Penicillin Resistance: The Chemistry of β-Lactamase Inhibition

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Soon after benzylpenicillin (1) was introduced into clinical practice at the end of World War II, the emergence of resistant strains of pathogenic microorganisms threatened its efficacy as an antibiotic. By 1949, more than half of all *Staphylococcus pyogenes* isolates from a U.K. hospital were resistant,[1] and the continued usefulness of this splendid addition to the meager armoury of chemotherapeutics was in doubt.[2] In this Account, we focus on the commonest cause of bacterial resistance to β-lactam antibiotics, the β-lactamases, and summarize what is known about the mechanism of action of the reagents that, in the past 8 years, have been discovered and synthesized to combat this defensive activity of resistant bacteria.

The voyage of a β-lactam antibiotic when it attacks a Gram-negative bacterium such as *E. coli* or a *Pseudomonad* is illustrated in Figure 1. To start with, the antibiotic must cross the outer membrane by passive diffusion through channels formed by the "porin" proteins. These channels do present some barrier to free access to the intermembrane space (the periplasm), though small hydrophilic species (of $M_r < 650$ or so) appear to penetrate most readily.[3] The antibiotic then crosses the cell wall, which is a cross-linked peptidoglycan net upon which the structural integrity of the cell depends (bacteria have no mechanism to balance their internal osmolarity with the outside). The cell wall is presumed to present no further barrier to the periplasm: the "holes" in this network are large enough to allow the free passage of a small-molecule antibiotic.

The β-lactam must then cross the periplasm on its way to the targets of its action, which are the inner membrane enzymes that are responsible for the biosynthesis of the cell wall.[4] If the bacterium carries the gene for the synthesis of a β-lactamase, then the periplasm may contain several thousand copies of this enzyme. For example, an *E. coli* cell carrying the plasmid RP4 contains about 65 000 molecules of β-lactamase in the periplasm,[5] which may catalyze the hydrolysis of the penicillin 1 to the harmless monocyclic penicilloic acid product 2 (Figure 2). Finally, if the antibiotic has managed to penetrate the periplasm and has survived the crossing to the inner membrane, it can inactivate one or more of the enzymes involved in cell-wall synthesis by acylating (at least in some cases[6]) a serine hydroxyl group at the active site. While different β-lactam antibiotics appear to have different targets,[7] and while the exact "killing sites" (and the subsequent events that result in cell lysis and death[8]) are still under discussion, there is no doubt that interference of cell-wall biosynthesis is the primary bacteriocidal act of a β-lactam antibiotic.

There are clearly several ways in which a bacterial population can become resistant.[9] First, the target enzymes may become less susceptible to acylation and inactivation. Second, changes in outer membrane permeability governed by the porins may so limit the access of the antibiotic to the periplasm that target enzyme activity remains high enough to allow cell growth and division. Third, alterations in the activities of other enzymes that are responsible for lysis of cells whose cell-wall synthetic apparatus has been blocked by the β-lactam may lead to cell stasis rather than cell death. Finally, the appearance of a β-lactamase may result in the hydrolytic destruction of the antibiotic in the periplasm before it can reach its target. Of all the types of resistance the last is the most common,[9] and a solution to this problem is critical for the continuing clinical utility of β-lactams as antibacterial agents. This challenge is especially important in light of the promiscuous exchange of the genetic information that codes for β-lactamase synthesis, both within and among bacterial populations.

There are two ways to overcome the destructive action of the β-lactamase. The first is to alter the structure of the β-lactam, rendering it insensitive to hydrolysis by the β-lactamase while maintaining its potency as an antibiotic. The second approach uses a reagent that incapacitates the β-lactamase, in synergy with a β-lactam antibiotic that would otherwise be rapidly destroyed by the enzyme. The first strategy *avoids* the β-lactamase, and the second *neutralizes* it. Avoidance first became a viable approach when the deacylated penam, 6-aminopenicillanic acid (1, R = H), was produced both microbiologically[10] and by de novo synthesis,[11] and when the corresponding cephem 7-aminocephalosporanic acid was made from the natural product cephalosporin C.[12] The availability of these

Jeremy Knowles is an Englishman for whom we have already twice had occasion to publish a biographical sketch: *Acc. Chem. Res.* **1972**, *5*, 155 and *Acc. Chem. Res.* **1977**, *10*, 105. He is now Amory Houghton Professor of Chemistry and Biochemistry at Harvard University.

(1) Barber, M.; Rozwadowska-Dowzenko, M. *Lancet* **1948**, *2*, 641–644.
(2) It must be said, however, that the resistance rate was much higher for isolates from hospital in-patients (68%) than from out-patients (12.5%): Forbes, G. B. *Br. Med. J.* **1949**, *2*, 569–571.
(3) Nikaido, H. In "β-lactam Antibiotics"; Salton, M. R. J., Shockman, G. D., Eds.; Academic Press: New York, 1981, pp 249–260.
(4) Blumberg, P. M.; Strominger, J. L. *Bacteriol. Rev.* **1974**, *38*, 291–335.
(5) Easton, C. J., unpublished work.
(6) Yocum, R. R.; Waxman, D. J.; Rasmussen, J. R.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2730–2734.
(7) Spratt, B. G. *Philos. Trans. R. Soc., Ser. B* **1980**, *289*, 273–283.
(8) Tomasz, A. *Annu. Rev. Microbiol.* **1979**, *33*, 113–137.
(9) Sykes, R. B. In "β-lactam Antibiotics"; Salton, M. R. J., Shockman, G. D., Eds.; Academic Press: New York, 1981; pp 199–214.
(10) Batchelor, F. R.; Doyle, F. P.; Nayler, J. H. C.; Robinson, G. N. *Nature (London)* **1959**, *183*, 257–258.
(11) Sheehan, J. C.; Henery-Logan, K. R. *J. Am. Chem. Soc.* **1959**, *81*, 5838–5839.
(12) Newton, G. G. F.; Abraham, E. P. *Biochem. J.* **1956**, *62*, 651–658. Loder, B.; Newton, G. G. F.; Abraham, E. P. *Biochem. J.* **1961**, *79*, 403–408.

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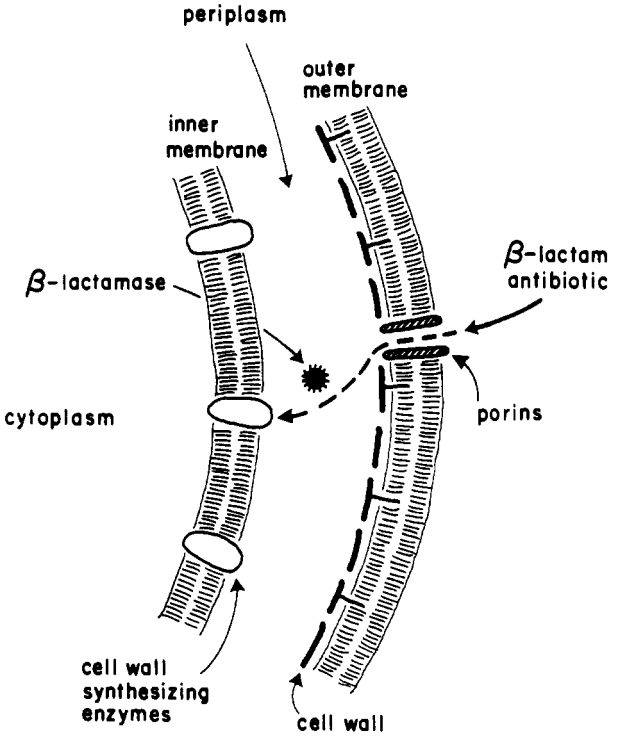


Figure 1. Section through the cell envelope of a Gram-negative bacterium, illustrating the voyage of a β-lactam antibiotic.

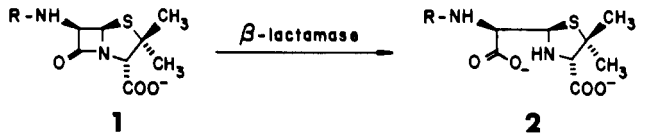


Figure 2. Action of the β-lactamase on a penam (1) to yield a penicilloic acid (2). R is an acyl group.

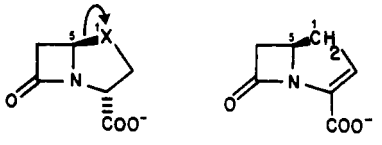
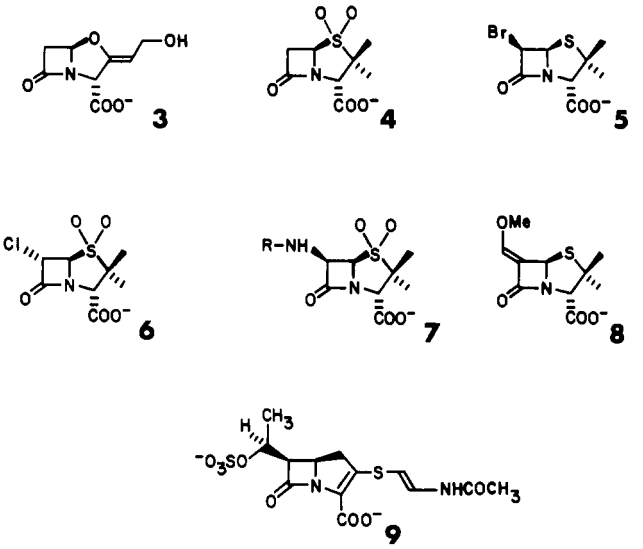


Figure 3. Two classes of mechanism-based β-lactamase inhibitors.

amino compounds allowed the semisynthesis of an immense number of acylated penams and cephems varying in their *N*-acyl side chains. Yet it was often found that molecules more resistant to the β-lactamase were also less good as antibiotics, thus frustrating the modification. This finding is unsurprising, since at least some of the enzymes of cell-wall biosynthesis are acylated by penams at a unique serine residue in a peptide that shows convincing homology[6] with the serine residue involved in acyl-enzyme formation by the β-lactamase,[13] and we might expect that it would not be trivial to find β-lactams that would rapidly acylate the cell-wall-synthesizing enzymes and yet not interact with the (probably evolutionarily related) β-lactamase.

The second, neutralizing, strategy for overcoming β-lactamase-derived bacterial resistance was therefore pursued. It was hoped thus to extend the range of good (but β-lactamase-sensitive) antibiotics so that they would be effective against resistant strains. The first success came in 1976 with the isolation of clavulanic acid[14] (3), which was capable of protecting susceptible



β-lactam antibiotics against the hydrolytic action of the bacterial β-lactamase. Indeed, a combination of amoxycillin and clavulanate ("Augmentin") is now in clinical use. The finding of clavulanate was quickly followed by reports of the discovery and synthesis of a number of other reagents (e.g., 4 through 9) having powerful inhibitory properties toward β-lactamases.[15] Mechanistic investigation of the interaction of these molecules with the β-lactamase has shown that they are all "suicide" or "mechanism-based" reagents, in the sense that they are recognized by the enzyme as potential substrates yet lead, in a diversion from the normal course of the hydrolytic reaction, to inhibition and/or inactivation of the enzyme.[16,17] As will become

(13) Fisher, J. F.; Belasco, J. G.; Khosla, S.; Knowles, J. R. *Biochemistry* **1980**, *19*, 2895–2901. Kiener, P. A.; Knott-Hunziker, V.; Petursson, S.; Waley, S. G. *Eur. J. Biochem.* **1980**, *109*, 575–580. Cartwright, S. J.; Coulson, A. F. W. *Philos. Trans. R. Soc., Ser. B* **1980**, *289*, 370–372. Anderson, E. G.; Pratt, R. F. *J. Biol. Chem.* **1981**, *256*, 11401–11404; **1983**, *258*, 13120–13126.

(14) Brown, A. G.; Butterworth, D.; Cole, M.; Hanscomb, G.; Hood, J. D.; Reading C.; Rolinson, G. N. *J. Antibiot.* **1976**, *29*, 668–669.

(15) For example, penicillanic acid sulfones (English, A. R.; Retsema, J. A.; Girard, A. E.; Lynch, J. E.; Barth, W. E. *Antimicrob. Agents Chemother.* **1978**, *14*, 414–419. Cartwright, S. J.; Coulson, A. F. W. *Nature (London)* **1979**, *278*, 360–361. Fisher, J. F.; Knowles, J. R. In "Enzyme Inhibitors as Drugs"; Sandler, M., Ed.; MacMillan: London, 1980; pp 209–218. Fisher, J. F.; Charnas, R. L.; Bradley, S. M.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2726–2731); 6-halopenams (Pratt, R. F.; Loosemore, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4145–4149. Knott-Hunziker, V.; Orlek, B. S.; Sammes, P. G.; Waley, S. G. *Biochem. J.* **1979**, *177*, 365–367. Von Daehne, W. *J. Antibiot.* **1980**, *33*, 451–452); carbapenems (Kahan, J. S.; Kahan, F. M.; Goegelman, R.; Currie, S. A.; Jackson, M.; Stapley, E. O.; Miller, T. W.; Miller, A. K.; Headlin, D.; Mochales, S.; Hernandez, S.; Woodruff, H. B. *Abstr. 16th Interscience Conf. Antimicrob. Agents Chemother., Chicago* **1976**, 227. Brown, A. G.; Corbett, D. F.; Eglington, A. J.; Howarth, T. T. *J. Chem. Soc., Chem. Commun.* **1977**, 523–525. Maeda, K.; Takahashi, S.; Sezaki, M.; Iinuma, K.; Naganawa, H.; Kondo, S.; Ohno, S.; Umezawa, H. *J. Antibiot.* **1977**, *30*, 770–772. Okamura, K.; Sakamoto, M.: Ishikura, T. *J. Antibiot.* **1980**, *33*, 293–302. Nakayama, M.; Iwasaki, A.; Kimura, S.; Mizoguchi, T.; Tanabe, S.; Murakami, A.; Watanabe, I.; Okuchi, M.; Itoh, H.; Saino, Y.; Kobayashi, F.; Mori, T. *J. Antibiot.* **1980**, *33*, 1388–1390); and sulfenimines (Gordon, E. M.; Chang, H. W.; Cimarusti, C. M.; Toeplitz, B.; Gougoutas, J. Z. *J. Am. Chem. Soc.* **1980**, *102*, 1690–1702).

(16) Omitted from consideration in this *Account* are the penam isocyanates (Ogawara, H.; Umezawa, H. *J. Antibiot.* **1974**, *27*, 567–569), reagents such as phenylpropynal (Schenkein, D. P.; Pratt, R. F. *J. Biol. Chem.* **1980**, *255*, 45–48), alkyl sulfates such as izumenolide (Liu, W.-C.; Astle, G.; Wells, J. S.; Trejo, W. H.; Principe, P. A.; Rathnum, M. L.; Parker, W. L.; Kocy, O. R.; Sykes, R. B. *J. Antibiot.* **1980**, *33*, 1256–1261), and boronic acids (Kiener, P. A.; Waley, S. G. *Biochem. J.* **1978**, *169*, 197–204. Beesley, T.; Gascoyne, N.; Knott-Hunziker, V.; Petursson, S.; Waley, S. G.; Jaurin, B.; Grundström, T. *Biochem. J.* **1983**, *209*, 229–233). These materials, even if affinity reagents, can be presumed to be less specific than the mechanism-based group of compounds and do not, moreover, pose such interesting problems at the enzyme level.

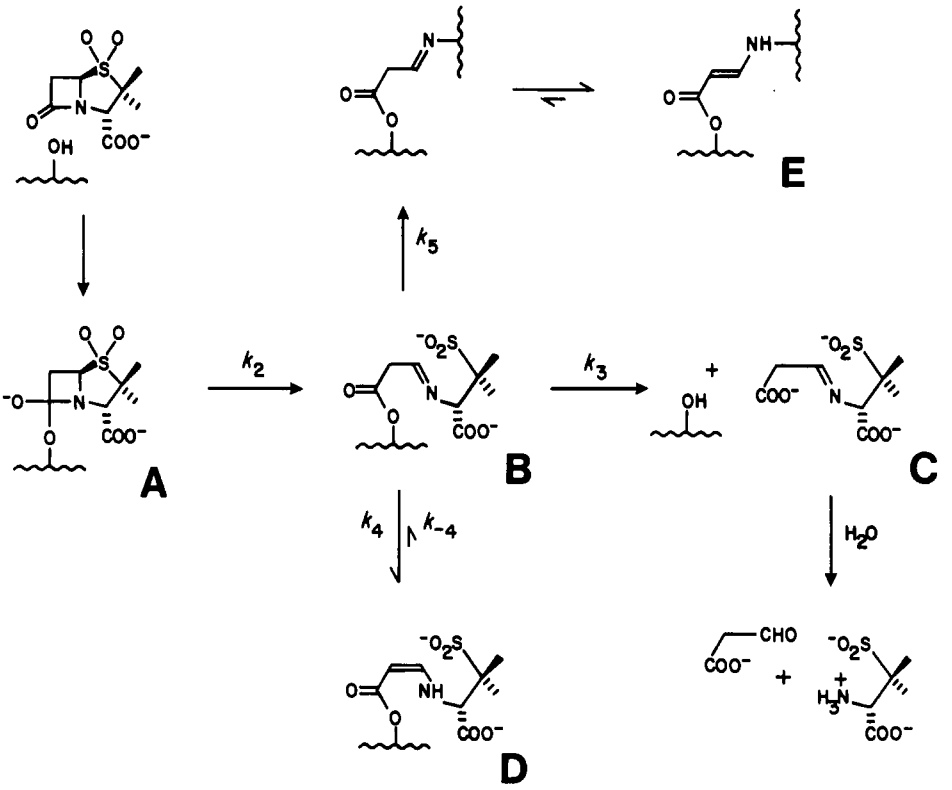


Figure 4. Interaction of penicillanic acid sulfone (4) with the β-lactamase.

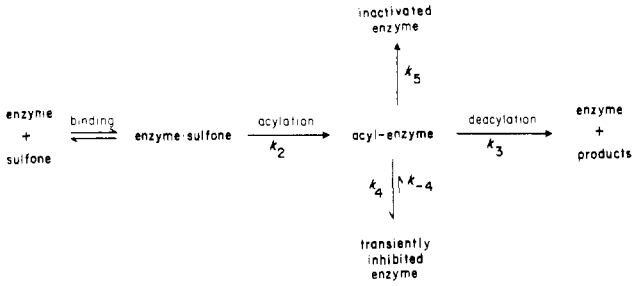
evident from what follows, all of these mechanism-based inhibitors of the β-lactamase fall into two classes: those that contain a heteroatom at the 1-position which may act as a leaving group from carbon-5, and those such as the carbapenems that do not (see Figure 3). We shall discuss the interaction of one example of each of these classes with the purified plasmid-encoded TEM β-lactamase. The sequence of this protein[18] and of its gene[19] are known, and crystallographic studies are in progress.[20] This β-lactamase is responsibe for a large proportion of β-lactam resistance in clinical isolates, but it must be emphasized that there are several classes of β-lactamase and considerable variation in specificity and kinetic behavior of the enzymes even within one class.[21] So while the nature of the overall pathways followed by substrates and inhibitors is clear (see below), the relative importance of particular intermediates depends upon the way in which, for a given enzyme, the different species partition among the various routes open to them.

Penicillanic Acid Sulfone (4). Although clavulanic acid (3) is historically the archetype for the class I inhibitors,[22] the nature of the interaction of penicillanic acid sulfone 4 is simpler and has been more thoroughly investigated,[23] and this will be presented here. When 4 is added to the enzyme and the remaining catalytic activity is followed with time, three distinct processes can be discerned. First, the sulfone is a substrate in the sense that the enzyme catalyzes the hydrolytic opening of the β-lactam ring. Second, 4 is an inhibitor of the enzyme: at pH 8, about 10 molecules of sulfone are consumed as the enzyme accumulates into a transiently inhibited state. Third, interaction of the enzyme with 4 ultimately leads to irreversible inactivation. Since we know from other work[13] that the normal course of the enzyme-catalyzed hydrolysis of β-lactam substrates involves an acyl-enzyme intermediate, the three reactions of 4 can be formulated as shown in Scheme I. What chemistry does this scheme conceal?

In the hydrolytic reaction of 4, the product is not the monocyclic penicilloic acid corresponding to 2, but malonsemialdehyde and the sulfinate of penicillamine.[23] That is, the action of the enzyme results in the cleavage of *three* bonds in 4 and the fragmentation of the molecule into two parts. This is readily rationalized in Figure 4, in which the active-site serine hydroxyl group attacks the β-lactam carbonyl to form a tetrahedral intermediate A, which then collapses to the acyl-enzyme B. (This collapse presumably occurs in a stepwise fashion, since the reverse reaction would otherwise be a 5-endo-trig closure on an imine rather than on an iminium ion.) This formulation accommodates the well-known instability of thiazolidine sulfones.[24] If the acyl-enzyme B follows the normal course of deacylation, the imine C will be liberated, and this will then hy-

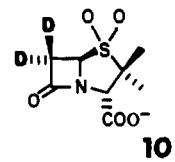
(17) Cartwright, S. J.; Waley, S. G. *Med. Res. Rev.* **1983**, *3*, 341–382.
(18) Ambler, R. Scott, G. K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3732–3736.
(19) Sutcliffe, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3737–3741.
(20) Knox, J. R.; Kelly, J. A.; Moews, P. C.; Murthy, N. S. *J. Mol. Biol.* **1976**, *104*, 865–875.
(21) Hamilton-Miller, J. M. T., Smith, J. T., Eds. "β-lactamases"; Academic Press: London, 1979.
(22) Fisher, J. F.; Charnas, R. L.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2180–2184. Charnas, R. L.; Fisher, J. F.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2185–2189. Charnas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3214–3219. Reading, C.; Hepburn, P. *Biochem. J.* **1979**, *179*, 67–76. Durkin, J. P.; Viswanatha, T. *J. Antibiot.* **1978**, *31*, 1162–1169. Cartwright, S. J.; Coulson, A. F. W. *Nature (London)* **1979**, *278*, 360–361.
(23) Labia, R.; Lelievre, V.; Peduzzi, J. *Biochim. Biophys. Acta* **1980**, *611*, 351–357. Fisher, J. F.; Charnas, R. L.; Bradley, S. M.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2726–2731. Brenner, D. G.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3680–3687. Kemal, C.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3688–3695.
(24) Woodward, R. B.; Neuberger, A.; Trenner, N. R. In "The Chemistry of Penicillin"; Clarke, H. T., Johnson, J. R., Robinson, R., Eds.; Princeton University Press: Princeton, NJ, 1949; Chapter 14.

Scheme I. Kinetic Pathways for the Interaction of Penicillanic Acid Sulfone with the β-Lactamase



drolyze spontaneously to give the two observed products. The second reaction of 4 is as a transient inhibitor of the enzyme. This inhibition is slower than the hydrolytic process, and it is easy to isolate enzyme (after a few minutes' incubation with 4) that is more than 90% in the transiently inhibited form. This form of the enzyme shows a large increase in absorbance at 290 nm ($\epsilon \sim 20\,000$),[23] which is consistent with the formation of the enamine D as the more stable tautomer of the imine B. This tautomerization (Figure 4) accommodates both the kinetic and spectral characteristics of the transiently inhibited enzyme. Finally, in a reaction that is much slower than either the hydrolytic process or the accumulation of enzyme into the transiently inhibited form D, the enzyme is irreversibly inactivated. Some 7000 molecules of 4 are hydrolyzed before all catalytic activity is lost, and the inactivated enzyme has a new absorption at 280 nm ($\epsilon \sim 16\,000$)[23] which is similar to (though not identical with) the new chromophore in the transiently inhibited species D. The appearance of the new absorption correlates with inactivation of the enzyme. When radiolabeled 4 (tritiated in one of the C-2 methyl groups) is used to inactivate the enzyme, the purified inactive protein has less than 5% of the radiolabel expected on the basis of a stoichiometric inactivation reaction.[25] These results can be explained by postulating that the third fate of the acyl-enzyme B is a transimination reaction by an enzyme lysine residue. Such a reaction would lead to the new chromophore E (Figure 4), and an inactivated enzyme in which two active site residues have been cross-linked. In the species E, half of the suicide weapon has been discarded post mortem.

Perhaps the most convincing confirmation that Figure 4 properly represents the interaction of 4 with the enzyme is the rather bizarre consequence of using the dideuterated sulfone, **10**. When the β-lactamase is



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incubated with this labeled analogue, both the hydrolytic reaction and the irreversible inactivation reaction are *accelerated* by about threefold.[23] These increases in rate are indeed due to the normal expression of a

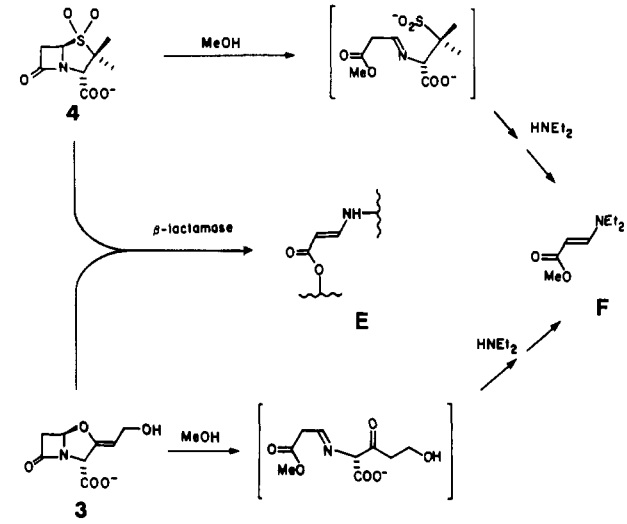


Figure 5. Analogies between the interaction of penicillanic acid sulfone (4) and clavulanic acid (3) with the β-lactamase and with methanolic diethylamine.

primary isotope effect and arise as follows. Aside from small secondary effects, acylation (k_2, Scheme I), deacylation (k_3), and inactivation (k_5[26]) will not be affected by the isotopic substitution. Yet the tautomerization of B to D (k_4) will be slowed by a normal primary isotope effect. Since the isotope that is removed in the conversion of B to D will then rapidly exchange with the unlabeled solvent, k_{-4} will always involve ^{1}H and be the same for both 4 and **10**. That is, the *only* isotopically sensitive step is k_4. The consequence of this is that when **10** is used, less of the enzyme is tied up as the transiently inhibitied species D, and 3 times as much enzyme is available for the other two processes, hydrolysis and inactivation. This is one of rather few examples in the literature[27] of what Jencks[28] has called the transmogrification of an isotope effect.

That the transformations of Figure 4 are chemically reasonable and provide the framework for all the class I β-lactamase inactivators is illustrated in Figure 5. Treatment of either clavulanic acid[29] or the sulfone 4[25] with methanolic diethylamine leads to the enamine F, presumably by methanolysis of the β-lactam followed by transimination (Figure 5), in precise analogy to the production of the inactivated enzyme E shown in Figure 4. Indeed, the fact that the single species of inactivated enzyme[25] deriving from the sulfone 4 is identical on isoelectric focusing with one of the three forms of inactivated enzyme[22] from clavulanate 3 suggests that part of the inactivation chemistry is common to both reagents and only involves the three carbon atoms (of the β-lactam ring) that are shared by both 3 and 4. While other reagents in class I, for example, the β-bromopenam 5, clearly have other fates,[30] these are

(25) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1984**, *23*, 5833–5839. In contrast to the loss of the ^{3}H label in the TEM enzyme from *E. coli* after inactivation with [^{3}H]penicillanic acid sulfone, Mezes et al. report a stoichiometry of 0.82 for the *B. cereus* I enzyme after inactivation with 6β-[[(trifluoromethyl)sulfonyl]amino][^{3}H]penicillanic acid sulfone (Mezes, P. S. F.; Clarke, A. J.; Dimtrienko, G. I.; Viswanatha, T. *J. Antibiot.* **1982**, *35*, 918–920).

(26) We may assume that transimination from B (Figure 4) is slower than the subsequent tautomerization to E.

(27) For example: Keefe, J. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1983**, *105*, 265–279. Samuelson, A. G.; Carpenter, B. K. *J. Chem. Soc., Chem. Commun.* **1981**, 354–356.

(28) Jencks, W. P. Enzymes Gordon Conference, July 1980.

(29) Davies, J. S.; Howarth, T. T. *Tetrahedron Lett.* **1982**, 3109–3112.

(30) Pratt, R. F.; Loosemore, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4145–4149. Knott-Hunziker, V.; Orlek, B. S.; Sammes, P. G.; Waley, S. G. *Biochem. J.* **1979**, *177*, 365–367. Cohen, S. A.; Pratt, R. F. *Biochemistry* **1980**, *19*, 3996–4003. Orlek, B. S.; Sammes, P. G.; Knott-Hunziker, V.; Waley, S. G. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2322–2329.

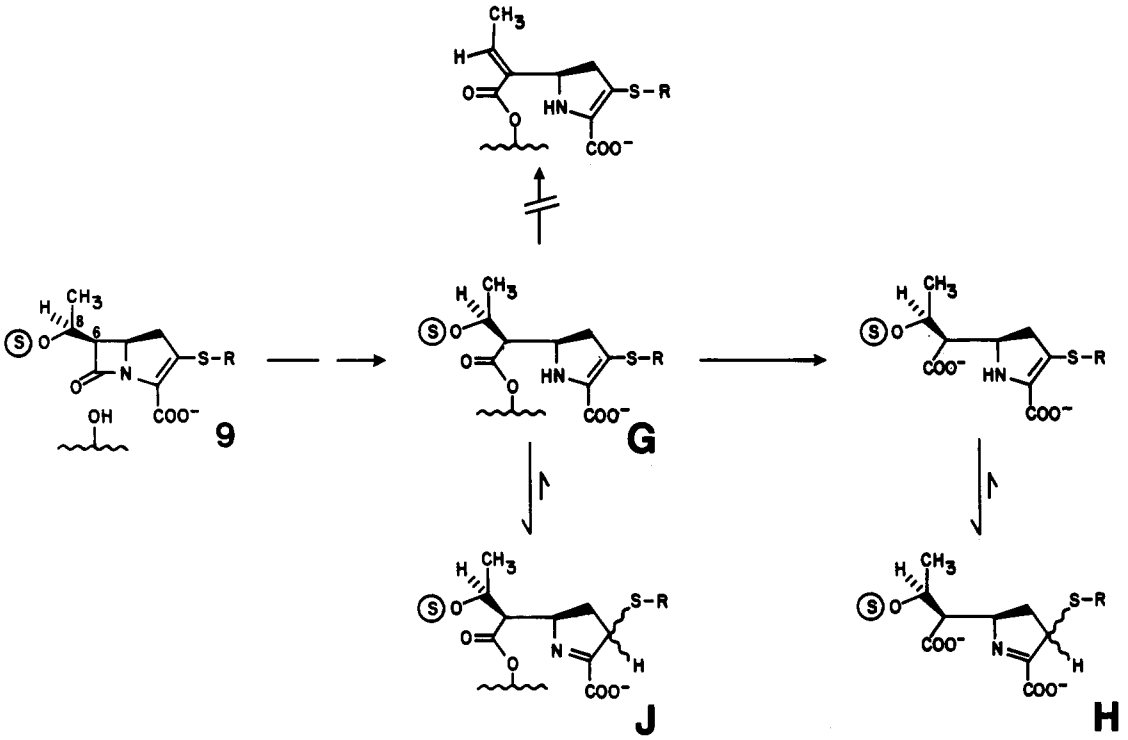


Figure 6. Interaction of a carbapenem (MM13902) with the β-lactamase.

effectively opportunistic diversions[31] from the intermediates illustrated in Figure 4.

Olivanic Acid (9). The effectiveness of a number of carbapenems as inhibitors of the β-lactamase in vivo[32] is obviously not accommodated by chemical events analogous to those outlined in Figure 4 that require cleavage of the bond between positions 1 and 5 (Figure 3). These materials constitute a separate class of anti-β-lactamase reagents. Since it was known that sulfato compounds such as **9** are good inhibitors of the enzyme whereas the unsulfated analogues are not, we originally suggested[33] that inhibition might be due to the formation of an α,β-unsaturated acyl-enzyme by elimination and loss of sulfate between carbon 6 and carbon 8 (see Figure 6). This suggestion was proved wrong, however, when we found that sulfate ion is *not* released when **9** interacts with the enzyme.[34] Indeed, the inhibited enzyme retains 1 mol of sulfate ester per mol of protein. Yet from the kinetics of the inhibition of β-lactamase by **9**, it is clear that there is transient inhibition of the enzyme. Further investigation[35] has shown that the first-formed acyl-enzyme, G (Figure 6), may either hydrolyze to the Δ^2-pyrroline which then

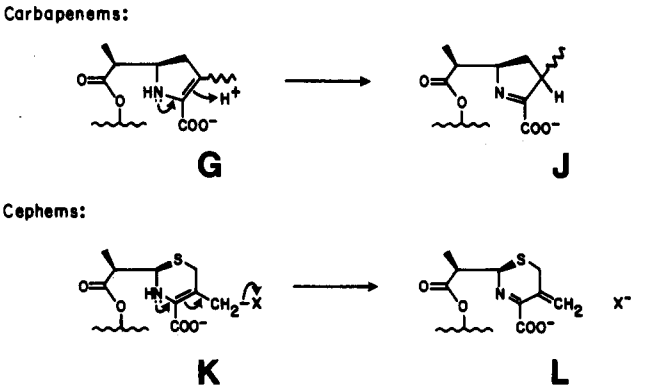


Figure 7. Formal analogy between the tautomerization of the acyl-enzyme from a carbapenem and elimination of HX from an acyl-enzyme from a cephem.

rapidly isomerizes to the more stable Δ^1 product, H, or may tautomerize on the enzyme to yield the Δ^1-acyl-enzyme J. Why the presence of a sulfate ester is required for inhibition and why the Δ^1 tautomer is more refractory to deacylation are questions that cannot be answered until a high-resolution structure for the enzyme becomes available. It seems clear, however, that once again the enzyme is being inhibited because of a chemical diversion from the normal course of the catalyzed hydrolytic reaction. Chemically, the tautomerization of the enamine G to the imine J would seem also to be possible for cephems with good leaving groups at C-3′ (see Figure 7), and one might expect that such cephems could be β-lactamase inhibitors, too. Recent work from Pratt's laboratory has nicely shown, indeed, that this is true.[36] Cephems with good leaving groups at C-3′ do yield a transiently inhibited enzyme in which the acyl-enzyme K (Figure 7), loses X^- to give L, which is slower to deacylate than K. When X is not a good

(31) Compound **5**, indeed, is the most expeditious inactivator, for which (for the *B. cereus* I enzyme) *every* turnover leads to enzyme inactivation. Species B, in this case, has a thiolate nucleophile that is efficiently trapped by the nearby bromoester, resulting in the formation of a stable dihydrothiazine label on the enzyme's active site serine residue.[30] This species is also formed when N_2 rather than bromide ion is the leaving group, as with the diazo compound derived from 6-aminopenicillanic acid (Patil, G. V.; Day, R. A. *Biochim. Biophys. Acta* **1973**, *293*, 490–496. Heckler, T. G.; Day, R. A. *Biochim. Biophys. Acta* **1983**, *745*, 292–300). It is clear from these results that cross-linking of active-site residues is not a sine qua non for enzyme inactivation and that a misalignment of the acyl-enzyme's ester group with respect to the catalytic groups is enough to prevent deacylation.

(32) Cole, M. *Philos. Trans. Soc., Ser. B* **1980**, *289*, 207–223.

(33) Fisher, J. F.; Belasco, J. G.; Charnas, R. L.; Khosla, S.; Knowles, J. R. *Philos. Trans. R. Soc., Ser. B* **1980**, *289*, 309–319.

(34) Charnas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2732–2737.

(35) Easton, C. J.; Knowles, J. R. *Biochemistry* **1982**, *21*, 2857–2862.

(36) Faraci, W. S.; Pratt, R. F. *Biochemistry*, in press.

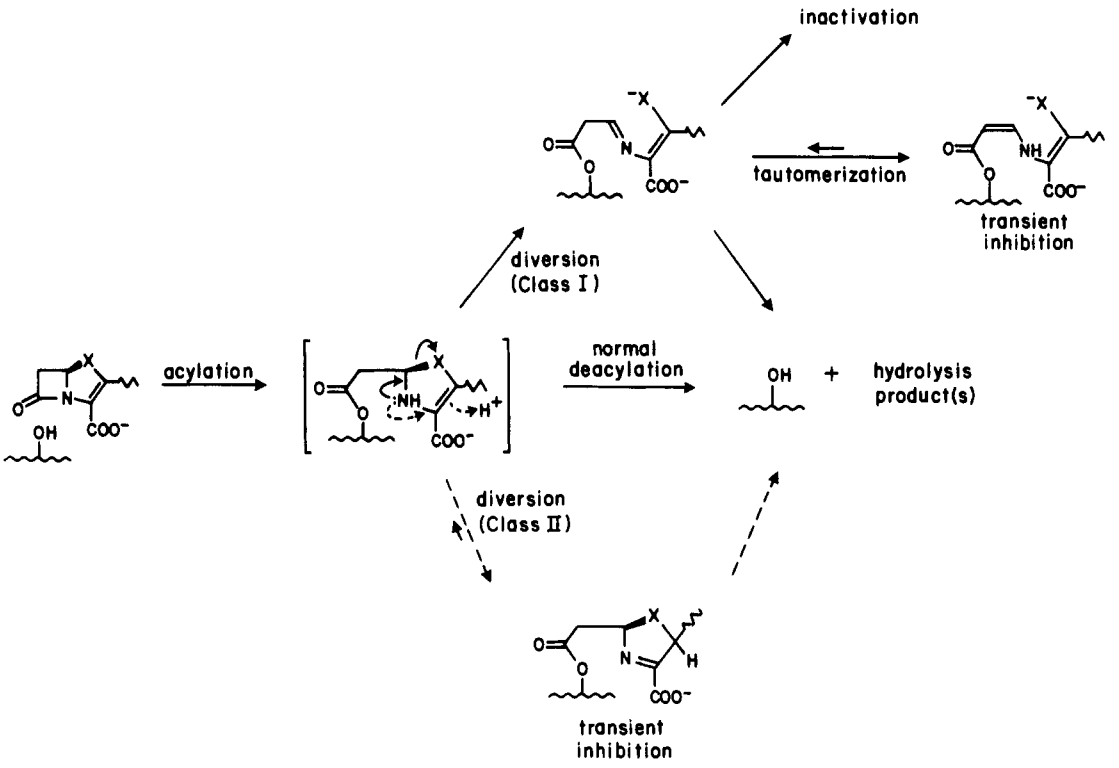
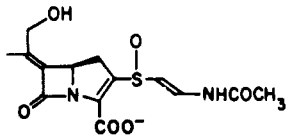


Figure 8. Generalized scheme for the mechanism-based inhibitors of the β-lactamase.

leaving group, no long-lived acyl-enzyme is formed, there is no transient inhibition of the enzyme, and X^- is lost later from the released cephalosporoate product.[37]

In passing, we should stress that the carbapenems appear not to lead to any *irreversible* loss of β-lactamase activity. When all the β-lactam has been consumed, all the catalytic activity of the enzyme returns. The fact that several of the carbapenems are effective in vivo in synergy with susceptible β-lactam antibiotics emphasizes the obvious pharmacokinetic point that irreversible inactivation of an enzyme is not a sine qua non for an effective chemotherapeutic agent. Provided that the β-lactamase is blocked by a tightly binding and slowly reacting substrate, the enzyme will be preoccupied for long enough to allow the antibiotic to reach its target. Antiaircraft missiles are not much use if the bombers have already passed them.

Generalized Scheme for Mechanism-Based Inactivators. Essentially all that has been discussed above can be combined into a single general scheme that incorporates the important features of both classes of β-lactamase inhibitors (see Figure 8). Figure 8 emphasizes the diversionary aspect of these inhibitors in the sense that as soon as the acyl-enzyme forms, either (for the class I systems) the five-membered ring can open and allow other chemical events that preoccupy the enzyme or (for the class II systems), the enamine may tautomerize to a species that deacylates more sluggishly. Consideration of this formulation suggests a number of possible new strategies for β-lactam inhibition. For example, we know that many of the class I inhibitors rapidly form the transiently inhibited species (D in Figure 4) in which the acyl-enzyme is stabilized by becoming an α,β-unsaturated system with a terminal heteroatom (nitrogen, in D). Would it not be advantageous to design a β-lactam that would lead *directly* to such an α,β-unsaturated ester? Accordingly, we synthesized the 6-(methoxymethylene)penam 8, which indeed shows the predicted behavior of slower turnover and slower reactivation, compared with either clavulanic acid or with penicillanic acid sulfone.[38] While the efficiency of 8 as useful inhibitor in vivo is compromised by a relatively high K_m and poor penetration of the outer membrane of Gram-negative bacteria, the point at issue lies (at least for this Account!) in the rational design of inhibitors for a particular enzyme target. Two β-lactams analogous to 8 have also recently been reported: asparenomycin A (11)[39] and



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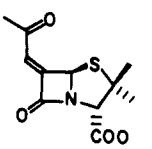

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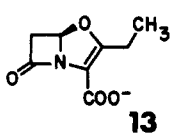

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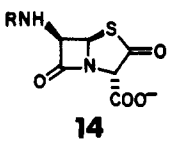

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6-(acetylmethylene)penicillanic acid (12),[40] each of which shows interesting inhibitory properties towards the β-lactamase. Other β-lactamase inhibitors, the

(37) Page, M. I. *Acc. Chem. Res.* **1984**, *17*, 144–151. Faraci, W. S.; Pratt, R. F. *J. Am. Chem. Soc.* **1984**, *106*, 1489–1490. Page, M. I.; Proctor, P. *J. Am. Chem. Soc.* **1984**, *106*, 3820–3825.

(38) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1984**, *23*, 5839–5846.

(39) Tanaka, K.; Shoji, J.; Terui, Y.; Tsuji, N.; Kondo, E.; Mayama, M.; Kawamura, Y.; Hattori, T.; Matsumoto, K.; Yoshida, T. *J. Antibiot.* **1981**, *34*, 909–911. Murakami, K.; Doi, M.; Yoshida, T. *J. Antibiot.* **1982**, *35*, 39–45.

(40) Arisawa, M.; Then, R. L. *J. Antibiot.* **1982**, *35*, 1578–1583. Arisawa, M.; Adam, S. *Biochem. J.* **1983**, *211*, 447–454. Arisawa, M.; Then, R. L. *Biochem. J.* **1983**, *209*, 609–615.

design of which was presumably based on mechanistic considerations such as those illustrated in Figure 8, are the oxapenem 13[41] and the thiolactone 14.[42]

One facet of β-lactamases that is not included in Figure 8 and is perhaps improperly understated in this Account is that these enzymes are rather floppy. Indeed, many aspects of the kinetic behavior of β-lactamases with both substrates and inhibitors can be accounted for in terms of conformational flexibility, which may not only stabilize particular forms of acyl-enzyme that are not obviously less labile chemically (e.g., J or L) but may also lead to the switching of the enzyme into a conformation appropriate to a subgroup of structurally similar β-lactam substrates.[43]

One of the more noble aims of medicinal biochemistry is to understand the details of cellular processes so well that specified enzymic reactions can be inhibited by the design of appropriately specific reagents rather than by the refinement of hopeful "lead" molecules obtained from a random screen. The search for chemotherapeutic agents would be more satisfying (never mind more rapid) if it were *rational*. It is important, therefore, to ask whether the enzymology presented in this Account is relevant to what actually goes on in a bacterial cell. Does the β-lactamase in the periplasm of a growing cell interact with (say) clavulanic acid in the same way as the purified enzyme in a cuvette? Preliminary experiments suggest that it does.[44] When a growing culture of *E. coli* is exposed to sublethal amounts of clavulanic acid, a "steady state" is reached where each daughter cell inherits the same number of molecules of active β-lactamase as its parent did, and one can calculate from the level of enzyme activity and the known generation time of the bacteria, the half-life of the periplasmic enzyme. Happily, this turns out to be almost identical with the half-life of the pure isolated enzyme when exposed to saturating levels of clavulanate, which gives one some confidence that the enzymological results *are* applicable to the situation in the bacterium. While any therapeutically valuable reagent must, of course, satisfy many other requirements such as stability and permeability, this confidence at least allows the primary evaluation of new β-lactamase inhibitors to be made cleanly with the purified enzyme.

Nearly 20 years ago, Tipper and Strominger suggested that β-lactam antibiotics were structural mimics of the C-terminal D-alanyl-D-alanine residues of the peptide chain of uncross-linked peptidoglycan.[45] The last D-alanine is replaced in a transpeptidation reaction by an amino group from a neighboring chain, resulting in the formation of an interchain cross-link. The antibiotic was postulated to acylate the transpeptidase, thereby blocking further cross-linking. Subsequent work has provided overall support for this view, even though there are now known to be several targets for β-lactam action.[7] When bacterial membranes, or whole

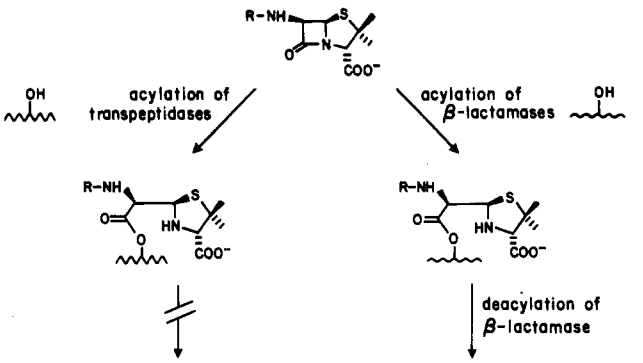

Figure 9. Acylation of the transpeptidases and β-lactamases by a penam.

bacteria, are incubated with a radiolabeled penicillin, several membrane proteins are specifically acylated. In *E. coli*, for example, there are at least seven of these penicillin binding proteins (PBPs), which vary in molecular weight from 40 000 to 91 000. It seems likely that the four larger ones, some of which, at least, are bifunctional transglycosylases–transpeptidases,[46] are the targets of β-lactam-induced cell death. The smaller PBPs are D-alanyl-D-alanine transpeptidases and carboxypeptidases, and while not being the targets for cell death are also acylated by β-lactam antibiotics on a unique serine residue that lies in a sequence[6] having some homology with the serine in β-lactamases that forms the acyl-enzyme intermediate.[13] There are suggestions that the transpeptidase domains of the larger PBPs may also have an analogous serine residue,[47] which supports Pollock's original suggestion[48] that the PBPs and the β-lactamases are distantly related in evolutionary terms. Functionally, both groups of proteins are acylated by β-lactam antibiotics, though only for the β-lactamases is *de*acylation also a rapid process.[49] This functional relationship has recently been put on much firmer ground by Pratt, who has formally demonstrated catalysis by β-lactamase of transpeptidation reactions involving D-alanine.[50] Thus the PBPs accept the β-lactam substrates of β-lactamases, and the β-lactamases can carry out the transpeptidation reactions catalyzed by the PBPs. This latter finding may turn out to be especially important in liberating the design of both antibioitics and β-lactamase inhibitors from the constraints of the β-lactam structure. The likely evolutionary relationship does, moreover, suggest a possible change in strategy for the killing of resistant bacteria that make a β-lactamase. If, as shown in Figure 9, the major functional difference between the transpeptidase antibiotic targets and the β-lactamases is that the latter have acquired the ability to deacylate, then a reagent that acylates both types of enzyme *but does not deacylate* would simultaneously inactivate the β-lactamase *and* kill the cell by blocking the cross-linking of the

(41) Cherry, P. C.; Newall, C. E.; Watson, N. S. *J. Chem. Soc., Chem. Commun.* **1978**, 469–470.
(42) Shimizu, B.; Saito, A.; Nishimura, T.; Nakahara, M. *Chem. Abstr.* **1981**, *92*, 181171.
(43) Citri, N.; Samuni, A.; Zyk, N. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1048–1052. Pain, R. H.; Virden, R. In "β-Lactamases"; Hamilton-Miller, J. M. T., Smith, J. T., Eds.; Academic Press: London, 1979; pp 141–180.
(44) Easton, C. J.; Knowles, J. R. *Antimicrob. Agents Chemother.* **1984**, *26*, 358–363.
(45) Tipper, D. J.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *54*, 1133–1141.
(46) For example: Nakagawa, J.; Tamaki, S.; Matsuhashi, M. *Agric. Biol. Chem.* **1979**, *43*, 1379–1380.
(47) Spratt, B. G.; Hirota, Y., private communications.
(48) Pollock, M. *Proc. R. Soc. B* **1971**, *179*, 385–401.
(49) The acylation of some of the PBPs is not, in fact, irreversible, and slow loss of the penicilloyl group often occurs, either by hydrolysis to the penicilloate **2** or by cleavage of the bond between C-5 and C-6 to yield the *N*-acylglycine and *N*-formylpenicillamine (Hammerström, S.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3463–3467.
(50) Pratt, R. F.; Govardhan, C. P. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1302–1306.

linear peptidoglycan. It may, indeed, be easier to block the hydrolytic deacylation catalyzed by the β-lactamase than to make the possibly more subtle distinction between the acylation of what appear to be related families of enzymes. We must be prepared to face a bacterial answer to the present armoury of β-lactamase-insensitive antibiotics and β-lactamase inactivators that is likely to be selected for by the necessary use (and unnecessary abuse) of these splendid antibacterial agents.

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