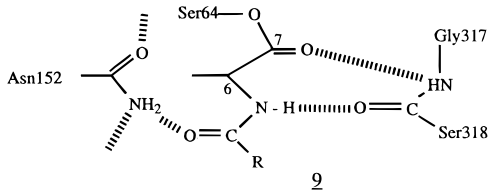
Scheme 5



In terms of active site interactions, it is believed that substrates bind to the P99 β-lactamase with the amido side chain hydrogen bonded across the active site cleft, between a backbone carbonyl of the B3 β-strand on one side and the side chain of Asn 152 on the other, as in the acyl-enzyme **9**.[23] Both of these elements



are important features of the active site. The adjacent Ser 318 NH of the β-strand represents one hydrogen-bond donor of the oxyanion hole, and Asn 152 is hydrogen bonded to the conserved Lys 67 and thence to the other catalytic residues.[25] In the absence of an amido side chain, such as is so in APA and in the unsubstituted penicillanic acid itself,[26] k_{cat} is some 50 times less and k_{cat}/K_m some 10^3 times less than that of a good substrate such as benzylpenicillin. Although a carbamate oxygen of **4** could provide interaction with Asn 152, the low reactivity of **4** with the enzyme suggests that it does not, perhaps because of the poor interaction of the other carbamate oxygen, replacing R, in a site better suited to hydrophobic substituents. Rotation around the single bonds C6−C7 and C_6−N could place the carbamate of **4** further into the solvent where it would be favorably hydrated. The net effect may be to give an acyl enzyme no more stable than that of APA and hence the minimal effect of bicarbonate on K_m. In solution, however, the carbamate would be in a suitable position to attack the C-7 ester carbonyl group from the Re face to initiate the deacylation of Scheme 5. It is also from the Re face that the deacylating water would approach in normal deacylation, catalyzed, in the mechanism of Oefner et al.,[23,25] by the phenoxide ion of Tyr 150. That the carbamate does obstruct the approach of other nucleophiles to the Re face is demonstrated by the data also shown in Figure 2 where it is seen that methanol is unable to attack acyl enzyme **8**. The small amount of methanol acceleration of the rate of reaction of **4** that is observed (Figure 2) can be explained in terms of methanolysis of the residual APA not present in solution as the carbamate.

7-Aminocephalosporanic Acid (ACA). Many of the phenomena recounted above with respect to APA were also observed with ACA. In $NaHCO_3$ solutions an additional molecular species is observed in ^{1}H NMR spectra of ACA, where, as with APA, the C_6−H and C_7−H protons have moved downfield, from 4.78 and 5.07 ppm respectively in the absence of HCO_3^- to 5.07 and 5.45 ppm respectively in its presence. That the latter resonance is that of C_7−H was made clear by its change in appearance from a doublet (J = 4.7 Hz) in HCO_3^- to a double doublet (J = 3.1, 4.7 Hz) in $H^{13}CO_3^-$. The ^{13}C

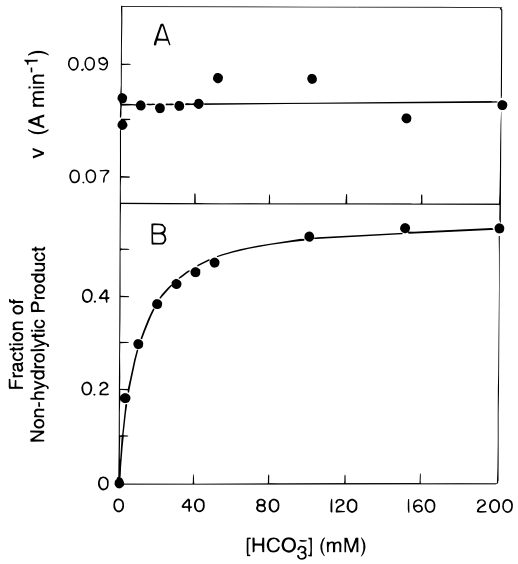
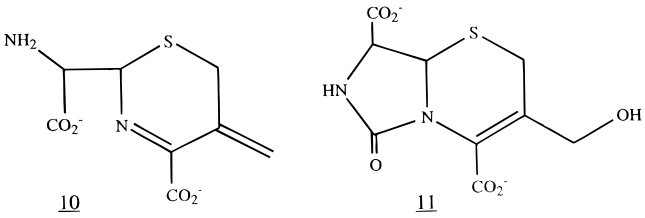


Figure 3. Effect of HCO_3^- concentration on the rate of disappearance of APA (0.75 mM) catalyzed by the P99 β-lactamase (1.5 μM) (A) and on the fraction of non−hydrolytic products of the reaction (B).

spectrum in $H^{13}CO_3^-$ shows a strong resonance at 163.0 ppm. Thus it is clear that ACA, like APA, reacts with carbon dioxide to form substantial amounts of carbamate at neutral pH.

On addition of the P99 β-lactamase to a solution of ACA (5 mM) in HCO_3^- (0.1 M), complicated changes to the ^{1}H NMR spectrum occurred. Apart from the normal hydrolysis product **10**,[27] several other cephalosporin-derived species were observed as a function of time, some of them not primary products. Their identity was not further pursued although it might be noted that there seems no literature precedent to an analog of **6** from cephalosporins. It seems likely, however, that one of the



products might be **11**. Certainly, however, there was clear evidence of novel products derived from the ACA carbamate.

Steady state kinetic parameters for turnover of ACA by the P99 β-lactamase were $k_{cat} = 0.89 \pm 0.06$ s^{-1} and $K_m = 0.29 \pm 0.04$ mM; ACA is a much poorer substrate of this enzyme than are typical cephalosporins.[17a] In 0.2 M HCO_3^-, the steady state parameters were $k_{cat} = 0.93 \pm 0.02$ s^{-1} and $K_m = 0.18 \pm 0.01$ mM, little different from in the absence of HCO_3^-. In particular, k_{cat} seems essentially unchanged. The latter was verified by measurement of the rate of disappearance of ACA (0.75 mM) followed at 285 nm, as a function of HCO_3^- concentration (0−0.2 M), where essentially no change was observed (Figure 3A). That different products were being formed, however, was clear by the decrease in absorbance at 320 nm, i.e. of the concentration of **10**, at the end of the reaction, as the HCO_3^- concentration increased. These data are shown in Figure 3B. The solid line of this figure is obtained from fitting Scheme 3 to the data with $K_m = 0.29$ mM, $K_{mc} = 0.18$ mM, $K_c = 14.0$ mM, $k_a = (k_b + k_c) = 0.9$ s^{-1}, and $k_c/(k_b + k_c) = 0.57$. Comparison with the data for APA described above indicates that the carbamate of

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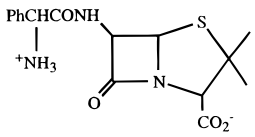
ACA is somewhat less stable with respect to ACA than is **4** with respect to APA under the prevailing conditions (pK_a values of APA and ACA are very similar[28]), and that the rearrangement of an enzyme substrate complex with intramolecular carbamate participation is less facile with respect to hydrolysis with ACA than APA.

The lack of effect of HCO_3^- on k_{cat} raised the possibility that acylation of the enzyme by ACA might be rate determining under conditions of substrate saturation, although this would be contrary to all other experiences with cephalosporins.[17a] A methanolysis experiment however (0.75 mM ACA, 0.17 M HCO_3^-, Figure 2B) certainly seemed to reaffirm rate-determining deacylation. The effect of methanol on ACA turnover was very similar to that on APA (Figure 2A). The absence of an effect of HCO_3^- on ACA turnover therefore seems to reflect a coincidental similarity of rate constants ($k_a = k_b + k_c$). Methanol also had some effect on the rate of reaction of ACA in the presence of HCO_3^- (Figure 2B). The acceleration cannot be explained by the amount of free ACA but must also reflect some methanolysis of the carbamate (some 35% of total turnover). This would correlate well with the amount of hydrolysis of the carbamate acyl enzyme revealed by Figure 3B, demonstrating again the relatively less facile rearrangement pathway in ACA than in APA.

Other β-Lactams. ^{1}H NMR spectra of ampicillin, **12**, and cephaloglycine, **13**, each at 10 mM concentration, in 0.2 M $NaHCO_3$, showed the presence in each case of a species other than the parent β-lactam, not present in phosphate buffer, and thus likely to be the carbamate. In each case, approximately 60% conversion had occurred under these conditions. The addition of the P99 β-lactamase led in each case to two products, one of them the expected hydrolysis product (by comparison with the single product observed in phosphate buffer) and the other, mostly likely, the corresponding carbamate. The latter conclusion was supported by the effect of adding the same amount of $NaHCO_3$ to the reaction products generated in phosphate buffer, which led to formation, again in each case, to the same additional product in similar quantity. Thus although carbamates are formed with **12** and **13**, they do not lead to novel products. They may however affect the observed reaction rate.

Other β-Lactamases. Class A β-lactamases are structurally both similar and different to the class C enzymes, of which the P99 β-lactamase is an example.[29] They differ especially in the functional group of the active site believed to act as a general



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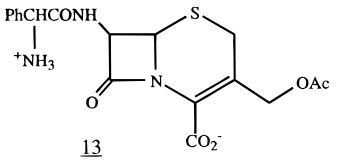

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base in catalysis, particularly of deacylation, the carboxylate of Glu 166 in class A β-lactamases,[18,30] and the anionic form of the side chain of Tyr 150 in class C.[23,25] It was of interest therefore to compare class A and C enzymes with respect to their interaction with the APA carbamate since the major effect was seen in deacylation. ^{1}H NMR spectra of APA (10 mM) and ACA (5 mM) in 0.1 M $NaHCO_3$ after addition of the TEM β-lactamase were identical to those observed in the absence of HCO_3^-, *viz.* spectra of **2** and **10**, respectively. The spectrum of **2** was also generated on addition of the PC1 β-lactamase to APA in HCO_3^-. Apparently intramolecular participation by carbamate (**8**) cannot compete with hydrolysis of the acyl enzyme in the class A active site. This is perhaps not surprising with the TEM β-lactamase which is particularly efficient at catalyzing penicillin hydrolysis,[9] but is more striking in the case of the PC1 enzyme. This β-lactamase is generally not a good catalyst of penicillin hydrolysis, from the point of view of k_{cat} at least (comparable to the class C P99 enzyme), and deacylation is rate determining under conditions of saturating substrate.[31] Nonetheless, the PC1 β-lactamase behaves like its fellow class A enzyme, the TEM β-lactamase, and differs from the P99 enzyme in that penicilloyl-enzyme intermediates are *not* susceptible to methanolysis.[16] This suggests that external nucleophiles do not have access to the acyl group of class A penicilloyl enzymes. The normal deacylation sequence is thought to involve Glu 166 carboxylate-catalyzed attack by an occluded water molecule on the Si face of the acyl group. The inability of class A enzymes to generate **6** and **11** from APA and ACA carbamates in the manner discussed in connection with Scheme 5 is consistent with these observations.

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