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Inhibition of the RTEM β-Lactamase from *Escherichia coli*. Interaction of the Enzyme with Derivatives of Olivanic Acid†

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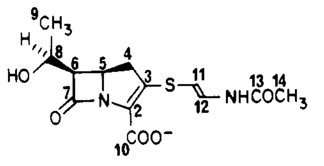
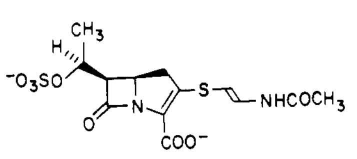
ABSTRACT: From chemical and kinetic studies of the interaction of the RTEM β-lactamase from *Escherichia coli* with three derivatives of olivanic acid, MM22382 (**1**), MM13902 (**2**), and MM4550 (**3**), a mechanism for the inhibition of the enzyme by these compounds is proposed: the interaction proceeds by formation of an acyl-enzyme, the Δ^2-pyrroline, which may either deacylate or undergo tautomerization to the more tightly bound Δ^1-pyrroline. The ability of olivanic acids to inhibit the enzyme thus depends on the partitioning of the acyl-enzyme to the Δ^1-pyrroline (a process that competes with the normal hydrolytic pathway) and on the rate of regeneration of free enzyme from this complex.

Many members of the carbapenem family of β-lactams are effective as antibiotics against β-lactamase-producing bacteria (e.g., Basker et al., 1980). To be effective, these species must be resistant to the enzyme-catalyzed hydrolysis reaction. We report here studies on the interaction of one group of carbapenems, the olivanic acids (Butterworth et al., 1979; Brown et al., 1977; Hood et al., 1979), with the purified plasmid-encoded RTEM[1] β-lactamase, to evaluate how these antibiotics protect themselves against the hydrolytic action of the enzyme.

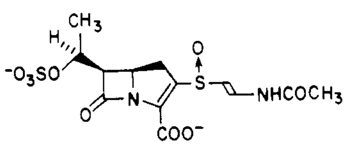
In our earlier report on the interaction of MM22382 (**1**) and MM13902 (**2**) with the β-lactamase, Schemes I and II (A or B) were shown to be the minimal kinetic schemes necessary to accommodate the behavior of these two olivanic acid derivatives, respectively (Charnas & Knowles, 1981). In the present work we probe the nature of these interactions in more detail and propose a scheme that accommodates the observed behavior. We also report studies on the interaction of the β-lactamase with a third olivanic acid derivative, MM4550 (**3**).



MM22382 **1**



MM13902 **2**



MM4550 **3**

Experimental Procedures

Materials

Olivanic acid derivatives, MM13902, MM4550, and MM22382, were generous gifts of Beecham Pharmaceuticals, Betchworth, Surrey, United Kingdom. MM13902, as the sodium salt, was a pale yellow crystalline solid. MM4550, as the sodium salt, was an off-white powder. MM22382, as the sodium salt, was an orange powder. These materials were stored desiccated at −20 °C. Solutions were prepared by weight by using a Cahn 25 electrobalance. Ultraviolet spectroscopic measurements were made in 0.1 M sodium phosphate buffer, pH 7.0, at 30 °C by using a Perkin-Elmer 554 or 575 spectrophotometer. MM13902 has λ_{max} at 223 and 304 nm (ϵ = 14 100 and 15 200 M^{-1} cm^{-1}, respectively), MM4550 has λ_{max} at 237 and 282 nm (ϵ = 13 500 and 12 000 M^{-1} cm^{-1}, respectively), and MM22382 has λ_{max} at 225 and 304 nm (ϵ = 13 500 and 13 300 M^{-1} cm^{-1}, respectively).

HPLC[1] was carried out on a Waters Associates chromatograph equipped with a differential ultraviolet detector operating at 254 nm, using a μBondapak C_{18} reverse-phase column (0.39 × 30 cm) and eluting with water (0.4 mL min^{-1}). The retention times of MM13902, MM4550, and MM22382 were 35, 29, and 52.5 min, respectively.

TLC was carried out on silica (Analtech) eluted with 1-butanol/methanol/water (4:1:2) and on cellulose (Eastman-Kodak) eluted with 2-propanol/water (7:3). The R_f values of MM13902, MM4550, and MM22382 were 0.6, 0.45, and 0.75, respectively, on silica and 0.85, 0.75, and 0.9, respectively, on cellulose.

Minor decomposition of each of the olivanic acid derivatives occurred on storage, giving rise to two products in each case, as determined by HPLC and TLC. The olivanic acids were purified by HPLC so that, except where noted, they were >95% pure when used.

^1H and ^{13}C NMR spectra were recorded on a Varian CFT20, a Varian XL100, a Jeol FX270, or a Bruker WM300 instrument.

The purification of the TEM-2[1] β-lactamase that was used has been described previously (Charnas & Knowles, 1981).

Methods

The procedures used to investigate the kinetics of the interaction of MM4550 with the β-lactamase have been described previously in the report on the interaction of MM13902 with the enzyme (Charnas & Knowles, 1981). Reactions were studied by following the absorbance change at 282 nm, and calculations were made on the basis of the measured $\Delta\epsilon$ at 282 nm of 10 100 M^{-1} cm^{-1}. The buffer used in experiments in-

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[1] Abbreviations: RTEM specifies the source of the plasmid [see Datta & Kontomichalou (1965)] and TEM-2 specifies the enzyme [see Sutcliffe (1978)]; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography.

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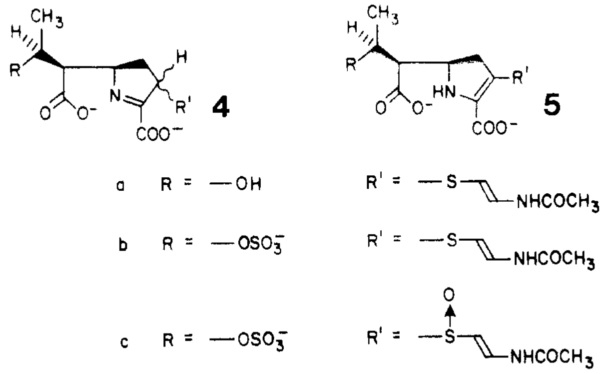
volving product isolation was 10 mM $Et_3NH{\cdot}HCO_3$, pH 7.0.

Enzyme-Catalyzed Hydrolysis of MM22382 (**1**). A solution of enzyme (100 μL, 1 μM) in buffer was added to a solution of **1** (0.25 mg, 0.75 μmol) in buffer (20 mL). The mixture was incubated at 30 °C for 0.5 h, then cooled, and concentrated to ~2 mL. For removal of the enzyme, the residue was applied to a Bio-Gel P-2 gel filtration column (1.5 × 20 cm) at 4 °C, eluting with buffer. Fractions eluting between 0.7 and 2.0 column volumes were pooled, aqueous NaOH (15 μL, 0.1 M) was added to prevent acid-catalyzed decomposition of the product, and the mixture was freeze-dried to give **4a** as a white powder: UV max 262 nm (ϵ 10600 M^{-1} cm^{-1}); TLC, R_f values of 0.8 on cellulose and 0.65 on silica; HPLC, retention time 13.5 min.



Enzyme-Catalyzed Hydrolysis of MM13902 (**2**) *and MM4550* (**3**). To a solution of enzyme (200 mL, 2.5 μM) in buffer, incubated at 30 °C, were added portions of **2** or **3** (0.3 mg, ~0.7 μmol) at hourly intervals for 10 h. After a further 2 h at 30 °C, the mixtures were worked up as described for the hydrolysis of **1** by the enzyme. Hydrolysis of **2** gave **4b** as a white powder: UV max 262 nm (ϵ 12000 M^{-1} cm^{-1}); TLC, R_f values of 0.7 on cellulose and 0.55 on silica; HPLC, retention time 9 min; ^1H NMR, see Table I. Hydrolysis of **3** gave **4c** as a white powder: UV max 248 nm (ϵ 16300 M^{-1} cm^{-1}); TLC, R_f values of 0.65 on cellulose and 0.4 on silica; HPLC, retention time 8 min; ^1H NMR, see Table I.

Base-Catalyzed Hydrolysis of MM22382 (**1**), *MM13902* (**2**), *and MM4550* (**3**). An aqueous solution of **1**, **2**, or **3** (0.2–0.3 mg, 0.3 mM) and NaOH (0.6 mM) was incubated at 30 °C for 12 h, and the solution was then freeze-dried. The products were identified as **4a** from **1**, **4b** from **2**, and **4c** from **3**, by comparison with the products obtained by enzyme-catalyzed hydrolysis. So that the product samples for ^{13}C NMR could be obtained, the same procedure was followed, except that 15 mg of **2** or **3** was used. The samples used in this experiment each contained ~15% of the two impurities formed on storage, one of which was shown by HPLC to be the normal hydrolysis product. The freeze-dried reaction products were chromatographed on a column (1 × 20 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated with 10 mM $Et_3NH{\cdot}HCO_3$ at 4 °C, eluting with a linear gradient (10–300 mM) of aqueous $Et_3NH{\cdot}HCO_3$ (200 mL), pH 7.0. Fractions containing **4** (**b** or **c**), as determined by HPLC, were pooled, NaOH (2 molar equiv) was added, and the mixtures were then freeze-dried. Analysis by ultraviolet, TLC, HPLC, and ^1H NMR showed that the isolated material was **4** (**b** or **c**), and ^{13}C NMR spectra were recorded (see Table II).

Results and Discussion

In our earlier investigation (Charnas & Knowles, 1981) the interaction of the RTEM β-lactamase with **1** and **2** was studied. Compound **1** behaves simply as a good substrate of the enzyme, and the kinetic scheme shown in Scheme I suffices to describe the observed behavior. In contrast, **2** is a poor substrate and an excellent inhibitor of the enzyme. The inhibition derives from a branching of the normal hydrolytic pathway in which the acyl-enzyme intermediate partitions to a transiently stable, inactive form of the enzyme. In the experiments described below we have investigated the interaction of MM4550 (**3**) with the β-lactamase and extended our studies on the interaction of **1** and **2** with the enzyme.

Scheme I: Minimal Kinetic Pathway for the Interaction of **1** and β-Lactamase[a]

$$e + s \rightleftharpoons e{\cdot}s \longrightarrow a\text{-}e \longrightarrow e + p$$

[a] e, free enzyme; s, **1**; e·s, the Michaelis complex of e and **1**; a-e, the acyl-enzyme from **1**; p, the product of enzymatic hydrolysis of **1**.

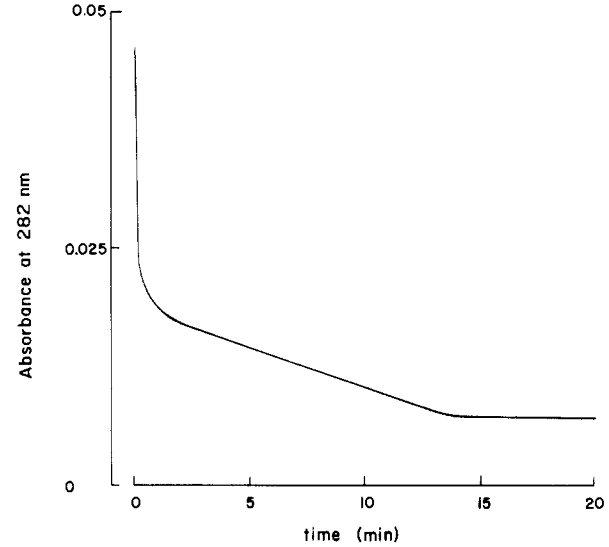


FIGURE 1: Changes in absorbance at 282 nm accompanying the hydrolysis of MM4550 (**3**) by β-lactamase. MM4550 (3.9 μM) was incubated with β-lactamase (2 μM) in 0.1 M sodium phosphate buffer, pH 7.0, at 30 °C.

Interaction of the β-Lactamase with MM4550 (**3**). Incubation of β-lactamase with **3** results in the disappearance of the chromophore at 282 nm and the appearance of a new chromophore at 248 nm. The kinetic characteristics of this interaction are complicated by a second-order reaction between **3** and the enzyme that is significant only at concentrations of **3** above about 5 μM. This reaction does not involve the enzyme's active site, since β-lactamase that has been inactivated by clavulanic acid (Fisher et al., 1978) still causes the loss of A_{282nm} with a rate constant of about 25 M^{-1} s^{-1}. At lower concentrations of **3** (<5 μM), however, the dominant reaction is zero order in substrate and first order in enzyme and has a rate constant of $(6.5 \pm 0.5) \times 10^{-4}$ s^{-1}. All kinetic experiments were done with **3** at <5 μM to avoid complications from the nonspecific reaction of the protein with **3**. At these concentrations the first-order reaction predominates, and minor contributions from the nonspecific second-order process are easily subtracted.

The interaction of the sulfoxide **3** with the enzyme is similar to that observed earlier with **2** (Charnas & Knowles, 1981). The time course of the reaction is biphasic: a rapid initial phase gives way over ~2 min to a slower steady-state reaction that proceeds at a constant rate until **3** is exhausted (Figure 1). The rate constant for the rapid phase of the reaction is $(8 \pm 2) \times 10^{-2}$ s^{-1} and is independent both of the concentration of **3** (above 1 μM) and of the molar excess of **3** over enzyme (from 2- to 20-fold). The steady-state reaction has a rate

Scheme II: Minimal Kinetic Pathways for the Interaction of **2** or **3** and β-Lactamase[a]

$$\begin{tikzcd}
e + s \arrow[r, shift left] & e\cdot s \arrow[l, shift left] \arrow[r] & a\text{-}e \arrow[r] \arrow[d, shift left] & e + p & \text{(A)} \\
 & & X \arrow[u, shift left] & &
\end{tikzcd}$$

$$\begin{tikzcd}
e + s \arrow[r, shift left] & e\cdot s \arrow[l, shift left] \arrow[r] & a\text{-}e \arrow[r] \arrow[d] & e + p & \text{(B)} \\
 & & X \arrow[r] & e + p' &
\end{tikzcd}$$

[a] e, free enzyme; s, **2** or **3**; e·s, the Michaelis complex; a-e, the acyl-enzyme; p and p′, products after deacylation; X, the enzyme intermediate responsible for the transient inhibition of the enzyme.

constant of (6.5 ± 0.5) × 10^{-4} s^{-1}. When the trace of A_{282nm} vs. time is extrapolated back to time zero, the "burst" size is approximately 1.4 times that of the enzyme concentration on a molar basis. As we have shown earlier for **2** (Charnas & Knowles, 1981), a burst size that is greater than 1.0 cannot be accommodated by the kinetic pathway shown in Scheme I. [As before, the trivial explanation that **3** contains a small amount of a rapidly reacting impurity is eliminated by the facts (a) that the burst size is independent of the concentration of **3** at a fixed enzyme level and (b) that subsequent additions of **3** to an incubation of **3** with the enzyme do not produce a second burst.] The observation of a rapid burst reaction in which more than 1 molar equiv of substrate is consumed can be accommodated by a branched pathway such as is shown in part A or B of Scheme II. In these schemes an intermediate X accumulates, this species deriving from an intermediate on the normal hydrolytic pathway. During the burst phase of the reaction, at least 1.4 hydrolytic turnovers occur before essentially all the enzyme has accumulated as X. The rate of the subsequent steady-state reaction is then governed by the rate of regeneration of free enzyme from X, either via the acyl-enzyme (Scheme IIA) or directly (Scheme IIB).

The proportion of enzyme that, during the steady-state enzyme-catalyzed hydrolysis of **3**, is tied up in the form of covalent intermediates (acyl-enzyme and X) can be estimated from the remaining catalytic activity measured immediately after a sample of an incubation of enzyme with **3** is diluted into an assay solution containing benzylpenicillin. The initial rate of benzylpenicillin hydrolysis provides a measure of the instantaneous concentration of free, active enzyme. Thus when **3** is mixed with the enzyme, there is a rapid decrease in enzyme activity to less than 1% of that present initially. The rate of activity loss has a half-time of about 8 s, corresponding to a first-order rate constant (k_{inact}) of 9 × 10^{-2} s^{-1}. This is in excellent agreement with the rate constant of 8 × 10^{-2} s^{-1} derived from the observed changes in A_{282nm} during the burst. It is evident that the rapid phase of the reaction produces complexes between enzyme and **3** that are catalytically inactive, as expected from Scheme II. The value of k_{inact} is constant at concentrations of **3** greater than 0.1 μM, and it is therefore clear that the inactivation process follows the expected saturation behavior.

Analysis of the intial rates of enzyme inactivation at low concentrations of **3** shows that the inactivation rate is half-maximal at a substrate concentration of ~1 nM. This value of $K_{m(inact)}$ is in good agreement with the K_i value of ~1 nM determined in direct competition experiments with benzylpenicillin and indicates that, as with **2**, inactivation of the enzyme occurs at substrate concentrations that may be physiologically relevant. The value of $k_{inact}/K_{m(inact)}$ for **3** is about 10^8 M^{-1} s^{-1}, which, as a second-order rate constant for the inactivation process, shows that **3** is ~100-fold more efficient than **2** in the inactivation of the RTEM β-lactamase at low concentrations.

As with **2**, the inactivation of β-lactamase by **3** is reversible, and after long incubations, full recovery of enzyme catalytic activity is observed. When measured directly after all the **3** has been consumed, or by dilution of an incubation of **3** with enzyme into an assay mixture, the catalytic activity of the enzyme against benzylpenicillin returns in a first-order manner. The rate constant for the recovery of catalytic activity, k_{react}, is (4.4 ± 0.3) × 10^{-4} s^{-1}, with the activity rising from <1% to 100% of the original value, within the limits of experimental detection.

The reaction that leads to recovery of enzyme activity (k_{react}) is somewhat slower than the steady-state turnover rate (k_{cat}). This difference in rate constants *requires* a branched pathway: the linear pathway of Scheme I cannot accommodate a rate of substrate turnover that is faster than the rate of recovery of enzyme catalytic activity after all of the substrate has been consumed. In terms of the pathways of parts A and B of Scheme II, the recovery of activity at the end of the reaction represents the decomposition of the intermediate X to regenerate free enzyme, which is slower than the deacylation rate of the acyl-enzyme.

In summary, while Scheme I adequately describes the interaction of **1** with the β-lactamase, the minimal kinetic scheme necessary to accommodate the behavior of compounds **2** and **3** with the enzyme is Scheme II (A or B). Why is there this difference?

Compounds **2** and **3** differ from **1** by the existence of a sulfate ester on C-8. In our earlier study we investigated the possiblity of sulfate elimination from the acyl-enzyme from **2** to give an α,β-unsaturated acyl-enzyme of greater hydrolytic stability than the first-formed saturated ester [see Scheme III of Charnas & Knowles (1981)]. It was found, however, that sulfate ion is *not* released during the interaction of **2** with the enzyme. The most persuasive interpretation of the differences in the observed behavior of **1**, **2**, and **3** is that deacylation is much faster in the case of **1**, the acyl-enzyme from which is so short-lived that formation of the inhibited species X does not compete significantly with deacylation. According to this view, the behavior of **1** is just a special case of the generalized formulation of Scheme II (A or B). Indeed, with **1** at very high substrate-to-enzyme ratios (400 000:1), some biphasic character of the interaction is discernible.

Product Studies. In an attempt to understand the chemical events that are outlined in Scheme II, we undertook product studies of the interaction of **1**, **2**, and **3** with the β-lactamase. The products deriving from the interaction of each of these olivanic acids with the β-lactamase have been isolated, and the characteristics of these products are presented under Experimental Procedures and in Table I. For comparison, the properties of the parent olivanic acids are also included. In each case the enzymic hydrolysis was performed under conditions such that the contribution from nonenzymic hydrolysis was <10%. The products from base-catalyzed hydrolysis were also isolated and characterized.

The products of enzymic and nonenzymic hydrolysis of the carbapenems are identical, as judged by their ultraviolet and ^{1}H NMR spectra and their behavior on HPLC and TLC. The data indicate that the hydrolytic product is in each case a mixture of the Δ^1-pyrrolines (**4**) epimeric at C-3. From their chromatographic behavior it is clear that the products of hydrolysis are more polar than the parent carbapenems. The differences in the ultraviolet absorptions of the olivanic acids

Table I: Chemical Shifts and Coupling Constants in ^{1}H NMR Spectra[a]

	δ from TMS (J in Hz)			
proton	MM13902 (**2**)[b]	Δ^1-pyrroline (**4b**)[c]	MM4550 (**3**)[b]	Δ^1-pyrroline (**4c**)[c]
3-CH		4.34 m		~4.8 m[e]
4-CH$_2$	3.29 dd (8.5, 18), 2.94 dd (9.5, 18)	2.21 m, 2.66 m	3.46 dd (9, 18.5), 2.99 dd (10.5, 18.5)	2.18 m, 2.57 m
5-CH	4.25 ddd (5.5, 8.5, 9.5)	4.22 m	4.37 ddd (6, 9, 10.5)	4.50 m [4.40 m]
6-CH	3.78 dd (5.5, 9)	2.84 dd (7, 7) [2.89 dd (7, 7)]	3.88 dd (6, 9)	2.96 dd (7, 7) [2.88 dd (7, 7)]
8-CH	4.75 dq (9, 6)	~4.8 m[e]	4.97 dq (9, 6.5)	~4.8 m[e]
9-CH$_3$	1.47 d (6)	1.44 d (6) [1.46 d (6)]	1.45 d (6.5)	1.46 d (6) [1.48 d (6)]
11-CH	7.07 d (14)	7.09 d (14) [7.06 d (14)]	7.18 d (14)	7.48 d (14) [7.60 d (14)]
12-CH	5.98 d (14)	5.72 d (14) [5.70 d (14)]	6.24 d (14)	5.97 d (14) [6.14 d (14)]
14-CH$_3$, NH[d]	2.00 s	2.04 s [2.06 s]	2.05 s	2.10 s [2.14 s]

[a] Spectra were recorded in D_2O by using sodium 3-(trimethylsilyl)-1-propanesulfonate (TMS) as an internal reference. [b] Values reported by Brown et al. (1977). [c] Where the chemical shifts of the epimers, for any particular proton, are different, that of the minor component is listed in brackets. [d] Amide proton exchanges (Brown et al., 1977). [e] Signal partially obscured by HDO resonance.

and their respective hydrolysis products are expected. The higher wavelength absorptions of the β-lactams are characteristic of the unsaturated sulfides (**1** and **2**) and sulfoxide (**3**), and the changes in the λ_{max} values upon hydrolysis are consistent with the Δ^1–Δ^2 conversion. Further evidence for the epimeric Δ^1-pyrrolines (**4**) is obtained from NMR spectra of the hydrolysis products from **2** and **3**.[2] The ^{1}H NMR spectra (Table I) show that in each case the product is a mixture of two similar components, with each component having new signals from 3-CH and major shifts of the signals from 4-CH$_2$, 6-CH, and 11-CH. The ^{13}C NMR spectra of the base-catalyzed hydrolysis products from **2** and **3** (Table II) are consistent with the proposed structures, and although it was impractical to obtain ^{13}C NMR spectra of the enzymic hydrolysis products, the enzymic and nonenzymic hydrolysis products are identical by all other criteria.

The results described above are in good agreement with the report by Maeda et al. (1977) of the products from the nonenzymic hydrolysis of a carbapenem (MC696-SY2-A) (**6**) which is believed to have the same structure as **3**. These workers established that the two epimeric Δ^1-pyrrolines were the main products from hydrolysis of **6**. The ^{13}C and ^{1}H NMR spectra of **6** and of its hydrolysis products show almost identical relative chemical shifts as we have observed for **3** and the products of its hydrolysis, though the absolute shifts of the ^{1}H NMR spectra differ by approximately 0.5 ppm. We may therefore conclude that with each of the olivanic acids, enzymic hydrolysis results in the formation simply of a mixture of the two Δ^1-pyrrolines (**4**) epimeric at C-3.

The Δ^1-pyrrolines obtained from the enzymic hydrolysis are also an equilibrium mixture of the C-3 epimers. Whereas base-catalyzed hydrolysis of the β-lactam gives a nonequilibrium ratio of C-3 epimers of **4**, neutralization rapidly effects equilibrium. Hydrolysis in D_2O under basic conditions results in deuterium incorporation at C-3, and this deuterium washes out in H_2O at neutral pH. Conversely, deuterium incorporation at C-3 occurs rapidly on incubation of unlabeled **4** at neutral pH in D_2O. This suggests that the Δ^1–Δ^2-pyrroline tautomerization is rapid near neutrality, but relatively slow at high pH. The Δ^1-pyrroline (**4**) is evidently strongly favored thermodynamically over the Δ^2 isomer (**5**), since no **5** can be detected by NMR either at neutral pH or under basic conditions. A preference for the Δ^1-pyrroline in analogous compounds has previously been reported (e.g., Hausler & Schmidt, 1979).

Table II: Chemical Shifts in ^{13}C NMR Spectra[a]

	δ from TMS			
carbon	MM13902 (**2**)[b]	Δ^1-pyrrolines (**4b**)[c]	MM4550 (**3**)[b]	Δ^1-pyrrolines (**4c**)[c]
2	144	170.4 (171.2)[d]	140	169.1 (169.3)[d]
3	128	52.7 (52.2)	139	72.9 (71.2)
4	37	33.6 (33.0)	29	25.5
5	58	70.9 (72.0)	59	70.6 (71.2)
6	54	59.9 (59.6)	55	59.9 (59.7)
7	169	173.3 (174.5)[d]	166	168.6[d]
8	74	77.3	74	77.2 (77.1)
9	19	18.9 (18.1)	19	19.1 (18.1)
10	178	178.6 (178.4)[d]	177	178.2[d]
11	103	101.8 (102.7)	112	111.0 (108.1)
12	131	133.0 (131.7)	135	135.8
13	172	172.2[d]	173	173.5[d]
14	23	22.8	23	23.2

[a] Spectra were recorded in D_2O by using dioxane as an internal standard. [b] Values reported by Brown et al. (1977). [c] Where the chemical shifts of the epimers, for any particular carbon, are different, that of the minor component is listed in parentheses. [d] Assignments within any vertical column may be reversed.

Since the enzyme-catalyzed reactions are conducted near neutrality where there is rapid epimerization at C-3, we cannot say what species is released by the enzyme. It is clear, however, that the primary product is one of the epimeric Δ^1-pyrrolines (**4**), the Δ^2-pyrroline (**5**), or a mixture of these species.

Nature of the Inactive Complex. Given that enzymic hydrolysis of the olivanic acids yields the epimeric Δ^1-pyrrolines from the corresponding Δ^2 parent species, what is the nature of the transiently inhibited enzyme intermediate? Both the

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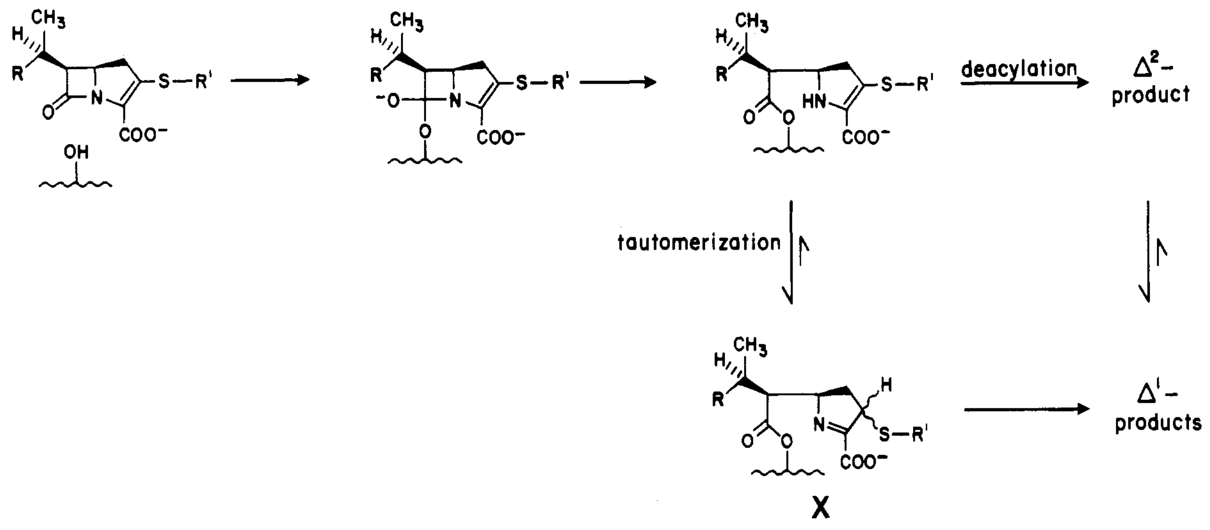
[2] Lack of material prevented an NMR analysis of the product from **1**.

Scheme III: Tentative Scheme That Accommodates the Interaction of Olivanic Acids with β-Lactamase



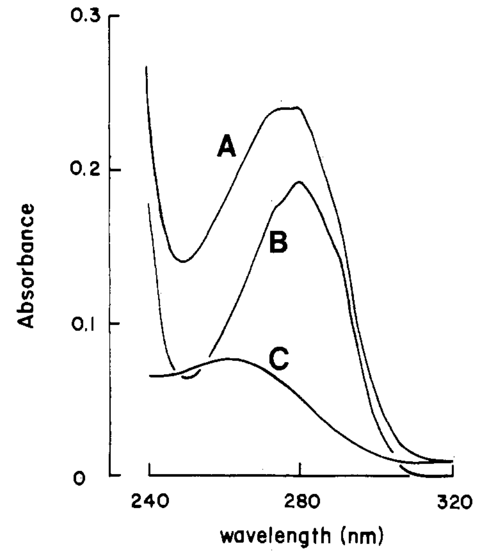


FIGURE 2: Ultraviolet spectra (A) of the inhibited enzyme species X (see Scheme II) (6 μM) obtained by gel filtration at 4 °C (Bio-Gel P-2, 1.5 × 17 cm) in 10 mM *N*-ethylmorpholinium acetate buffer, pH 7.0, of a mixture of enzyme (10 μM) and MM13902 (**2**) (100 μM) in buffer (1 mL) that had been incubated at 30 °C for several minutes, (B) of free enzyme (6 μM) in buffer, and (C) of the difference between (A) and (B). All spectra recorded during the reactivation of X (from <20% to >95% activity, assayed by measuring the rate of hydrolysis of benzylpenicillin and based on the total activity of free enzyme added to the incubation mixture) are superimposable on (A).

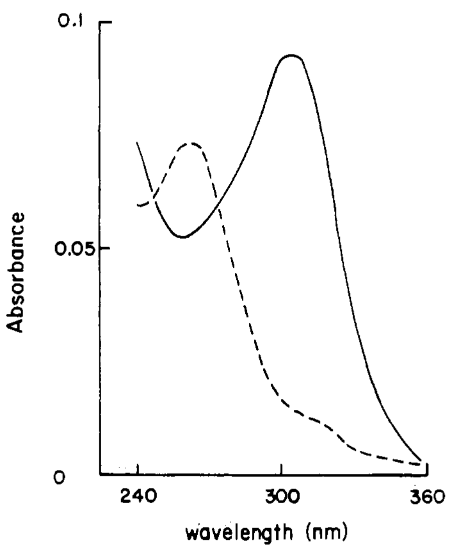


FIGURE 3: Ultraviolet spectra of MM13902 (**2**) (6 μM) (—) and of its hydrolysis product, **4b** (6 μM) (---), each in 10 mM *N*-ethylmorpholinium acetate buffer, pH 7.0, at 30 °C.

acyl-enzyme and the intermediate X must be the Δ^1- or Δ^2-pyrroline, or a species that can readily rearrange to one or other of them. The simplest formulation consistent with all the results appears to be that illustrated in Scheme III, where collapse of the first-formed tetrahedral intermediate produces the acyl-enzyme as a Δ^2-pyrroline. This intermediate may undergo deacylation or in a competing process tautomerize to the more stable Δ^1-pyrroline. This Δ^1-pyrroline may deacylate directly (Scheme IIB) or tautomerize back to the bound Δ^2-pyrroline, which then deacylates via the normal hydrolytic path (Scheme IIA).

The inhibited enzyme species X (obtained from an incubation of enzyme with **2**) was isolated by gel filtration according to the procedure of Charnas & Knowles (1981) to obtain evidence in support of this postulate. Upon deacylation of this species, and repeated gel filtration to remove enzyme, the recovered product was the Δ^1-pyrroline (**4b**) as deduced from its spectroscopic and chromatographic properties. The ultraviolet spectrum of the inhibited enzyme before and during reactivation was also studied (Figure 2), and it was found that *no changes* in the spectrum occurred during the reactivation process. Indeed, it is clear that the difference in the ultraviolet spectra of free enzyme and inhibited enzyme X (Figure 2) closely resembles the spectrum of the Δ^1-pyrroline (**4b**) (Figure 3) and is quite different from the spectrum of the Δ^2 substrate (**2**) itself (Figure 3). It is thus evident that the inhibited enzyme species X contains the carbapenem moiety bound as a Δ^1-pyrroline, as shown in Scheme III.

In qualitative terms, the observed behavior of the carbapenems **2** and **3** could be accommodated by postulating a slow substrate-induced enzyme conformational change of the kind proposed by Citri and co-workers (Samuni & Citri, 1975; Citri et al., 1976). Thus the nonstoichiometric burst would represent the transition of the enzyme into a form appropriate for the hydrolysis of those carbapenems, and the recovery of enzyme activity when an incubation with carbapenem is diluted into a solution containing benzylpenicillin would derive from the isomerization of the enzyme back to the more active state appropriate for benzylpenicillin hydrolysis. However, aside from the sharp difference in the behavior of **1** from that of **2** and **3**, the recovery reaction (of **2**) is accelerated by (and is first order with respect to) hydroxylamine and has a rate constant of 5.6×10^{-2} M^{-1} s^{-1} (Charnas & Knowles, 1981). Analogously, the recovery of activity from incubations of **3** with enzyme is accelerated by hydroxylamine, this reaction having a second-order rate constant of 3.3×10^{-2} M^{-1} s^{-1}. These results are not readily accommodated by a scheme involving substrate-induced conformational changes but are entirely consistent with the picture presented in Scheme III.

The chemical identity of the acyl-enzyme (Scheme III), while most logically the Δ^2-pyrroline, is unproven. Indeed, there will be little difference in the inherent chemical stabilities of the ester linkages of the acyl-enzyme (the Δ^2-pyrroline) and of X (the Δ^1-pyrroline), and it is possible that the acyl-enzyme is the C-3 epimer of X. Moreover, we cannot distinguish between parts A and B of Scheme II. These points are not particularly important, however, since the ability of the carbapenems to inhibit the enzyme clearly depends on the rate of formation of the Δ^1-pyrroline, X, and on the rate of release of free enzyme from this complex.

From the mechanism outlined here, it is evident that carbapenems will be β-lactamase inhibitors if the formation of the Δ^1-pyrroline species competes effectively with the deacylation process. The olivanic acids are one class of the larger group of carbapenem antibiotics, all of which may well follow the path of Scheme III. These include the thienamycins (Leanza et al., 1979; Kahan et al., 1979; Tally et al., 1978; Kropp et al., 1980), PS-5, PS-6, and PS-7 (Okamura et al., 1978–1980; Sakamoto et al., 1980), the carpetimycins (Nakayama et al., 1980), the epithienamycins (Stapley et al., 1981; Cassidy et al., 1981), C-19393 S_2 and H_2 (Nozaki et al., 1981; Okonogi et al., 1981; Imada et al., 1980; Harada et al., 1980), and asparenomycin A (Tanaka et al., 1981). The results of our preliminary studies on the interaction of asparenomycin A, thienamycin, *N*-acetylthienamycin, and *N*-formimidoylthienamycin with β-lactamase are consistent with the branched pathway shown in Scheme III (C. Easton, J. Fisher, E. Jaffe, and C. Kemal, unpublished results). Elucidation of the mode of lactamase inhibition exhibited by these compounds may aid the search for new antibiotics suitable for treating infections of β-lactamase-producing bacteria.

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