Mechanism of Inhibition of the Class C β-Lactamase of *Enterobacter cloacae* P99 by Cyclic Acyl Phosph(on)ates: Rescue by Return

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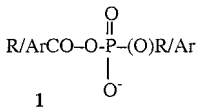
Abstract: As previously described (Pratt, R. F.; Hammar, N. J. *J. Am. Chem. Soc.* **1998**, *120*, 3004.), 1-hydroxy-4,5-benzo-2,6-dioxaphosphorinone(3)-1-oxide (salicyloyl cyclic phosphate) inactivates the class C β-lactamase of *Enterobacter cloacae* P99 in a covalent fashion. The inactivated enzyme slowly reverts to the active form. This paper shows that reactivation involves a recyclization reaction that regenerates salicyloyl cyclic phosphate rather than hydrolysis of the covalent intermediate. The inactivation, therefore, is a slowly reversible covalent modification of the active site. The thermodynamic dissociation constant of the inhibitor from the inactivated enzyme is 0.16 μM. Treatment of the inactivated enzyme with alkali does not produce salicylic acid but does, after subsequent acid hydrolysis, yield one molar equivalent of lysinoalanine. This result proves that salicyloyl cyclic phosphate inactivates the enzyme by (slowly reversible) phosphorylation of the active site serine residue. This result contrasts sharply with the behavior of acyclic acyl phosphates which transiently inactivate the P99 β-lactamase by acylation (Li, N.; Pratt, R. F. *J. Am. Chem. Soc*. **1998**, *120*, 4264.). This chemoselectivity difference is explored by means of molecular modeling. Rather counterintuitively, in view of the relative susceptibility of phosphates and phosphonates to nucleophilic attack at phosphorus, 1-hydroxy-4,5-benzo-2-oxaphosphorinanone(3)-1-oxide, the phosphonate analogue of salicyloyl cyclic phosphate, did not appear to inactivate the P99 β-lactamase in a time-dependent fashion. It was found, however, to act as a fast reversible inhibitor ($K_i = 10$ μM). A closer examination of the kinetics of inhibition revealed that both on and off rates (9.8×10^3 s^{-1} M^{-1} and 0.098 s^{-1}, respectively) were much slower than expected for noncovalent binding. This result strongly indicates that the inhibition reaction of the phosphonate also involves phosphylation of the active site. Hence, unlike the situation with bacterial DD-peptidases covalently inactivated by β-lactams, the P99 β-lactamase inactivated by the above cyclic acyl phosph(on)ates can be rescued by return. Elimination of the recyclization reaction would lead to more effective inhibitors.

Introduction

The resistance of bacteria to β-lactam antibiotics largely arises from the production of β-lactamases.[1] These defensive enzymes catalyze the hydrolysis of β-lactams and thus destroy the antibiotic activity. The existence of β-lactamases was recognized even before the introduction of these antibiotics into medicine,[2] and the search for inhibitors of these enzymes began immediately thereafter.[3] Now, some sixty years later, the general problem of β-lactamases has still not been solved. Although many inhibitors have been discovered, few have proven sufficiently effective to be introduced into clinical practice as synergists of existing β-lactams. Many of these inhibitors, including those presently in clinical practice, are mechanism-based inhibitors and β-lactams themselves;[4] β-lactamases are, of course, well-known to be able to develop resistance to β-lactams.

Another group of inhibitors that have been investigated in recent years and which are not β-lactams are transition state analogues. These acyclic molecules are actually analogues of transition states for hydrolysis of acyclic ester substrates. They include a variety of boronates[5] and phosphonates. The latter molecules have been investigated for some time in this laboratory.[6] They are particularly effective against class C β-lactamases which are an acute problem at present.[7] They act, in general, by covalent phosphonylation of the nucleophilic serine of the β-lactamase active site.[8,9] The acyl phosph(on)ates (**1**) represent a new group of phosph(on)ate inhibitors, having activity against both class A and class C β-lactamases.[10,11] Depending on the enzyme and the structure of the



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compound, either acylation or phosphylation, or both, of the enzyme active site has been observed. The acyl derivatives can slowly hydrolyze to free enzyme and carboxylate, and thus, in this mode of reaction, compounds **1** are poor but inhibitory substrates. Phosphylation, although less efficient than acylation

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(1) Medeiros, A. A. *Clin. Infect. Dis.* **1997**, *24* (Suppl. 1), 519.
(2) Abraham, E. P.; Chain, E. *Nature* **1940**, *146*, 837.
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(6) Pratt, R. F. *Science* **1989**, *246*, 917.
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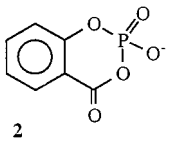
with the compounds surveyed to date,[10,11] produces a considerably more refractory derivative, leading effectively to irreversible inactivation of the enzyme. As would be expected on the basis of chemical reactivity,[12,13] phosphylation is more facile when **1** is a phosphonate than when it is a phosphate.

We have also described the reactivity of cyclic acyl phosphate **2** with these enzymes.[5] This molecule reacts with representative class A and C β-lactamases as well as with the DD-peptidase of *Streptomyces* R61 to yield inert complexes. We had anticipated that the breakdown of these complexes to free enzyme would be slower than that of acyl enzymes derived from **1**. This expectation, which was in fact realized, was based on the (*ex post facto*) rationale for the effectiveness of penicillin itself where acylation of transpeptidase active sites leads to an inert acyl enzyme, partly at least because the thiazolidine leaving group remains attached to the acyl moiety and obstructs deacylation. The latter could also be true for an acyl enzyme (or a phosphoryl enzyme) derived from **2**.



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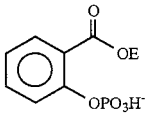
Our original report concerning the discovery of **2** did not reach a conclusion as to whether the covalent complex of **2** with any particular enzyme was an acyl enzyme, **3**, or a phosphoryl enzyme, **4**. The present paper addresses this point.



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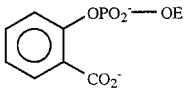

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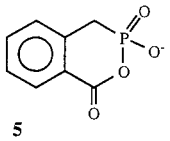
As one approach to this problem, we prepared and examined the enzyme inhibitory abilities of **5**, the phosphonate analogue of **2**. The rationale here was that if **2** phosphylated the enzyme, **5** should do so more rapidly, whereas, if acylation were involved, the reactivity of **5** should be very similar to or perhaps less than that of **2**. The pattern of reactivity of acyclic analogues



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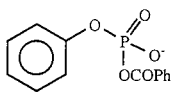

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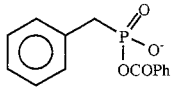

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6 and **7** was also examined for comparison. We conclude that, in contrast to the acyclic analogues **6** and **7**, cyclic compounds **2** and **5** primarily react with the class C β-lactamase of *Enterobacter cloacae* P99 by phosphylation of the nucleophilic active site serine residue. Further, dephosphylation to regenerate the free enzyme occurs by recyclization to the cyclic phosph(on)ate.

Results and Discussion

Reaction Between 2 and the P99 β-Lactamase. Cyclic acyl phosphate **2** has been shown to inactivate several β-lactam-recognizing enzymes and, in particular, the class C β-lactamase of *Enterobacter cloacae* P99.[14] The results from the initial experiments with this enzyme were interpreted in terms of

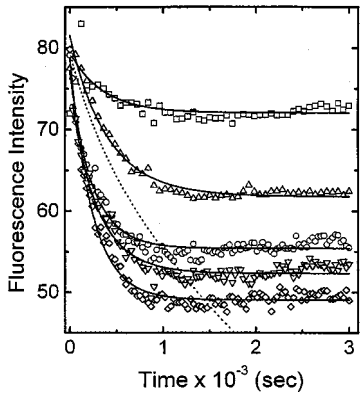


Figure 1. Time-dependent changes in fluorescence intensity on mixing acyl phosphate **2** (0.26 μM) with 0.1 μM (□), 0.2 μM (△), 0.3 μM (○), 0.4 μM (▽), and 0.5 μM (◇) concentrations of the P99 β-lactamase. The solid lines were calculated on the basis of Scheme 2 as described in the text. The dotted line corresponds to the progress curve for reaction of 0.26 μM **2** with 0.1 μM enzyme as calculated on the basis of Scheme 1.

Scheme 1

$$E + I \xrightarrow[5.6\times10^{3}\,s^{-1}M^{-1}]{k_i} E\text{-}I \xrightarrow[8.2\times10^{-4}\,s^{-1}]{k_r} E + P$$

Scheme 1. The cyclic phosphate, I in this scheme, reacted in a second-order process ([I] ≤ 15 μM) to yield an intermediate that slowly broke down, regenerating free enzyme and, implied but not proven, the hydrolysis product of **I**. Evidence for the covalent nature of E−I was provided by the decrease in fluorescence of **2** on its reaction with the enzyme to form E−I,[14] a change also observed on the spontaneous hydrolysis of **2**.

Subsequently, we realized that complete turnover did not accompany the addition of enzyme to **2**. This is seen in the results shown in Figure 1. It appears that the reaction of **2** with the enzyme stops short of completion, a result seen very clearly at the lower enzyme concentrations, for example, with 0.1 μM enzyme in Figure 1. This is not solely a function of stoichiometry and burst kinetics, because, although a decrease in fluorescence occurs, representing formation of E−I, no subsequent decrease in fluorescence is seen which would represent the expected breakdown of E−I and complete turnover of **2**. Scheme 1 would, in fact, predict the dotted line for reaction of 0.26 μM **2** with 0.1 μM enzyme. This clearly does not fit the data (the upper trace in Figure 1).

Fluorescence spectra of reaction mixtures at the end of the reaction period shown in Figure 1 suggested that they contained unreacted **2** (λ_{ex} = 310 nm, λ_f = 370 nm). The reaction with enzyme had therefore stopped prior to complete turnover of **2**. Further evidence that **2** was still present after the fluorescence change had ceased was achieved by adding a $KHCO_3/K_2CO_3$ buffer to the reaction mixture (pH 7.5) to achieve a 0.1 M total carbonate concentration and a pH of 9.5. Figure 2A shows the initial decrease in fluorescence intensity on reaction of 0.4 μM enzyme with 0.26 μM **2**. On then changing the pH to 9.5, a further exponential decrease in fluorescence was observed (Figure 2B) from which a rate constant of 8.4×10^{-4} s^{-1} was derived. When **2** alone was treated with the same buffer, a very similar fluorescence decrease was observed for which a rate constant of 7.9×10^{-4} s^{-1} was obtained. These observations support the proposition that after the reaction of 0.4 μM β-lactamase with 0.26 μM **2** had apparently reached completion at pH 7.5, a considerable amount of the original **2** remained in solution and was not turned over by the enzyme in the time frame expected from the k_r value (Scheme 1).

(12) Hudson, R. F.; Keay, L. *J. Chem. Soc.* **1960**, 1859.
(13) Hays, H. R. *J. Org. Chem.* **1971**, *36*, 98.
(14) Pratt, R. F.; Hammar, N. J. *J. Am. Chem. Soc.* **1998**, *120*, 3004.

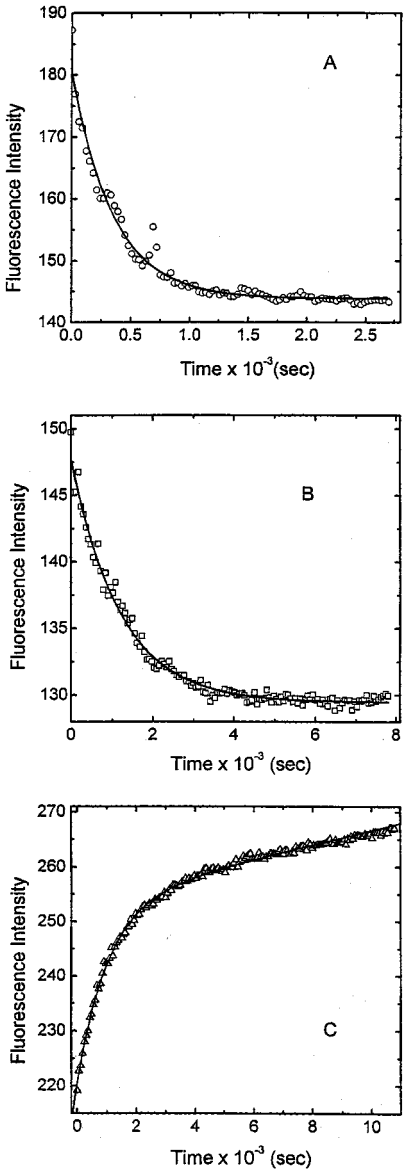
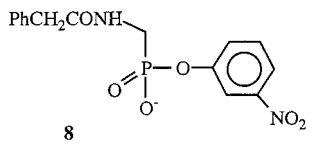


Figure 2. Fluorescence intensity changes: A, on reaction of **2** (0.26 μM) with the P99 β-lactamase (0.4 μM); B, on adjustment of the pH to 9.5 after the reaction in A; C, on addition of the phosphonate **8** (100 μM) to the reaction mixture after the reaction in A.

The implication of these results is that the reaction of E with I proceeds, not to complete hydrolysis of I, but to a position of equilibrium (Scheme 2). In this reaction scheme, k_r corresponds not to turnover but to reformation of I from E−I.

To provide further evidence for this revised view of the interaction of **2** with the enzyme, the following experiment was conducted. Enzyme (0.4 μM) and **2** (0.26 μM) were mixed in a cuvette and the reaction monitored by fluorescence until no further change occurred (Figure 2A). To this reaction mixture, sodium *m*-nitrophenyl *N*-(phenylacetyl)aminomethyl phosphonate **8** (100 μM) was added. This is an irreversible, covalent inhibitor of the P99 β-lactamase which phosphonylates the active site serine residue.[8,9] At a concentration of 100 μM, this



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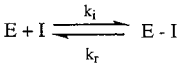
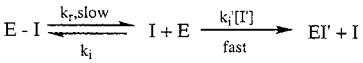
phosphonate would inactivate the P99 β-lactamase with a pseudo-first-order rate constant of 0.23 s^{-1}. On addition of **8** to the reaction mixture, the fluorescence change shown in Figure 2C was observed. The fluorescence intensity at 400 nm increased exponentially with a rate constant of 9.4×10^{-4} s^{-1}. (The reason for the subsequent slow linear increase in fluorescence, which was included as a linear term in the curve fitting, is not known at present but may represent some denaturation of the inactivated enzyme.) The emission spectrum at the end of the exponential phase was again identical to that of **2**. These observations can be interpreted in terms of Scheme 3, where I′ represents **8**. According to this scheme, I′ reacts rapidly and irreversibly with free E, and E−I dissociates in the rate-determining step to restore equilibrium; eventually, all E−I has dissociated and all E is inactivated by I′. The observed rate constant should be k_r, because $k_i'[I'] \gg k_i$. The value of k_r obtained by this method is gratifyingly close to that obtained from previous direct return of activity experiments, viz. 8.2×10^{-4} s^{-1}.[14] This result therefore provides convincing evidence for Scheme 2.

Scheme 2



Scheme 3



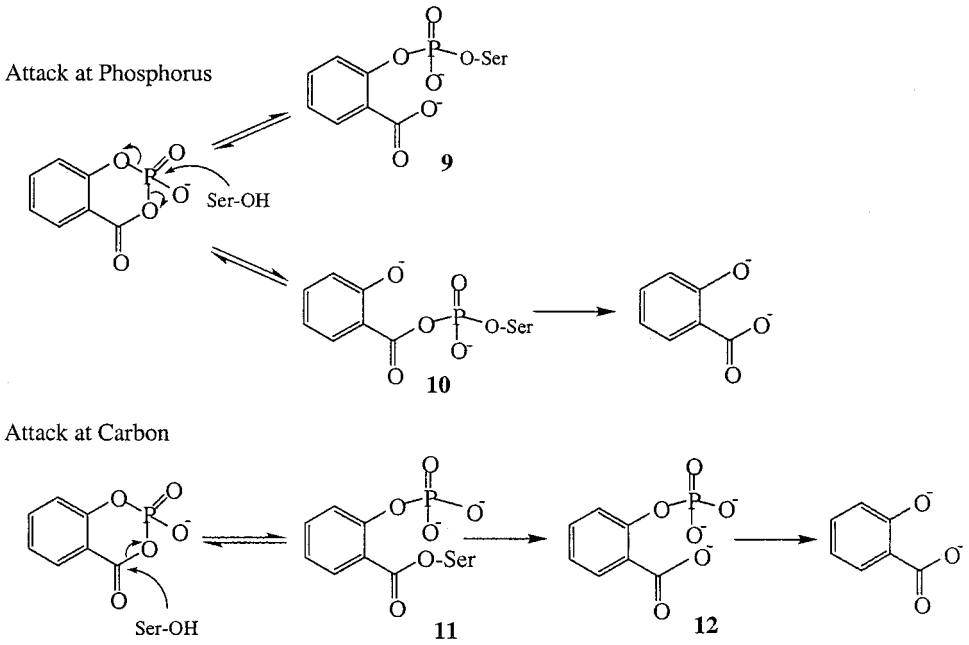
The data of Figure 1 were then fitted to Scheme 2 by means of the Dynafit program.[15] The best fit obtained is indicated by the solid lines in the figure. The values of k_i and k_r thus obtained were 7350 s^{-1} M^{-1} and 1.14×10^{-3} s^{-1}, respectively, in general agreement with previous estimates,[14] although slightly different because the assumed reaction scheme is different. The thermodynamic dissociation constant of E−I ($K_i = k_r/k_i$) is, thus, 0.16 μM.

Chemical Nature of the Covalent Complex between 2 and the P99 β-Lactamase. The covalent complex E−I may represent either acyl enzyme **11** or a phosphoryl enzyme (Scheme 4). Two types of phosphoryl enzyme, **9** and **10**, may be envisaged. Certainly, the carboxylate (from attack at phosphorus, upper path) would be expected to be a better leaving group than the aryloxide (from attack at phosphorus, lower path), and thus **9** the more likely phosphoryl enzyme, but the direction of ring opening could be dictated by the active site.

Distinction between these possibilities was achieved by treatment of E−I with sodium hydroxide, as described in the Experimental Section. Acyl phosphate **10** would be expected to yield salicylate directly. The HPLC analysis of the reaction products gave no evidence of any salicylate on treatment of E−I with hydroxide. Both **9** and **11** should give salicyl phosphate **12**, but by different routes. Alkaline hydrolysis of **11** should regenerate the active site serine residue after acyl oxygen fission. In contrast, in alkaline solution, the facile route to salicyl phosphate from **9** would be by way of elimination, yielding a dehydroalanine residue on the protein as the other product.[8] As previous work in this laboratory has demonstrated, generation of dehydroalanine at the P99 β-lactamase active site by this type of elimination of phosph(on)ate leads to generation of lysinoalanine.[8] This product presumably arises by facile addition of the adjacent Lys-67 amine group to dehydroalanine derived from Ser-64 by elimination. Amino acid analysis of the hydroxide-treated E−I revealed 1 mol of lysinoalanine per mole

(15) Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260.

Scheme 4

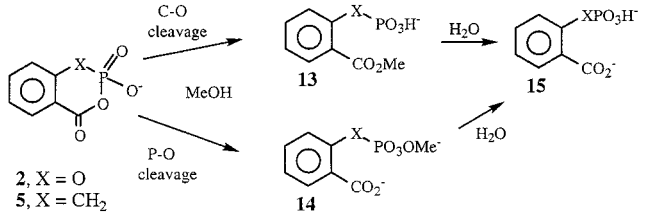


of protein (1.20 and 1.14 mol on the basis of 44 alanine and 29 leucine residues, respectively). The enzyme alone, treated with hydroxide in the same way, yielded no lysinoalanine. This result strongly suggests that E−I is phosphoryl enzyme **9** and that the active site serine hydroxyl is phosphorylated. Although it is possible that **10** or **11** could rearrange into **9** on hydroxide treatment, the simpler interpretation seems more likely.

Kinetics and Mechanism of Reaction of the Phosphonate 5 with the P99 β-Lactamase. The phosphonate analogue of **2** was prepared, as described in the Introduction, to probe further into the inhibition mechanism and, perhaps, to obtain a more effective inhibitor. The synthesis of **5** was straightforward, as described in the Experimental Section (Scheme 8). The hydrolysis of **5** could be followed spectrophotometrically at 286 nm (ϵ = 1410 cm^{-1} M^{-1}; the hydrolysis product absorbed maximally at 271 nm, ϵ = 600 cm^{-1} M^{-1}). The pseudo-first-order rate constant of its disappearance in 50 mM sodium hydroxide was 0.058 s^{-1}. Under the same conditions, the rate constant for hydrolysis of **2** (monitored at the same wavelength) was 0.21 s^{-1}. This difference, favoring **2**, suggests nucleophilic attack by hydroxide ion at the carbonyl group and C−O cleavage (the phosphate of **2** would be a better leaving group −lower conjugate acid pK_a− than the phosphonate of **5**; attack at phosphorus should favor phosphonate **5** over phosphate **2**[13,14,16]). This conclusion was supported by the finding that the products obtained by addition of **2** to a mixture of 0.1 M sodium hydroxide and methanol (1:4) had ^{1}H NMR spectra containing a singlet peak at 3.52 ppm, which disappeared on brief heating in aqueous hydroxide ion. A similar peak arose and fell during the reaction of **5**. This strongly suggests a methyl carboxylate product (**13**, Scheme 5) in each case, arising from nucleophilic attack at the carbonyl group, rather than methyl phosph(on)ate **14** which should be more stable and give rise to a methyl doublet, coupled to phosphorus, in the NMR spectrum. Thus, alkaline solvolysis of both **2** and **5** appears to involve C−O cleavage. This result is in accord with precedent for acyclic acyl aryl phosphates.[17]

In contrast to the above result, the rate constant for hydrolysis of **5** at around neutral pH, viz. 1.59 × 10^{-4} s^{-1} (20 mM phosphate buffer, pH 7.5), is considerably greater than that for **2** (2.11 × 10^{-5} s^{-1}). This is closer to the result that would be expected for phosphyl attack and P−O bond cleavage.[13,14,16] Solvolysis of **2** and **5** in a mixture of 20 mM phosphate at pH 7.5 and methanol (1/1) yielded different results. The product mixture from **2** contained both hydrolysis and carbonyl methanolysis products, the latter (**13**, X = O) distinguished by a singlet at 3.91 ppm which disappeared on heating the solution in the NMR tube. In contrast, the solvolysis mixture from **5** initially contained two products in roughly equal amounts. One, distinguished by a singlet at 3.93 ppm and a doublet at 3.47 ppm (J = 21.5 Hz), is likely to be the carbonyl methanolysis product (**13**, X = CH_2), while the second, characterized by doublets at 3.47 ppm (J = 10.4 Hz) and 3.36 ppm (J = 21.2 Hz), probably corresponds to the phosphonyl transfer product, **14**, X = CH_2. Both of these compounds slowly hydrolyzed to a common product, characterized by a doublet at 3.17 ppm (J = 20.8 Hz), characteristic of the complete hydrolysis product, **15**. Although it is not known at present whether the phosphyl transfer product from **5** arises from attack by water alone or from buffer catalyzed hydrolysis, there is clear evidence that nucleophilic attack at the phosphonyl group of **5** may be as facile as at the carbonyl center under certain circumstances. It is likely, for example, that neutral nucleophiles (e.g., water) may show a greater preference than anionic nucleophiles (e.g., hydroxide ion) for attack at the anionic phosphonyl center.

Scheme 5



In very sharp distinction to the results with **2**,[14] the phosphonate analogue **5**, when incubated with the P99 β-lactamase, did not cause inactivation of the enzyme. At first, this seemed very surprising. The structural similarity between **2** and **5** made it seem unlikely that **5** would not react with the enzyme at all.

(16) Rahil, J.; Pratt, R. F. *Biochem. J.* **1993**, *296*, 389.
(17) DiSabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400.

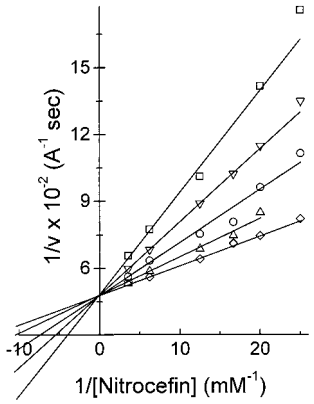


Figure 3. Double reciprocal plot for inhibition of the P99 β-lactamase-catalyzed turnover of nitrocefin by acyl phosphonate **5** at concentrations of 0 μM ($\diamond$), 3 μM ($\triangle$), 8 μM ($\bigcirc$), 15 μM ($\triangledown$), and 25 μM ($\square$). The lines represent the fit to the data of a competitive inhibition model as described in the text.

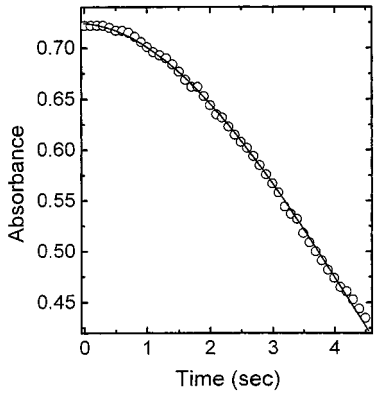
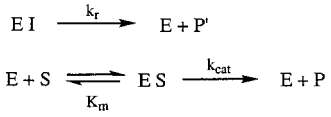


Figure 4. Return of β-lactamase activity on mixing a mixture of enzyme (1.5 μM) and **5** (20 μM) with the substrate cephalothin (300 μM). The absorbance of cephalothin at 284 nm as a function of time is shown. The change in slope with time reflects the increase in enzyme activity. The line was calculated on the basis of Scheme 6 as described in the text.

If the enzyme were to attack at phosphorus, as the emerging results with **2** suggested would be likely, the rationale originally employed in the design of **5** suggested that it should be a more effective inhibitor than **2**. The enzyme did not appear to catalyze hydrolysis of **5** either.

This dilemma was resolved when it was found that **5**, although not a slowly reversible inhibitor as was **2**, behaved, under manual-mixing steady-state conditions, as a fast reversible competitive inhibitor (Figure 3), with a K_i of (10.1 $\pm$ 0.6) μM. In view of all precedents, this would represent surprisingly tight noncovalent binding for a molecule of structure **5**. The unlikelihood of this explanation was confirmed by experimental measurements of initial rates of cephalothin turnover by the P99 β-lactamase in the presence of **2** (0$-$100 μM; see Experimental Section for details) where no indication of inhibition was observed. It seemed unlikely that **5** but not **2** would bind noncovalently at micromolar concentrations. Consequently, the possibility of fast, reversible covalent binding by **5** was considered. Evidence for this was obtained from a stopped-flow experiment when a time-dependent return of activity of the β-lactamase against cephalothin was observed on mixing a combination of the enzyme and **5** with cephalothin (Figure 4). Interpretation of these data in terms of Scheme 6 yielded the value of the rate constant, k_r, for dissociation of the complex of **5** with the β-lactamase. The value obtained, 0.098 s^{-1}, could

Scheme 6

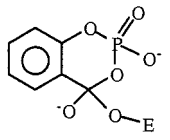


then be combined with the K_i value above to give the rate constant k_i for association of the enzyme and **5** (Scheme 2); a value of 9.8 $\times$ 10^3 s^{-1} M^{-1} for k_i was thus obtained. The magnitude of these rate constants is certainly more suggestive of a covalent interaction than a noncovalent interaction between enzyme and inhibitor.

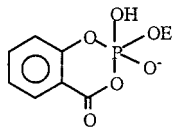
Acyclic Analogues. Compounds **6** and **7** represent, to some degree, acyclic analogues of **2** and **5**, respectively. They appear to interact differently, however, in a quantitative sense at least, to their cyclic counterparts. Compound **6** is a substrate of the P99 β-lactamase by an acyl-transfer reaction where a transiently stable benzoyl enzyme intermediate is generated.[10,11] Phosphonate **7**, synthesized as described in the Experimental Section, turned out, like other acyclic acyl phosphonates,[10,11] to also be a substrate, although poorer than **6** (k_{cat}/K_m values of **6** and **7** are 6070 s^{-1} M^{-1} and 226 s^{-1} M^{-1}), but forming the same benzoyl enzyme intermediate (k_{cat} = 0.011 s^{-1}). Like other acyclic phosphonates, **7** also slowly and irreversibly inactivated the enzyme, presumably by phosphonylation[11] (k_i = 22 s^{-1} M^{-1}). Acylation by **6** is thought to be facile because of favorable interaction of the negatively charged phosphate leaving group with the electropositive active site.[9,11] Phosphylation of the active site by **2** and **5** is clearly much more facile than by **6** and **7**. Possible reasons for this are discussed below. It is interesting to note, however, that the second-order rate constant for enzyme acylation by **6** is very similar to that of phosphylation by **2**.

Molecular Modeling

Computational models for the tetrahedral intermediate of acylation **16** and the pentacoordinated intermediate of phosphorylation **17** of the P99 β-lactamase by **2** were constructed as described in the Experimental Section. These were studied



16



17

as approximations to the transition states of the acylation and phosphorylation reactions, respectively. Molecular dynamics and several starting conformations were employed to find the most likely structures, as also described in the Experimental Section. Calculation of the E_{int} parameter[18] for a variety of energy-minimized structures led to the structures of Figure 5 as the most favorable intermediates for the two reactions. Both appear to involve strong interactions with the active site functional groups of the enzyme. For example, the acylation tetrahedral intermediate (Figure 5A) displays nominal hydrogen bonding (heavy atom distances < 3 Å) between Tyr-150 O$_\xi$ and Ser-64 O$_\gamma$, Lys-67 N$_\epsilon$ and Ser-64 O$_\gamma$, and Lys-67 N$_\epsilon$ and a phosphoryl O$^-$. The Asn-152 side chain also seems well-placed to hydrogen bond to a phosphoryl oxygen, although the N$-$O distance is 3.17 Å in the structure shown. The tetrahedral oxyanion is firmly held in the oxyanion hole (backbone N$-$H moieties of Ser-64

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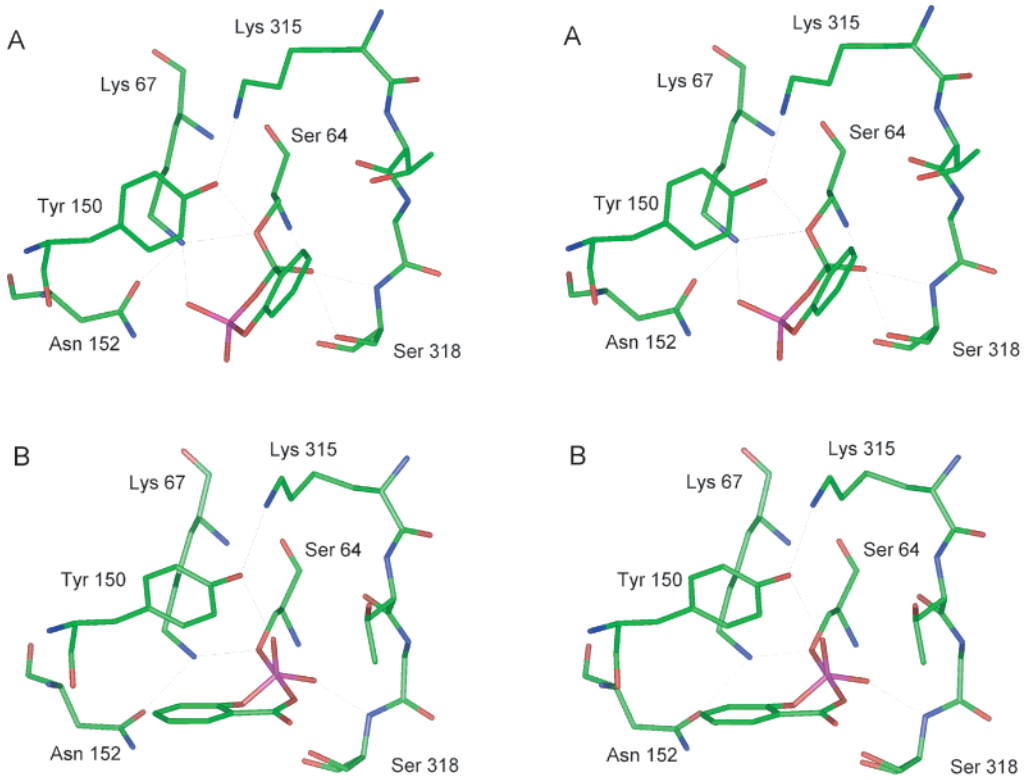


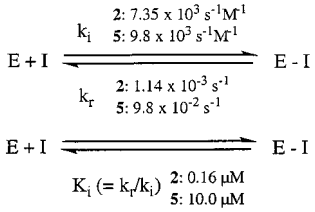
Figure 5. Energy-minimized model structures of an acylation tetrahedral intermediate (A) and a pentacoordinated phosphorylation intermediate (B) which may occur on interaction of the acyl phosphate **2** with the P99 β-lactamase.

and Ser-318). The intermediate thus appears to be well-stabilized by the enzyme, and activation of the leaving group would be provided by its interaction with Lys-67.

Similarly, the pentacoordinated intermediate of phosphorylation (Figure 5B) appears to interact closely with the enzyme active site. There appear to be hydrogen bonds between Lys-67 N_ϵ and Ser-64 O_γ and from Tyr-150 O_ξ to the phosphoranyl POH; the phosphoranyl PO^- is accommodated in the oxyanion hole. No functional group appears to interact with the leaving group, but a carboxylate, directed as it is, out of the active site into solution, should not require general acid catalysis to depart.

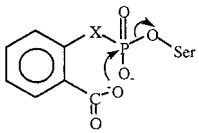
It is not possible to choose between **16** and **17** on the basis of the structures in Figure 5 or the semiempirical calculations leading to them. Experiment, as discussed, strongly supports **17**. It is possible, however, that this result is obtained by default in the sense that it is only the phosphorylation product that is inert enough to be detected. It is still possible, in view of the results from the solvolysis experiments where acylation appeared to be the dominant reaction of **2**, and of the apparently well-stabilized structure in Figure 5A, that acylation of the P99 β-lactamase by **2** is very facile. If, for example, k_i for acylation were 10-fold larger than for phosphorylation, say 7×10^4 s^{-1} M^{-1} (cf. Scheme 6) and k_r were greater than 7 s^{-1}, which would preclude its detection in manual mixing experiments, then K_i for **2** as a fast, covalent, acylation reagent would be >100 μM. The latter number is in accord with the upper limit established by our stopped-flow experiment (see above). A rapid intramolecular deacylation reaction would certainly be in accord with the structure of Figure 5A. The phosphate leaving group of the acylation reaction is directed down into the protein and is likely to be held there in position for facile deacylation by the Lys-67 ammonium ion. Even if this scenario were true, however, the enzyme would be predominantly phosphorylated rather than acylated when both reactions had reached equilibrium.

Scheme 7



Summarizing Discussion

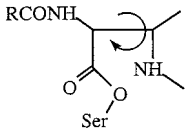
The evidence described above strongly suggests that the P99 β-lactamase does not catalyze the hydrolysis of cyclic phosph(on)ates **2** and **5** but is reversibly phosphylated, and thus inhibited, by them. Quantitatively, the results are summarized by Scheme 7. These results show that **5** does phosphylate the enzyme faster than **2**, but not by a significant amount. Given that the reaction does involve enzyme phosphylation, this result may reflect a weaker productive noncovalent binding of **5** to the enzyme prior to reaction. The greater reactivity of the phosphonate is seen, however, in k_r which, if enzyme phosphylation is involved, also describes a phosphyl transfer reaction (**18**). The rather counterintuitive result of the relative sizes of these rate constants, therefore, is that the phosphonate is a poorer inhibitor of the enzyme, both kinetically (it forms a more labile complex) and thermodynamically (K_i).



18a, X = O
18b, X = CH_2

The reversible phosphylation of the P99 β-lactamase by **2** and **5** is reminiscent of the reversible formation of the cyclic

phosphate intermediate in ribonuclease catalysis.[19] Analogies among enzyme inhibitors are the reversible inhibition of chymotrypsin[20] and papain[21] by cyclic sulfonates and of chymotrypsin and thrombin by various lactones.[22,23] The β-lactams themselves supply a counterexample, however. They covalently inhibit bacterial DD-peptidase by acylation of the active site serine to give the inert complex, **19**. This slowly deacylates by hydrolysis or fragmentation,[24] but there is no evidence of recyclization. This may be due, in part, to the high thermodynamic barrier to ring closure of bicyclic β-lactams,[25] but it may also, in some cases at least, reflect other factors. For



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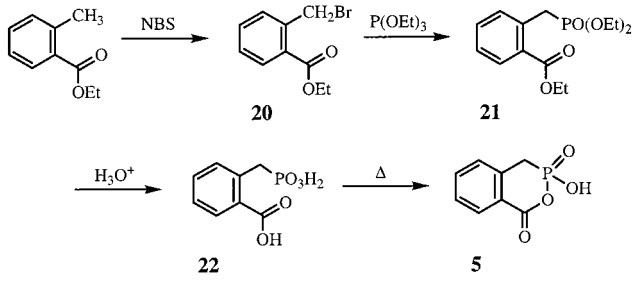
example, on acylation of the *Streptomyces* R61 DD-peptidase by cephalosporins, rotation around the C6−C7 bond of the β-lactam occurs (**19**), presumably to relieve steric stress between the β-substituents on C6 and C7.[26] Recyclization would not directly be possible from this acyl enzyme conformation. On the other hand, an acyl enzyme generated from the reaction of penicillin binding protein 2x of *Streptococcus pneumoniae* with cefuroxime appears to be well-placed for recyclization[27] (although it should be noted that this structure, like the R61 examples, does not represent the initial acyl enzyme but that of a subsequent complex where the 3′-leaving group has been eliminated and a Δ^1-dihydrothiazine ring formed). The phosphyl enzyme intermediates generated from **2** and **5** (**18**) apparently retain access to a conformation where recyclization is possible. A more effective inhibitor than **2** might include functionality to promote conformational relaxation after phosphylation that would better hinder or prevent recyclization and regeneration of the enzyme.

Acyl phosph(on)ates **2** and **5** thus inhibit the P99 β-lactamase most likely by phosphylation of the active site. The enzyme is unable to catalyze the hydrolysis of these species to restore the enzyme, but the latter can be achieved by a recyclization reaction which restores the cyclic acyl phosph(on)ates: rescue by return. These compounds therefore function as reversible inhibitors. Experiments to expand the kinetic and thermodynamic repertoire of these molecules in the direction of more effective inhibitors are in progress. There is evidence that even **2** does enhance β-lactam activity in *in vitro* microbiological assays.[28]

Experimental Section

Materials. The β-lactamase of *Enterobacter cloacae* P99 was purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. Chemical reagents for synthesis were generally purchased from Aldrich Chemical Co. Lysinoalanine was purchased from Sigma Chemical Co. Salicyloyl cyclic phosphate **2** and its acyclic analogue **6** were synthesized previously in this laboratory.[10,14]

Scheme 8



1-Hydroxy-4,5-benzo-2-oxaphosphorinanone(3)-1-oxide Dicyclohexylammonium Salt (5). This compound was prepared by a route beginning with ethyl *o*-toluate (Scheme 8). Bromination of this material with *N*-bromosuccinimide gave substituted benzylbromide **20**. This was reacted with triethyl phosphite to give phosphonate diester **21** which was hydrolyzed to diacid **22**. The synthesis of **22** was largely as described by Gasuti et al.[29] The acid was cyclized by azeotropic dehydration to give cyclic acyl phosphonate **5**, which was isolated as the dicyclohexylammonium salt.

Ethyl *o*-toluate (20 g, 122 mmol) and *N*-bromosuccinimide (21.7 g, 122 mmol) were mixed with 81 mL of carbon tetrachloride. Benzoyl peroxide (20 mg) was added as a catalyst. The flask was irradiated with visible light from a 150 W tungsten lamp, while the mixture was heated under reflux for 3 h. At that time, the reaction mixture was cooled to room temperature. The precipitated solid was removed by vacuum filtration, and the remaining solvent was removed by rotary evaporation to give the brominated product **20** as a deep yellow liquid (33.1 g). ^{1}H ($CDCl_3$) δ 7.90 (d, J = 7.5 Hz, 1H), 7.49 (t, J = 7.5 Hz, 1H), 7.48 (t, J = 7.5 Hz, 1H), 7.40 (d, J = 7.5 Hz, 1H), 4.99 (s, 2H), 4.42 (q, J = 7.1 Hz, 2H), 1.44 (t, J = 7.1 Hz, 3H). This material was used without further purification.

Ester **20** was heated under reflux with an equimolar amount of triethyl phosphite at 160 °C for 3 h. The reaction mixture was cooled overnight and then vacuum distilled to give phosphonate ester **21** (bp 170−174 °C, 0.1 Torr) as a clear yellow liquid in 82% yield (two steps). ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 7.7 Hz, 1H), 7.44 (d, J = 7.7 Hz, 1H), 7.41 (t, J = 7.7 Hz, 1H), 7.30 (t, J = 7.7 Hz, 1H), 4.39 (q, J = 7.6 Hz, 2H), 4.00 (quint, J = 7.6 Hz, 4H), 3.82 (d, J = 23 Hz, 2H), 1.41 (t, J = 7.6 Hz, 3H), 1.21 (t, J = 7.6 Hz, 6H).

The phosphonate **21** was added to 500 mL of 6 M HCl and the mixture heated under reflux for 3 days. The HCl/H_2O was removed by rotary evaporation, and the white solid thus obtained was recrystallized from water in 62% yield. The product diacid **22** exhibited a broad melting point of 194−202 °C (lit.[29] mp 177 °C). ^{1}H (D_2O) δ 7.83 (d, J = 8.1 Hz, 1H), 7.56 (t, J = 8.1 Hz, 1H), 7.41 (m, 2H), 3.57 (d, J = 21.9 Hz, 2H). ν_{max} (KBr) 1659s (C═O), 1271s (P═O).

Solid acid **22** (200 mg) was heated under reflux in 50 mL xylene in a Dean and Stark apparatus. The solid dissolved in boiling xylene, and drops of water were noted in the collection arm of the apparatus after 5 h, signifying cyclization. At this stage, an equimolar amount of dicyclohexylamine was added to the reaction mixture. A colorless salt precipitated as the solution was cooled. The solid was recrystallized from cyclohexane/benzene, yielding the amine salt of pure cyclized product **5**. Melting point 163−165 °C. ^{1}H NMR ($CDCl_3$) δ 9.06 (br, 2H), 8.21 (d, J = 8 Hz, 1H), 7.51 (t, J = 8 Hz, 1H), 7.38 (t, J = 8 Hz, 1H), 7.28 (d, J = 8 Hz, 1H), 3.29 (d, J = 18 Hz, 2H), 2.82 (br, 2H), 1.1−2.0 (m, 20H). ^{31}P NMR (^2H_2O) δ 10.52 (t). ν_{max} (KBr) 1713s (C═O), 1251s (P═O). Anal. Calcd for $C_{20}H_{29}NO_4P$: C, 63.31; H, 7.97; N, 3.69; P, 8.16. Found: C, 63.40; H, 8.08; N, 3.64; P, 8.80. ESMS (H_2O) m/z 560.9 (M + $2C_{l2}H_{23}NH^+$) 560.8.

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Sodium Benzoyl Benzylphosphonate (7). This compound was prepared by a general method described previously,[2] beginning with the silver salt of benzylphosphonic acid and benzoyl chloride in a 1:2 molar ratio. The diester thus obtained was hydrolyzed to give the desired sodium salt of the monoester, which was recrystallized from water. ^{1}H NMR (D_2O) δ 7.98 (d, J = 7.7 Hz, 2H), 7.69 (t, J = 7.4 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H), 7.23−7.33 (m, 5H), 3.39 (d, J = 21 Hz, 2H). ^{31}P (^2H_2O) δ 18.73. ν_{max} (KBr) 1701s (C═O), 1286s (P═O).

Analytical and Kinetic Methods. The concentration of enzyme in stock solutions was determined spectrophotometrically.[11] All enzyme kinetics experiments were performed at 25 °C in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.5.

Kinetics of Reaction of the P99 β-Lactamase with 2. Fluorescence measurements were obtained from a FluoroMax-2 (Instruments S.A., Inc.) spectrofluorimeter. Samples of salicyloyl cyclic phosphate **2** were excited at 310 nm, and the emission was monitored between 340 and 450 nm, where the maximum was at 370 nm. After addition of the P99 β-lactamase, emission intensity at 400 nm was followed as function of time. The data from these experiments were fitted to Scheme 2 using the program Dynafit.[15]

Initial velocities of cephalothin (200 μM) hydrolysis by the P99 β-lactamase (40 nM) in the presence of several concentrations (0−100 μM) of **2** were measured with a Durrum D-110 stopped-flow spectrophotometer at 278 nm over 2 s. This experiment was designed to detect any fast noncovalent binding of the enzyme to **2** at these concentrations without the complication of the covalent reaction.

Kinetics of Reaction of the P99 β-Lactamase with 5. The inhibition constant (K_i) for **5** as an inhibitor of the P99 β-lactamase was obtained from spectrophotometric measurements of initial velocities of nitrocefin (at several concentrations: 40, 50, 60, 80, 160, and 280 μM) hydrolysis in the presence of several concentrations of **5** (0−25 μM). Nitrocefin hydrolysis was monitored by the absorbance of product formation at 482 nm. Initial velocities of nitrocefin hydrolysis were fitted to a competitive inhibition model by the method of Cleland.[30]

The rate of breakdown of the complex of the enzyme with **5** to restore free enzyme was obtained from the following experiment. Return of activity of the β-lactamase against cephalothin was monitored spectrophotometrically at 284 nm. Thus, a solution of **5** (20 μM and 40 μM, in separate experiments) and β-lactamase (1.5 μM) was mixed with cephalothin (300 μM), and the decrease in absorbance due to cephalothin hydrolysis was measured for 5 s in the stopped-flow spectrophotometer. A rate constant, k_r, for the return of activity against cephalothin, as the complex dissociated, was obtained by fitting the experimental data to Scheme 6 using the program Dynafit as described previously.[11,15] In this Scheme, S represents cephalothin, EI the enzyme/**5** complex, and P and P′ represent the respective products of cephalothin hydrolysis and dissociation of I from EI. A control experiment where cephalothin (300 μM) hydrolysis in the presence of the β-lactamase (1.5 μM) alone was performed gave k_{cat} and K_m values for cephalothin of 780 s^{-1} and 20 μM, respectively.

Kinetics of Reaction of 7 with the P99 β-Lactamase. Kinetic parameters for interaction of the β-lactamase with **7** were determined as described previously for similar acyclic acyl phosphonates.[11] Thus, K_m was obtained as the inhibition constant (K_i) from an experiment where **7** was used to inhibit turnover of a good substrate (cephalothin). Initial velocities of cephalothin (200 μM, K_m = 20 μM, monitored spectrophotometrically at 278 nm) hydrolysis in the presence of several concentrations of **7** (0−200 μM) were fitted to a competitive inhibition model by the method of Cleland.[30]

(30) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

Scheme 9

$$E + I \underset{K_m}{\rightleftharpoons} EI \xrightarrow{k_{cat}} E + P$$

$$E + I \xrightarrow{k_i} E\text{-}I$$

To obtain the inactivation rate constant (k_i) and turnover number, k_{cat}, the absorbance of **7** (50 and 100 μM, in separate experiments) with time in the presence of the enzyme (1.07 μM) was monitored at 240 nm. The data were analyzed by means of the Dynafit program using Scheme 9 as described previously.[11] In the fitting of these progress curves, K_m was fixed at the value obtained as described above.

Characterization of the β-Lactamase−Inhibitor (2) Complex. HPLC Analysis. Product analysis of the enzyme−inhibitor (**2**) complex after base hydrolysis was obtained from a HPLC method using a Rainin Rabbit-HPX solvent delivering system, a Machery−Nagel Nucleosil C18 column, and a Gilson HM/HPLC UV−vis detector. The mobile phase was 0.05 M acetonitrile, adjusted to pH 3.5 with phosphoric acid.

P99 β-lactamase (20 μM) was partly inactivated by incubation with **2** (10 μM) for 30 min at room temperature. Excess β-lactamase was used to ensure that essentially all of **2** was converted to the enzyme−inhibitor complex. The sample was then treated with NaOH (final concentration 0.2 M) and incubated for another 90 min. It was then filtered through an ultracentrifuge tube (Ultrafree-MC filters 5000 NMWL from Millipore). The filtrate (15 μL aliquots) was then injected into the HPLC column at a flow rate of 1.5 mL/min with the detector set at 232 nm. Authentic salicyclic acid was used as a standard. Thus, salicyclic acid (10 μM) was treated the same as **2** before passing through the HPLC column; that is, it was treated with NaOH and filtered through an ultracentrifuge tube. The retention time of salicylic acid was 7.9 min. Also, 15 μL of **2** (10 μM) alone was injected into the column as a control.

Lysinoalanine Analysis. The P99 β-lactamase (10 μM) was inactivated by incubation with **2** (20 μM) for 10 min at room temperature. Excess of **2** was used to ensure that essentially all of the enzyme was converted to the enzyme−inhibitor complex. The enzyme, diluted with MOPS buffer to 10 μM final concentration, was used as a control. The solutions were then made 0.2 M in NaOH and held at room temperature for 1.5 h. Each of these was then dialyzed separately against 0.01 M HCl for 24 h (two changes) to remove excess of **2**. Aliquots (100 μL) were then freeze-dried in acid washed tubes and submitted for amino acid analysis to the Yale Protein and Nucleic Acid Chemistry Facility. Standard solutions of lysinoalanine were prepared for quantitation. Lysinoalanine was identified by its retention time (41.7 min) on the amino acid analysis column.

Molecular Modeling. The computations were set up essentially as previously described[11,18] and run on an SGI Octane 2 computer with Insight II 2000 (MSI, San Diego, CA). The starting point for the simulations was the crystal structure of the P99 β-lactamase with a phosphonate inhibitor covalently attached to the active site serine residue (PDB file 1bls[9]). A variety of initial conformations were explored by means of molecular dynamics, and the significantly populated conformations arising were selected and subjected to energy minimization. The conformations shown in Figure 5 were the lowest energy carbonyl and phosphoryl adducts found, where the energies compared were E_{int} values[18] which take into account interactions between ligand and protein, residue−residue interactions, and internal residue energies.

Acknowledgment. This research was supported by the National Institutes of Health.

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