Mechanism of Reaction of Acyl Phosph(on)ates with the β-Lactamase of *Enterobacter cloacae* P99†

Kamaljit Kaur and R. F. Pratt*

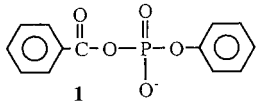
Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

Received September 25, 2000; Revised Manuscript Received January 24, 2001

ABSTRACT: A series of acyl phosph(on)ates has been prepared to more closely examine the details of the interactions of this class of molecule with β-lactamases. In general, they were found to react with the class C β-lactamase of *Enterobacter cloacae* P99 in two ways, by acylation and by phosphylation. The acyl-enzymes generated by the former reaction were transiently stable with half-lives of between 3 and 45 s, of comparable lifetime therefore to those generated by the inhibitory β-lactams cefotaxime, cefuroxime, and cefoxitin. On the other hand, phosphylation led to a completely inactive enzyme. In general, the second-order rate constants for acylation (k_{cat}/K_m) were larger than for phosphylation (k_i). As expected on chemical grounds, phosphylation was found to be relatively more effective for the phosphonates than the phosphates. The acyl phosphates were much more effective acylating agents however. The acylation reaction was found to be enhanced by hydrophobic substituents in both the acyl and leaving group moieties. Thus, the most reactive compound in this series was benzo[*b*]thiophene-2-carbonyl 2′-naphthyl phosphate with a K_m value of 0.15 μM and a k_{cat} of 0.2 s^{-1}; k_{cat}/K_m is therefore 1.3×10^6 s^{-1} M^{-1}, making this compound the most specific acyclic acylation reagent for this β-lactamase yet described. Significant substrate inhibition by this compound suggested that further binding regions may be available for exploitation in inhibitor design. A linear free energy analysis showed that the transition states for acylation of the β-lactamase by aroyl phosphates are analogues of the corresponding aryl boronic acid adducts. Molecular modeling suggested that the aroyl phosphates react with the P99 β-lactamase with the aroyl group in the side chain/acyl group site of normal substrates and the phosphate in the leaving group site. In this orientation, the phosphate leaving group interacts strongly with Lys 315.

Bacterial resistance to β-lactam antibiotics arises largely because of the susceptibility of these molecules to hydrolysis catalyzed by β-lactamases (*1*). The problem of the β-lactamases in β-lactam chemotherapy has been met, with considerable success in the short term, by application of β-lactamase-resistant β-lactams and of β-lactamase inhibitors. The β-lactamase inhibitors in current clinical practice, however, for example, clavulanic acid, penicillanic acid sulfone, and tazobactam, another penicillanic acid sulfone, are themselves β-lactams, and thus, like the β-lactam-resistant β-lactams, are susceptible to hydrolysis by the mutant β-lactamases selected for by β-lactam chemotherapy (*2*, *3*). Thus, new chemical classes of antibiotic and β-lactamase inhibitor are of continued interest. Recent research in this laboratory (*4*) has identified simple acyl phosph(on)ates, **1** and **2**, as a new class of molecules that interact covalently with the active sites of β-lactam-recognizing enzymes (β-lactamases and the DD-peptidases of bacterial peptidoglycan biosynthesis; the latter enzymes are the targets of β-lactam antibiotics).

Preliminary investigation (*4*) suggested that these molecules have the ability to both phosphylate and acylate the β-lactamase active site, with greatest reactivity against a typical class C β-lactamase. In this paper, we expand on the theme of **1** and **2** in a structure/activity study and explore more fully the mechanism of reaction of these molecules with a class C β-lactamase.



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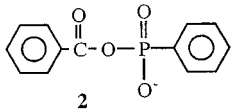

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EXPERIMENTAL PROCEDURES

Materials. The β-lactamase of *Enterobacter cloacae* P99 was obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. α-Chymotrypsin, electric eel acetylcholinesterase, bovine intestine mucosal phosphodiesterase, and other biochemical reagents were obtained from Sigma Chemical Co. Cephalothin was a gift from Eli Lilly and Co. Chemical reagents for synthesis were generally purchased from Aldrich Chemical Co. Benzo[*b*]thiophene-2-carbonyl chloride was obtained from Maybridge Chemical Company Ltd, Cornwall, U.K. Aryl boronic acids were purchased from Lancaster. The Bradford assay reagent (*5*) was purchased from Bio-Rad.

Acyl Phosph(on)ates. The silver salt of phenyl phosph(on)ate or a substituted phenyl phosph(on)ate (3.0 mmol)

† This research was supported by National Institutes of Health Grant AI-17986.

* To whom correspondence should be addressed: Dr. R. F. Pratt, at the above address; Fax: (860) 685-2211; E-mail: rpratt@wesleyan.edu.

10.1021/bi002243+ CCC: $20.00
Published on Web 03/23/2001

Table 1: Analytical Wavelengths and Rate Constants of Background Hydrolysis of the Acyl Phosph(on)ates **3**−**19**

compound	wavelength (nm)	$\Delta\epsilon$ ($M^{-1}\,cm^{-1}$)	($k_0 \times 10^7 s^{-1}$)[a]
3	244	9610	3.74
4	244	7800	2.97
5	240	8260	0.97
6	260	4470	45.8
7	240	5250	22.2
8	294	11890	19.8
9	264	250	132.0
10	294	11840	5.7
11	294	12850	1.4
13	244	5580	5.8
14	296	9280	7.1
15	244	10710	14.3
16	244	9140	11.7
17	240	16060	6.6
18	240	6350	7.9
19	244	6150	1.34

[a] Rate constant for spontaneous hydrolysis of **3**−**19** in 20 mM MOPS buffer, pH 7.5, at 25 °C.

and benzoyl or a substituted benzoyl chloride (6.0 mmol) were stirred together in acetonitrile (15 mL) overnight under nitrogen at room temperature following the procedure of Laird and Spence (*6*). The resulting reaction mixture was added dropwise into a stirred solution of sodium bicarbonate (3.0 mmol) in H_2O (2.0 mL) at 0 °C. Stirring was continued for 30−45 min at room temperature. More acetonitrile (50 mL) was added to the solution, which was then cooled for 1 h at 0 °C. The precipitate that appeared was collected and recrystallized from water to give the pure sodium salt of the acyl phosph(on)ate (55−60% yield). All of the compounds were synthesized using the above procedure except for sodium 2,6-dimethylbenzoyl phenyl phosphate where the silver salt of 2,6-dimethylbenzoic acid and phenyl dichlorophosphate were stirred together to give the corresponding diester. The diester was hydrolyzed to give the desired sodium salt of the monoester as described above.

Sodium Benzoyl p-Nitrophenyl Phosphate (**3**). ^1H NMR (^2H_2O) δ 7.43 (d, $J = 8.8$ Hz, 2H, Ar*H*CO), 7.56 (t, $J = 7.5$ Hz, 2H, Ar*H*CO), 7.74 (t, $J = 7.0$ Hz, 1H, Ar*H*CO), 8.07 (d, $J = 7.7$ Hz, 2H, Ar*H*OP), 8.28 (d, $J = 8.3$ Hz, 2H, Ar*H*OP); ^{31}P (^2H_2O) δ −15.67; ν_{max} (KBr) 1717s (C═O). Anal. calcd for $C_{13}H_9NNaO_7P$: C, 45.24; H, 2.63; N, 4.06; P, 8.97. Found C, 45.31; H, 2.87; N, 4.17; P, 9.12.

Characterization of the other compounds prepared is presented as Supporting Information.

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were measured with a Hewlett-Packard 8452A spectrophotometer. The concentrations of stock enzyme solutions were obtained spectrophotometrically (*7*). All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5. Steady-state kinetic parameters were obtained as described below. Thus, k_{cat} values for compounds **3**−**5** and **8**−**11** were obtained spectrophotometrically, at the wavelengths listed in Table 1, from total progress curves. In these experiments, the initial concentration of the compounds was typically between 30 and 100 μM to obtain a good estimate of V_{max}. Since the K_m values of all of the compounds were too low to be determined directly from these progress curves, they were obtained as inhibition constants (K_i) from experiments where the acyl phosphates were used to inhibit turnover of a good substrate

Scheme 1

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

$$EI \xrightarrow{k_r} E + P'$$

Scheme 2

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

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

$$I \xrightarrow{k_o} P$$

(cephalothin). Initial velocities of cephalothin [200 μM, K_m = 20 μM (*8*), monitored at 278 nm] hydrolysis in the presence of several concentrations of each acyl phosph(on)ate were fitted to eq 1 (which assumes a competitive relationship between the inhibitor I and the substrate S) by a nonlinear least squares procedure to obtain K_i which was taken to be equal to K_m for the phosph(on)ate as a substrate.

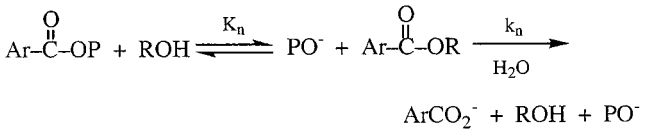
$$v = v_o(K_m + [S])/\{K_m(1 + [I]/K_i) + [S]\} \qquad (1)$$

In eq 1, v represents the initial velocity in the presence of the inhibitor and v_o that in its absence.

Rates of breakdown of transiently stable intermediates to restore free enzyme were obtained in the case of acyl phosphate **13** and acyl phosphonates **15** and **16** from incubation of mixtures of appropriate concentrations of enzyme and inhibitor (the concentration of the former much less than that of the latter). After two minutes of incubation at room temperature, aliquots of these reaction mixtures were diluted into assay cuvettes containing the substrate cephalothin at essentially saturating concentration (1 mM). The rate of cephalothin hydrolysis, as monitored at 288 nm, increased with time due to breakdown of the intermediate to free enzyme. Rate constants for this return of activity, k_r, were obtained by fitting the experimental data to Scheme 1 using the numerical simulation/least-squares fitting program Dynafit (*9*). In this scheme, S represents cephalothin, EI the transient intermediate whose chemical nature is discussed below, and P and P′ their respective hydrolysis products. For the fitting procedure, values of k_{cat} and K_m for cephalothin were fixed at 250 s^{-1} and 20 μM, respectively. The value of k_{cat} was obtained from progress curves for hydrolysis of cephalothin (1 mM) alone in the presence of the P99 enzyme (5 nM). The K_m value of cephalothin was taken to be 20 μM (*8*, *10*).

In the cases of **6**, **7**, **13**, and the acyl phosphonates **15**−**19**, a more complicated reaction scheme involving competing turnover and inhibition was required (see Scheme 2). To obtain the inactivation rate constant (k_i) and k_{cat}, the absorbance of the inhibitor, I, (several concentrations in separate experiments) with time in the presence of the enzyme was monitored, and the data were analyzed by means of the Dynafit program (*9*). For each compound, K_m was fixed at the value obtained from above (see Table 2). For **13**, **15**, and **16**, k_{cat} was fixed at the value obtained from the above return of activity experiment. A slightly more complicated scheme (Scheme 5, see discussion) was used to fit

Scheme 3



the progress curves of compound **14** with enzyme. In this case, the values of K_m and K_{si} were fixed during this fitting. The value of K_m was obtained from the inhibition experiment described above. The value of K_{si} was obtained from initial velocities of turnover of **14** at high concentrations (5−120 μM) using eq 2 which is appropriate, in terms of Scheme 5, under these conditions.

$$v = V_{max}/(1 + [I]/K_{si}) \quad (2)$$

The solid line shown in Figure 3 was obtained from Dynafit (*9*), using the parameters of Scheme 5 obtained in this way.

Inhibition constants for aryl boronic acids were obtained from measurements of initial velocities of cephalothin (200 μM) hydrolysis in the presence of several concentrations of each boronate; competitive inhibition was assumed (*11*).

Compound **6** was also tested as an inhibitor and/or substrate of α-chymotrypsin, acetylcholinesterase, and phosphodiesterase. To test for inhibition, α-chymotrypsin (10 μM) was incubated with **6** (1 mM) in 0.1 M Tris-HCl buffer, pH 7.8, at 25 °C. The enzyme was assayed spectrophotometrically (410 nm) against succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (*12*) as a function of time. Similarly, acetylcholinesterase and phosphodiesterase were also tested with **6**. Thus, acetylcholinesterase (1 μM) and phosphodiesterase (4 μM) were incubated with **6** (1 mM) separately in 20 mM MOPS buffer, pH 7.5, at 25 °C. The enzymes were assayed spectrophotometrically against acetylthiocholine iodide (250 nm) and bis(*p*-nitrophenyl) phosphate (398 nm), respectively. Controls without inhibitor were also followed in each case. Turnover of **6** by α-chymotrypsin, acetylcholinesterase, and phosphodiesterase was determined from the spectrophotometric progress curves of reaction of **6** with each of these enzymes separately.

Linear Free Energy Corrections. The parameters k_n and K_n of eqs 9 and 11 (see Results and Discussion) relate to the reactions of Scheme 3 (see also Scheme 7). They will vary, in principle, with the aroyl group, largely due to electronic effects. This variation was taken into account by means of eqs 3 and 4. In these equations, $k_{n,o}$ and $K_{n,o}$ represent the rate and equilibrium constants for the reference compound where aroyl is benzoyl, and the δ term represents the correction for the electronic effect of other aroyl groups.

$$\ln k_n = \ln k_{n,o} + \delta \ln k_n \quad (3)$$

$$\ln K_n = \ln K_{n,o} + \delta \ln K_n \quad (4)$$

These correction terms for each aroyl group were estimated from linear free energy relationships taken from the literature. The reaction attributed to the rate constant k_n involves the hydrolysis of alkyl benzoates in neutral water. The effect of aromatic substituents on this rate constant is given by the Hammett equation $\delta \ln k_n = \rho\Delta\sigma$. The value of ρ for this correction was approximated by the average of the ρ values for acid-catalyzed hydrolysis of alkyl benzoates (+0.03) and for alkaline hydrolysis (+2.5) (*13*). The rationale for this procedure is that ρ is a measure of the effective charge on the reacting molecule in the transition state (*14*). Under acid conditions, the transition state will be positively charged and under alkaline conditions the transition state will be negative. Under neutral conditions where water is the nucleophile and acid/base catalyst, the charge on the transition state will lie between these two extremes. Since we were unable to find a directly applicable value for this parameter from the literature, the average of the two extremes was taken as a reasonable estimate. This correction was applied to **6**, **7**, and **13**. An *o*-OMe σ value of −0.39 (*15*) was taken to calculate the correction term for **13**. A correction for the electronic effect of the benzothiophene group of **10** was obtained using data for the hydrolysis of the methyl esters of heterocyclic carboxylates (*16*). No correction (with respect to **1**) was applied in the case of **11**. Similarly, the reaction attributed the equilibrium constant K_n involves alcoholysis of aroyl phosphates. Substituents in the aroyl moiety of these molecules would be expected to have little effect on the equilibrium constant of the reaction (*17*) and thus $\delta \ln K_n$ was taken to be zero. From this analysis, the corrections ($\delta \ln k_n$) applied were 0, −2.07, −1.89, −0.60, 0, and +1.04 to the data for compounds **1**, **6**, **7**, **10**, **11**, and **13**, respectively.

^{1}H NMR Turnover Experiment. A solution of **6** (1 mM) in 20 mM sodium bicarbonate in D_2O was prepared and its NMR spectrum was taken. To this solution, the P99 β-lactamase (0.55 mg, 10 μM) was added and another NMR spectrum was obtained after 20 h. Another sample of **6** (1 mM) was prepared in 20 mM sodium bicarbonate in D_2O and 2.5 M CD_3OD. After taking an NMR spectrum of this sample, the P99 β-lactamase (0.58 mg) was added and a final NMR spectrum obtained after 20 h.

Phosphorus Assays. The P99 β-lactamase (1 mL, 12 μM) was inactivated by incubating it with the phosphates (3−4 mM) for 10−15 h or phosphonates (0.5 mM) for 1−2 h, at room temperature in both cases. These concentrations and times were based on calculations derived from the kinetic parameters of Table 2. Protein was then precipitated from the solution by the addition of trichloroacetic acid (5%) to it and allowing the resultant mixture to stand in an ice bath for 30 min. The mixture was then centrifuged and the solid precipitate was washed four times with 5% aqueous trichloroacetic acid. The solid protein was then redissolved in 1 N NaOH and the alkaline solution was incubated overnight at 37 °C. It was then neutralized with 1 N HCl. The resulting solution was assayed for phosphate by the procedure of Hess and Derr (*18*). A mixture of sodium phenyl phosphate with the enzyme was used as a control and sodium phosphate was used as a standard. Protein concentration was measured using the Bradford assay (*5*) with the native β-lactamase as the standard. This assay procedure yielded anomalous results with **14**, as described in Results and Discussion. These arose because of precipitation of the hydrolysis product of **14**, 2-naphthyl phosphate, on acidification of the reaction mixture with trichloroacetic acid. An alternative procedure was therefore tried where the protein was isolated and washed twice by ultrafiltration before acidification.

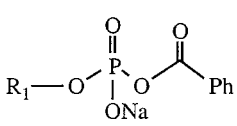
Phosphorus was assayed in phosphonate complexes by the procedure of Kapoulas et al. (*19*). Thus, the P99 β-lactamase (1 mL, 12.1 μM) was inactivated by incubation with **15** (0.5

mM) for 1 h at room temperature. After the precipitation and washing of the protein with trichloroacetic acid, it was redissolved in 100 μL of 70% $HClO_4$ and heated at 160−170 °C for 3 h. After the solution was restored to room temperature, 200 μL of H_2O was added. The resulting solution was assayed for phosphate as described above. Phenylphosphonic acid was used as the standard.

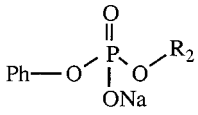
Computational Methods. The computations were set up essentially as previously described (*20*) and run on an IBM 3CT computer with INSIGHT II 97.0 (MSI, San Diego, CA). The starting point for the P99 β-lactamase structural simulations was the crystal structure of the enzyme with a phosphonate inhibitor covalently attached to the active site serine residue (PDB file 1bls; *21*). This was transformed into the benzoyl-enzyme tetrahedral intermediate, **21**, with the leaving group (phenyl phosphate) still attached, by means of the Builder module of INSIGHT II. MNDO charges were employed for the benzoyl phenyl phosphate ligand, while charges on the protein were assigned by INSIGHT II. The enzyme−ligand complex was then hydrated by a 15 Å sphere of water centered at the active site serine O_γ. Two different orientations, the benzoyl side chain placed in the leaving group site and the benzoyl group placed in the β-lactam side chain site, were separately employed as starting structures for dynamics runs. From the results of molecular dynamics runs of up to 20 ps at temperatures of 300 and 1000 K, likely conformations of the side chain and leaving group were assessed. During the dynamics runs at 300 K, the ligand, water, and the protein were permitted to move. At 1000 K, the protein backbone was restrained and the water was omitted. In these calculations, Lys 67 and Lys 315 were cationic, Tyr 150 was neutral, and the tetrahedral intermediate was dianionic (phosphate and oxyanion). Significantly populated conformations were chosen from these runs, and the energy of each was minimized in the CV force field by means of 1000 steps by the method of steepest descents followed by 2000 steps of conjugate gradients. In this minimization procedure, the entire system, protein, ligand, and water, was freed from constraint. The final derivative of the energy with respect to structural perturbation was then in the range of 0.02−0.03 kcal/Å. Bond distances and interaction energies, including those of all nonbonded interactions, could then be obtained from the minimized structures by means of the Discover program. Residues included in the E_{int} calculations were Ser 64, Lys 67, Tyr 150, Asn 152, Lys 315, Thr 316, Gly 317, and Thr 318. These have previously been shown to contribute the most to discrimination between tetrahedral ligands (*20*). The debenzoylation tetrahedral intermediate **22** was treated in the same way.

RESULTS AND DISCUSSION

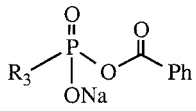
The molecules synthesized to expand our understanding of the affinity of the prototype compounds **1** and **2** for β-lactam-recognizing enzymes have the structures **3**−**19**. These were prepared as the sodium salts as described above in Experimental Procedures. Thus, reaction of a silver phosph(on)ate with an acyl chloride (2 eq) or of a silver carboxylate (2 eq) with a phosphoryl chloride led to the diacyl phosph(on)ate. This was not isolated but hydrolyzed immediately to the anion of the mono-acyl compound (**3**−**19**).



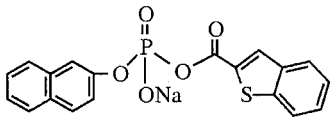
R_1 = p-nitrophenyl **3**
2-naphthyl **4**
n-hexyl **5**



R_2 = p-nitrobenzoyl **6**
m-nitrobenzoyl **7**
cinnamoyl **8**
phenylacetyl **9**
benzo[b]thiophene-2-carbonyl **10**
4-biphenylcarbonyl **11**
2,6-dimethylbenzoyl **12**
o-methoxybenzoyl **13**



R_3 = p-nitrophenyl **15**
m-nitrophenyl **16**
2-naphthyl **17**
4-biphenyl **18**
n-hexyl **19**



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Reactions of Acyl Phosph(on)ates. In neutral aqueous solution, these compounds are, as previously noted (*4*), rather surprisingly stable. The rate constants for their spontaneous hydrolysis, determined spectrophotometrically (Table 1), in 20 mM MOPS buffer, pH 7.5, are also given in Table 1, from which half-lives of between 0.6 and 80 days can be calculated. The shorter half-lives correspond to compounds with electron-withdrawing acyl substituents, as, for example, in **6**, and with aliphatic rather than aromatic acyl groups, as, for example, in **9**. This pattern of reactivity rather suggests hydrolysis by attack of water/hydroxide at the acyl rather than the phosphyl center.

^{1}H NMR studies showed that addition of the P99 β-lactamase to compounds **3**−**19** led to hydrolysis of these compounds. For example, in the case of **6**, the NMR spectrum after the enzyme-catalyzed reaction, showed the presence of phenyl phosphate [δ 7.08 (t,1H), 7.18 (d, 2H), 7.33 (t, 2H)] and *p*-nitrobenzoate [δ 7.98 (d, 2H), 8.27 (d, 2H)]. Reaction of **6** in the presence of the P99 β-lactamase and 2.5 M $^2H_4$-methanol, yielded a third product, identified by comparison of its spectrum with that of an authentic sample, as $^2H_3$-methyl *p*-nitrobenzoate [δ 8.17 (d, 2H), 8.30 (d, 2H)], where the ratio of the latter to *p*-nitrobenzoate was approximately 1:1. This strongly suggests that turnover of **6** by the P99 β-lactamase involves an acyl (rather than a phosphoryl) transfer reaction and thus an acyl-enzyme intermediate. Methanol is well-known to act as an alternative nucleophile toward the acyl-enzymes that accumulate during turnover of β-lactams (*22*) and depsipeptides (*23*) by class C β-lactamases.

These compounds were also found to interact with other hydrolases. For example, chymotrypsin slowly catalyzed the hydrolysis of **6**, with formation of a transiently stable intermediate which hydrolyzed with a rate constant of 10^{-3} s^{-1}. This intermediate is presumably *p*-nitrobenzoyl chymotrypsin for which Vishnu and Caplow report a hydrolysis rate constant of 9.1×10^{-4} s^{-1} at 25 °C (*24*). Acetyl

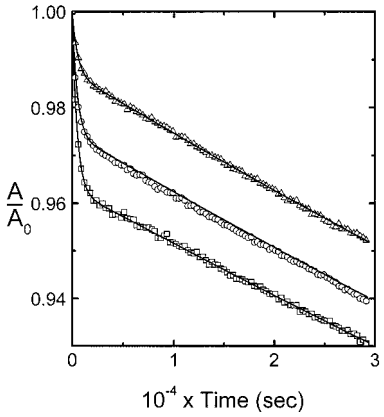


FIGURE 1: Inactivation of the P99 β-lactamase (45 nM) by **6**. Shown are changes in the absorption of **6**, as a fraction of the initial absorption (□, 40 μM; ○, 70 μM; △, 100 μM), as a function of time. The initial burst indicates turnover of **6** by the enzyme, decreasing to zero with time. The slow rates at long times represent the spontaneous hydrolysis of **6**. The points are experimental and the lines were calculated from Scheme 2.

cholinesterase and phosphodiesterase turned over **6** more rapidly, with k_{cat}/K_m values of 80 and 5800 s^{-1} M^{-1} respectively. Acyl phosphates in general are thus not specific substrates/inhibitors of β-lactamases.

Spectrophotometric studies showed that reaction of the β-lactamase with **3**−**19** led to either complete hydrolysis of the acyl phosph(on)ate or to complete inactivation of the enzyme, depending on the compound involved and its concentration relative to that of the enzyme. For example, Figure 1 shows the absorption changes of various concentrations of **6** on addition of the β-lactamase. The burst corresponds to turnover of ca. 90 molecules of **6** per molecule of enzyme. After the burst, the enzyme was inactive and the subsequent slow decrease in absorption corresponds to spontaneous hydrolysis of **6**. These data were fitted to Scheme 2 as described in Experimental Procedures, yielding values for k_{cat} for turnover and k_i for inactivation. The values of these parameters for **3**−**19** are given in Table 2. Also presented in this Table are the previously acquired data (*4*) for compounds **1** and **2**.

Compounds **3**−**5** and **8**−**11** were completely turned over by the enzyme with no inactivation observed, at least at acyl phosphate concentrations up to 50 μM. On the other hand, inactivation was observed by the phosphates **6**, **7**, **12**−**14**, and the phosphonates **15**−**19**. To determine whether the inactivation reaction involved phosphylation of the enzyme, phosphorus analyses of the inactivated enzymes were performed. All precedents would suggest that the active site serine (Ser 64) would be the site of any such phosphylation (*21*). Thus, enzyme inactivated by an acyl phosphate was precipitated by trichloroacetic acid, washed, redissolved in 1 M sodium hydroxide, and incubated at 37 °C overnight to release phosphate from the active site serine (*25*, *26*). Similarly, the inactivated enzyme after treatment with an acyl phosphonate was analyzed for phosphorus. In this case, treatment with perchloric acid (*19*) rather than sodium hydroxide was used to release phosphate.

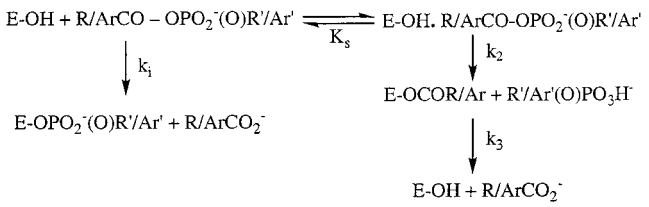
The results from the phosphorus assays suggest that the instances of irreversible inactivation of the P99 β-lactamase by **3**−**19** arose by enzyme phosphylation. The native enzyme and a sample incubated with 3.7 mM phenyl phosphate contained no phosphorus. On the other hand, enzyme inactivated by the same concentration of **6** contained, in two determinations, 1.09 and 1.14 atoms of phosphorus per active site (these figures assume an enzyme 65% active; *24*). A similar value (1.29) was obtained from **7**. The assay with the phosphonates **15** and **16** revealed 1.32 and 1.28 phosphorus atoms per active site, respectively. In contrast, the enzyme inactivated by **14** contained 9.9 and 10.5 (two determinations) atoms of phosphorus per molecule of enzyme. We believe that the latter result reflects incomplete removal of the very hydrophobic **14** and its hydrolysis product 2-naphthyl phosphate by the washing procedure used. When the enzyme was washed twice by ultrafiltration, the

Table 2: Rate Constants for Turnover of **1**−**19** by the P99 β-Lactamase and for Inhibition

compound	structure	k_{cat} (s^{-1})[a]	K_m (μM)[a]	$k_{cat}/K_m \times 10^{-4}$ (s^{-1} M^{-1})[a]	k_i ($s^{-1}M^{-1}$)[a]	K_i (μM)[b]
1[c]	$PhOP(O_2^-)OCOPh$	0.017	2.8	0.607	0	5.8 ± 1.2
2[c]	$PhP(O_2^-)OCOPh$	0.020	2.9	0.690	70	
	$R_1OP(O_2^-)OCOPh$					
3	R_1=*p*-nitrophenyl	0.0110 ± 0.0002	1.8 ± 0.4	0.644	≤2[e]	
4	R_1=2-naphthyl	0.013 ± 0.001	0.7 ± 0.2	1.75	≤1[e]	
5	R_1=*n*-hexyl	0.0110 ± 0.0003	8.8 ± 1.3	0.125	≤1[e]	
	$PhOP(O_2^-)OR_2$					
6	R_2=*p*-nitrobenzoyl	0.17 ± 0.06	2.7 ± 0.5	6.27	700 ± 100	2.0 ± 0.4
7	R_2=*m*-nitrobenzoyl	0.21 ± 0.02	2.7 ± 1.2	7.88	440 ± 60	2.7 ± 0.8
8	R_2=cinnamoyl	0.050 ± 0.001	1.7 ± 0.3	3.04	≤1[e]	
9	R_2=phenylacetyl	0.260 ± 0.002	8.3 ± 1.2	3.10	≤0.04[c]	
10	R_2=benzo[*b*]thiophene-2-carbonyl	0.15 ± 0.01	0.64 ± 0.07	23.2	≤5[e]	0.19 ± 0.03
11	R_2=4-biphenylcarbonyl	0.023 ± 0.001	0.33 ± 0.04	6.83	≤3[e]	0.65 ± 0.25
12[d]	R_2=2,6-dimethylbenzoyl				20.9 ± 1.5	
13	R_2=*o*-methoxybenzoyl	0.060 ± 0.005	109 ± 13	0.0562	3.6 ± 0.4	15.7 ± 3.1
14	R_1=2-naphthyl					
	R_2=benzo[*b*]thiophene-2-carbonyl	0.20 ± 0.04	0.15 ± 0.01	131	1813 ± 3	
	$R_3P(O_2^-)OCOPh$					
15	R_3=*p*-nitrophenyl	0.018 ± 0.004	144 ± 16	0.0125	25 ± 3	
16	R_3=*m*-nitrophenyl	0.014 ± 0.002	78 ± 15	0.0179	65 ± 19	
17	R_3=2-naphthyl	0.019 ± 0.002	35 ± 4	0.0551	115 ± 5	
18	R_3=4-biphenyl	0.018 ± 0.001	13 ± 3	0.117	89 ± 4	
19	R_3=*n*-hexyl	0.011 ± 0.001	47 ± 3	0.0226	11.1 ± 0.1	

[a] These constants refer to the parameters of Scheme 2. [b] Inhibition constant for the analogous aryl boronic acid. [c] Taken from ref *4*. [d] No turnover observed. [e] Limits estimated by assuming no more than 10% inactivation during the experiment.

Scheme 4



phosphorus incorporation fell to 3.9 atoms/molecule. The more complicated kinetics of turnover of **14** (see below) also probably relate to the hydrophobicity of **14**.

Compound **12**, where acylation (and deacylation) would be sterically hindered, showed slightly different behavior than the others of Table 2. The β-lactamase was slowly and irreversibly inactivated by this compound, but the protein produced by this reaction contained no phosphorus. The inert derivative thus appears to be an acyl-enzyme. Apparently, although acylation of the enzyme by **12** is slow, deacylation is even slower.

The experiments reported above suggest that the interaction of the P99 β-lactamase with acyl phosphates likely follows the paths shown in Scheme 4. In principle, the noncovalent intermediate could partition to give both turnover and inactivation. But given that turnover involves acyl transfer and inactivation phosphyl transfer at Ser 64, the separate paths of Scheme 4 seem more likely.

Kinetics of the Reactions between Acyl Phosph(on)ates and the β-Lactamase. Previous experiments have indicated that the rate of turnover of **1** and **2** at saturating concentrations by the P99 β-lactamase is limited by deacylation, i.e., $k_{cat} = k_3$ (Scheme 2) (*4*). The evidence for this included the close similarity of k_{cat} values for **1** and **2**, corresponding to deacylation of a common benzoyl-enzyme intermediate, and the acceleration in rates produced by the addition of the alternative nucleophile methanol. It can be seen from Table 2 that the k_{cat} values for all the benzoyl phosph(on)ates, **3**−**5** and **15**−**19**, are very similar to each other and to those of **1** and **2**. This strongly suggests that deacylation is rate-determining for these compounds also. This conclusion is further supported by the observation of a delayed return of activity on diluting reaction mixtures of **3**−**19** with the β-lactamase into a substrate solution as described in the Experimental Procedures. Figure 2, for example, shows the results of such an experiment with **16**, where the calculated rate constant for return of activity was 0.0136 s^{-1}. The close similarity between this value and that of k_{cat} for this substrate (Table 2) is strong evidence for accumulation of a covalent acyl-enzyme. Finally, the steady-state hydrolysis of **10** (12 μM) was accelerated by methanol (0−2.5 M) to an extent similar to that for **1** (data not shown). Numerical treatment of these data by the usual method (*27*) yielded a partition ratio, methanolysis vs. hydrolysis, for the acyl enzyme of 25 ± 2. This is the same, within experimental uncertainty, as the values obtained with **1** (*4*) and with *N*-(phenylacetyl)-glycyl depsipeptides (*27*). The acyl-enzyme derived from **10** is therefore as accessible to methanol as those from these other substrates.

The acyl-enzymes derived from reaction of the P99 β-lactamase with acyclic depsipeptides can also be specifically intercepted by D-amino acids to give peptide products (*28*). The presence of D-amino acids also increases steady-state initial rates of substrate disappearance under $S_o \geq K_m$ conditions. As part of the present work, we checked whether amino acids could also accelerate deacylation of the aroyl-enzymes that arose by reaction of the P99 enzyme with typical acyl phosphates. Reaction of both **1** (50 μM) and **6** (50 μM) was accelerated by D-alanine (0−200 mM) but not by L-alanine. Thus stereospecific aminolysis of aroyl-enzyme derivatives can also occur. More interestingly however, reaction of **6** was not accelerated by D-phenylalanine (0−50 mM). The latter amino acid is a much better acyl-acceptor than D-alanine when the acyl group is the more enzyme-specific *N*-(phenylacetyl)glycyl (*28*). This unexpected result was expanded by examination of the effect of D-amino acids on reaction of **10** (12 μM) in the presence of the P99 β-lactamase. In this case, the observed rate was not accelerated by either D-alanine (0−200 mM) or D-phenylalanine (0−50 mM); presumably, aminolysis of the acyl-enzyme did not occur in these cases. The stereospecific aminolysis reaction at this active site must therefore involve subtle interactions between the enzyme and both the acyl group and the D-amino acid.

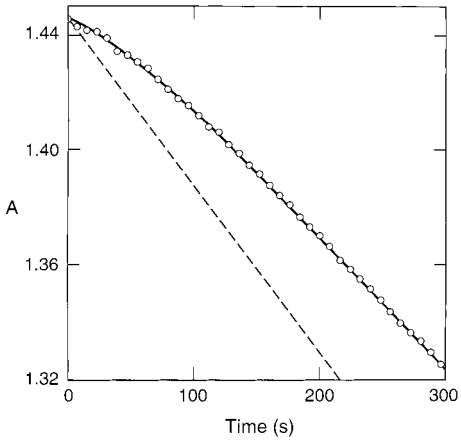


FIGURE 2: Return of activity of the P99 β-lactamase (0.23 μM) after incubation with **16** (100 μM) for 2 min. The absorbance of cephalothin at 288 nm as a function of time is shown after addition of an aliquot (22 μL) of the reaction mixture to a cuvette containing cephalothin (1 mM, 1.0 mL). The increase in slope with time reflects the increase in enzyme activity. The points are experimental and the line was calculated from Scheme 1. The dashed straight line represents the activity of the enzyme with cephalothin alone.

Thus, acylation of the P99 β-lactamase by **1**−**19** produces a transiently stable (half-lives vary from 2.7 to 45 s) acyl-enzyme species, stable enough to quite seriously discommode the enzyme. The lifetimes of these acyl-enzymes are of comparable magnitude to those generated by reaction of the P99 β-lactamase with the inhibitory β-lactams cefotaxime, cefuroxime, and cefoxitin, for example (*29*). A number of the acyl phosph(on)ates also irreversibly inactivate the enzyme by phosphylation. Acylation rates (k_{cat}/K_m) are, in general, considerably larger than phosphylation rates (k_i), even for the phosphonates where more efficient phosphylation might have been expected. Inactivation of the enzyme by acylation thus seems the more effective strategy.

Some trends emerge from the data of Table 2. The more electron-withdrawing *p*-nitrophenyl phosphate leaving group of **3** than that of **1** does not significantly affect the acylation rate (k_{cat}/K_m). This rate, however, is increased by the larger, more hydrophobic 2-naphthyl phosphate leaving group of **4**. The aliphatic hexyl phosphate leaving group, present in

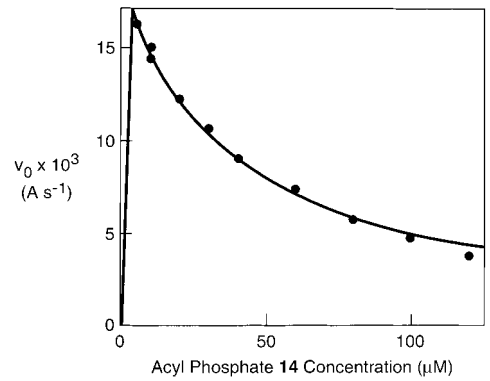


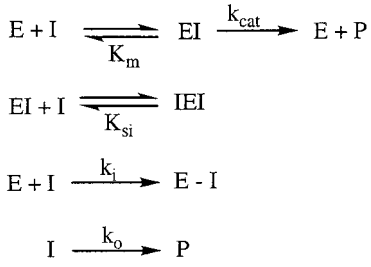
FIGURE 3: Substrate inhibition of the P99 β-lactamase (0.10 μM) by **14**. The points are experimental and the line is calculated from Scheme 5.

the acyl phosphate **5** and the acyl phosphonate **19**, produced a less effective acylating agent than the parent benzoyl compounds **1** and **2**, respectively. An electron withdrawing group on the acyl moiety, as in **6** and **7**, did produce faster acylation, but also, by a similar factor, a faster deacylation (k_{cat}). The larger, more hydrophobic acyl side chains on **8**−**11**, particularly on the benzothiophene derivative **10**, did increase the acylation rate over that of **1**. Steric hindrance of the acyl group by the ortho-substituents present in **12** and **13** significantly impeded acylation. Although deacylation of the *o*-methoxy derivative, as in **13**, actually appeared to be faster than the unsubstituted benzoyl-enzyme, the 2,6-dimethyl substitution pattern present in **12**, strongly depressed deacylation (and acylation). The phosphates were, in general, much more effective acylating agents than the phosphonates. This difference may reflect the poorer leaving group in phosphonates (higher conjugate acid pK_a values) and/or their different distributions of negative charge and hydrophobic surface.

The major conclusion from the above, at least with respect to the development of a more effective reagent, was that hydrophobic substituents, on both the acyl group and the leaving group, enhanced acylation. These leads were combined and the molecule **14** was prepared. Gratifyingly, the contribution of the acyl group and the leaving group appeared to be additive with the result that **14**, with a k_{cat}/K_m of 1.3×10^6 s^{-1} M^{-1}, is the best of the compounds yet investigated, and in fact the most active acylating agent of this enzyme yet discovered, with the exception of the better bicyclic β-lactams (*29*, *30*).

Reaction of **14** with the P99 β-lactamase was more complicated than observed with the other compounds. Figure 3 shows a plot of initial velocity vs. concentration for this compound. There is clear evidence of substrate inhibition. The data of Figure 3 were fitted to Scheme 5 as described in the experimental section. The value of the substrate inhibition constant, K_{si}, thus obtained, was 38 ± 4 μM. Although this is 2 orders of magnitude greater than the K_m for productive binding, it still represents impressive affinity for the enzyme by a second molecule of **14**. The results from the phosphate assay on the enzyme inactivated by **14** also suggested that nonproductive association may occur. It is perhaps not surprising that a molecule as hydrophobic as **14** might find more than one binding site on a protein, but it is certainly interesting that its binding at a site other than the active site leads to inhibition of the enzyme. The "parent" compounds of **14**, viz. **4** and **10**, did not show any sign of substrate inhibition at concentrations up to 50 μM. The aggregation problems of **14** could probably be alleviated by hydrophilic substituents on one or both of the aromatic rings.

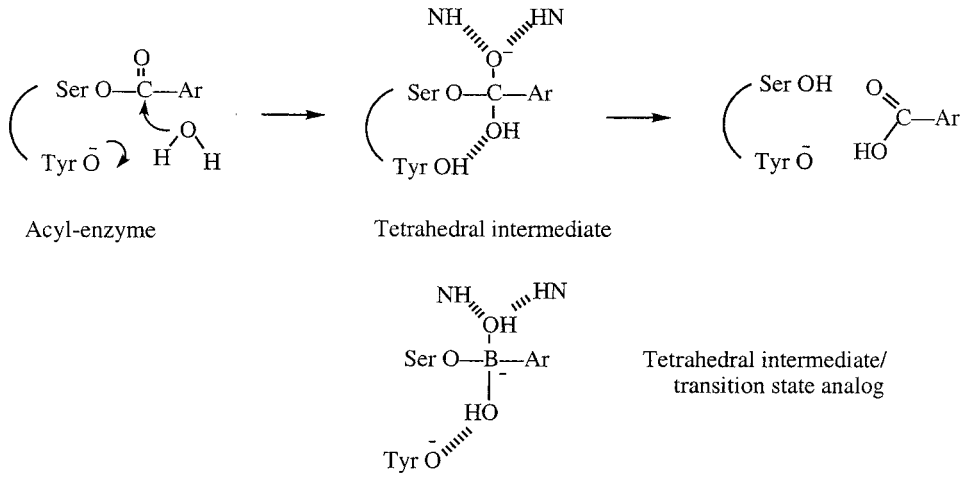
Scheme 5



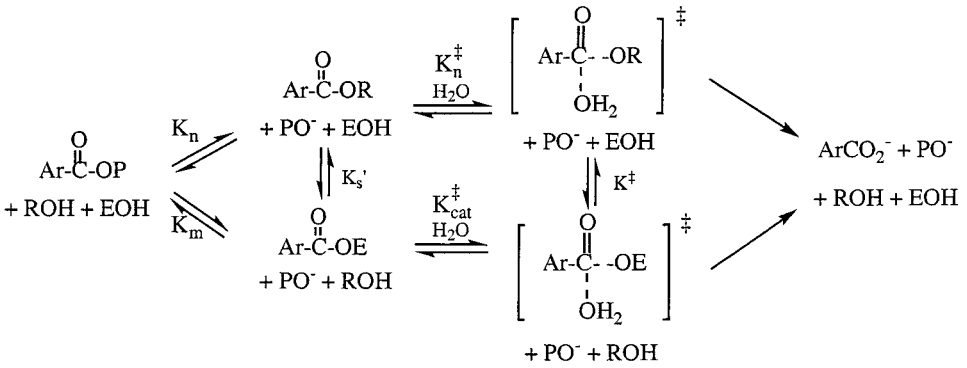
The Relationship between Aroyl Phosph(on)ates and Aryl Boronates: Linear Free Energy Relationships. The ability of aroyl phosph(on)ates to efficiently acylate the P99 β-lactamase points to significant affinity between these compounds and the active site. As discussed previously (*4*), part of this affinity certainly arises from interactions between the active site, which is significantly positively charged (*21*), and the negatively charged phosph(on)ate leaving group. Beyond this, however, it seems that aromatic acyl groups also have a positive influence on the interaction, during acylation at least. This is an interesting result because of the significant difference in structure between these acyl groups and the acylamido substituents of specific substrates. It does however correlate with the known affinity of class C β-lactamases for aryl boronic acids, $ArB(OH)_2$. The inhibitory ability of these latter compounds against β-lactamases was first demonstrated by Waley and co-workers (*31*) and has been recently extended by Shoichet and co-workers (*11*, *32*, *33*). The latter group has produced two crystal structures of aryl boronates bound to a class C β-lactamase (*32*, *33*). These structures demonstrate that the boronates are bound as tetrahedral, presumably anionic, adducts to the active site serine (Ser 64 O_γ). Furthermore, one of the boronate hydroxyls occupies the oxyanion hole of the β-lactamase active site which, in the P99 enzyme, is composed of the backbone NH groups of Ser 64 and Ser 318 (*21*). The boronate complex can be seen as a transition state analogue for the hydrolysis of aroyl-β-lactamases (Scheme 6). Thus the complementarity of the boronate studies and the present work is evident.

Dissociation (inhibition) constants for complexes of boronic acids containing the same aryl groups as compounds **1**, **6**, **7**, **10**, **11**, and **13** were determined and are given in Table 2. The most effective boronate inhibitor [as demonstrated by Shoichet et al. (*11*) and hence incorporated into this study in **10** and **14**] and also the most specific aroyl phenyl phosphate (k_{cat}/K_m) was the benzothiophene derivative. The relationship between the aroyl phosphate turnover parameters and boronate binding can be seen from the thermodynamic cycle depicted in Scheme 7. In Scheme 7, ROH represents a small molecule aliphatic alcohol with comparable nucleophilicity to the active site serine hydroxyl of the β-lactamase. The upper pathway represents the nonenzymatic route of hydrolysis of aroyl phosphate to products mediated by the alcohol ROH. The alcohol reacts with the substrate and displaces the phosphate leaving group PO^- yielding the intermediate alkyl ester $ArCO_2R$. This then

Scheme 6



Scheme 7



hydrolyzes in the second step of the reaction, by way of the transition state shown, to the hydrolysis products. A similar double displacement reaction occurs in the enzyme-catalyzed lower pathway where nucleophilic attack on the substrate by the active site serine hydroxyl displaces the leaving group and forms the acyl-enzyme $ArCO_2E$. In the second step, the acyl-enzyme is hydrolyzed, passing through the transition state shown, to products, including the regenerated enzyme. Since deacylation is rate-determining for this substrate, K_m encompasses formation of the acyl-enzyme.

This scheme represents the relationships between the uncatalyzed and enzyme catalyzed reaction in terms of transition state theory. This approach for an enzyme-catalyzed reaction with a covalent intermediate was proposed by Lienhard (*34*) and subsequently affirmatively reviewed by Wolfenden (*35*) and by Kraut (*36*). In the present instance, the uncatalyzed reaction is divided into two steps to allow the transition state comparison to be as close as possible. In accordance with transition state theory, formation of the transition state in each case is represented as a pseudo-equilibrium process. The constant $K^{\ddagger}$ however does not formally represent a dissociation process but rather an equilibrium constant expressing the relative affinity of a transition state for two catalysts, an alcohol and the β-lactamase (*35*). The degrees of bond making and breaking and the positions of the protons in these transition states is not meant to be defined in Scheme 7.

The thermodynamic cycle of Scheme 7 leads directly to eq 5.

$$K^{\ddagger} = \frac{K_n^{\ddagger} K_n}{K_{cat}^{\ddagger}/K_m} \tag{5}$$

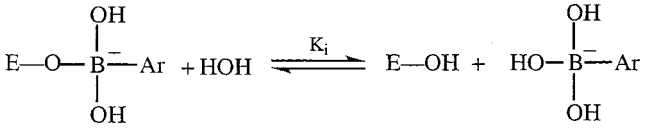
According to transition state theory, eq 6 is true, and therefore eq 7 follows.

$$K_n^{\ddagger}/K_{cat}^{\ddagger} = k_n/k_{cat} \tag{6}$$

$$K^{\ddagger} = \frac{k_n K_n}{k_{cat}/K_m} \tag{7}$$

In eq 7, k_n represents the second order rate constant of the uncatalyzed reaction of the substrate with the alcohol ROH and k_{cat} represents the first-order catalytic constant of the enzyme-catalyzed reaction. If it is assumed that adducts of aryl boronates with the β-lactamase closely resemble, structurally and electronically, the transition states for deacylation of the analogous aroyl-enzymes, then the equilibrium of Scheme 8 will be a direct analogue of the reaction with equilibrium constant $K^{\ddagger}$ of Scheme 7. The linear free energy relationship of eq 8 would then follow; in this equation, δ refers to analogous structural perturbations in the acyl phosphonates and boronates such as, in this case,

Scheme 8



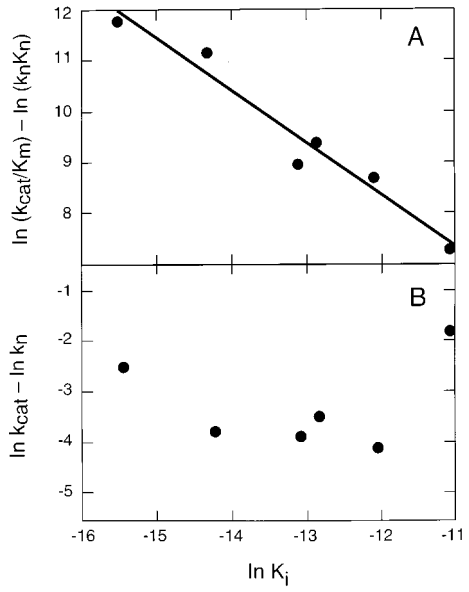


FIGURE 4: (A) Correlation between the rates of acylation (k_{cat}/K_m) of the P99 β-lactamase by acyl phosphates, **1**, **6**, **7**, **10**, **11**, and **13**, and the dissociation constants of analogous aryl boronate complexes (K_i). The points are experimental and the line shows the fit to eq 9. (B) Correlation between rates of turnover (k_{cat}) by the P99 β-lactamase of the above acyl phosphates and the boronate dissociation constants.

variation in the aromatic ring of the acyl group and aryl boronate.

$$\delta \ln K^{\ddagger} = \delta \ln K_i \quad (8)$$

From eqs 7 and 8, eq 9 follows.

$$\delta [\ln(k_{cat}/K_m) - \ln(k_n K_n)] = -\delta \ln K^{\ddagger} = -\delta \ln K_i \quad (9)$$

A plot of $[\ln(k_{cat}/K_m) - \ln(k_n K_n)]$ vs $\ln K_i$ would then be expected to yield a straight line of slope -1. Scheme 7 also generates eq 10.

$$K^{\ddagger} = K_{s'}(k_n/k_{cat}) \quad (10)$$

Given eqs 8 and 10, eq 11 follows. Plots of the left-hand

$$\delta [\ln k_{cat} - \ln k_n] = \delta [\ln K_{s'} - \ln K^{\ddagger}] = \delta [\ln K_{s'} - \ln K_i] \quad (11)$$

sides of eqs 9 and 11 vs. $\ln K_i$ are shown in Figure 4. The corrections to terms on the left-hand sides of these equations, arising from electronic effects, $\ln k_n K_n$ and $\ln k_n$, respectively, were calculated as described in Experimental Procedures. A least-squares straight line of slope -1.02 ($r = 0.978$) is drawn through the points in the former plot (Figure 4, panel A). This fit suggests that eq 9 and Scheme 7 do accommodate the data. This result shows that the effects of structural perturbations in the aromatic ring of the boronates on the strength of their binding to the enzyme are very similar to those produced by the same perturbation in the aroyl phosphate on achieving the acylation transition state (vs. free enzyme and ligand); thus, the boronic acid adducts do resemble the transition states for turnover of benzoyl substrates. On the other hand, the plot of ($\ln k_{cat} - \ln k_n$) vs $\ln K_i$ (eq 11, Figure 4, panel B) shows considerable scatter.

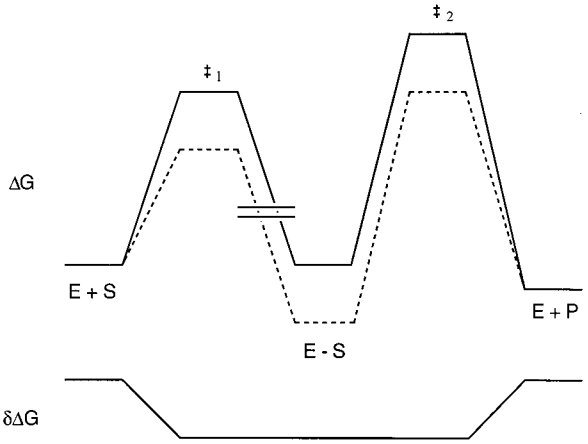


FIGURE 5: Perturbation of the free energy profile for turnover of an aroyl phenyl phosphate by the P99 β-lactamase on change of the aroyl moiety.

The points corresponding to m- and p-substituted benzoyl derivatives (compounds **1**, **6**, **7**, and **11**, with ordinate values around -4 on the plot) appear to show little variation, although those arising from the perhaps more sterically demanding compounds **10** and **13** do have different values. If only the upper right point (corresponding to **13**) is omitted, a straight line of slope 0.38 ($r = 0.82$) is obtained. Despite this, we think that these data are best interpreted to indicate that in the simple *m*- and *p*-substituted benzoyl systems, $\ln K_{s'} - \ln K_i$ (eq 11) is close to constant and thus that the substitutions affect the acyl-enzyme and the deacylation transition state to a similar extent. A combination of these interpretations of Figure 4, panels A and B, would yield the free energy profile for acyl phosph(on)ate turnover shown in Figure 5, where structural perturbations in the aryl group are close to uniformly expressed along the reaction coordinate, i.e., the strength of binding of the aryl group to the enzyme does not change significantly along the reaction coordinate, a uniform binding situation.

Although Scheme 7 was proposed quite some time ago for enzymes employing a covalent intermediate, it appears to have seen little application to real data. In contrast, the analogous scheme for noncovalent intermediates has been widely used to assess the quality of transition state analogue inhibitors (see ref *37* for an early example). It is gratifying therefore to be able to provide an example of the former type. Another example, published very recently, is that of Pressmore and Raines (*38*), who investigated the validity of vanadate complexes as transition state analogues of ribonuclease. In this case, the enzyme rather than the inhibitor was subject to perturbation. Although the interpretation of k_{cat}/K_m in general is still under discussion (*39*−*41*), the existence of linear free energy correlations that can be interpreted in terms of Scheme 7 and its analogues is not. Interpreted in terms of Scheme 7, the present results show that aryl boronates, which are well-established β-lactamase inhibitors, react with the P99 β-lactamase to yield good analogues of the transition states for turnover of aroyl phosph(on)ates by this enzyme.

Structural Models. A final important issue is that of the orientation of acyl phosph(on)ates as substrates in the active site. A specific β-lactamase substrate is oriented primarily by interactions with the enzyme of the amido side chain, the carbonyl oxygen of the scissile bond, and the leaving

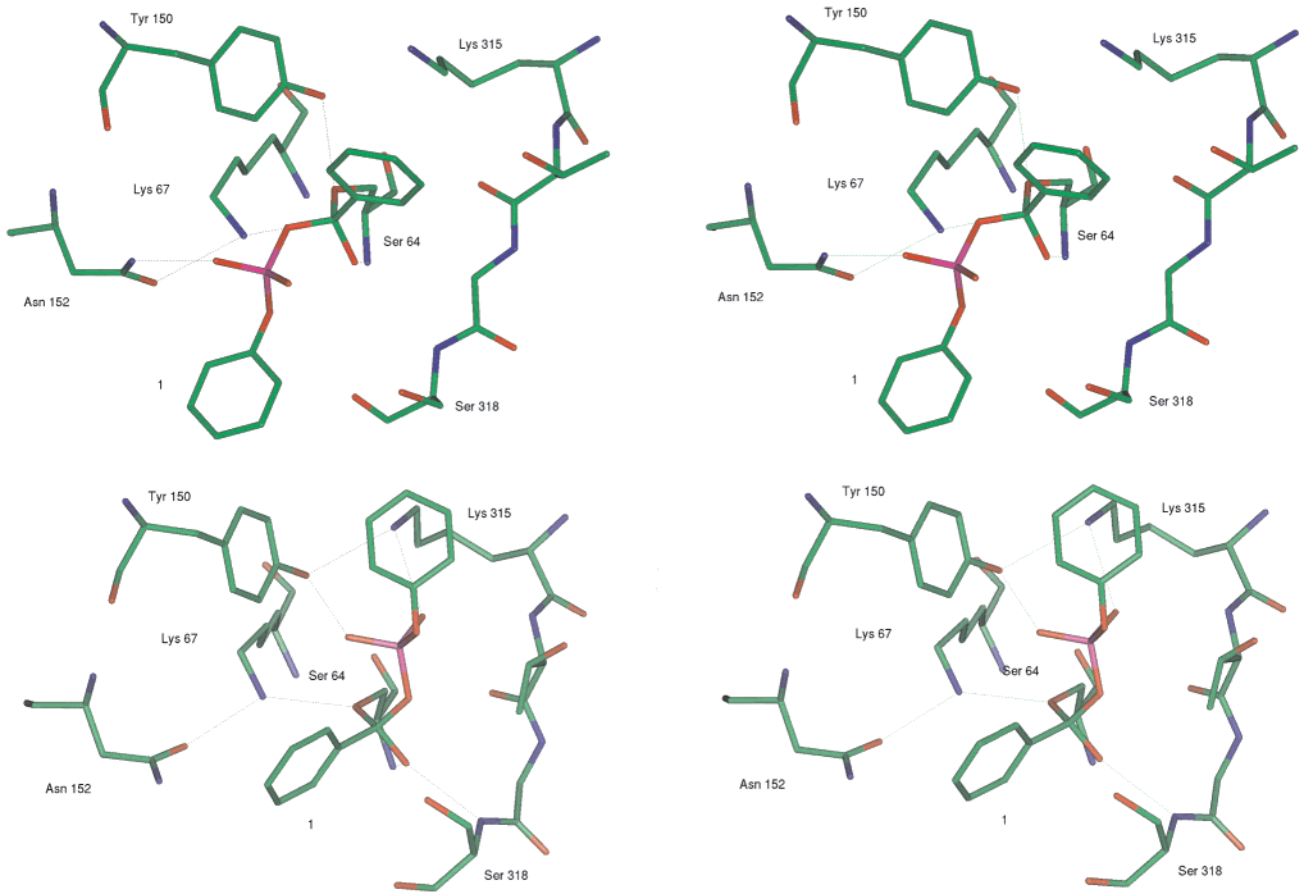
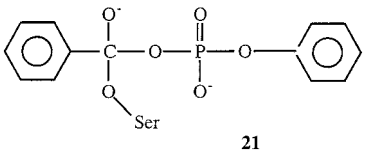


FIGURE 6: Stereoviews of optimized acylation tetrahedral intermediate structures formed on interaction of the P99 β-lactamase with the acyl phosphate **1**. Upper pair: The aroyl group in the leaving group site ("up") and the phosphate leaving group in the side chain site ("down"). Lower pair: The inverse orientation with the aroyl group down and the leaving group up.

group carboxylate (*21*, *42*). Since acyl phosph(on)ates lack two of these three features, the preferred and productive dispositions [which might be different for individual acyl phosph(on)ates] of these molecules in the active site are not self-evident. The modeling studies and crystal structures of Shoichet et al. (*11*, *32*, *33*) who have addressed the same issue, are clearly relevant, at least with respect to the deacylation step. The boronate modeling studies suggested that the phenyl group and substituted phenyl groups could, perhaps equally well, occupy either the side chain or leaving group sites of a normal substrate, whereas larger aryl groups, such as biphenyl or benzothiophene, should prefer the side chain site. The crystal structure of the *m*-aminobenzene boronic acid complex revealed the aryl group in the leaving group site (*32*) while that of the benzothiophene-2-boronic acid demonstrated that it lay in the side chain site (*33*).

Our computer modeling experiments, carried out as described in Experimental Procedures, were designed to compare the two possible orientations of the acylation tetrahedral intermediate **21**, both qualitatively, in terms of the nature of the interactions with the enzyme, and quantitatively, in terms of the relative interaction energies between ligand and enzyme in the two orientations. The final, energy-minimized structures achieved by these prodecures, corresponding to the two alternative orientations of the substrate, are shown in Figure 6, where the lower pair has the aryl group of the benzoyl moiety in the side chain site and the upper pair has it in the leaving group site. The substrate appears to be quite well accommodated in the active site in both orientations. In both cases the oxyanion is in its hole, although more tightly in the former case, and the aromatic rings are adjacent to the hydrophobic residues Tyr 150, Tyr 221, Leu 119, and Leu 293. In the former case (Figure 6, lower pair), Ser 64 O_γ appears to be hydrogen-bonded to the ammonium group of Lys 67, as seen in previous models of the tetrahedral intermediates (*43*), and the phosphate oxygen atoms are hydrogen-bonded to Tyr 150 OH and Lys 315. The structure could be seen as an analogue of the transition state for either formation or breakdown of the tetrahedral intermediate during acylation. In the alternative orientation (Figure 6, upper pair), Tyr 150 seems hydrogen-bonded to Ser 64 O_γ while the ammonium group of the Lys 67 is within hydrogen-bonding distance of the bridging phosphoryl oxygen and Asn 152 within that of a nonbridging phosphoryl oxygen.



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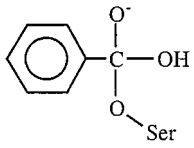
A choice between the above two alternatives is not easily made by inspection of Figure 6. The orientation shown in Figure 6, lower pair, seems the more likely however on the basis of the following two points. First, calculation shows that the interaction energies between substrate and protein, E_{int}, which also take into account intrasubstrate and intraprotein interactions (*20*), are considerably larger for the

orientation of Figure 6, lower pair, than for that of Figure 6, upper pair (−590 kcal/mol and −557 kcal/mol, respectively; the difference appears to derive mainly from the electrostatic interaction between the phosphate group and the positive charge of Lys 315). Second, the modeling and crystal structure of aryl boronates by Shoichet et al. (*11*, *32*, *33*) show that although smaller aroyl groups may fit into the active site in either orientation, larger ones, and in particular the benzothiophene derivatives such as present in compounds **10** and **14**, should only fit in the orientation of Figure 6, lower pair because of steric problems in the alternative mode. The linear correlation of Figure 4 suggests that there is no difference in mechanism between the smaller and larger aroyl groups and thus it seems likely that acylation of the β-lactamase by acyl phosphonates involves the mode of binding indicated by Figure 6, lower pair.

From the point of view of deacylation, the orientation of the aroyl group in the acyl-enzyme that would derive from the tetrahedral intermediate of Figure 6, lower pair, also seems better since it allows the incoming water to attack from the same direction, catalyzed by Tyr 150, as in normal substrates.



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In fact, the results from the combination of molecular dynamics and molecular mechanics procedures described in Experimental Procedures and performed on the tetrahedral intermediate **22** suggested that the more favorable structure did have the aroyl group in the side chain site (as in Figure 6, lower pair) rather than the leaving group site. This result was evident both from inspection of the optimized structures and from the calculated E_{int} values. The former structure contained all of the hydrogen-bonding and electrostatic interactions observed in the phosphonate (*21*) and boronate (*32*, *33*) crystal structures and in computed substrate models (*20*, *43*), while the latter had fewer hydrogen bonds, very noticeably in the oxyanion hole for example, and greatly reduced electrostatic interaction between the oxyanion and Lys 315 because the latter residue was displaced by the phenyl ring.

General Conclusions. These studies show that acyl phosph(on)ates, in general, are both substrates and irreversible inhibitors of a typical class C β-lactamase. Turnover, as a substrate, involves fast acylation followed by slower deacylation, whereas inactivation involves phosphylation. In general, acylation is far more rapid than phosphylation. The most strongly interacting of the compounds described in this paper, **14**, rapidly acylates the enzyme ($k_{cat}/K_m = 1.25 \times 10^6$ s^{-1} M^{-1}) and forms a tight acyl-enzyme complex ($K_m =$ 0.l5 μM). Since the acyl-enzyme has a half-life of 4 s, the enzyme is also transiently inhibited during steady-state turnover. Acylation is enhanced by hydrophobic groups in both the acyl and the leaving group moieties of the acyl phosph(on)ate. This affinity of the class C β-lactamase active site for hydrophobic species has not previously been seen as clearly, although it was certainly indicated, by the aryl boronic acid studies (*11*). It should be an important feature of the future design of inhibitors for these enzymes. Mechanistically, the aroyl phosph(on)ates can be thought of as substrates whereof the aryl boronate inhibitors are transition state analogues. The aromatic ring of the aroyl group seems to be rather uniformly bound along the acylation−deacylation reaction coordinate, as illustrated by Figure 5. Models of these compounds at the active site, based on the available crystal structures of the enzymes and their aryl boronate complexes suggest that the more likely mode of binding and reaction has the acyl group in the side chain site and the phosph(on)ate group in the leaving group site. It seems possible that sufficiently active compounds of this type might be achievable for possible clinical application although their susceptibility to other hydrolases would have to be controlled by careful design.

ACKNOWLEDGMENT

We are grateful to Mr. Rajesh Nagarajan who synthesized the phosphonic acids employed in the syntheses of **15**−**18**; the details of these syntheses will be published elsewhere.

SUPPORTING INFORMATION AVAILABLE

Characterization data for the compounds **4**−**19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI002243+