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β-Lactamase inactivation by mechanism-based reagents

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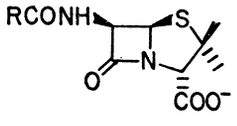
The mechanistic pathway followed by the *E. coli* R_{TEM} β-lactamase has been studied with a view to clarifying the mode of action of a number of recently discovered inactivators of the enzyme. There is clear evidence that the β-lactamase-catalysed hydrolysis of the 7-α-methoxycephem, cefoxitin, proceeds via an acyl-enzyme intermediate. An analysis of the inactivation reactions of all the known β-lactam derivatives that result in irreversible loss of enzyme activity permits the identification of three structural features required for a β-lactamase inactivator. The application of these principles suggests a new group of mechanism-based inactivators of the enzyme: the sulphones of *N*-acyl derivatives of 6-β-aminopenicillanic acid that are themselves poor substrates for the enzyme. These sulphones are powerful inactivators of the β-lactamase.

Introduction

While bacterial resistance to the lethal consequences of exposure to β-lactam antibiotics can arise in a number of ways, by far the most common is the acquisition of the capacity to make a β-lactamase. β-Lactamases catalyse the hydrolysis of the β-lactam ring in penicillins (**1**) and cephalosporins (**2**) with great efficiency, resulting in the formation of the harmless penicilloic acids, or 'cephalosporoic acids' (Hamilton-Miller *et al.* 1970). Since the β-lactamase gene is often carried on a plasmid, the promiscuity of bacteria in terms of plasmid transfer poses a special threat to the continuing therapeutic utility of antibiotics that are sensitive to β-lactamases. It is therefore a matter of some importance both to find β-lactamase-resistant antibiotics and to discover reagents that by inactivating these enzymes will extend the antibacterial spectrum of β-lactams to include those species that produce β-lactamases.



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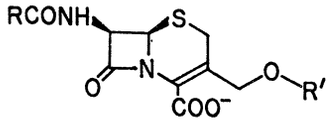

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Despite a number of attempts over the past two decades to synthesize β-lactamase inactivators, it is only relatively recently that successful reagents have been found. In the light of our experience with other hydrolytic enzymes, it is rather curious that efforts to make simple affinity labelling reagents have failed, and that the first example of a potentially useful β-lactamase inactivator should have been isolated from natural sources (Brown *et al.* 1976). Since it will be argued later that all the known inactivators of the enzyme are of the 'suicide' type (where the enzyme catalyses early steps in the normal hydrolytic reaction to produce an intermediate that can react abnormally, and lead to inactivated enzyme (Abeles & Maycock 1976; Walsh 1977)), it is important to establish what is known about the mechanism of the normal hydrolytic reaction catalysed by the β-lactamases.

The mechanism of β-lactamases

Much more information is available about the structure of β-lactamases than about their mechanism. The sequences of the enzyme from three Gram-positive species (*Staphylococcus aureus* (Ambler 1975), *Bacillus licheniformis* (Ambler & Meadway 1969) and *Bacillus cereus* (β-lactamase I: Thatcher 1975*a*)), and from one Gram-negative bacterium (*Escherichia coli* (Ambler & Scott 1978; Sutcliffe 1978)) are known, and work is under way on the crystal structures of three of these proteins (Knox *et al.* 1976; Aschaffenburg *et al.* 1978; D. W. Green, referred to in Thatcher 1975*b*). Further, a number of studies aimed at defining amino acids that are essential to catalytic activity have been published. Modification by such unselective reagents as iodine, tetranitromethane, carbodiimide, triethyloxonium fluoroborate, *N*-bromosuccinimide, diethylpyrocarbonate and iodoacetate, and photooxidation, have all led to loss of β-lactamase activity, and in two cases (nitro-Tyr of the *S. aureus* enzyme (Bristow & Virden 1978) and carboxymethyl-His of the *E. coli* enzyme (Scott 1973)) the modified residue has been located in the sequence. It has long been known that β-lactamases are partially or completely inactivated by some of their substrates (Citri & Garber 1961; Crompton *et al.* 1962; Dyke 1967) and specific 'active-site directed' reagents have been synthesized, including the diazotization products of 6-aminopenicillanic acid (Patil & Day 1973) and of ampicillin (Durkin *et al.* 1977), and the C-3 isocyanates of various penams (Ogawara & Umezawa 1974; Ogawara 1977), but the sites of attack of these reagents have not yet been identified. Quinacillin has been found to be attached to the *S. aureus* enzyme when incubated with enzyme and then quenched from the steady state with denaturants, and up to 0.7 mol of quinacillin per mole of enzyme is covalently bound (Virden *et al.* 1975). Unfortunately, it appears that quinacillin is attached to at least eight peptides after proteolytic digestion of the denatured labelled enzyme (Virden *et al.* 1978), and this finding has not provided useful clues about the mechanism of action of the enzyme. Recently, the *B. cereus* I enzyme has been treated with 6-β-bromopenicillanic acid (Pratt & Loosemore 1978; Knott-Hunziker *et al.* 1979*a*), which stoichiometrically inactivates the enzyme in a reaction that is slower in the presence of substrate. This reagent evidently labels the enzyme cleanly, resulting in attachment to the hydroxyl group of Ser-44 (Knott-Hunziker *et al.* 1979*b*).

The steady-state kinetics of a wide variety of substrates and some competitive inhibitors of β-lactamases from various sources have been examined. These studies have, for the most part, served to define the substrate selectivity of the enzymes rather than the reaction mechanism. It appears (Samuni & Citri 1975; Citri *et al.* 1976) that the kinetics of substrate hydrolysis are not always described accurately by the Michaelis–Menten equation, and it has been suggested that the conformation of β-lactamases may adjust to accommodate substrates of different size, in a relatively slow transition that can be detected by the biphasic reaction of a different substrate added subsequently. The mechanistic significance of these effects is unknown. The pH dependence of the steady-state parameters of the *B. cereus* enzyme with benzylpenicillin has also been determined (Waley 1975).

None of the above studies bears directly upon the most basic mechanistic question that one can ask of a hydrolytic enzyme: does the catalytic reaction proceed via a covalent acyl-enzyme intermediate, or is the susceptible carbonyl group attacked by a water molecule in a pathway where the enzyme acts as a general base? A glance at the pathways followed by the proteases (which also, after all, hydrolyse an amide bond) shows two distinct classes: the first, exemplified by the serine and thiol protease families (Kraut 1977; Lowe 1970), forms well defined and easily

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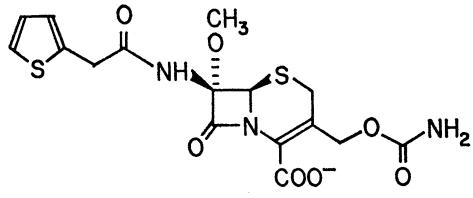
detectable acyl-enzymes; and the second, exemplified by pepsin and carboxypeptidase (Fruton 1970; Kaiser & Kaiser 1972) does not. For the latter, the question of acyl-enzyme intermediates is still somewhat controversial (the only hint of acyl-enzyme formation comes from studies at temperatures below 0 °C (Makinen *et al.* 1976)) and there seems no obvious reason why, if such intermediates do form, they should remain so secluded. In the light of the wholesale failure to detect acyl-β-lactamases by any of the battery of methods that have so successfully exposed the acyl-enzymes of the serine and the thiol proteases (these methods include direct spectrophotometric observation of 'burst' kinetics, and the use of alternate nucleophiles such as [^{14}C]methanol or hydroxylamine to trap the putative intermediate), the prejudice against an acyl-enzyme pathway for the β-lactamases has understandably been gaining ground. An acyl-enzyme seems, indeed, to be energetically not only unnecessary but undesirable when the substrate is as thermodynamically unstable as a β-lactam. The view that β-lactamases use a general base mechanism gains some support from rapid-kinetic studies on the hydrolysis of a chromophoric cephalosporin by the *S. aureus* enzyme. The fact that below pH 6 proton release accompanies the formation of the enzyme–product complex, was taken to favour the general base pathway (S. Halford, referred to in Thatcher 1975*b*). For many substrates of β-lactamase, however, the rate of hydrolysis is so rapid (approaching, in terms of k_{cat}/K_m, the value for a diffusion-limited process) that reaction intermediates and elementary steps in the enzyme-catalysed reaction have not been detected. We have therefore studied cefoxitin, the 7-α-methoxy-cephem (**3**), whose hydrolysis is catalysed by the *E. coli* enzyme more than 10^5 times slower than, for instance, phenoxymethyl penicillin. While there is the ever-present risk that a relatively poor substrate may provide results that are not of universal mechanistic validity, it can hardly be claimed that the use of modified substrates (even *p*-nitrophenylacetate, that over-indulged favourite) has not illuminated the mechanism of other hydrolases.



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When a portion of an incubation mixture of cefoxitin with the *E. coli* β-lactamase is removed to an assay cuvette containing a high concentration of an excellent penicillin substrate, it is observed that the rate at which the new substrate is hydrolysed is initially close to zero. The reaction then accelerates over several minutes, finally reaching a velocity equal to that which would have been observed immediately had there been no cefoxitin present in the first incubation. Cefoxitin is therefore not an inactivator of the enzyme, but a substrate that, during the course of its enzymatic hydrolysis, accumulates in a transient complex whose breakdown to active enzyme can be followed after the dilution. The kinetic competence of this transient complex and its identity as an acyl-enzyme intermediate have been established by a number of chemical, kinetic and spectroscopic experiments.

The transient complex may be isolated intact by cooling an incubation of enzyme and cefoxitin to 0 °C, from the steady state at 30 °C, or it may be intercepted by denaturation of the enzyme–cefoxitin complex with sodium dodecyl sulphate. In the latter case a stable, covalent enzyme–cefoxitin species is isolated with a stoichiometry of 1.0 ± 0.1 mol of cefoxitin per mole of enzyme. Most importantly, examination of the pre-steady-state hydrolysis of cefoxitin by the

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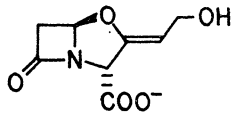
enzyme shows a kinetic 'burst' corresponding to the acylation of the active site, whose time course, along with the previously determined rate constant for the breakdown of the transient complex, allows us to extract the following rate constants:

$$E + C \rightleftharpoons E{\cdot}C \xrightarrow{2.2\times10^{-2}\,s^{-1}} E\text{–}C \xrightarrow{0.48\times10^{-2}\,s^{-1}} E + P,$$

where E is enzyme, C is cefoxitin and P is product). The validity of this kinetic scheme is confirmed by the agreement between the calculated and observed values for k_{cat}, the proportion of enzyme as E–C at the steady state, and the size of the 'burst'. By analogy with the proteases (and, indeed, with the membrane DD-carboxypeptidases (Duez *et al.* 1978) and transpeptidases (Fuad *et al.* 1976) from a number of bacterial species that appear to be acylated by β-lactam antibiotics (Kozarich & Strominger 1978; Rasmussen & Strominger 1978)), it seems probable that E–C is an acyl-enzyme. This view is directly supported by Fourier-transform infrared measurements, that show a transient species absorbing at 1753 cm^{-1} (as expected for an α-methoxyester) which disappears with a time course consistent with the above kinetic scheme. These experiments, taken together, show that the hydrolysis of cefoxitin by the *E. coli* β-lactamase proceeds via an acyl-enzyme intermediate, and constitute the first clear evidence for such an intermediate in reactions catalysed by β-lactamases. The probability that formation of an acyl-enzyme is a general phenomenon even for β-lactam substrates for which the rate of breakdown of the acyl-enzyme is faster than its rate of formation, greatly simplifies the interpretation of the mode of action of the known inactivators of the enzyme. These inactivators are discussed below.

The inactivation of β-lactamases

Since the report of the discovery of clavulanic acid (**4**) in 1976 (Brown *et al.* 1976), four other inactivators of β-lactamases have been reported. Since the levels of detail to which these molecules have been studied are rather different, their interaction with the enzyme will be considered separately. We shall then summarize the common features that seem to be required for an effective inactivator.



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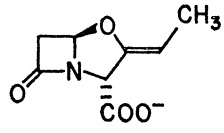

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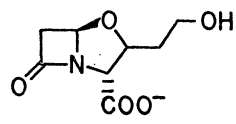

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Clavulanic acid (**4**)

We have shown earlier (Fisher *et al.* 1978; Charnas *et al.* 1978) that clavulanic acid interacts with the β-lactamase from *E. coli* in three ways. First, clavulanate is a substrate for the enzyme and presumably suffers the normal hydrolytic fate of a β-lactam. Secondly, a transient intermediate is formed that slowly regenerates free enzyme. The third interaction involves the irreversible inactivation of the enzyme. The first two phenomena (i.e. β-lactam hydrolysis and transient complex formation) mean that more than 100 molecules of clavulanate are required to inactivate one enzyme molecule. On isoelectric focusing of the completely inactivated enzyme, three protein bands can be discerned, each of which is distinct from the native enzyme. A striking feature of both the inactivated enzyme and the transient complex is a marked increase in the absorbance at about 280 nm that correlates with the loss of enzyme activity. On treatment of

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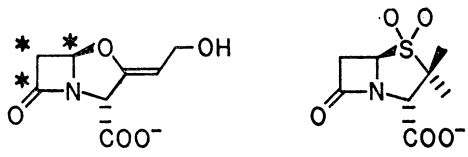

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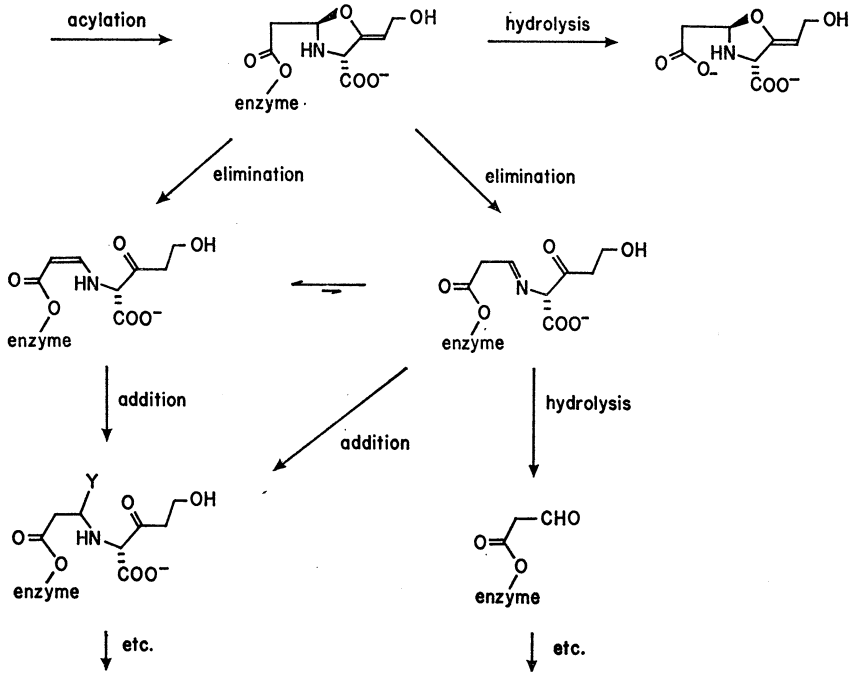
the inactivated enzyme with hydroxylamine, about one-third of the enzyme activity is restored, and one of the three protein bands seen on isoelectric focusing returns to the position of the native enzyme. The rate of recovery of enzyme activity is consistent with (but certainly does not prove) the hydroxylaminolysis of an *O*-acyltyrosine. The behaviour of 9-desoxyclavulanic acid (**5**) (Howarth *et al.* 1977) is almost identical to that of clavulanate itself, both in kinetic and protein-chemical terms. This rules out mechanisms that involve the allylic alcohol group of the parent molecule, **4**. The dihydro derivative (**6**) (Brown & Howarth 1976) does not inactivate the enzyme. Finally, the use of clavulanic acid (**7**) labelled with ^{14}C at C-5, C-6 and C-7 yields inactive enzyme containing 0.82 ± 0.03 mol inactivator per mole of enzyme. This stoichiometry is unchanged whether the inactivation is performed at 0 °C (when the middle band of inactivated enzyme constitutes 67 % of the inactive material), at 30 °C (when the three bands of inactive enzyme are in about equal proportion) or at 44 °C (when the species with the lowest p*I* constitutes 61 % of the inactive material). These data suggest that in *each* of the three inactive species, at least the three labelled carbons of **7** remain covalently attached to the enzyme.



SCHEME 1. Some possible pathways for the inactivation of β-lactamase by clavulanic acid (**4**).

The aspects of the interaction of clavulanic acid and the β-lactamase outlined above can be accommodated by the kinds of reaction illustrated in scheme 1. Acylation of the enzyme (possibly on the 'normal' hydrolytic pathway, cf. cefoxitin, above) may be followed by a β-elimination reaction that results in the ene-aminocarbonyl derivative. Such materials are known

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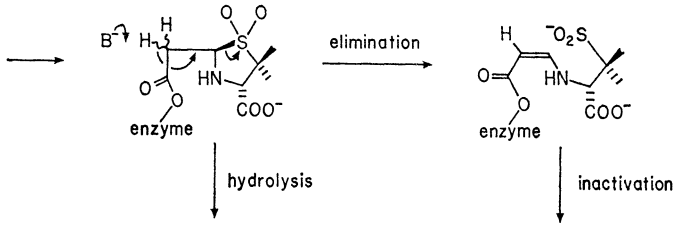
to absorb very strongly near 280 nm. Whether scheme 1 will turn out to represent correctly the pathways of the clavulanate inactivation reaction remains to be seen; there is certainly good chemical and enzymological precedent for the suggestions therein.

Penicillanic acid sulphone (*CP-45,899*) (**8**)

This material, first reported by English *et al.* (1978), is both a substrate and an irreversible inactivator of the *E. coli* β-lactamase. The hydrolysis of **8** can be monitored at 235 nm ($\Delta\epsilon$ = 2500) and has a k_{cat} of 1.2 s^{-1}. The K_m is 0.9 μM (as determined by using **8** as a competitive substrate and monitoring benzylpenicillin hydrolysis at 240 nm) which makes **8** a potent short-term inhibitor of the enzyme. Incubation of the β-lactamase with a large molar excess (5000-fold) of **8**, however, leads to irreversible inactivation of the enzyme. The first-order rate constant for enzyme inactivation is quite small (2.6×10^{-4} s^{-1}), which means that **8** suffers a considerable amount of hydrolytic turnover before enzyme inactivation. Neither exhaustive dialysis nor gel filtration restores activity, and the inactive enzyme shows a large increase in absorbance at 280 nm (cf. clavulanic acid, above). Hydroxylamine treatment leads to the return of about 25 % of the catalytic activity with a rate constant (0.12 M^{-1} s^{-1}) nearly identical to that for the clavulanate reactivation. Many of the features of the interaction of **8** with the enzyme are thus strikingly reminiscent of the behaviour of clavulanate, and it is tempting to suggest that **8** is a β-lactamase inactivator (whereas the parent 6-desaminopenicillanic acid is not) by virtue (*a*) of the greater acidity of the protons at C-6 and (*b*) of the weakening of the bond between sulphur and C-5, both of which are consequences of the oxidation of sulphur to the sulphone. The generation of a chromophoric entity (scheme 2) analogous to that suggested in scheme 1 is therefore to be expected. Once again, it should be emphasized that this is only one of a number of possible fates of **8**.



SCHEME 2. Possible pathway for the inactivation by penicillanic acid sulphone (**8**).

6-β-*Bromopenicillanic acid* (**9**)

It has recently been noted independently by Pratt & Loosemore (1978) and by Waley and co-workers (Knott-Hunziker *et al.* 1979*a*) that whereas 6-α-bromopenicillanic acid is without effect on β-lactamase, incubation at high pH allows epimerization at C-6 to produce a small (approx. 10 %) equilibrium proportion of the 6-β-bromo compound, which is an inactivator of the enzyme. The inactivated protein develops a chromophore with a λ_{max} of 314 nm after denaturation (Knott-Hunziker *et al.* 1979*a*). This value is rather characteristic of a 2,3-dihydro-1,4-thiazine, to which it is known that methyl 6-chloropenicillanate rearranges in sodium methoxide in methanol (McMillan & Stoodley 1966, 1968). Should it be established that the protein-bound chromophore does indeed arise from the dihydro-thiazine, then the identification by Waley's group (Knott-Hunziker *et al.* 1979*b*) of serine-44 as the site of attachment suggests that the pathway of inactivation for 6-β-bromopenicillanate involves acyl-enzyme formation

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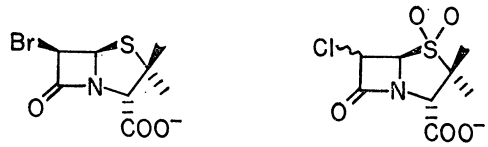
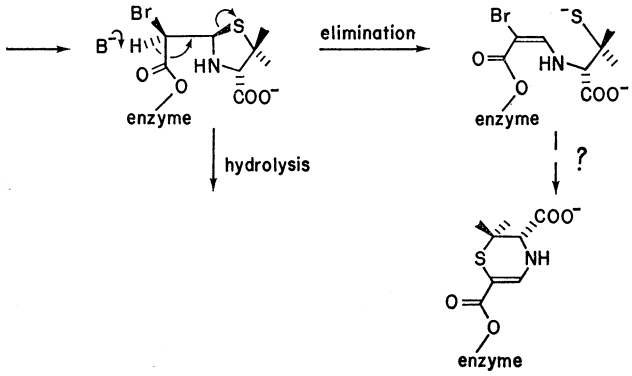


9 **10**

at Ser-44 followed by skeletal rearrangement of the acyl-enzyme (scheme 3). The appearance of this chromophore adds credence to the view that β-lactamases are 'serine enzymes' that proceed via acyl-enzyme intermediates. The analogy with clavulanate (**4**) and the sulphone (**8**) may be close, since *anti*-elimination of the 6-α-proton (which is made more acidic by the 6-β-bromo substituent) may be the route by which enzyme inactivation results.



SCHEME 3. Possible pathway for the inactivation of β-lactamase by 6-β-bromopenicillanic acid (**9**).

6-*Chloropenicillanic acid sulphone* (**10**)

Very recently, Cartwright & Coulson (1979) have reported that incubation of the β-lactamase from *Staphylococcus aureus* with 6-α-chloropenicillanic acid sulphone leads to inactivation of the enzyme. Since both the sulphone group and the 6-chloro substituent will increase the acidity of the 6-β-proton (compared with 6-desaminopenicillanic acid and with **9**), we may expect rapid epimerization at C-6 at neutral pH (Pant & Stoodley 1978). Allowing this, the formal schemes proposed for **4**, **8** and **9** may readily apply to compound **10**. Epimerization to the 6-β-chloro compound will yield a β-lactamase substrate, the acyl-enzyme from which can suffer a facile *anti*-elimination across the bond between C-5 and C-6. This provides the chromophore analogous to those suggested for **4**, **8** and **9**, and observed by Cartwright & Coulson for **10**.

Olivanic acid (**11**)

The almost simultaneous discovery of two examples of a new skeletal class of β-lactams, the 1-carbapenems, provided two interesting species: olivanic acid (**11**) (Brown *et al.* 1977; Maeda *et al.* 1977) and thienamycin (**12**) (Kahan *et al.* 1976; Albers-Schönberg *et al.* 1978). Perhaps the most striking feature of thienamycin is the α-stereochemistry at C-6, while olivanic acid has the 'normal' substitution on the β face. Olivanic acid is extremely effective in synergy with susceptible β-lactam antibiotics, and may act by inactivation of the β-lactamase. In contrast, thienamycin is a very powerful broad-spectrum antibiotic. Until these materials become available and enzymological studies become feasible, we may only speculate. But in scheme 4 we suggest that acyl-enzyme formation from olivanic acid may be followed by β-elimination of the 6-α-proton to produce a Michael acceptor at the active site in what would be a

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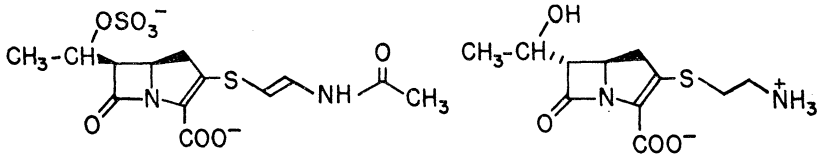

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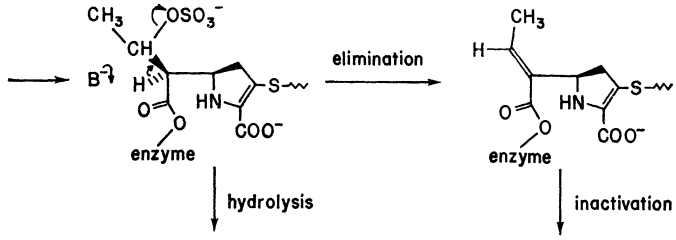
reasonable 'suicide' pathway. (We should not, perhaps, overstress the importance of Michael acceptors, for it may be simply that the β-elimination reactions proposed in schemes 1, 2 and 4 merely serve to stabilize the acyl-enzyme against subsequent hydrolytic breakdown.)



SCHEME 4. Suggested pathway for the inactivation of β-lactamase by olivanic acid derivatives (**11**).

Methicillin sulphone (**13**) *and quinacillin sulphone* (**14**)

The discussion above may be summarized as follows. An effective β-lactam inactivator for β-lactamase must satisfy three criteria (see figure 1). First, the enzyme-catalysed hydrolysis of the reagent should proceed via an acyl-enzyme that hydrolyses only slowly, so that there is ample opportunity for the postulated β-elimination to occur within the acyl-enzyme. Secondly, there should be a 6-α-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.

(*a*) appropriate β-lactam for acyl-enzyme formation

(*b*) 6-α-proton of adequate acidity

(*c*) leaving group for *anti*-elimination

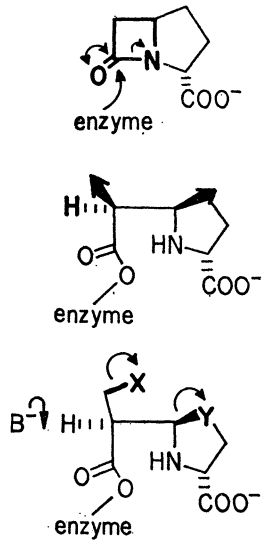


FIGURE 1. Features of mechanism-based β-lactamase inactivators.

These three conditions can be simply met by oxidation of the sulphur atom in 6-β-acylamino-penams that are *poor* substrates of the β-lactamase. Poor substrates are most likely to yield long-lived acyl-enzyme intermediates, and the sulphone group not only acidifies the 6-α-proton

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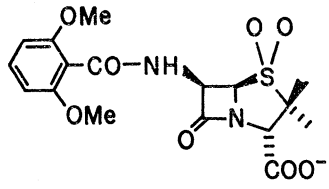

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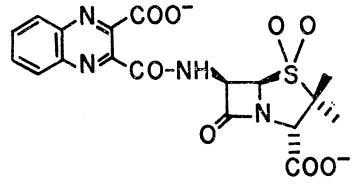

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(see, for example, Pant & Stoodley 1978) but also provides a better leaving group (a sulphinate) for the putative β-elimination step. We have, accordingly, investigated the behaviour of the sulphones of two poor β-lactamase substrates, methicillin and quinacillin, with the enzyme. It is evident that the expectations presented above are borne out by the data shown in table 1. The sulphone of a *good* substrate, phenoxymethyl penicillin, does not inactivate the enzyme, whereas the sulphones of the poor substrates are effective inactivators of the enzyme. These results support the mechanistic proposals made in the schemes, and the validity of the criteria used in the choice of these sulphone reagents.

TABLE 1. KINETIC CHARACTERISTICS OF THE INTERACTION OF VARIOUS PENAM SULPHONES WITH THE β-LACTAMASE FROM *E. COLI* R_{TEM}

compound	k_{cat} for the parent penam / s^{-1}	$t_{\frac{1}{2}}$ for inactivation by the sulphone / min	number of hydrolytic turnovers before enzyme inactivation
penicillin V sulphone	1000	—	> 100000
penicillanic acid sulphone (**8**)	40	44	4500
methicillin sulphone (**12**)	10	~ 1	22500
quinacillin sulphone (**13**)	7	~ 1	400

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