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6-(Methoxymethylene)penicillanic Acid: Inactivator of RTEM β-Lactamase from *Escherichia coli*†

Daniel G. Brenner and Jeremy R. Knowles*

ABSTRACT: The *Z* and *E* isomers of 6-(methoxymethylene)-penicillanic acid have been synthesized, and their interaction with the RTEM β-lactamase has been studied. The *Z* isomer is an inhibitor and an inactivator of the enzyme, and there is some similarity between its behavior and that of other mechanism-based inactivators such as clavulanic acid and the penam sulfones. Kinetic analysis of the interaction of the enzyme with the *Z* isomer has allowed a detailed evaluation of the factors that are important in the design of anti-β-lactamase agents. In contrast to the *Z* compound, the *E* isomer of 6-(methoxymethylene)penicillanic acid is not a substrate, an inhibitor, or an inactivator of the enzyme.

The most common basis for bacterial resistance to β-lactam antibiotics is the existence of an enzyme, the β-lactamase, that catalyzes the hydrolysis of the penicillin to the corresponding penicilloic acid. In the past 7 years, a number of naturally occurring and semisynthetic β-lactam derivatives have been reported that inhibit the β-lactamase and thus have the potential of preventing the enzyme from destroying hydrolytically sensitive but otherwise potent antibiotics. The mechanisms of the interaction of several of these inhibitors with isolated β-lactamases have been studied, and the characteristics of the inhibitory processes have been delineated (Knowles, 1983). Nearly all of the inhibitors belong to the class of "mechanism-based" or "suicide" reagents, in which the hydrolytic reaction is diverted from its normal course at an intermediate stage. For the two compounds most thoroughly investigated, clavulanic acid (Fisher et al., 1978; Charnas et al., 1978; Labia & Peduzzi, 1978; Cartwright & Coulson, 1979) and penicillanic acid sulfone (English et al., 1978; Fisher & Knowles, 1980; Brenner & Knowles, 1981, 1984; Kemal & Knowles, 1981), it appears that the enzyme recognizes and

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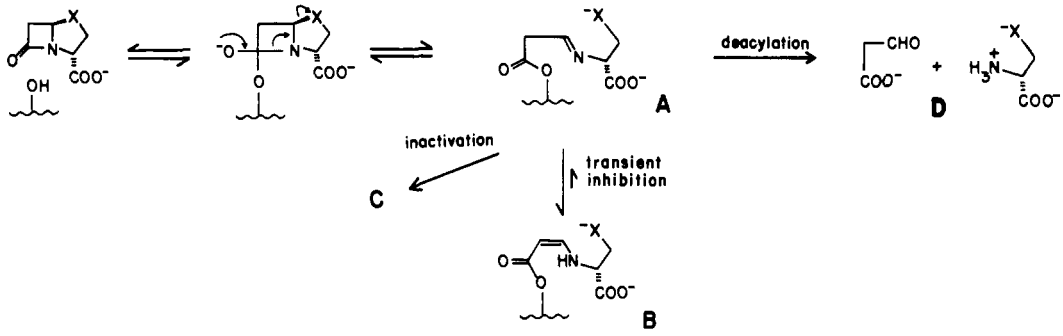
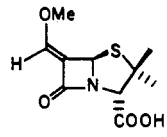
Scheme I: Generalized Scheme for Interaction of β-Lactam Derivatives Such as Clavulanic Acid and Penam Sulfones with TEM-2 β-Lactamase



is acylated by the β-lactam (Fisher et al., 1980). The acyl-enzyme then suffers one of three fates: hydrolysis to regenerate the free enzyme, rearrangement that leads to transient inhibition, or further reaction that results in irreversible inactivation of the enzyme. As illustrated in Scheme I, the active site serine residue attacks the β-lactam carbonyl group, and the resulting tetrahedral intermediate then collapses to the acyl-enzyme. With clavulanic acid and penicillanic acid sulfone, the bond between C-5 and the heteroatom at the 1-position is weak enough to allow cleavage of the five-membered ring, so the acyl-enzyme has the imine structure A (Scheme I). Kinetic and spectroscopic studies have shown that the transiently inhibited form of the enzyme has the enamine ester structure B, which is more stable to hydrolytic breakdown than the unconjugated ester A. The stability of such esters accounts for the phenomenon of transient inhibition in the case of clavulanate and penicillanic acid sulfone, though the enamine ester that forms (as a dihydrothiazine) from the interaction of β-bromopenicillanic acid and the β-lactamase is so stable that it results in irreversible enzyme inactivation (Pratt & Loosemore, 1978; Orlek et al., 1979; Knott-Hunziker et al., 1979a,b, 1980; Loosemore et al., 1980; Cohen & Pratt, 1980). In contrast, although the acyl-enzyme formed from carbapenems of the olivanate series can also undergo an enamine–imine tautomerization that leads to transient inhibition of the β-lactamase (Easton & Knowles, 1982), this tautomerization is remote from the acyl-enzyme ester linkage, and the transient inhibition may here derive simply from the inappropriate positioning of the scissile ester bond with respect to the enzyme's catalytic groups. In all these cases, however, the effectiveness of the inhibition of the enzyme is limited by the fact that the first-formed acyl-enzyme must tautomerize to a species (often a conjugated unsaturated ester, e.g., A → B, Scheme I), the deacylation rate of which is slower than that of the normal hydrolytic pathway (A → D, Scheme I).

In light of the above, it seemed possible that if the original penam were designed so that the *first-formed* acyl-enzyme was a conjugated unsaturated ester, an efficient inhibitor of the β-lactamase might result. We report here the inhibitory properties of the *Z* and *E* isomers of 6-(methoxymethylene)penicillanic acid (**1** and **2**). The essential feature



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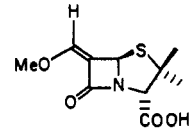


2

of these β-lactams is the 6-vinyl substituent terminally substituted with a heteroatom, which, in the presence of β-lactamase, could lead *directly* to acyl-enzymes having many features of what are known to be transiently inhibited forms of the enzyme. While functionalization of a penam in this manner might lead to an effective transient inhibitor of the β-lactamase, it was also possible that, by virtue of the conjugation in the parent penam, the β-lactam carbonyl would become too stable toward nucleophilic attack and react only very slowly with the enzyme. This possibility was deemed unlikely on the basis that the additional strain energy in the four-membered ring due to the exocyclic methylene group would *favor* nucleophilic attack by the active site serine on the β-lactam carbonyl, since this changes the hydridization at C-7 from sp^2 to sp^3 and releases, in part, some of the strain energy.

Experimental Procedures

Materials

β-Lactamase was from *Escherichia coli* W3110 carrying the RP4 plasmid (Matthew & Hedges, 1976). The TEM-2[1] enzyme was purified essentially as described earlier (Fisher et al., 1980) and had a specific activity of 4300 units/A_{280nm}. A unit of activity is that amount of enzyme that will catalyze the hydrolysis of 1 μmol of benzylpenicillin/min at 30 °C in 100 mM potassium phosphate buffer, pH 7.0. The enzyme was >95% homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

6-(Methoxymethylene)penicillanic Acid. The crystalline benzyl ester of 6-oxopenicillanic acid was prepared by oxidation of the corresponding 6α-alcohol (Chandrasekaran et al., 1977) and purified by flash chromatography (Still et al., 1978) on silica. This material was allowed to react with the anion of (methoxymethyl)trimethylsilane at −100 °C (Magnus & Roy, 1982), to yield a mixture of the two diastereoisomeric silyl alcohol adducts deriving from attack at the C-6 carbonyl exclusively from the less hindered α-face [see, e.g., Lo & Sheehan (1972, 1975)]. The two trimethylsilyl adducts were separated chromatographically, and the hydroxy group was acetylated so as to provide a better leaving group for the β-elimination. The benzyl ester group was removed by hydrogenolysis, and the acetylated acids were then treated with cesium fluoride in dimethyl sulfoxide (Magnus & Roy, 1982) to yield the penams **1** and **2**. These isomers were separated and purified by reverse-phase high-pressure liquid chromatography. The details of the synthesis of **1** and **2** will be reported elsewhere (Brenner, 1984).

Methods

Ultraviolet measurements were made on a Perkin-Elmer 554 or 575 instrument equipped with a thermostated cell block.

Enzyme-Catalyzed β-Lactam Hydrolysis. All kinetic runs were performed in 100 mM potassium phosphate buffer, pH

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

7.0, 30 °C. The reaction of **1** (9 μM) with β-lactamase (2 μM) was followed conveniently at 245 nm, where the $\Delta\epsilon$ is 7800 M^{-1} cm^{-1} (the intact penam has ϵ 12400 M^{-1} cm^{-1}). Higher molar ratios of substrate to enzyme were necessary to achieve the steady state for k_{cat} determinations. Typical concentrations were as follows: penam **1**, 500 μM; β-lactamase, 0.22 μM. No change in the absorbance at the λ_{max} (252 nm) of **2** (9 μM to 4 mM) upon incubation with the enzyme (2 μM) was observed.

Enzyme Inhibition and Inactivation. The inhibition of the β-lactamase by the enol ether **1** was followed by assay of the remaining enzyme activity. The enzyme (10–40 μL of a 22 μM solution) was incubated with **1** (20–50 μL of a 4.7 mM solution). Portions (5 μL) were withdrawn at appropriate intervals and mixed with a buffered solution (3.0 mL) of benzylpenicillin (3 mM), and the hydrolysis of the latter was followed at 240 nm. The final concentration of the enol ether in the assay mixture was less than 3 μM.

To study the irreversible inactivation of the β-lactamase by the enol ether **1**, the enzyme (2 μL of a 22 μM solution) was incubated with **1** (0.2 mL of a 512 μM solution) at 30 °C. At appropriate intervals, portions (10 μL) of the incubation mixture were first diluted into a solution (1.0 mL) of buffered benzylpenicillin (3 mM). A portion (0.1 mL) of this solution was then mixed in a cuvette with a buffered solution (0.9 mL) of benzylpenicillin (3 mM). This solution was then incubated for 1 h at 30 °C, before monitoring the hydrolysis of the benzylpenicillin at 240 nm. The final concentration of the enol ether in the assay mixture was 0.5 μM.

Enzyme Reactivation. To investigate the reactivation of β-lactamase that had been transiently inhibited by **1**, the enzyme (2 μL of a 22 μM solution) was incubated with **1** (0.2 mL of a 512 μM solution) for 12 min (in order to attain the steady state). A portion (10 μL) of the incubation was first diluted into a buffered solution (1.0 mL) of benzylpenicillin (3 mM), from which a portion (0.1 mL) was withdrawn and further diluted into a buffered solution (0.9 mL) of benzylpenicillin (3 mM). The hydrolysis of the benzylpenicillin was then monitored at 240 nm. The data from the reaction progress curves were plotted according to the method of Glick et al. (1978), and the rate constant for enzyme reactivation was determined.

Number of Hydrolytic Events before Enzyme Inactivation. β-Lactamase (10 μL of a 22 μM solution) was mixed with **1** (20 μL of a 2–8 mM solution). After the reaction was complete (20 h), the residual enzymatic activity was measured by diluting a portion (2.5 μL) of the incubation mixture into a buffered solution (3.0 mL) of benzylpenicillin (2 mM), and the hydrolysis of the latter was followed at 240 nm. From the linear plot of the initial molar ratio of enol ether to enzyme vs. the fraction of catalytic activity remaining, the number of molecules of the enol ether required to effect complete enzyme inactivation was determined by extrapolation.

Results and Discussion

The penams **1** and **2** have characteristic ultraviolet chromophores: the *E* isomer has λ_{max} 252 nm and ϵ 10300 M^{-1} cm^{-1}, and the *Z* isomer has λ_{max} 245 nm and ϵ 12400 M^{-1} cm^{-1}. The enol ether functionality in these compounds is conjugated with the β-lactam carbonyl, and the hydrolysis of the β-lactam rings may be monitored directly by measuring the change in the absorbance at 252 or at 245 nm, respectively. When an 8-fold molar excess of the *Z* isomer (**1**) is incubated with the β-lactamase, there is a decrease in the absorbance at 245 nm having $\Delta\epsilon$ 7800 M^{-1} cm^{-1}. In contrast, *no* change is observed in the ultraviolet spectrum of the *E* isomer (**2**) under the same conditions. Furthermore, in the presence of **2** at concentrations up to 4 mM, there is no effect on the catalytic activity of the β-lactamase toward benzylpenicillin. It appears that only the *Z* isomer is recognized by the β-lactamase: the *E* isomer does not interact detectably with the enzyme.

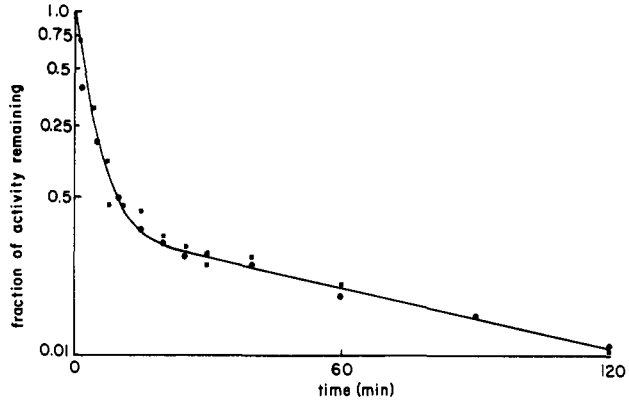


FIGURE 1: Semilogarithmic plot of the instantaneous β-lactamase activity remaining in incubation mixtures of the enzyme with saturating levels (400 μM) of **1**. The circles and squares are from replicate experiments.

The *Z* isomer (**1**) acts as a competitive inhibitor of the enzyme-catalyzed hydrolysis of benzylpenicillin (the reaction is followed only for short times, before significant amounts of **1** have been hydrolyzed, and before enzyme activity is reduced), and a value for the inhibition constant K_i (equivalent to the K_m value for **1** when it is a substrate) of 39 μM is found. This value is much higher than those for such potent β-lactamase inhibitors as penicillanic acid sulfone (0.8 μM; Brenner & Knowles, 1981) or clavulanic acid (<1 μM; Fisher et al., 1978). However, when saturating levels of **1** (>400 μM) are incubated with the enzyme, there is a sharp *decrease* in the rate of disappearance of the chromophore at 245 nm during the first 12 min or so of the incubation, despite the fact that the enzyme is saturated with **1** during the whole of this period. These data would be consistent with a simple linear reaction pathway in which an acylation step (over 12 min) is followed by a slower deacylation that would become rate-limiting overall after the steady state had been reached. Further experiments, however, rule out this simple interpretation.

The β-lactamase was incubated with 700-fold molar excess of **1**, and the catalytic activity of the enzyme was measured at various times by monitoring the rate of hydrolysis of benzylpenicillin in a solution into which a sample of the incubation mixture had been diluted. The enzyme activity remaining after different incubation times is shown in Figure 1. The catalytic activity of the enzyme decreases with time in a first-order fashion during the first 12 min of the reaction, and at later times, the further decrease in activity occurs more slowly (Figure 1). Examination of the behavior of the enzyme in the presence of different initial concentrations of the enol ether **1** (Figure 2) revealed that when **1** is in less than 700-fold molar excess over the enzyme, there is a recovery of enzymatic activity after an interval that is dependent upon the initial ratio of substrate to enzyme. This observation is reminiscent of the behavior of penicillanic acid sulfone and of clavulanic acid with the β-lactamase: the enzyme undergoes multiple turnovers but in the process becomes transiently inhibited and (over longer periods) is irreversibly inactivated.

There are two additional observations that give insight into the interactions of the enol ether **1** with the β-lactamase. The first is that, judging by the change in absorbance observed for

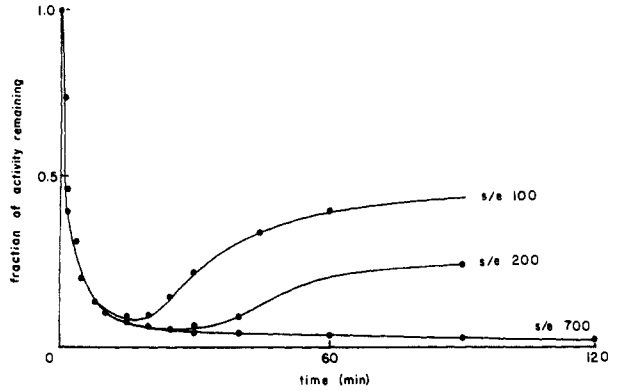


FIGURE 2: Time dependence of the instantaneous β-lactamase activity on incubation of the enzyme with 6-[(*Z*)-methoxymethylene]-penicillanic acid. The penam (s) was incubated with the enzyme (e) in various initial molar ratios, and at appropriate intervals, portions were withdrawn and assayed for remaining enzyme activity.

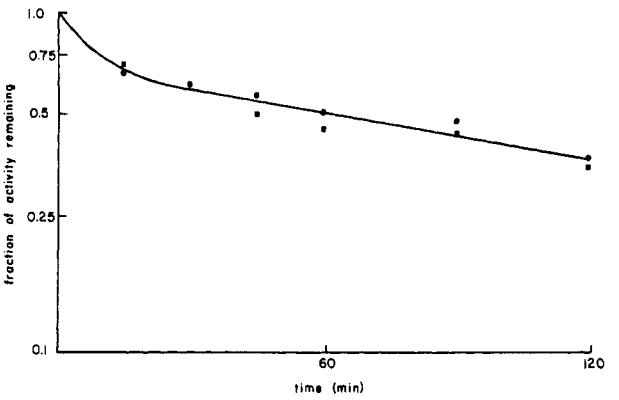
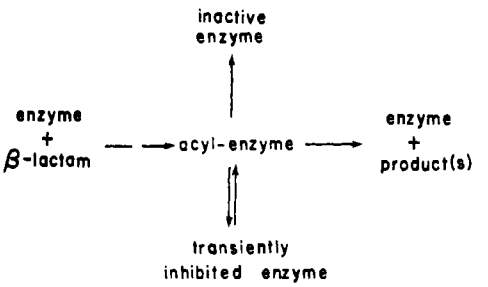


FIGURE 3: Semilogarithmic plot of the time course of the irreversible inactivation of the β-lactamase by saturating levels (400 μM) of **1**. The circles and squares are from replicate experiments.

Scheme II: Minimal Kinetic Scheme for Interaction of 6-[(*Z*)-Methoxymethylene]penicillanic Acid with β-Lactamase



the enol ether, approximately 280 turnovers have already occurred by the end of the first phase of the reaction (that is, after about 12 min). Because the enzyme undergoes multiple turnovers during this initial period, the slow accumulation of the acyl-enzyme intermediate on an unbranched pathway cannot account for the observed behavior. Second, when a mixture of β-lactamase and the enol ether **1** is preincubated for at least 12 min and then diluted into a solution of benzylpenicillin, the rate of hydrolysis of the benzylpenicillin *accelerates* with time, reaching a limiting value that depends on the period of the preincubation with **1**. On the basis of this acceleration in the hydrolysis rate of benzylpenicillin, a rate constant for the recovery of enzymatic activity (the reactivation process), k_{react}, was calculated to be 0.04 min^{-1}. The value of k_{cat} obtained from the decrease in absorbance of the enol ether (the measurement being made at the same time as the dilution for the k_{react} determination) is 3.6 min^{-1}. When corrected for the estimated proportion of active enzyme (at the time of the measurement, some 30% of the enzyme had been irreversibly inactivated), this value becomes 5.6 min^{-1}. Since the overall turnover rate (as measured by k_{cat}) is much faster than the reactivation rate (as measured by k_{react}), the process measured by k_{react} cannot be part of the overall turnover reaction, and a branched pathway must be involved. This phenomenon is analogous to that observed in the interaction between penicillanic acid sulfone and the β-lactamase.

Finally, the time course of the irreversible inactivation of the enzyme was investigated. The enzyme was incubated with a large excess of **1**, and portions were withdrawn at appropriate intervals and diluted into a solution of benzylpenicillin. After incubation with the benzylpenicillin for at least 90 min (that is, five half-lives for the reactivation of the transiently inhibited enzyme), the final catalytic activity of the enzyme was measured. These results are plotted in Figure 3. It is evident that the rate of irreversible inactivation of the enzyme is more rapid during the first 10 or 15 min than it is at later times. There is a minimum molar ratio of substrate to enzyme needed to inactivate the enzyme completely. This ratio represents the average number of hydrolytic turnovers of substrate prior to a single inactivation event, that is, the ratio of k_{cat}/k_{inact}. Taking both k_{cat} (5.6 min^{-1}; see above) and k_{inact} (0.013 min^{-1}; from Figure 3) from measurements during the slower phase of the reaction (that is, at least 12 min after the beginning of the incubation), the value of this ratio is 430. An independent measurement of this quantity was made by incubating the enzyme for 18 h with molar ratios of substrate to enzyme of less than 500. A plot of the residual catalytic activity of the enzyme from these incubations vs. the initial molar ratio of substrate to enzyme yields a value of 500 for the number of substrate molecules consumed before enzyme inactivation. This is in reasonable agreement with the ratio of k_{cat}/k_{inact} determined independently.

On the basis of the observations described, a minimal kinetic scheme can be proposed for the interaction of the enol ether **1** with the β-lactamase (Scheme II). In this scheme, the acyl-enzyme partitions three ways, to liberate product and regenerate free enzyme, to cause transient inhibition of the enzyme, or to inactivate the enzyme irreversibly. During the approach to the steady state, the enzyme accumulates into the transiently inhibited form (Figure 1). As a result, there is less acyl-enzyme available to partition toward irreversible inactivation, and the rate of this process should therefore *decrease* with time, as observed (Figure 3).

In order to understand better the interaction of enol ether **1** with the β-lactamase, an analytical solution was sought to the pathway depicted in Scheme II, in the hope of gaining support for the kinetic pathway and of finding values for the rate constants involved. This approach seemed possible since inspection of Figure 1 shows that the slopes of the two phases of the curve differ by more than 10-fold, and the data in this figure should easily be fitted to the sum of two exponentials. The solid line in Figure 1 represents the fit of the data in this manner. [An estimation of the analogous rate constants was not made in our earlier studies of penicillanic acid sulfone and clavulanic acid since the approach to the steady state is in both of these cases too rapid to be easily measured at pH 7.0.]

The pathway in Scheme II can, however, be simplified if the analysis is limited to conditions where the enzyme is saturated with the β-lactam throughout the entire reaction. This reduces Scheme II to Scheme III (Appendix), and the latter system has three time constants that are each functions of the rate constants of the scheme. The solution to the differential equations that relate the rate constants to the time

Scheme III: Simplified Kinetic Scheme for Interaction of 6-[(*Z*)-Methoxymethylene]penicillanic Acid with β-Lactamase

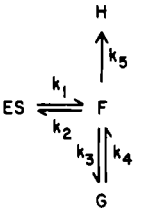


Table I: Kinetic and Rate Constants for Interaction of 6-[(*Z*)-Methoxymethylene]penicillanic Acid with β-Lactamase

	obsd	calcd[a]		obsd	calcd[a]
k_{cat} (min^{-1})	5.6	6.5	k_{inact} (min^{-1})	0.013	
K_m (μM)	39		k_{react} (min^{-1})	0.044	0.037
k_{inhib} (min^{-1})	0.30		hydrolysis/ inactivation	500	430

	calcd[b]		calcd[b]
k_1 (min^{-1})	68	k_4 (min^{-1})	0.037
k_2 (min^{-1})	230	k_5 (min^{-1})	0.047
k_3 (min^{-1})	0.76		

[a] From the individual rate constants derived from the analytical solution presented in the Appendix. [b] See Scheme III (Appendix).

constants is presented in the Appendix. The derived rate constants are listed in Table I, and the variation of the various enzyme species with time is shown in Figure 4. It can be seen that there is a rapid initial burst in the concentration of the acyl-enzyme F, which then falls to a steady-state level after 10–15 min. This fall is accompanied by a rise in the concentration of the transiently inhibited species G to its steady-state level, which then slowly decreases as the enzyme accumulates into the irreversibly inactivated form H. Also apparent from this figure is that the rate of formation of the inactivated enzyme decreases with time, as observed experimentally. Since the simplified analytical solution (see Appendix) does not take account of any variations in substrate concentration, this treatment cannot describe a reaction under subsaturating conditions. In principle, however, further confirmation of Scheme II could be obtained by performing computer simulations to reproduce the observed behavior of the more diagnostic curves of Figure 2 where the enzyme is *not* saturated with the β-lactam during the whole reaction. When iterative computer simulations were attempted, it was found that the large differences in the size of the time constants for different steps in the reaction made the computational time prohibitively long. From the arguments presented, however, it is evident that Scheme II is the simplest that can accommodate the observed behavior of **1** with the β-lactamase.

In the light of the similarity between the generalized scheme (Scheme I) for the interaction of β-lactams such as clavulanic acid and penicillanic acid sulfone with the enzyme, and the evident kinetic behavior of 6-[(*Z*)-methoxymethylene]-penicillanic acid (**1**) (Scheme II), what *chemical* changes describe the interaction of **1** with the enzyme? Upon mixing the β-lactamase with an 8-fold molar excess of **1**, the previously mentioned decrease in A_{245nm} is followed by the appearance of a new chromophore (with a rate constant of about 0.2 min^{-1}) at 302 nm (ε 12 200 M^{-1} cm^{-1}). This chromophore at 302 nm has a half-life of about 6 h. Because the new chromophore develops *after* the disappearance of the absorbance of **1** at 245 nm and because only a negligible proportion of the enzyme is inactivated under these conditions, the new chromophore at 302 nm must be associated with a structure that derives from the first-formed product of enzymic hydrolysis. A reasonable explanation for this behavior is that, after enzymatic

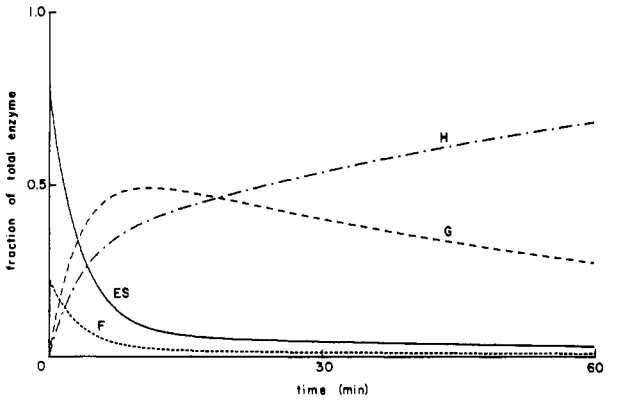


FIGURE 4: Calculated time course for the intermediates in the reaction of **1** with the β-lactamase. The designations ES, F, G, and H refer to the enzyme–substrate complex, the acyl-enzyme, the transiently inhibited enzyme, and the irreversibly inactivated enzyme, respectively (see Scheme III). The curves were generated from the equations derived in the Appendix with the rate constants listed in Table I.

deacylation, the thiazolidine ring of the first-formed penicilloic acid product (U in Figure 5) opens to give an iminium thiolate (V, Figure 5). The enol ether in V is cross-conjugated to the protonated imine and to the carboxylate. The cross-conjugation of this species could account for the observed chromophore at 302 nm (Silverstein et al., 1974). Such opening of the thiazolidine ring is known to occur with penicilloic acids, as judged by the epimerization that occurs at C-5 [presumably via the iminium thiolate (Pesek & Frost, 1975; Kiener & Waley, 1978)] and by formation of a dihydrothiazine at the active site of the β-lactamase on inactivation by 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Orlek et al., 1979). With benzylpenicilloic acid, the equilibrium lies well on the side of the thiazolidine (no imine can be detected by ^1H NMR), whereas the corresponding thiazolidine sulfones are too unstable to be isolated and exist completely in the imine form. It seems reasonable that the dicarboxylic acid U, formed from **1**, should also exist largely as the acyclic protonated imine, stabilized by conjugation with the enol ether function.

The chemistry suggested above for the free enzymatic hydrolysis product from the enol ether may be applicable to the enzyme-bound intermediates as well. Figure 5 shows a hypothetical scheme that accommodates all our observations on the interaction of the enol ether **1** with the β-lactamase. Nucleophilic attack by the active site serine-70 on the β-lactam carbonyl group results in acylation of the enzyme by the penam to give the acyl-enzyme R, in precise analogy with the known behavior of normal β-lactam substrates with the enzyme (Fisher et al., 1980). The acyl-enzyme R then partitions in one of three ways: to deacylation (restoring enzyme activity), to the transiently inhibited form of the enzyme, or to irreversibly inactivated enzyme. We suggest that transient inhibition may result from the reversible opening of the thiazolidine ring to form the thiolate S. Finally, irreversible inactivation could occur by Michael addition of an enzymic nucleophile to the α,β-unsaturated system in R, with the subsequent expulsion of methanol to give a β-aminoacrylate T. This last path has chemical precedent in the transetherification of enol ethers (Watanabe & Cohen, 1957; Watanabe, 1957) and enzymic precedent in the transimination proposed for the inactivation of β-lactamase by clavulanic acid and penam sulfones (Knowles, 1983). While it must be emphasized that the nature of the enzyme-bound species in Figure 5 is speculative, these formulations are, at least, consistent with all our spectroscopic and kinetic observations and with the known behavior of such materials.

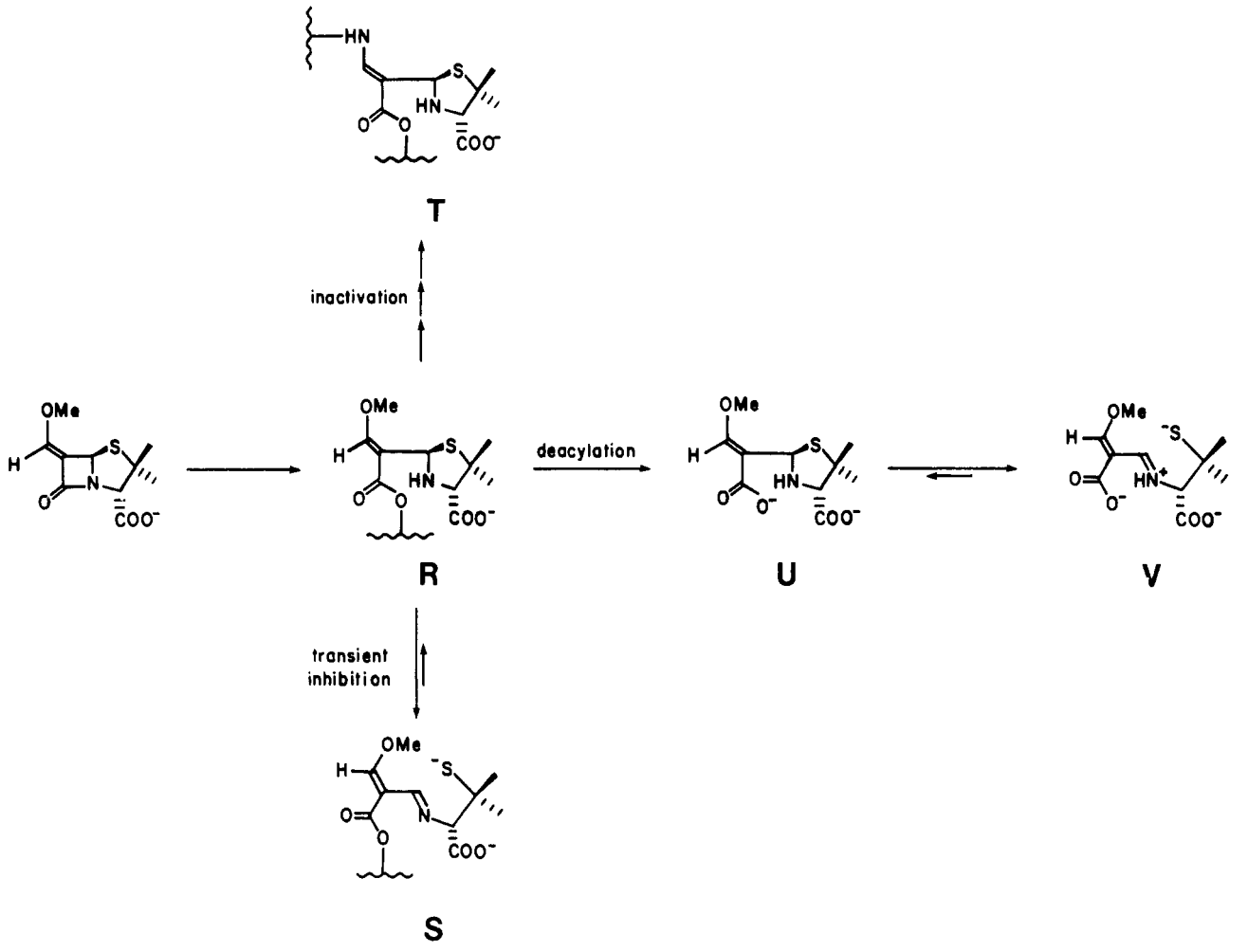
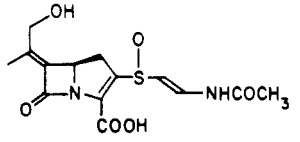


FIGURE 5: Hypothetical scheme for the interactions of **1** with the β-lactamase.

Very recently, two β-lactamase inactivators have been reported that are related to 6-(methoxymethylene)penicillanic acid in having substituted methylene groups at the 6-position. The first of these is asparenomycin A (**3**) (Tanaka et al., 1981),



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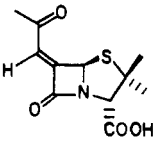

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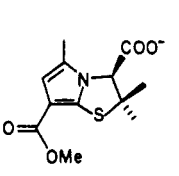
which is a naturally occurring carbapenem isolated from *Streptomyces*. This compound acts as a broad-spectrum antibiotic against many non-β-lactamase producing organisms. In addition, synergistic activity was observed with a combination of **3** and ampicillin against various ampicillin-resistant bacteria, presumably as a result of inhibition of the β-lactamase by **3**. From other studies, it was concluded that **3** progressively inhibits the β-lactamases produced by Gram-negative bacteria, by acylation of the enzyme. The mode of inhibition by **3** could initially involve an acyl-enzyme (similar to that pictured in Figure 5) stabilized by the substituted ethylidene group through conjugation with the serine ester. Although there are other pathways available for inhibition of β-lactamase by carbapenems once the enzyme has been acylated (for example, by tautomerization of the enamine; Easton & Knowles, 1982), an α,β-unsaturated acyl-enzyme (which is necessarily formed if the β-lactam is to be hydrolyzed by the enzyme) could account for the inhibition kinetics observed.

The second β-lactamase inhibitor in this class that has been investigated is a semisynthetic compound, 6-(acetylmethylene)penicillanic acid (**4**) (Arisawa & Then, 1982, 1983; Arisawa & Adam, 1983). This β-lactam was found to form a 1:1 complex with the β-lactamase after brief incubation with the enzyme. Reaction of **4** with equimolar hydroxylamine produces the oxime exclusively, demonstrating the reactivity of the ketone in this molecule. Additionally, methoxide reacts with **4** to yield the substituted pyrrole **5**, and two likely



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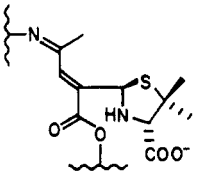

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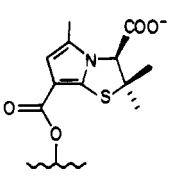

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structures, **6** and **7**, for the inhibited enzyme were proposed. These suggestions are all consonant with the proposed chemistry presented here for the interaction of **1** with the β-lactamase.

Conclusions

The enol ether **1** belongs to a new structural class of β-lactam. This penam is an inactivator of the β-lactamase, and there is considerable similarity between its behavior with the enzyme and that of the other mechanism-based inactivators such as clavulanic acid and the penam sulfones. The salient feature of the interaction of all of these compounds with the

enzyme is an acyl-enzyme intermediate that has fates open to it other than normal hydrolysis. While the efficiency of such reagents in preventing the β-lactamase from hydrolyzing β-lactam antibiotics in vivo may be compromised by such factors as stability and accessibility to the β-lactamase, kinetic studies with the purified enzyme determine whether the necessary characteristics of enzyme inhibition and/or inactivation are fulfilled. Such investigation also provides some insight into the factors that are important in the design of reagents that preoccupy the β-lactamase.

Acknowledgments

We are grateful to W. J. Albery for guidance with the analytical solution presented in the Appendix and to D. Purich for help and advice.

Appendix

The derivation and equations here pertain to Scheme III. This scheme has been simplified from Scheme II (main paper) by making the assumption that the enzyme is saturated with substrate throughout the reaction; that is, all of the free enzyme is bound as the Michaelis complex. [It should be noted that under these conditions, k_2 (Scheme III) really represents the rate constant for the deacylation step that regenerates free enzyme.]

The rate equations relating to Scheme III, with lower case letters to represent the concentrations of the different species, are

$$\frac{d[es]}{dt} = -k_1[es] + k_2[f]$$

$$\frac{d[f]}{dt} = k_1[es] + k_4[g] - (k_2 + k_3 + k_5)[f]$$

$$\frac{d[g]}{dt} = k_3[f] - k_4[g]$$

$$\frac{d[h]}{dt} = k_5[f]$$

These differential equations can be solved by the Laplace transform method (Roberts, 1977). At $t = 0$, $[es] = [e_0]$ and $[f] = [g] = [h] = 0$. With barred lower case letters for the transformed time-dependent species, the transformed equations are

$$s[\bar{es}] - [e_0] = -k_1[\bar{es}] + k_2[\bar{f}]$$

$$s[\bar{f}] = k_1[\bar{es}] + k_4[\bar{g}] - (k_2 + k_3 + k_5)[\bar{f}]$$

$$s[\bar{g}] = k_3[\bar{f}] - k_4[\bar{g}]$$

$$s[\bar{h}] = k_5[\bar{f}]$$

where s is the Laplace operator. Solving for $[\bar{es}]/[e_0]$, one obtains

$$[\bar{es}]/[e_0] = [s^2 + s(k_2 + k_3 + k_4 + k_5) + k_4(k_2 + k_5)]/[s^3 + s^2(k_1 + k_2 + k_3 + k_4 + k_5) + s[k_1(k_3 + k_4 + k_5) + k_4(k_2 + k_5)] + k_1k_4k_5]$$

This equation can be written in the form

$$\frac{[\bar{es}]}{[e_0]} = \frac{s^2 + sx + y}{(s + \alpha)(s + \beta)(s + \gamma)} \qquad (A1)$$

with $\alpha > \beta > \gamma$ and where

$$\alpha + \beta + \gamma = k_1 + k_2 + k_3 + k_4 + k_5$$

$$\alpha\beta\gamma = k_1k_4k_5$$

$$\alpha^{-1} + \beta^{-1} + \gamma^{-1} = k_2/(k_1k_5) + 1/k_1 + 1/k_4 + 1/k_5 + k_3/(k_4k_5)$$

$$x = k_2 + k_3 + k_4 + k_5$$

and

$$y = k_4(k_2 + k_5)$$

Equation A1 has a corresponding inverse Laplace transform, which can be written as

$$\frac{[es]}{[e_0]} = \frac{\alpha^2 - \alpha x + y}{(\alpha - \beta)(\alpha - \gamma)}e^{-\alpha t} + \frac{\beta^2 - \beta x + y}{(\beta - \alpha)(\beta - \gamma)}e^{-\beta t} + \frac{\gamma^2 - \gamma x + y}{(\gamma - \alpha)(\gamma - \beta)}e^{-\gamma t}$$

The variation in [es] with time should therefore be a triple exponential:

$$[es]/[e_0] = Xe^{-\alpha t} + Ye^{-\beta t} + Ze^{-\gamma t} \qquad (A2)$$

This result for ES is readily extended to the other intermediates F–H.

An analytical solution to the system can be used to solve for the rate constants only if all three time constants (α, β, and γ) as well as the preexponential factors (X, Y, and Z) can be derived from experimental observations. From inspection of the data in Figure 1 (which measures the proportion of enzyme as ES and F), it is apparent that the initial phase governed by the first exponential is too rapid to be observed. Although the first time constant (α) could not be determined experimentally, the rate constant could still be evaluated. The values of β, γ, B, and C can be obtained graphically from the data in Figure 1 (Fersht, 1977). In order to solve for the rate constants, it is necessary to express them first in terms of the parameters β, γ, Y, and Z. From the expressions for Y and Z and by using the fact that $X = \alpha + \beta + \gamma - k_1$, it can be shown that

$$k_1 = \alpha(1 - Y - Z) + \beta Y + \gamma Z$$

$$y = (\alpha - \gamma)\beta Z + (\alpha - \beta)\gamma Y + \beta\gamma$$

$$y/(\alpha\beta\gamma) = (1/k_1)(1 + k_2/k_5) = (\gamma^{-1} - \alpha^{-1})Z + (\beta^{-1} - \alpha^{-1})Y + \alpha^{-1}$$

If k_1 and $k_2 \gg k_3$, k_4, and k_5 and then since $\alpha \gg \beta$ and γ, we can write

$$\frac{k_1}{k_2} = \frac{1 - Y - Z}{Y + Z}$$

It can also be shown that

$$k_5 = \frac{Y + Z}{(1 - Y - Z)(\gamma^{-1}Z + \beta^{-1}Y)}$$

$$k_4 = \frac{\beta Z + \gamma Y}{Y + Z}$$

$$k_3 = \left(\frac{\beta^{-1}Z + \gamma^{-1}Y}{Y + Z} - \frac{Y + Z}{\beta Z + \gamma Y}\right)\left(\frac{\beta\gamma}{1 - Y - Z}\right)$$

Additionally, the molar ratio of substrate to enzyme (p) needed to inactivate the enzyme irreversibly, is known. The value of p is equal to the number of turnovers prior to inactivation of the enzyme, that is, k_{cat}/k_{inact}. This value of k_{cat}/k_{inact} must equal the ratio of the rate constants that determine the partitioning between hydrolysis and inactivation, i.e., k_2/k_5. Thus

$$k_{cat}/k_{inact} = k_2/k_5 = p$$

This equality can be used to solve explicitly for k_1 and k_2:

$$k_1 = \frac{p}{\gamma^{-1}Z + \beta^{-1}Y}$$

$$k_2 = \frac{p(Y + Z)}{(1 - Y - Z)(\gamma^{-1}Z + \beta^{-1}\gamma)}$$

Finally, a value for α can also be obtained since

$$\alpha = \frac{k_1 k_4 k_5}{\beta\gamma}$$

All of the rate constants can therefore be evaluated numerically, and these results are presented in Table I. This analysis also permits an expression for k_{cat} to be derived. The k_{cat} observed is one that is measured after the first two exponentials have decayed to zero. At this point, the system is at a steady state (ss). The loss of enzyme to an irreversibly inactivated form is negligible at this point, and the effective concentration of enzyme can be represented by $[e_0] - [h_{ss}]$, where $[h_{ss}]$ is the concentration of H at the beginning of this period and is equal to the value of the y intercept in Figure 3. The equation for $[f]/[e_0]$ (and likewise for $[g]/[e_0]$ and $[h]/[e_0]$) can then be approximated as

$$\frac{[f_{ss}]}{[e_0]} = \frac{k_1 k_4 - k_1\gamma}{(\gamma - \alpha)(\gamma - \beta)} e^{-\gamma t}$$

Thus

$$\frac{[f_{ss}]}{[e_0] - [h_{ss}]} = \frac{\gamma}{k_5}$$

Then since

$$v = k_2[f_{ss}]$$

$$k_{cat} = \frac{v}{[e_0] - [h_{ss}]} = \frac{k_2}{k_5}\gamma \qquad \text{(A3)}$$

Since $k_{inact} = \gamma$, $k_{cat}/k_{inact} = k_2/k_5$, as expected.

From eq A3 the value of k_{cat} is calculated to be 6.5 min^{-1}. The value of k_{cat} determined experimentally is 5.6 min^{-1}. The rate constant, k_{react}, was also measured after the effects from the first two exponentials could be neglected. At this point, most of the active (or potentially active) enzyme is bound in the transiently inhibited form (88%), with very little as the acyl-enzyme (3%). The remaining 9% is bound as the Michaelis complex. The reactivation rate of this mixture is therefore predominantly that of transiently inhibited enzyme. Given that the ratio k_2/k_4 (from the data in Table I) is about 300, it is apparent that the transiently inhibited enzyme (G) upon returning to the acyl-enzyme form (F) will regenerate active enzyme. The rate constant for enzyme reactivation is therefore that for the conversion of G to F, which is k_4. The experimentally determined k_{react} (0.044 min^{-1}) is close to the predicted k_4 (0.037 min^{-1}). The agreement of the experimental values with those predicted by the analytical solution supports the adequacy of Scheme II in describing the interactions of the enol ether with the β-lactamase.

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