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COMMENTARY

ON THE MECHANISM OF ACTION OF ANTIBIOTICS WHICH ACT AS IRREVERSIBLE ENZYME INHIBITORS

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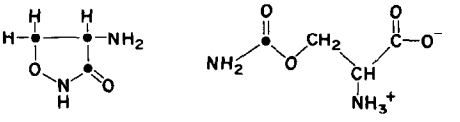
A particularly attractive mechanism for the specific and irreversible inhibition of enzymes involves the use of substrate molecules which are converted into chemically reactive inhibitors by the target enzyme. Once converted into a reactive inhibitor, the molecule forms a covalent bond with the enzyme, inactivating it. Such inhibitors have been termed "k_{cat}" inhibitors since they require catalytic conversion into their active form by the target enzyme [1]. This type of inhibitor is capable of great specificity by virtue of the fact that the agent is not rendered chemically reactive until activated by the target enzyme, and hence the possibility of an undesired chemical reaction(s) with other biomolecules is circumvented. Since many antibiotics are specific and irreversible enzyme inhibitors, it was natural to ask whether or not they might function by a "k_{cat}" mechanism. In this article the mechanisms of action of three chemically distinct classes of antibiotics are analyzed: (1) the penicillins and cephalosporins, (2) D-cycloserine and *O*-carbamyl-D-serine, and (3) the diazocarboxyl-containing antibiotics, L-azaserine, L-diazoöxonorleucine and L-diazoöxonorvaline. In all three cases it is shown that these drugs act by a unified chemical mechanism which requires activation by the target enzyme.

D-Cycloserine and O-carbamyl-D-serine

O-carbamyl-D-serine is an irreversible inhibitor of alanine racemase, a pyridoxal phosphate-linked enzyme responsible for the synthesis of D-alanine from its enantiomorph [2–4]. D-Cycloserine also irreversibly inhibits this enzyme but, in addition, irreversibly inhibits a host of other pyridoxal-linked, amino acid-metabolizing enzymes [2–5]. Phenotypically, both inhibitors appear to function by preventing cell wall formation in bacteria, which could very well be a consequence of their preventing the synthesis of D-alanine, a required intermediate in cell wall biosynthesis [2–4]. Of great interest here is the fact that both molecules have been demonstrated to be irreversible enzyme inhibitors of pyridoxal-linked enzymes. Inspection of the structures of cycloserine and *O*-carbamylserine (shown below on the left and



right respectively) reveals no clues as to what the chemically reactive groups might be. Therefore, these molecules are not likely to be inactivating agents of the affinity-labeling type [6]. However, when one regards these molecules as substrates for the enzyme, the mechanisms of inhibition become clear. All of the enzymes irreversibly inhibited by these two drugs are pyridoxal phosphate-linked, which involves Schiff base formation between the drug and cofactor followed by an α C—H bond cleavage [7, 8]. With cycloserine as substrate, the conversion shown in Fig. 1 occurs [9].

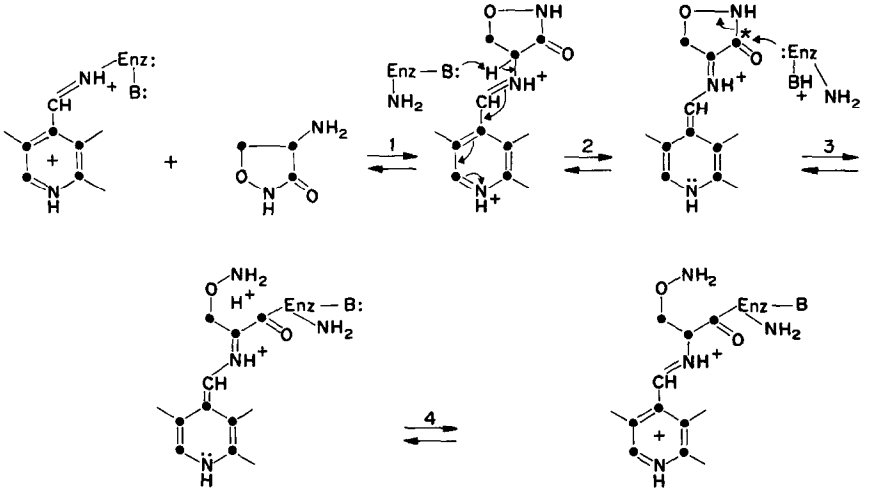


Fig. 1. Enzymatic inhibition with cycloserine as substrate.

In step 2, a highly reactive acylating agent (3) is generated from its unreactive cycloserine counterpart. This molecule (3) is a powerful acylating agent as a consequence of the strain introduced into the five-membered ring and the fact that a positive charge is introduced adjacent to the polarized carbonyl carbon (C*, Fig. 1). This reagent could then enter into a chemical reaction with an active-site nucleophile, irreversibly inhibiting the target enzyme.

O-carbamyl-D-serine is thought to inhibit alanine racemase irreversibly by the mechanism shown in Fig. 2. Again the drug is converted into the active reagent by the enzyme and hence must first be a substrate. In this instance, the α C—H bond is cleaved (e.g., reaction 2 of Fig. 2), but the electrons are subverted from their normal course to an elimination route with the genesis of the highly reactive Michael acceptor [10], *M*. This reactive intermediate can then engage in a Michael reaction with an active site nucleophile, resulting in the irreversible inhibition of the enzyme. The carbamyl group is, of course, an excellent leaving group so that equation 2 (Fig. 2) represents a quite favorable conversion.

What evidence exists in the literature to substantiate the claim that the two drugs function by a k_{cat} mechanism? The mechanism of cycloserine action will be considered first. First of all, the inhibition of pyridoxal phosphate-linked enzymes with cycloserine is time dependent and, in a simple system, this observation is certainly suggestive of an irreversible mode of inhibition. Neither gel filtration nor continued dialysis reactivates the inactivated enzymes [11]. Thus, the enzymes are irreversibly inactivated by cycloserine. That this mode of irreversible inhibition requires catalytic conversion can be demonstrated by showing that cycloserine has no effect on the activity of the enzymes when they are in the pyridoxamine form [11]. This observation is crucial, because the mechanism drawn demands that cycloserine be a substrate. Clearly this requires Schiff base formation between the free amino group of the cycloserine and the aldehydic portion of the pyridoxal phosphate. This cannot occur when the cofactor is in the amine form; hence the first step of the enzymatic process is prevented and the cycloserine can no longer function as an irreversible inhibitor. In addition to these experiments, the structure activity relationships for cycloserine derivatives are wholly consistent with the mechanism as written [9, 11]. For example, alkylation or acylation of the free amino group of cycloserine destroys the activity of the inhibitor [9, 11]. This is expected, since a substitution of this type would prevent Schiff base formation. Finally, cleavage of the ring, as in aminoxy-D serine, results in the abolition of the irreversible component of the inhibition [9, 11].

As previously mentioned, the cycloserines are irreversible inhibitors of several pyridoxal phosphate-containing enzymes whose mechanism of action is similar to that of alanine racemase. This is expected, since many pyridoxal phosphate-containing enzymes share quite similar mechanistic pathways and the structure of cycloserine is such that it should be capable of being a substrate for many amino acid-metabolizing enzymes. Indeed, cycloserine has been shown to be an irreversible inhibitor of aspartate aminotransferase [5], alanine aminotransferase [5], asparagine aminotransferase [5] and γ-aminobutyric acid transaminase [5]. Importantly, the mechanism of action of these transaminases is identical to that

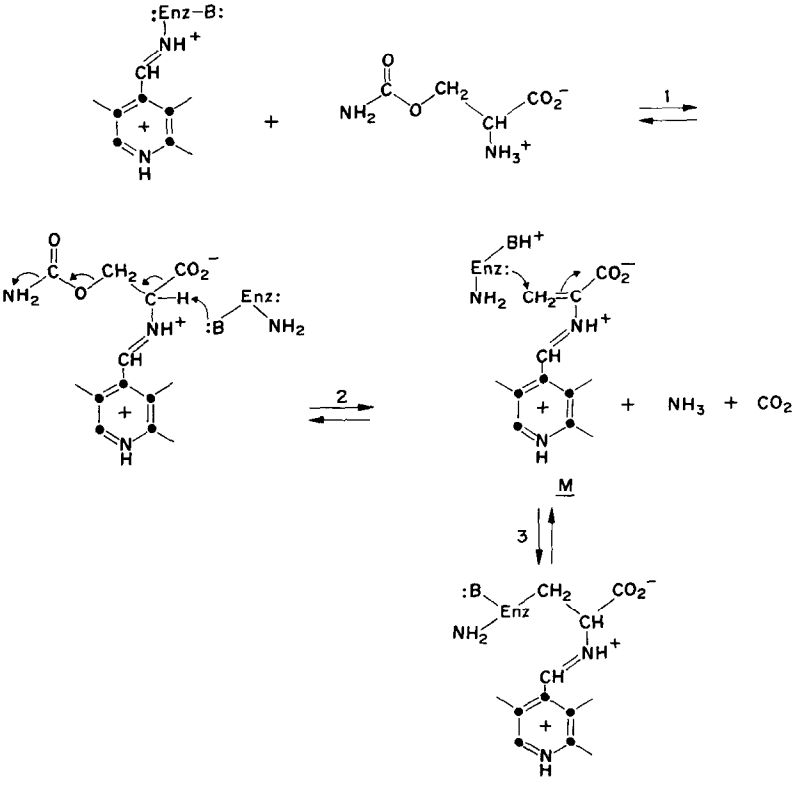


Fig. 2. Enzymatic inhibition with *O*-carbamyl-D-serine as substrate.

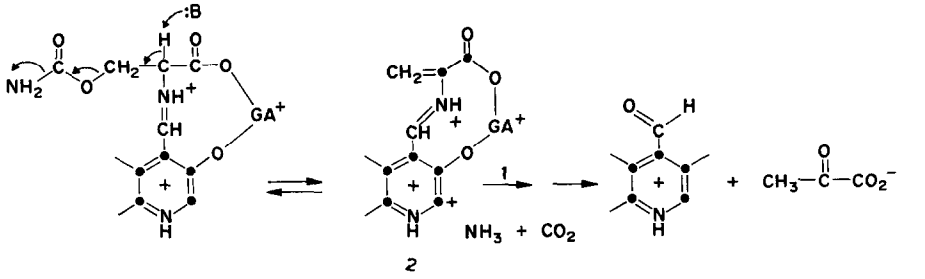


Fig. 3. Conversion of carbamyl serine into the assumed active intermediate, 2, by treatment with pyridoxal phosphate in the presence of gadolinium ion.

of alanine racemase through Schiff base formation followed by the crucial state where the α C—H of the substrate is cleaved.

Regarding *O*-carbamyl serine, the evidence here is a bit more scanty simply because the drug has not been as thoroughly studied as cycloserine. The drug is an irreversible inhibitor of alanine racemase and shows the typical time-dependent course of inactivation customary with these inhibitors [2–4]. Model studies show that carbamyl serine can be converted into the assumed active intermediate, 2, by simply treating it with pyridoxal phosphate in the presence of gadolinium ion [12] (Fig. 3). Therefore, the formation of the highly reactive 2 is a favored process with this molecule.

In conclusion then, both drugs are irreversible inhibitors of alanine racemase and, in addition, cycloserine has been also shown to be an irreversible inhibitor of several transaminases. What is important to recognize is that all of these enzymes are pyridoxal phosphate-linked and they share the same mechanistic pathways through α C—H bond cleavage, the crucial stage in the chemical activation of the drugs.

Diazocarboxyl-containing antibiotics (azaserine, 6-diazo-5-oxo-L-norleucine (DON) and 5-diazo-4-oxo-L-norvaline (DONV))

Each of these antibiotics is of interest in that they possess a biologically unusual diazoester or diazoketone moiety (Fig. 4). Azaserine and DON are generally considered to be glutamine analogues, whereas DONV is considered to correspond to asparagine [13]. These molecules function as irreversible enzyme inhibitors, with the diazo moiety being crucial to their mechanisms of action. In general, the drugs function *in vivo* by irreversibly inhibiting certain enzymes involved in the biosynthesis of purine and pyrimidines; hence the drugs have been used as anti-cancer agents. Before discussing the mode of action of these inhibitors, we would first like to summarize the salient chemical features of the diazo grouping, which will enable us to analyze their mechanism(s) of action.

The diazo grouping, as such, is essentially inert towards nucleophilic attack [14]. The moiety is generally thought to behave as a Lewis base rather than as a Lewis acid. Since all alkylating and acylating reagents used in protein modification are Lewis acids, one would think that diazo compounds would never function in this way. However, the diazo compounds are notoriously sensitive to acid attack [15]. For example, diazoacetic esters are rapidly decomposed by dilute, aqueous acid to release nitrogen and generate a glycollate ester (Fig. 5). This mechanism involves the intermediacy of the exceedingly reactive diazonium ion, 2, which can, of course, react with nucleophiles (Lewis bases) at an essentially diffusion-controlled rate. The important point is to realize that the diazoesters must first be protonated before they will react with a nucleophile. Therefore, in the instances where diazoesters or diazoketones function as irreversible enzyme inhibitors by an alkylation or acylation route, we would expect that prior protonation by the enzyme must be involved, to generate the reactive diazonium intermediate.

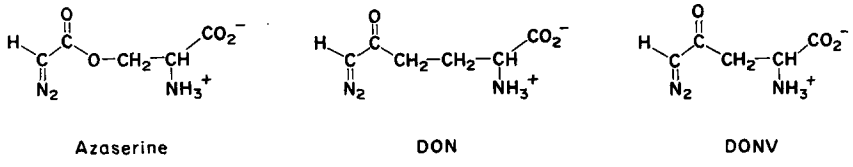


Fig. 4. Structure of azaserine, 6-diazo-5-oxo-L-norleucine (DON) and 5-diazo-4-oxo-L-norvaline (DONV).

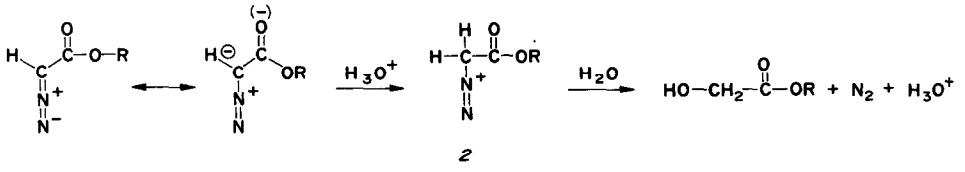


Fig. 5. Mechanism of decomposition of a diazoacetic ester by a dilute aqueous acid.

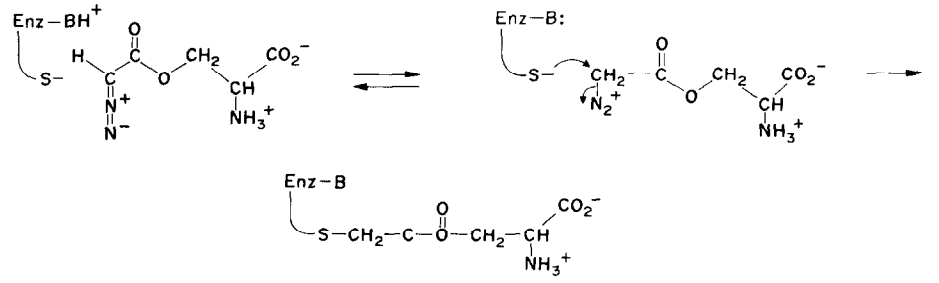


Fig. 6. Enzymatic protonation of and inactivation by azaserine.

With these facts in mind, we will describe the mechanisms of action of the diazo-containing antibiotics in cases where sufficient biochemical data are available. Azaserine has been the most thoroughly studied of the diazocarboxyl-containing antibiotics. This drug is an irreversible inhibitor of the enzyme that converts formylglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM) in the synthesis of purines [16, 17]. This reaction requires L-glutamine and ATP in addition to FGAR, and its interruption prevents purine biosynthesis, inasmuch as this step is an integral part of inosinic acid biosynthesis. Studies have demonstrated than an active-site cysteine group of formyl-GAR amidotransferase, the enzyme carrying out this conversion, is alkylated by azaserine [16, 17]. This is unusual in that alkyl mercaptans are not reactive towards diazoesters [16, 17], a point consistent with the view developed above concerning the lack of reactivity of diazoesters toward nucleophiles. Indeed, as was correctly pointed out by French *et al.* [16] and Dawid *et al.* [17], enzymatic protonation of azaserine should occur prior to sulfhydryl alkylation (Fig. 6). In Fig. 6, a residue other than the sulfhydryl group has been depicted as the group responsible for the acid-catalyzed protonation. It is not clear which group is involved in the protonation step. If the active-site cysteine is sufficiently acid, it, in fact, could be both the proton donor and the site of alkylation. What is clear, however, is that prior enzymatic protonation is required. DON will also irreversibly inhibit the transferase, presumably by the same mechanism. In general, DON would be expected to behave quite similarly to azaserine, and most experiments bear this out [13].

Studies on DONV, the diazo analogue of asparagine, are also relevant to the thesis presented in this article. DONV is an irreversible inhibitor of L-asparaginase from *Escherichia coli* [18]. DONV appears to have no effect on the various enzymes that utilize glutamine. The mechanism of the DONV inhibition of asparaginase is exceedingly interesting. The inhibitor is known both to react irreversibly with the active site of the enzyme and to be catalytically decomposed by the enzyme to nitrogen and 5-hydroxy-4-oxonorvaline (HONV) [19]. Aqueous environments favor the formation of HONV over inactivation. When dimethylsulfoxide (DMSO) is added, the ratio of hydrolysis to inactivation decreases. These results require a common intermediate for both hydrolysis to HONV and alkylation of the active-site residue of the enzyme. The diazonium ion (1, Fig. 7), which is produced by enzymatic protonation of DONV, is this intermediate. Solvents such as DMSO, which lower the water concentration, favor route 1 (alkylation of an active site residue) over route 2 (hydrolysis by water). The water required in the latter step is expected to be at the active site of the enzyme, since it is a hydrolase. The enzymatic protonation of the diazo compound is also of interest, since one would expect that a protonation step would be part of the normal enzyme mechanism in the hydrolysis of asparagine. Amide hydrolysis is, of course, strongly catalyzed by acid. In summary then, diazocarbonyl-containing antibiotics are active as irreversible enzyme inhibitors only in instances where they are first enzymatically protonated to afford the highly reactive diazonium ion intermediate.

With these results in mind, it might be assumed that the diazo-containing compounds would be powerful irreversible inhibitors of enzymes that normally function by acid catalysis. This is precisely the case with acid proteases. A host of diazoketones and diazoesters have been shown to be active-site directed irreversible inhibitors of the acid protease pepsin [20, 21]. These inhibitors probably function by esterifying a weakly acidic carboxyl group [20, 21]. Again the decomposition of the diazo moiety requires prior protonation by the enzyme.

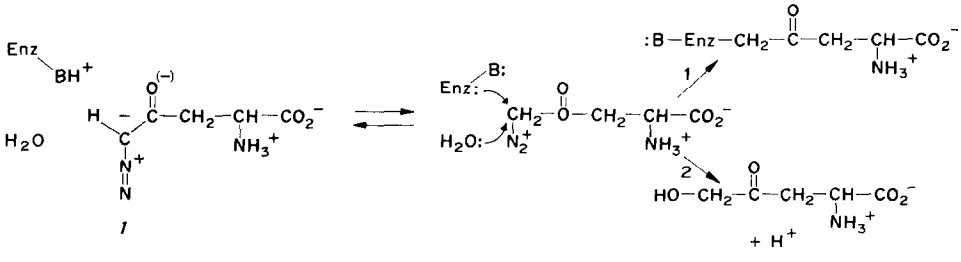
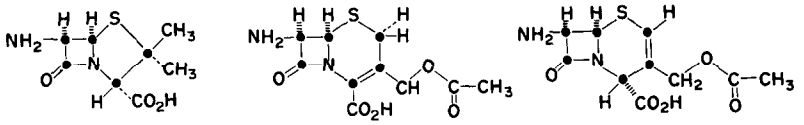


Fig. 7. Enzymatic protonation of and inactivation by DONV.



1 2 3

Fig. 8. Structure of 6-amino penicillanic acid, Δ^3-7-amino-cephalosporinic acid and Δ^2-7-amino-cephalosporinic acid.

No one has ever reported a diazoester or ketone to be an irreversible inhibitor of a serine (basic) protease. This is expected in light of the arguments presented above.

Penicillins and cephalosporins

The penicillins and cephalosporins are among the most thoroughly studied of all classes of antibiotics. The active penicillins are all derivatives of 6-amino penicillanic acid (structure 1, Fig. 8), and the active cephalosporins are all derivatives of Δ^3-7-amino-cephalosporinic acid (structure 2, Fig. 8). The Δ^2-7-amino-cephalosporinic acid (structure 3, Fig. 8) derivatives are inactive. Generally speaking, the penicillins are roughly an order of magnitude more active than the Δ^3-cephalosporins [22]. These drugs prevent cell wall biosynthesis in bacteria and they are thought to do this by preventing crosslinking of the peptidoglycan peptide chains, a terminal and crucial reaction in cell wall biosynthesis [23]. These drugs are assumed to inactivate irreversibly the as yet unisolated membrane-bound transpeptidase enzyme, which results in the lysis of growing cells. In the transpeptidization reaction, a terminal *d*-alanine on one chain is cleaved off in the process of crosslinking the two adjacent chains. It has been argued that the antibiotics, which inhibit this reaction, function by mimicking the D-alanyldipeptide portion of the acceptor molecule so that they can bind to the active site of the transpeptidase and react with an active-site residue to inhibit the enzyme [23]. It is thought that an active-site sulfhydryl group is acylated during the process of penicillin inhibition of cell growth [22, 24]. The strained β-lactam portion of the antibiotic is presumably involved in the site of the chemical reaction, which results in the irreversible inhibition of the enzyme. It is also likely that the sulfhydryl group that is acylated by penicillin is also involved in the normal transpeptidization [23]. The crucial issue to be understood is why penicillin and Δ^3-cephalosporin derivatives are so specific in their mode(s) of action. Their remarkable lack of toxicity is a corollary of this specificity. It is highly unlikely that these molecules are simply extraordinarily specific affinity-labeling agents of the transpeptidase [6]. Why, in fact, don't these β-lactam antibiotics react with other nucleophilic biomolecules like glutathione, before ever coming into contact with the transpeptidase?

The notion that penicillin* is an affinity-labeling agent is based on the assumption already alluded to—that the antibiotic is isosteric with the terminal D-alanyldipeptide of one of the chains [23]. However, careful inspection of models shows that the ground state conformation of penicillin differs significantly from the shape of the alanyl dipeptide (Fig. 9). The main reason for this is that the β-lactam nitrogen must remain pyramidal (SP^3) whereas the peptide bonds in dialanyl peptide are, of course, planar (SP^2). The reason why β-lactams, especially ring-fused ones, have non-planar (SP^2) peptide bonds is clear: planarity would require the introduction of further strain energy into an already highly strained system [24, 25]. X-ray structural data on the penicillins confirm the pyramidal nature of the β-lactam's nitrogen in these molecules [25]. As can be seen in Fig. 9., the pyramidal nature (SP^3) of the β-lactam nitrogen introduces the following important differ-

* What is stated here for the penicillin derivatives is also assumed to apply to the active cephalosporin derivatives.

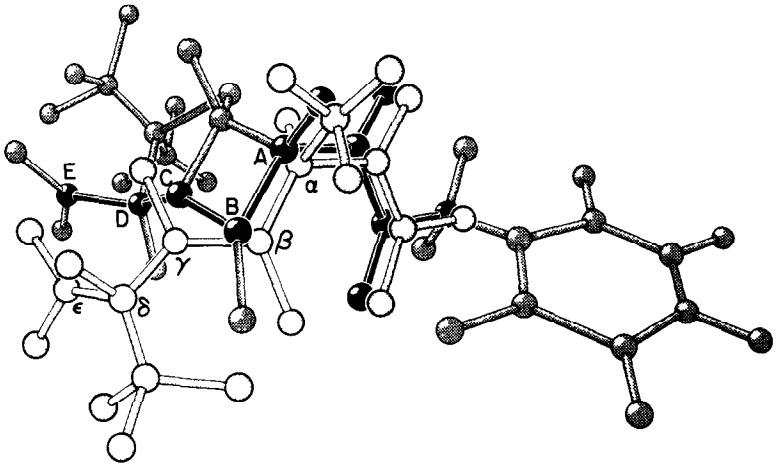
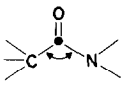


Fig. 9. Comparison of D-alanyl-dipeptide with benzyl penicillin. The molecule labelled α, β, γ, δ, ϵ is the dipeptide and A, B, C, D, E is the benzyl penicillin.

ences between penicillin and the peptide bond in the alanyldipeptide: the



bond angle of the β-lactam is 90·5°, whereas the corresponding bond angle in the dipeptide is 117° and the dihedral angle about the β-lactam nitrogen is 135·7° as opposed to the normal planar dihedral angle (180°) about the peptide bond in the alanyl dipeptide. In order for the same active site to accommodate both penicillin and the alanyl dipeptide portion of the substrate, the enzyme must either force the alanyldipeptide to "look like" penicillin or force penicillin to "look like" the dipeptide. This would require that either the peptide bond of the dipeptide to be cleaved would have to be distorted to a pyramidal shape or the β-lactam nitrogen of the antibiotic would have to be distorted to a planar shape. The former alternative has been proposed in a paper by Lee [26], which makes the point that if the normal mechanism of transpeptidase action involves the distortion of the peptide bond of the substrate, then penicillin could be viewed as being a "transition state" analogue of the normal enzymatic process and hence bind to the enzyme with an exceedingly low dissociation constant K_s [27]. Thus, the selectivity of penicillin action would be a consequence of selectivity in the K_s terms [1]. This possibility seems unlikely since this proposal would require that rotation about the peptide bond be a normal part of the transpeptidase's mode of action. The essence of it would be that this rotation would destroy the double-bond character of the peptide bond due to overlap between the free electron pair on the nitrogen and the carbonyl π system. Since the transpeptidase is simply a protease that uses an amine instead of water as an acceptor molecule, we would expect to see some evidence for this kind of mechanism in studies on other proteases. Very simply, there is none. If this mechanism were important, one would predict that unsubstituted amides would not be hydrolyzed by proteases because the enzyme would not have a "handle" by which to apply this torsional deformation. Unsubstituted amides happen to be perfectly good substrates for proteases. For example, α-chymotrypsin is perfectly capable of catalyzing the hydrolysis of the unsubstituted amides of *N*-acetyl-L-tyrosine and *N*-acetyl-L-tryptophane [28]. The second alternative alluded to earlier, that of the penicillin binding to the transpeptidase in a strained conformation, is of interest here. If the transpeptidase were to bind penicillin in a conformation which required the β-lactam nitrogen to approach planarity, the molecule would be rendered exceedingly reactive. Any strain energy applied by the enzyme in this manner would be directly translated into the chemical reactivity of the β-lactam peptide-like bond, since cleavage there would relieve the strain. Thus, the enzyme-bound antibiotic would be in a much higher energy state than the unbound form. Hence, the enzyme, by applying binding forces, would greatly increase the chemical reactivity of the penicillin by distorting it from its ground-state structure. The increased reactivity of the bound antibiotic would result in a much higher incidence of fruitful collisions with a propinquous active-site residue. This activation, of course, would be accomplished at the expense of a greater dissociation constant, but this need not be so important if the selectivity of penicillin is, indeed, based on chemical selectivity related to the "k_{cat}" mechanism.

This model is consistent with what is known about the activities of the various penicillin and cephalosporin derivatives. First of all, an intact β-lactam is required [18]. This is consistent with any model in which the antibiotic's effectiveness is based on its ability to be an irreversible enzyme inhibitor, since the chemical reaction occurs at the β-lactam. More interestingly, the intact β-lactam unit must be fused to another ring (either a five or a six). Molecules such as desthiobenzylpenicillins 1 and 2 (Fig. 10) have virtually no activity at all [18]. The stereochemistry of these products is the same as in penicillin. The fact that these molecules are virtually inactive is consistent with our hypothesis, since, not being fused-ring systems, they would start out with much less strain energy than penicillin or cephalosporin so that the extra strain brought about by enzymatic bond-angle deformation shouldn't be so important. This finding is also inconsistent with the idea that these antibiotics are "transition state" inhibitors. Furthermore, the free amino group of penicillin must be substituted and the free carboxylic acid grouping must remain intact [22, 24]. Presumably, these groupings are the "handle" through which the enzyme applies the bonding forces required to further strain the antibiotics. On the other hand, if penicillin is simply a "transition state" analogue or an affinity-labeling agent, removing this "handle" might reduce potency slightly, but not abolish it completely. Only the induced strain model introduced here would predict that this substitution would be crucial. In addition, substitution of a methyl group for a hydrogen on the β-lactam carbon bearing the amino group of penicillin results in the complete loss of antibiotic potency [29]. On the basis of assumed isosterism between the D-alanyldipeptide and penicillin, it was predicted that this substitution would increase the potency of the antibiotic [23].

Germane to the arguments in support of the induced strain are the observations concerning the relative reactivities of the penicillin group of antibiotics versus the Δ^3 and Δ^2-cephalosporin derivatives. As mentioned earlier, the penicillins are roughly an order of magnitude more active than Δ^3, and Δ^2 is inert. These results can only be rationalized on chemical reactivity grounds, for models indicate that the Δ^2 derivatives are structurally more similar to penicillin than are the Δ^3 derivatives [25]. Thus, the

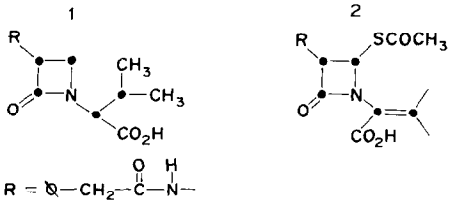


Fig. 10. Structure of desthiobenzylpenicillins 1 and 2.

order of antibiotic activity of the three series of drugs parallels their inherent chemical reactivity and is not governed by a presumed isosterism. Finally, studies on the inhibition of D-alanine carboxypeptidases by penicillin led to exceedingly interesting results which support the model presented here. Those bacterial carboxypeptidases which are irreversibly inhibited by the drug, such as the *B. subtilis* enzyme, bind the penicillin very poorly ($K_I \sim 10^{-3}$ M) [30]. On the other hand, those carboxypeptidases which are only competitively inhibited by penicillin, such as the enzymes from *E. coli* [31] and *Streptomyces* R 39 and K 11 [32], bind the drug very tightly (K_I values $\sim 10^{-8}$M). Since the energy required to strain the penicillin in our model must come from binding forces, we would of necessity expect relatively weak binding in those instances where covalent bond formation occurs.

All of the above results are consistent with a model that requires enzymatic activation of the already strained β-lactam antibiotics by further deformation. Thus, these antibiotics are viewed as strained molecules which can be further deformed upon binding to the target enzyme and hence rendered even more reactive than when in solution. It is to this factor that they owe their specificity of action and not to isosterism. That enzymes can deform small molecules into high energy conformations has been suggested by x-ray and mechanistic studies in connection with the mechanism of action of lysozyme [33]. Along these lines, it is of interest to note that a conduritol α-epoxide is an irreversible inhibitor of β-glucosidase, an enzyme that presumably functions by forcing the ground-state chair form of the sugar into a half chair [34]. If the bicyclic conduritol epoxide were bound like the substrate, it would be rendered exceedingly reactive, a view consistent with the experimental results.

Conclusion

In this paper I have attempted to demonstrate that selective, naturally occurring, irreversible enzyme inhibitors function by a mechanism somewhat more complicated than simple isosterism of inhibitor and substrate. I suggest that, in each case, the target enzyme can be considered to modify the putative inhibitor to render it more reactive at the active site, hence more selective. Note that the enzymatic activation of the β-lactam antibiotics is different in kind, but not in principle, from the activation described for the cycloserine or diazoketone antibiotics. Fundamentally, all of these enzymatic activations yield the same result: that of increasing the chemical reactivity of the bound versus unbound drugs. Whether the enzyme accomplishes this by chemical conversion or by binding forces is simply a matter of individual mechanism.

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