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

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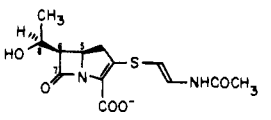
ABSTRACT: The interaction of the RTEM β-lactamase with two derivatives of olivanic acid has been studied. The compound MM22382 (**1**) behaves simply as a good substrate for the enzyme and is a relatively ineffective inhibitor. In contrast, the sulfate ester MM13902 (**2**) is a poor substrate and an excellent inhibitor of the enzyme. The inhibition derives from a branching of the normal hydrolytic pathway of the enzyme. At long times, all the catalytic activity of the enzyme returns. Free sulfate ion is not produced during the interaction with the enzyme, which rules out a mechanistic pathway involving β elimination between C-6 and C-8. The validity of a number of alternative schemes is assessed.

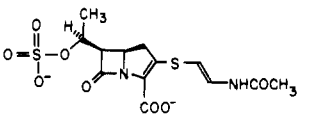
By catalyzing the hydrolysis of the β-lactam ring in penicillins and cephalosporins, the β-lactamases defend growing bacteria against the lethal effect of these antibiotics. The circumvention of β-lactamase-mediated resistance is of obvious clinical importance and has been achieved primarily through the development of β-lactams that possess both potent antibiotic activity and some resistance to hydrolysis by β-lactamases.

Although β-lactamases pose ready targets for inactivation, surprisingly little progress had been made toward the development of useful inactivating reagents until the discovery in 1976 of clavulanic acid (Brown et al., 1976), which is an inactivator of β-lactamases from a variety of Gram-negative and Gram-positive bacteria. The finding of clavulanic acid was followed by reports of a number of other β-lactamase inactivators from both natural and synthetic sources, but progress toward understanding their mode of action has been hampered both by the paucity of information about the normal hydrolytic pathway followed by β-lactamase and by the absence of studies at the molecular level on a number of the inhibitory molecules. However, our recent demonstration that the *Escherichia coli* β-lactamase proceeds via an acyl-enzyme intermediate (Fisher et al., 1980a) and the accumulated evidence on the mode of action of a number of the inactivators (Fisher et al., 1980b) have provided at least a silhouette of the necessary mechanistic features for β-lactamase inactivation. It appears that all the listed inactivators of β-lactamase are of the mechanism-based or "suicide" type, being β-lactam derivatives that suffer the first steps of the normal hydrolytic reaction, generating an acyl-enzyme from which inactive enzyme may derive.

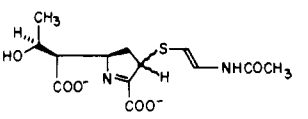
To test these ideas, we have investigated the two carbapenems MM22382 (**1**) and MM13902 (**2**) (Brown et al., 1977). [**1** is (5*R*,6*R*)-3-[(*E*)-(2-acetamidoethenyl)thio]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.] These compounds differ only in the substituent on C-8: the hydroxyl group in **1** is replaced in **2** by the sulfato function. We report here the minimal kinetic pathways that describe the interaction of **1** and **2** with the TEM-2[1] β-lactamase from *E. coli* and elaborate further upon the structural requirements for mechanism-based β-lactamase inactivators.



1 (MM 22382)



2 (MM 13902)



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Experimental Procedures

Materials

Olivanic acid derivatives, MM13902 and MM22382, were generous gifts of Beecham Pharmaceuticals, Betchworth, Surrey, U.K. MM13902 (5 mg), as the sodium salt, was a pale yellow crystalline solid. MM22382 (5 mg), as the sodium salt, was an orange powder. Both materials were stored desiccated at −20 °C. Except where noted, solutions of these compounds were prepared immediately before use in 0.1 M potassium phosphate buffer, pH 7.0, and kept at 0 °C.

β-Lactamase was from *Escherichia coli* W3310 carrying the RP4 plasmid (Matthew & Hedges, 1976). The enzyme is a TEM-2 β-lactamase (Sykes & Matthews, 1976) with a specific activity of 4200 μmol of benzylpenicillin hydrolyzed per A_{280nm} (enzyme absorption) per min (30 °C, pH 7.0). The enzyme was purified as described previously (Fisher et al., 1978) and was homogeneous (>95%) as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by isoelectric focusing in polyacrylamide gels in the presence or absence of 4 M urea.

Sodium olivanate solutions were prepared by weight by using a Cahn 25 electrobalance. Spectroscopic measurements were made in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C by using a Perkin-Elmer 554 or 575 spectrophotometer equipped with a multiple cell changer and five cell programmer. Enzyme activity was assayed by measuring the change in absorbance at 240 nm following the hydrolysis of benzylpenicillin (Samuni, 1975) in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C.

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received September 30, 1980.* This work was supported by the National Institutes of Health and the National Science Foundation.

‡ National Science Foundation Predoctoral Fellow.

[1] Abbreviations used: RTEM specifies the source of the plasmid [see Datta & Kontomichalou (1965)] and TEM-2 specifies the enzyme [see Sutcliffe (1978)].

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Methods

Enzyme Activity. Assays of enzyme after incubation with high concentrations of **2** were done as follows. A buffered solution of enzyme (10 μL, 100 μM) was mixed with a buffered solution of **2** (90 μL, 100–900 μM) and incubated at 30 °C. At appropriate intervals, samples (1 μL) were removed and mixed with a buffered solution of benzylpenicillin (1.0 mL, 1.4 mM) in an optical cuvette. The rate of hydrolysis of benzylpenicillin was measured at 240 nm after 1 min and indicates the amount of free active enzyme present. Assays of enzyme after incubation with low concentrations of **2** were done as follows. A buffered solution of enzyme (10 mL, 5 nM) was mixed with a buffered solution of MM13902 (10 mL, 25–200 nM) and incubated at 30 °C in a silanized flask. At appropriate intervals, samples (1.5 mL) were removed and mixed with a buffered solution of benzylpenicillin (1.5 mL, 2.8 mM) in an optical cuvette. The rate of hydrolysis of benzylpenicillin was then monitored at 240 nm. Assays of enzyme activity after incubation with **1** were done as follows. A buffered solution of enzyme (10 μL, 1 μM) was mixed with a buffered solution of **1** (90 μL, 100–2000 μM) and incubated at 30 °C. At appropriate intervals, samples (5 μL) were removed and mixed with a buffered solution of benzylpenicillin (1.0 mL, 1.4 mM) in an optical cuvette, and the hydrolysis of benzylpenicillin was followed at 240 nm.

*Enzyme-Catalyzed Hydrolysis of MM22382 (**1**).* The buffered enzyme solution (5 μL, 1 μM) was mixed with a buffered solution of **1** (5 μL, 1 mM) in 490 μL of 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C. The progress of the reaction was followed at 304 nm. The data were fitted to the integrated rate equation (Samuni, 1975), and on the basis of a measured $\Delta\epsilon$ at 304 nm of 10 500 M^{-1} cm^{-1}, values of k_{cat} and K_m were calculated. The K_m of **1** was obtained independently by estimating its K_i as a competitive substrate (inhibitor) against benzylpenicillin.

The buffered enzyme solution (10 μL, 1 μM) was mixed with a buffered solution of **1** (0–30 μL, 1mM) and of benzylpenicillin (300 μL, 1.4 mM) in 0.1 M potassium phosphate buffer, pH 7.0. The final volume was 3.0 mL. The changes in absorbance at 304 and 240 nm were monitored. Correction of the change at 240 nm (which was dominated by benzylpenicillin hydrolysis) for the hydrolysis of **1** (measured from the change at 304 nm) produced a time course for benzylpenicillin hydrolysis from which values of k_{cat} and K_m(app) were obtained as above. From these data, a K_i value for **1** was calculated.

*Enzyme-Catalyzed Hydrolysis of MM13902 (**2**).* The buffered enzyme solution (10–50 μL, 100 μM) was mixed with a buffered solution of **2** (10–70 μL, 1 mM) in 0.1 M potassium phosphate buffer, pH 7.0, such that the final volume was 0.5 mL. After achievement of the steady state (~30 min), the time course of the absorbance change at 304 nm was linear. After subtraction of the rate of the buffer-catalyzed reaction measured independently ($k = 0.9 \times 10^{-5}$ s^{-1}), the steady-state reaction rate was calculated on the basis of the measured $\Delta\epsilon$ at 304 nm of 10 000 M^{-1} cm^{-1}. The rate constant for the rate of approach to the steady state was determined by the method of Guggenheim (Gutfreund, 1972).

The effect of hydroxylamine on the reactivation of enzyme after incubation with **2** was determined as follows. The buffered enzyme solution (25 μL, 10 μM) was mixed with a buffered solution of **2** (25 μL, 100 μM) and incubated at 30 °C for 20 min. After this time, less than 8% of the initial enzymatic activity remained. A portion of the incubation mixture (25 μL) was then mixed with a buffered solution of hydroxylamine (5–25 μL, 100 mM) in 0.1 M potassium phosphate buffer, pH 7.0 (final volume 50 μL). At appropriate times, samples (4 μL) were taken for assay of enzyme activity by using benzylpenicillin as substrate.

Sulfate Assay. Free sulfate was determined by a modification of the method of Agterdenkas & Martinius (1964). Barium chloranilate was prepared by the dropwise addition of chloranilic acid (100 mL of a 100 mM solution in acetone/ethanol, 1:1 v/v) to barium acetate (90 mL, 0.1 M) over 1 h. The resulting purple precipitate was filtered, washed sequentially with water, ethanol, and ether, and then dried at 110 °C for 4 h. The finely divided precipitate (as a suspension, 0.15% w/v in 10 mM sodium acetate buffer, pH 4.5) was used as the reagent.

To follow the release of sulfate ion from incubations of enzyme with **2**, the buffered enzyme solution (500 μL, 50 μM) was mixed with a buffered solution of **2** (250 μL, 1 mM) in 10 mM *N*-ethylmorpholinium acetate buffer, pH 7.0, at 30 °C. At appropriate intervals, samples (100 μL) were removed and incubated with ethanol (300 μL) at room temperature for 2 h. The precipitated protein was removed by centrifugation (10000*g*, 10 min), and a portion (350 μL) of the supernatant was mixed with the barium chloranilate reagent (100 μL of suspension). The mixture was vortexed every 20 min, over 2 h, after which the precipitate was removed by centrifugation (14000*g*, 10 min). The absorbance of the supernatant of 332 nm was then determined and compared with calibration plots established with potassium sulfate.

To assay total sulfate from free or enzyme-bound **2**, samples were evaporated to dryness at 70 °C, dissolved in 100 mM HCl (50 μL), and incubated at 70 °C for 24 h. After evaporation to dryness at 70 °C, the residue was dissolved in 10 mM *N*-ethylmorpholinium acetate buffer, pH 7.0, and analyzed for sulfate as described above. Protein was removed from samples containing enzyme by ethanol precipitation as described above.

Results and Discussion

The kinetic and chemical processes responsible for the inhibition of β-lactamase activity by **1** are different from the more complex processes that characterize the interactions of **2** with the enzyme. The primary mode of β-lactamase inhibition by **1** derives from competitive behavior between **1** and other substrates, while **2** reversibly forms an inactive enzyme that slowly regenerates native β-lactamase. The experiments described below define the minimal kinetic schemes necessary to accommodate the behavior of the two olivanic acid derivatives with β-lactamase and place limits upon the chemical processes responsible for this behavior.

*Interaction of the β-Lactamase with MM22382 (**1**).* Incubation of β-lactamase with **1** results in the loss of the carbapenem chromophore at 304 nm and the concomitant appearance of a new chromophore at 262 nm (Figure 1). These spectral changes presumably reflect the hydrolytic opening of the β-lactam ring and the simultaneous formation of the Δ^1 pyrroline (**3**), by analogy with the known product of the nonenzymatic hydrolysis of other carbapenems (Maeda et al., 1977). The rate of the enzyme-catalyzed hydrolysis is proportional to the enzyme concentration and is characterized by a maximal rate from which a k_{cat} value of 15 ± 1 s^{-1} can be derived. When the progress curve at 304 nm is analyzed by using the integrated Michaelis equation, values for k_{cat} and K_m of 14 ± 1 s^{-1} and 3.5 ± 1 μM, respectively, are obtained. Comparison of the value of k_{cat}/K_m (4×10^6 M^{-1} s^{-1}) for **1** with those for carbenicillin (7×10^6 M^{-1} s^{-1}) and benzylpenicillin (98×10^6 M^{-1} s^{-1}), for example, shows that **1** is a

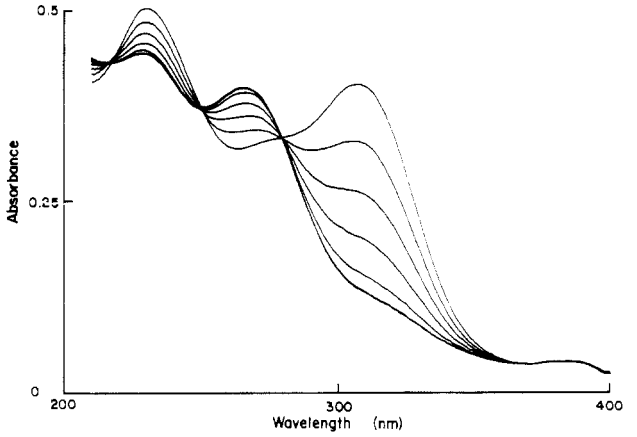


FIGURE 1: Spectral changes accompanying the hydrolysis of MM22382 by β-lactamase. MM22382 (40 μM) was incubated with β-lactamase (1 nM) in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C. Scans are shown for every 9 min.

Scheme I: Minimal Kinetic Pathway for the Interaction of MM22382 and β-Lactamase[a]

$$e + s \rightleftharpoons e{\cdot}s \xrightarrow{k_2} a{\cdot}e \xrightarrow{k_3} e + p$$

[a] Abbreviations used: e, free enzyme; s, MM22382; e·s, the Michaelis complex with MM22382; a·e, the acyl-enzyme from MM22382; p, the product of enzymatic hydrolysis of MM22382; k_2 and k_3, acylation and deacylation rate constants, respectively.

moderately good substrate for the RTEM β-lactamase.

The K_m for **1** is sufficiently low that this compound acts as a "competitive substrate" and decreases the rate of enzyme-catalyzed hydrolysis of a good substrate such as benzylpenicillin. The K_i value for **1** was found to be 4 ± 1 μM, in good agreement with the K_m value determined directly.

Preparations of **1** may also lead to enzyme inactivation, though it appears that the inactivation is not a primary event in the processing of **1** by the enzyme. Thus, (a) aged solutions of **1** (after incubation at 4 °C in 100 mM phosphate buffer, pH 7.0, for more than 24 h) produce more inactivation than freshly prepared solutions, (b) incubation mixtures containing identical molar ratios of substrate to enzyme (500:1) show more inactivation when the absolute concentration of substrate is higher (90 μM vs. 9 μM), and (c) the inactivation process continues long after the chromophore at 304 nm has been lost. These features argue against a simple partitioning at the active site leading to inactivation (Rando, 1975), and it seems likely that this phenomenon, which occurs less than once in 50 000 hydrolytic turnovers, derives from the presence of very small amounts of an inactivator that slowly forms from **1**.

The primary interaction of **1** with the β-lactamase is therefore as a reasonable substrate. Following our recent demonstration (Fisher et al., 1980a) that the mechanism of the RTEM enzyme involves an acyl-enzyme intermediate, the minimal kinetic scheme for **1** can be formulated as shown in Scheme I.

*Interaction of the β-Lactamase with MM13902 (**2**).* Incubation of a 10-fold molar excess of **2** with the lactamase leads to the disappearance of the chromophore at 304 nm, consequent upon the formation of the sulfato derivative of **3** as the first-formed product. In contrast to the simple behavior of **1**, however, the time course of the reaction is biphasic: a rapid initial phase gives way over several minutes to a slower steady-state reaction that can be followed until all of **2** has been consumed (Figures 2 and 3). The rate constant for the rapid phase of the reaction is (9.5 ± 0.5) × 10^{-3} s^{-1} and is independent of the concentration of **2** from 20 to 140 μM and

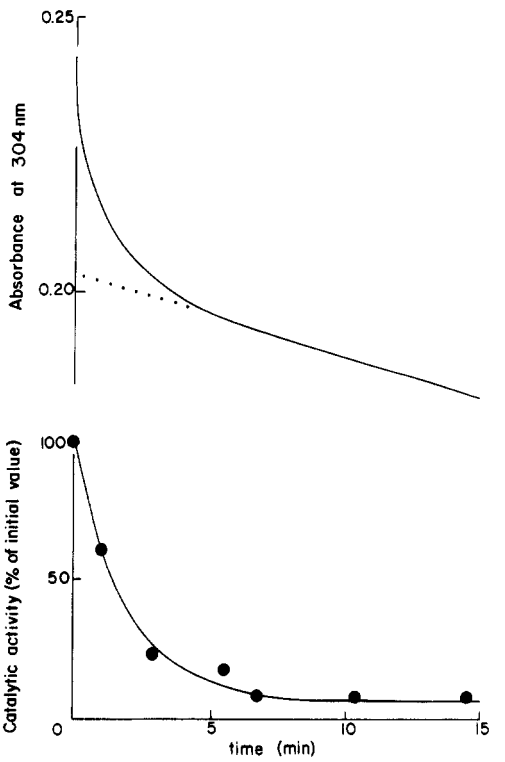


FIGURE 2: Early events in the hydrolysis of MM13902 by β-lactamase. MM13902 (20 μM) was incubated with β-lactamase (2 μM) in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C.

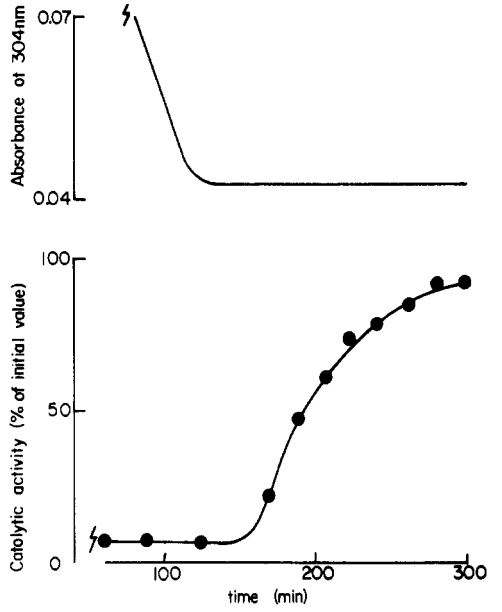
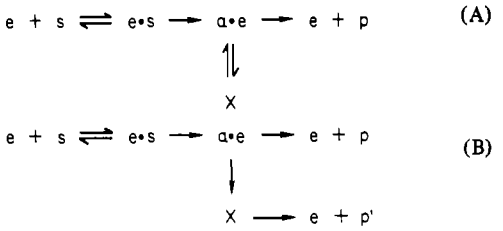


FIGURE 3: Late events in the hydrolysis of MM13902 by β-lactamase. The conditions were as for Figure 2.

of the molar excess of **2** over enzyme (from 10- to 70-fold). The steady-state reaction has a rate constant of (8.5 ± 0.5) × 10^{-4} s^{-1}. When the linear portion of Figure 2 is extrapolated back to time zero, the "burst" size is approximately 2.3 times that of the enzyme concentration on a molar basis. Provided that the steady-state reaction is fully established (and the linear portion of Figure 2 is truly linear), then a "burst" size greater than 1.0 cannot be accommodated by the kinetic pathway shown in Scheme I. In this scheme, if $k_3 \ll k_2$, a burst of 1.0 will be observed, and the burst size may fall below the stoichiometric value if k_3 is more nearly equal to k_2. There is no condition in which a burst that is larger than the stoichiometric

Scheme II: Possible Kinetic Pathways for the Interaction of MM13902 and β-Lactamase[a]



[a] Abbreviations used: e, free enzyme; s, MM13902; e•s, the Michaelis complex with MM13902; a•e, the acyl-enzyme from MM13902; p and p', products after deacylation; X, the enzyme intermediate responsible for the transient inhibition of the enzyme.

value can be obtained. [The trivial explanation that the substrate contains a small amount of a rapidly reacting impurity is eliminated by the facts that (a) the burst size is independent of the concentration of **2** at a fixed enzyme level and (b) subsequent additions of **2** to an incubation of **2** with the enzyme do not produce a second "burst".] The first phase of the reaction shown in Figure 2 must therefore represent *the accumulation of an enzyme intermediate* that derives from a species on the normal hydrolytic pathway that occurs before the rate-determining step. In Scheme II are shown two of the simplest schemes that can accommodate the large burst size as well as the enzyme activity results presented below. In either Scheme IIA or IIB, the species X represents a "waiting room", an intermediate state into which the enzyme accumulates. During the burst phase of the reaction, some 2.3 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 decomposition of X, either via the acyl-enzyme (Scheme IIA) or directly (Scheme IIB).

The simple picture derived above is supported by measurements of the enzyme activity during the hydrolysis of **2**. The catalytic activity of enzyme that has been incubated with **2** can be measured by diluting a small portion of incubation mixture into an assay solution of benzylpenicillin. The initial rate of benzylpenicillin hydrolysis then provides a measure of the instantaneous concentration of active enzyme. Incubation of β-lactamase with a 10-fold molar excess of **2** leads to a rapid decrease in enzyme activity to less than 10% of that present initially (Figure 2). The rate of activity loss has a half-time of about 90 s, corresponding to a first-order rate constant (k_{inact}) of 8×10^{-3} s^{-1}. This is in excellent agreement with the rate constant of 9.5×10^{-3} s^{-1} derived from the spectrophotometric changes shown in Figure 2. It is evident that the rapid phase of the reaction produces complexes between enzyme and **2** that are catalytically inactive, as expected from Scheme IIA,B. The value of k_{inact} is constant over a concentration range of **2** from 0.1 to 900 μM (at constant molar excess of **2** over enzyme), and the inactivation reaction thus follows the expected saturation behavior. Analysis of the initial rates of inactivation at very low concentrations of **2** shows that the inactivation rate is half-maximal when **1** is 10 ± 2 nM. This value of $K_{m(inact)}$ is in good agreement with the K_i value of 10 nM determined in competition experiments with benzylpenicillin and demonstrates that the in vitro inactivation occurs at concentrations of **2** that may be physiologically relevant. The value of $k_{inact}/K_{m(inact)}$ for **2** is about 1×10^6 M^{-1} s^{-1}, which, as an effective second-order rate constant for the inactivation process, emphasizes the efficiency of this molecule in the inactivation of the RTEM β-lactamase.

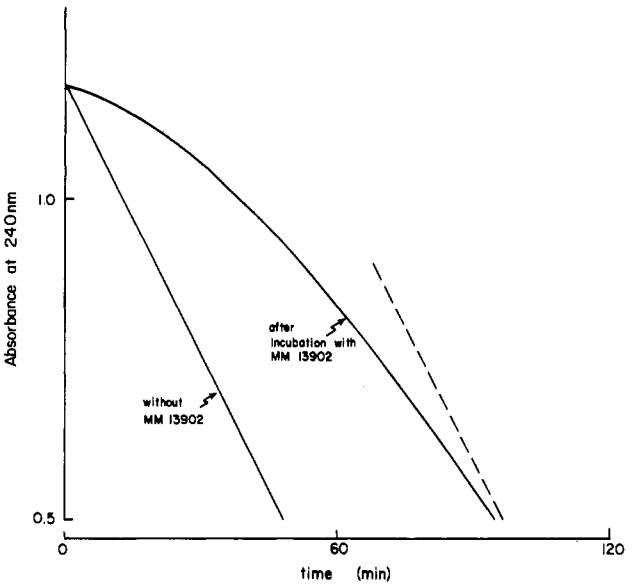


FIGURE 4: Recovery of β-lactamase activity after dilution from an incubation with MM13902 into benzylpenicillin. MM13902 (100 μM) was incubated with β-lactamase (10 μM) in 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C for 10 min. A portion (1 μL) of this mixture was then added to a solution (1.0 mL) of benzylpenicillin (1.4 mM). After being mixed, a portion (100 μL) of this solution was immediately added to an assay solution (2.9 mL) of benzylpenicillin (1.4 mM), and the absorbance was monitored at 240 nm.

The inactivation of β-lactamase by **2** is not, however, irreversible, and after long incubations, full recovery of enzyme catalytic activity is observed. This is shown in Figure 3 where the catalytic activity of the enzyme (measured directly, with benzylpenicillin as substrate) returns in a first-order manner, after all the **2** has been consumed (measured directly, from the absorbance at 304 nm). The recovery of the catalytic activity has a rate constant of $(3.2 \pm 0.2) \times 10^{-4}$ s^{-1}. The enzyme activity rises from less than 10% to more than 95% of its original value. Exactly analogous behavior can be seen if a portion of an incubation of enzyme with **2** is diluted into an assay solution of benzylpenicillin (Figure 4).

The initial gradient is low, showing that there is only a small proportion of free enzyme during the steady-state hydrolysis of **2**. The enzyme activity increases with time, the final activity being within 5% of that from an enzyme sample that had not been incubated with **2** (see Figure 4). The rate constant for this recovery of catalytic activity is identical with that measured at the end of the reaction, shown in Figure 3. Neither the rate nor the extent of the recovery of catalytic activity is affected by increasing the molar excess of **2** over enzyme.

The reaction that leads to recovery of enzyme activity ($k = 3.2 \times 10^{-4}$ s^{-1}) is less than half as fast as the steady-state turnover rate ($k = 8.5 \times 10^{-4}$ s^{-1}). This difference in rates 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 the substrate has been consumed. In terms of the pathways of Scheme II, the acyl-enzyme partitions somewhat more favorably toward e + p than it does toward X (this results in the more-than-stoichiometric rapid phase of the reaction), and 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 (k_3) of the acyl-enzyme].

The kinetic behavior of **2** as described above is open to an alternative interpretation, involving substrate-induced con-

formational changes of the kind proposed by Citri and coworkers (Samuni & Citri, 1975; Citri et al., 1976). According to this view, the first few turnovers of **2** by the enzyme would be accompanied by a conformational change to an enzyme form that hydrolyzed the substrate more slowly. This change would occur during several turnovers, accounting for the larger-than-stoichiometric burst phase. After all the substrate had been consumed, the enzyme would slowly relax back to its more active form, accounting for the slow recovery rate of enzyme activity. Although this theory is kinetically indistinguishable from the more classical pathways of Scheme II, the distinction can be made chemically. If the enzyme were, at the end of substrate hydrolysis, in a different conformational state of lower catalytic activity, we should not expect that the addition of hydroxylamine would affect the rate of its isomerization to the "native" state. If, in contrast, the recovery of enzyme activity at the end of the reaction derives from the direct breakdown of X to liberate active enzyme, an accelerating effect of added hydroxylamine would not be unexpected. In fact, hydroxylamine (10–50 mM) *does* accelerate the reappearance of enzyme activity from enzyme samples taken after **2** has been consumed. The recovery rate is first order in hydroxylamine and has a rate constant of 5.6×10^{-2} M^{-1} s^{-1}. [This may be compared with the rate of 12×10^{-2} M^{-1} s^{-1} for the hydroxylamine-mediated partial reactivation of β-lactamase that has been inactivated by an excess of clavulanic acid (Charnas et al., 1978; Fisher et al., 1978).] A substrate-induced catalytically altered conformation state of the enzyme does not readily account for our observations with the RTEM β-lactamase and **2**, and the pathways of Scheme II are more attractive in this case.

Nature of the Inactive Complex with MM13902 (2). On the basis of our work with clavulanic acid (Fisher et al., 1978; Charnas et al., 1978) and with other β-lactamase inactivators (Fisher & Knowles, 1980; Fisher et al., 1980b) and of studies in a number of other laboratories on, for instance, 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b) and 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979), we suggested that there were three features shared by all effective β-lactamase inactivators (Fisher et al., 1980b). First, the inactivating species should contain a β-lactam, the recognition of which by the enzyme leads to the formation of an acyl-enzyme intermediate. Secondly, there should be a 6α proton of an acidity adequate to allow a facile β elimination across the bond between C-5 and C-6. Finally, the reagent should contain a good leaving group for this β elimination. These features were shared by all those β-lactam derivatives having a hereto atom in the 1 position. [This group includes clavulanic acid, 6β-bromopenicillanic acid, penicillanic acid sulfone, 6-chloropenicillanic acid sulfone, and the sulfones of quinacillin and methicillin (Fisher & Knowles, 1980; Fisher et al, 1980b).]

In the case of the olivanic acids, it is unreasonable to suggest that the carbon–carbon bond between C-5 and C-1 would be broken in a β elimination, and the effectiveness of **2** as a β-lactamase inactivator led us to suggest (Fisher et al., 1980b) that a β elimination into the C-6 side chain (Scheme III) could accommodate the available information. From what has been presented above, the kinetic pathways followed by **1** (Scheme I) and **2** (Scheme II) would seem consistent with the more facile elimination of sulfate from **2** than of water from **1**.

Indeed, on this basis one could identify X as an α,β-unsaturated acyl-enzyme of greater hydrolytic stability than the first-formed saturated ester (see Scheme III). To test this hypothesis, we examined the liberation of sulfate ion from **2** during its interaction with β-lactamase.

Scheme III

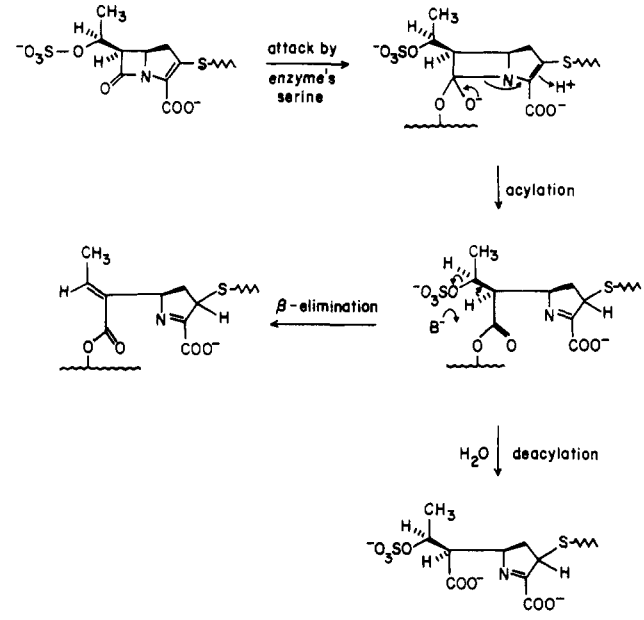


Table I: Determination of Sulfate Ion Deriving from MM13902 and Its Interaction with the β-Lactamase

	amount (nmol)	absorbance[a] at 322 nm
MM13902	15	0.21 ± 0.03
	30	0.40 ± 0.04
K_2SO_4	5	0.18 ± 0.03
	10	0.38 ± 0.04
	15	0.57 ± 0.04
	20	0.79 ± 0.03
	25	0.98 ± 0.05
MM13902 (hydrolyzed)[b]	25	0.93 ± 0.05
MM13902 plus K_2SO_4	30 + 10	0.78 ± 0.05
enzyme	3	0.02 ± 0.04
	22	0.10 ± 0.04
MM13902 plus enzyme	30 + 3 at 0 min	0.36
	30 + 3 after 10 min[c]	0.43
	30 + 3 after 75 min[d]	0.43
	30 + 3 after 150 min[e]	0.40
species X[f]	22	1.2 ± 0.05
species X after reactivation[g]	22	0.30 ± 0.02

[a] All absorbance measurements have been corrected for the background absorbance of 0.40, obtained when distilled water is added to the sulfate assay mixture. [b] 0.1 M HCl, 70 °C, 24 h. [c] 28% hydrolysis. [d] 68% hydrolysis. [e] 100% hydrolysis. Had sulfate ion been released in accord with Scheme III and the rate constants quoted in the text, an absorbance of 0.67 would have been expected. [f] Species X (Scheme II) was 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 (78 μM) and MM13902 (780 μM) in buffer (700 μL) that had been incubated at 30 °C for several minutes. The material was treated with 100 mM HCl for 24 h at 70 °C before sulfate assay (see Experimental Procedures). [g] Species X prepared as in *f* was incubated at 30 °C until >95% of the catalytic activity had been regenerated (12 h). This material was subjected to gel filtration as in *f*, and the protein fraction was then treated with 100 mM HCl for 24 h at 70 °C before sulfate assay (see Experimental Procedures).

When β-lactamase is incubated with a 10-fold molar excess of **2** and the release of free sulfate ion is monitored, no liberation of sulfate could be detected (Table I). Under the conditions of the experiments, about 15% of the expected amount of free sulfate could have been measured. For confirmation that the sulfate group remained attached to the carbon skeleton of **2** during and after the latter's hydrolysis

by the enzyme, a portion of an incubation of enzyme with **2** was taken from the steady state and subjected to gel filtration at 4 °C. The enzyme thus isolated showed 20% of the catalytic activity of a control from which **2** had been omitted. [This activity partly derives from the slow recovery of enzyme activity during the chromatography at 4 °C]. That this enzyme sample was indeed species X (Scheme II) was shown by the reappearance of more than 90% of the expected catalytic activity, at the appropriate rate ($t_{1/2} \sim 34$ min), when the sample was warmed to 30 °C. Sulfate assays of the protein fractions (after treatment with acid to hydrolyze the sulfate ester) showed that the inactive enzyme species X contains about 1.3 mol of sulfate per mol of protein (after correction for the small contribution of enzyme protein to the sulfate assay). The slight molar excess of sulfate evidently derives from the incomplete separation of protein from **2** during gel filtration. When the species X is incubated at 30 °C to allow the regain of >95% of the catalytic activity and then subjected to gel filtration, the amount of sulfate found after acid treatment is about 0.25 mol per mol of protein. The species X thus contains a stoichiometric amount (1.05 mol) of sulfate ester (see Table I). It is clear from these results that Scheme III does *not* describe the kinetic events that characterize the interaction of olivanic acids with the β-lactamase.

The two carbapenems investigated here differ markedly in their behavior toward the β-lactamase. **1** is merely a good substrate for the enzyme (Scheme I), yet the corresponding 8-sulfato derivative **2** is an inhibitor that forms a transiently stable intermediate X with the enzyme (Scheme II). How does the sulfate group affect this difference since we know that it does not act as a leaving group in the β-elimination process outlined in Scheme III? The existence of a sulfate ester on C-8 somehow diverts the acyl-enzyme to the transiently stable species X. One superficially attractive possibility is that the sulfate ester takes the place of the water molecule that would necessarily be involved in the deacylation step and adds to the carbonyl group of the acyl-enzyme to form (reversibly) a tetrahedral intermediate. The reasonable nucleophilicity of a sulfate oxygen (Backer & Dubsky, 1920), the formation of a six-membered ring, and the resulting tetrahedral adduct (which, by analogy with the "oxyanion hole" of the serine proteases, may be especially well stabilized by the enzyme) would all be consistent with this suggestion. It is true, however, that some carbapenems that *lack* a sulfate ester at C-8, such as PS-5 (Okamura et al., 1980; Fukagawa et al., 1980) and *N*-formimidoylthienamycin (Kahan et al., 1979), show kinetic behavior analogous to that of **2** (C. Kemal, unpublished results). While the differing stereochemistry at C-6 and at C-8 in the thienamycin series could be important, the addition of sulfate to the acyl-enzyme carbonyl group is clearly inadequate to accommodate the behavior of all carbapenems with the β-lactamase.

The most general, if inexplicit, proposal to account for the differences in the modes of interaction of different carbapenems is to invoke enzyme specificity. On this basis, the anionic sulfate group would interact with an appropriate cationic locus at the active site and in some way slow the deacylation reaction so as to allow the acyl-enzyme to suffer another chemical fate [perhaps the reversible attack of an enzyme nucleophile on the imine of the Δ^1 pyrroline (e.g., Meister, 1954)]. Whatever the detailed chemistry, however, it is clear from the present work that the *irreversible* inactivation of the β-lactamase is not a sine qua non for the effective protection of a β-lactam antibiotic. Provided that the β-lactamase is blocked by a tightly binding and slowly reacting substrate, in vivo synergy (that is, prevention of antibiotic hydrolysis by the β-lactamase) can be achieved. Recognition of these features may aid the search for new compounds that act as effective inhibitors of the β-lactamase.

Acknowledgments

We are especially grateful to F. P. Doyle, Esq., of Beechams, Ltd., for providing the samples of MM13902 and MM22382 that allowed us to carry out this investigation.

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