Inhibition of β-Lactamases by Monocyclic Acyl Phosph(on)ates†

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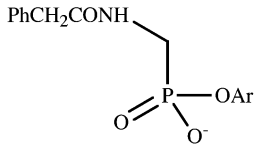
ABSTRACT: The cyclic acyl phosph(on)ates, 1-hydroxy-5-phenyl-2,6-dioxaphosphorinone(3)-1-oxide, its 4-phenyl isomer, and the phosphonate (2-oxo) analogue of the latter inhibited typical class A (TEM-2) and class C (*Enterobacter cloacae* P99) β-lactamases in a time-dependent fashion. No enzyme-catalyzed turnover was detected in any case. The interactions occurring were interpreted in terms of the reaction scheme $E + I \leftrightarrows EI \leftrightarrows EI'$, where EI is a reversibly formed noncovalent complex, and EI′ is a covalent complex. Reactions of the cyclic phosphates with the P99 β-lactamase were effectively irreversible, while that of the 4-phenyl cyclic phosphate with the TEM β-lactamase was slowly reversible. The 4-phenyl cyclic phosphate was generally the most effective inhibitor, both kinetically and thermodynamically, with second-order rate constants of inactivation of both enzymes around 10^4 s^{-1} M^{-1}. This compound also bound noncovalently to both enzymes, with dissociation constants of 25 μM from the P99 enzyme and 100 μM from the TEM. It is unusual to find an inhibitor equally effective against the TEM and P99 enzymes; the β-lactamase inhibitors currently employed in medical practice (e.g., clavulanic acid) are significantly more effective against class A enzymes. The results of lysinoalanine analysis after hydroxide treatment of the inhibited enzymes and of a ^{31}P nuclear magnetic resonance spectrum of one such complex were interpreted as favoring a mechanism of inactivation by enzyme acylation rather than phosphylation. Molecular modeling of the enzyme complexes of the 4-phenyl phosphate revealed bound conformations where recyclization and thus reactivation of the enzyme would be difficult. The compounds studied were turned over slowly or not at all by acetylcholinesterase and phosphodiesterase I.

Inhibition of β-lactamases remains a viable approach to salvaging the effectiveness of β-lactam antibiotics in medicine (*1*). Among non-β-lactam inhibitors, the phosphonates have shown promise, particularly with class C β-lactamases. Compounds such as **1** generate transition state analogue structures on reaction at the β-lactamase active site (*2*−*5*).



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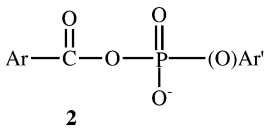
More recently, acyl phosph(on)ates, **2**, have also demonstrated interesting inhibitory activity, also predominantly against class C enzymes (*6*, *7*). These molecules have been



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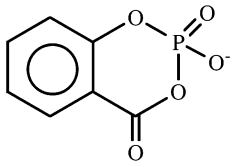
shown to both acylate and phosphylate the active site serine residue. The acylation activity is perhaps more interesting since it leads to quite inert acyl enzymes that cannot be efficiently achieved otherwise. For example, the half-life of the benzoyl derivative of the *Enterobacter cloacae* P99 enzyme is around 1 min at 25 °C. Extension of this lifetime by structural perturbation is likely to be possible.

The cyclic phosph(on)ates **3** and **4** have also been studied in this laboratory (*8*, *9*). They are notable in being reversible



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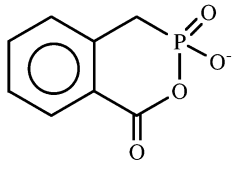

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covalent inhibitors of the P99 β-lactamase (Scheme 1) but not substrates (i.e., the enzyme does not catalyze their hydrolysis). Their mode of action can be conceptually compared with that of β-lactams as inhibitors of bacterial DD-peptidases (Scheme 2), where covalent modification of the active site serine by a cyclic acylating agent also leads to a hydrolytically inert enzyme−inhibitor complex. The situations differ in that acylation by β-lactams is irreversible. This is in part due to the unfavorable thermodynamics of reforming the β-lactam ring (*10*), but this problem is exacerbated by the unfavorable steric interaction between the eclipsed β-substituents A and B (Scheme 2) on the β-lactam ring. This, and the direct interactions between the acyl group and amine in the opened ring, lead to rotation around the single bond connecting the carbon atoms bearing

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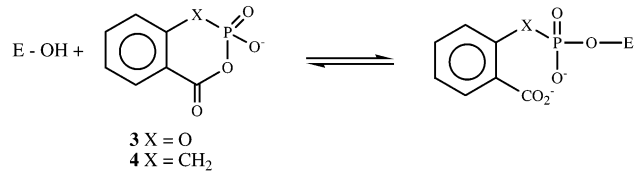
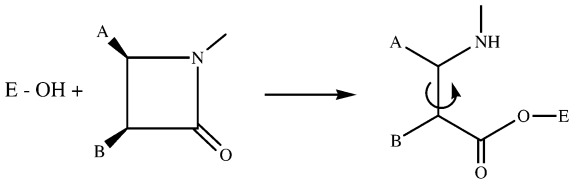
Scheme 1



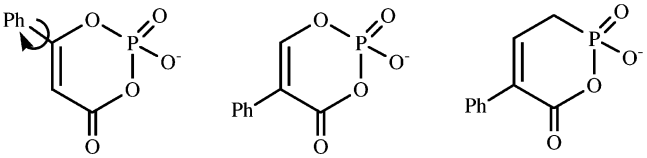
3 X = O
4 X = CH_2

Scheme 2



substituents A and B after the ring opens (Scheme 2). Such rotation is clearly evident in the acyl-enzymes formed between β-lactam-recognizing enzymes and β-lactams (*11*−*14*). In structural terms, the steric repulsion leads to separation of amine and acyl groups so that they are no longer suitably placed for recyclization.

In view of these considerations, we prepared the cyclic acyl phosph(on)ates **5**−**7** where we hoped that the additional degree of freedom incorporated beyond those available in **3** and **4** (see **5**) would allow these molecules to achieve conformations less likely to recyclize after reaction with the enzyme. Gratifyingly, the phosphates **5** and **6** were found to be irreversible inhibitors of the class C P99 β-lactamase. Further, **6** was found to inhibit the class A TEM β-lactamase as effectively as it did the class C enzyme. This is of considerable interest since the β-lactamase inhibitors currently in medical practice are very effective against class A enzymes but have little activity against the class C enzymes (*1*).



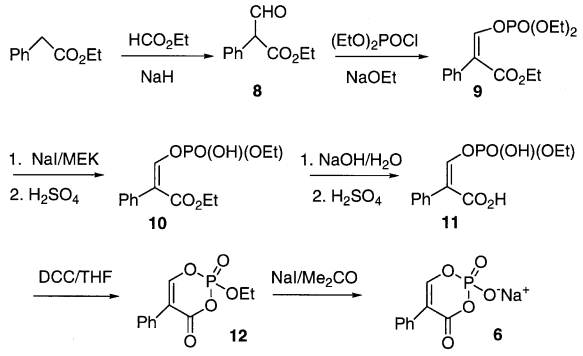
5 **6** **7**

EXPERIMENTAL PROCEDURES

Materials. The class C β-lactamase of *E. cloacae* P99 and the class A TEM-2 β-lactamase from *Escherichia coli* W 3310 were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. Cephalothin was a gift from Eli Lilly and Co. Phosphodiesterase I and acetylcholinesterase and their respective assay substrates, bis(*p*-nitrophenyl) phosphate and acetyl thiocholine, respectively, were purchased from Sigma.

1-Hydroxy-5-phenyl-2,6-dioxaphosphorinanone(3)-1-oxide Sodium Salt (5). This compound was prepared as described by Marecek and Griffith (*15*) and characterized as follows: ^{1}H NMR[1] (^2H_2O) δ 6.59 (s, 1H, ═CH), 7.81 (t, J = 7 Hz, 2H, ArH), 7.84 (t, J = 7 Hz, 1H, ArH), 8.18 (d, J = 7 Hz, 2H, ArH). IR (KBr) ν_{max} 1701.6 cm^{-1} (C═O), 1606.5, 1311.5, 1157.4, 1247 (P═O) [Lit. (*15*) ν_{max} 1700, 1570, 1310, 1160 cm^{-1}]. UV (20 mM MOPS, pH 7.5) λ_{max} 286.0 nm (ϵ = 1.95 × 10^4 cm^{-1} M^{-1}). MS (EI$^-$) m/z 225.1.

Scheme 3

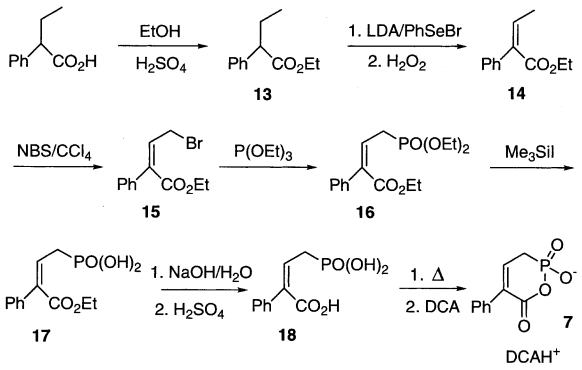


1-Hydroxy-4-phenyl-2,6-dioxaphosphorinanone(3)-1-oxide Sodium Salt (6). The preparation of this compound (Scheme 3) was analogous to that of **5**. Thus, ethyl 2-formyl-2-phenylacetate (**8**) was prepared by condensation of ethyl formate and ethyl phenylacetate as described by Becalli et al. (*16*) and purified by distillation. The product (33 g) was converted into diethyl 2-carbethoxystyryl-1-phosphate, **9**, in 69% yield by the general procedure of Craig and Moyle (*17*). The product, purified by distillation (bp 165−167 °C, 0.3 Torr), proved to be a mixture of the *E* and (required) *Z* isomers, where the latter comprised some 70% of the total. ^{1}H NMR (C^2H_3CN) δ 1.25−1.40 (m, 9H, CH_3), 4.08−4.35 (m, 6H, CH_2), 7.07 (d, J = 5.8 Hz, 0.7 H, *Z*═CH), 7.31−7.42 (m, 5H, ArH), 7.75 (d, J = 6.3 Hz, 0.3 H, *E*═CH). The *E* and *Z* isomers were distinguishable because of the upfield anisotropic shift of the vinyl proton of the latter caused by the adjacent (cis) phenyl ring (*18*, *19*). Attempts to separate these isomers by fractional distillation or by silica gel chromatography were unsuccessful, so the mixture was carried forward.

Conversion of diethyl 2-carbethoxystyryl-1-phosphate into **6** was then achieved by the procedure employed by Marecek and Griffith (*15*) to obtain **5**. First, treatment of the triester **9** (5 g) with sodium iodide in butanone yielded the corresponding phosphate monoester **10**. This was obtained in 90% yield after recrystallization from $CHCl_3$/hexane as a mixture of *Z* (60%) and *E* isomers. ^{1}H NMR (^2H_2O) *Z* isomer: δ 1.22−1.31 (m, 6H, CH_3), 4.01 (quint, J = 7.6 Hz, 2H, CH_2-OP), 4.30 (q, J = 7.2 Hz, 2H, CH_2OC), 7.32−7.45 (m, 5H, ArH), 7.13 (d, J = 7.1 Hz, 1H, ═CH). Alkaline hydrolysis of **10** yielded the carboxylic acid **11** in 85% isolated yield. ^{1}H NMR (^2H_2O) *Z* isomer (60%): δ 1.26 (t, J = 7.5 Hz, 3H, CH_3), 4.01 (quint, J = 7.5 Hz, 2H, CH_2), 7.14 (d, J = 7.0 Hz, 1H, ═CH), 7.33−7.55 (m, 5H, ArH). The carboxylic acid **11** was then cyclized to **12** by treatment with dicyclohexylcarbodiimide in tetrahydrofuran. This product was converted immediately into **6** by reaction with sodium iodide in acetone (*15*). The final product was purified by Sephadex G-10 chromatography with water as the eluent. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **6** in 14% yield from **11**. ^{1}H NMR (^2H_2O) δ 7.47−7.53 (m, 5H, ArH), 7.60 (d, J = 24.9 Hz, 1H, ═CH). IR (KBr) ν_{max} 1712.4 cm^{-1} (C═O). UV (20 mM

[1] Abbreviations: DCA, dicyclohexylamine; DCC, dicyclohexyl carbodiimide; DMSO, dimethyl sulfoxide; IR, infrared; MEK, methylethyl ketone; MOPS, 3-morpholinopropanesulfonic acid; MS(ES$^-$), mass spectrum (electrospray, negative ion); NBS, *N*-bromosuccinimide; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; UV, ultraviolet; standard 3-letter abbreviations for amino acids are used.

Scheme 4



MOPS, pH 7.5) λ_{max} 260 nm (ϵ = 10 200 cm^{-1} M^{-1}). MS (ES^-) *m*/*z* 225.1.

1-Hydroxy-4-phenyl-2-oxaphosphorinanone(3)-1-oxide Dicyclohexylammonium Salt (7). This phosphonate was prepared following the sequence shown in Scheme 4. First, ethyl 2-phenyl butanoate **13** was prepared by esterification of 2-phenylbutanoic acid (Aldrich) with ethanol in the presence of sulfuric acid as described by Breukelman et al. (*20*). The product was purified by distillation (bp 75−78 °C, 0.3 Torr) in 94% yield. The ester **13** (6.7 g) was dehydrogenated by treatment with benzeneselenyl bromide (Acros) and hydrogen peroxide as described by Reich et al. (*21*). The crude product was subjected to flash chromatography on silica gel where elution with 2% diethyl ether/hexane allowed separation of the *E* and *Z* (**14**) isomers of the product, which were present in essentially equal quantities. The yield of each was 2.0 g; thus, a total yield of 60% was achieved. ^1H NMR ($^2H_6$-DMSO) *Z* isomer: δ 1.25 (t, *J* = 6.9 Hz, 3H, CH_3CH_2), 1.93 (d, *J* = 7.7 Hz, 3H, CH_3CH_2), 4.27 (q, *J* = 6.9 Hz, 2H, CH_2CH_3), 6.36 (q, *J* = 7.7 Hz, 1H, $CHCH_3$), 7.2−7.4 (m, 5H, ArH). The alkene **14** was then brominated by *N*-bromosuccinimide in the presence of benzoyl peroxide and the light of a tungsten lamp (*22*). Thus, a mixture of **14** (4.45 g, 23.4 mmol), *N*-bromosuccinimide (Aldrich; 5.43 g, 30.5 mmol), benzoyl peroxide (6 mg), and carbon tetrachloride (16 mL) was heated under reflux and irradiation for 4 h. The reaction mixture was then cooled and filtered. The solvent was removed from the filtrate by rotary evaporation leaving the crude product. This was purified by flash chromatography (silica gel, 3% ether/hexane) yielding 0.8 g of the *Z*-bromide **15** and 2.9 g of the *E*-bromide (total yield 60%). ^1H NMR ($^2H_6$-DMSO) *Z* isomer: δ 1.26 (t, *J* = 7.1 Hz, 3H, CH_3), 4.31 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 4.36 (d, *J* = 8.6 Hz, 2H, CH_2Br), 6.53 (t, *J* = 8.6 Hz, 1H, =CH), 7.35−7.40 (m, 5H, ArH). Heating and irradiation of the *E*-isomer in carbon tetrachloride, as above, reestablished the *E*/*Z* equilibrium and allowed more of the *Z* isomer to be isolated.

The *Z*-bromide **15** (0.8 g, 3.0 mmol) was added to triethyl phosphite (0.54 g, 3.3 mmol), and the mixture was heated in the dark at 80 °C for 100 min. This procedure yielded the phosphonate product essentially quantitatively. Flash chromatography (silica gel, hexane/ethyl acetate/ether, 5:4:1) was again used to purify the product **16** and separate it from a small amount of the *E*-isomer that had been formed; at higher reaction times and temperatures, the *E*-isomer was in fact the major product. ^1H NMR ($^2H_6$-DMSO) δ 1.25 (t, *J* = 7.2 Hz, 3H, CH3), 1.24 (q, *J* = 7.0 Hz, 6H, 2 CH_3), 3.09 (dd, *J* = 8.1, 22.8 Hz, 2H, CH_2P), 4.03 (quint, *J* = 7.0 Hz, 4H, 2 CH_2), 4.25 (q, *J* = 7.2 Hz, 2H, CH_2), 6.16 (q, *J* = 8.1 Hz, 1H, =CH), 7.2−7.4 (m, 5H, ArH). The diethyl phosphonate **16** was then converted to the phosphonic acid **17** by treatment with trimethylsilyl iodide. Thus, **16** (0.4 g, 1.23 mmol) and trimethylsilyl iodide (Aldrich; 1.23 g, 6.17 mmol) were heated together in chloroform (15 mL) at 50 °C for 17 h. The ^1H NMR spectrum of a small aliquot showed the presence of the product and a small amount of starting material. A further 2 equiv of trimethylsilyl iodide was therefore added, and heating continued for a further 2 h. An NMR spectrum at this stage showed only product so, after the solution had cooled, 15 mL of water was added, and the mixture was stirred at room temperature for 2 h. All volatiles were then removed under vacuum. The ^1H NMR spectrum ($^2H_6$-DMSO) of the product **17** (0.3 g) was as follows: δ 1.20 (t, *J* = 6.9 Hz, 3H, CH_3), 2.85 (dd, *J* = 8.26, 22.1 Hz, 2H, CH_2P), 4.21 (q, *J* = 6.9 Hz, 2H, CH_2CH_3), 6.20 (q, *J* = 8.26 Hz, 1H, =CH), 7.2−7.4 (m, 5H, ArH). Alkaline hydrolysis (*18*) of **17** yielded the triacid **18**. A solution of **17** (0.3 g) with sodium hydroxide (10 equiv) in 3.0 mL of water was stirred at room temperature for 105 min. After the resulting solution had been extracted with ethyl acetate, it was acidified with 4 M H_2SO_4 to pH 1 and evaporated to dryness. The product **18** was then extracted into ethanol, separated from salt by filtration, and the solution evaporated to dryness. ^1H NMR (^2H_2O) δ 2.88 (dd, *J* = 8.3, 22.4 Hz, 2H, CH_2), 6.28 (q, *J* = 8.3 Hz, 1H, =CH), 7.3−7.4 (m, 5H, ArH).

Dehydration/cyclization of **18** to **7** was effected by heating a solution of **18** (100 mg) in xylene (20 mL) under reflux in a Dean and Stark apparatus for 100 min. At the end of the heating period, redistilled dicyclohexylamine (75 mg, 1 equiv) was added, and the solution was cooled. A colorless solid precipitated and was removed by filtration. More of essentially the same material was recovered by evaporation of the xylene. The required product **7** was purified by G-10 column chromatography at 4 °C using water as eluent. The product was isolated by lyophilization of the appropriate fractions and identified from UV absorption spectra; 8.6 mg was obtained from 40 mg of the crude reaction mixture. It was characterized as follows: ^1H NMR (^2H_2O) δ 0.8−1.8 (m, 20 H, dicyclohexylamine), 2.78 (dd, *J* = 5.4, 18 Hz, CH_2), 3.05 (m, 2H, dicyclohexylamine), 7.00 (d tr, *J* = 5.4, 30 Hz, 1H, =CH), 7.2−7.3 (m, 5H, ArH). IR (KBr) ν_{max} 1724 cm^{-1} (C=O). UV (20 mM MOPS, pH 7.5) λ_{max} 250 nm (ϵ = 2600 cm^{-1} M^{-1}). MS (ES^-) *m*/*z* 223.2. The proton NMR spectrum indicated that the final product contained 10% of the hydrolysis product **18**.

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 determined spectrophotometrically (*16*).

All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5. Rate and equilibrium constants for interaction of **5**−**7** with the enzymes under consideration were determined by methods described in detail in previous publications (*7*, *9*). In general, three types of experiment were employed.

(1) Second-order rate constants for reaction of **5**−**7** with the enzymes were directly obtained from measurements of enzyme activity as a function of time of incubation of enzyme and inhibitor together (the P99 β-lactamase with **5** and **6** and TEM with **5** and **7**), or indirectly, from total progress curves for reaction of enzyme and substrate in the presence of inhibitor (the P99 β-lactamase with **7** and the TEM β-lactamase with **6**). Cephalothin was the substrate for both experiments.

(2) Equilibrium constants for fast reversible binding were determined from initial rates of turnover of a substrate (cephalothin) in the presence of the inhibitor (the P99 and TEM β-lactamases with **5**). In cases where fast equilibrium binding was followed by a slow reaction, the equilibrium constant for the first step was determined from initial rate measurements using a stopped-flow (Durrum D-110) spectrophotometer (the P99 β-lactamase with **5** and **6** and the TEM β-lactamase with **6**).

(3) Dissociation rate constants for EI′ complexes were obtained from progress curves produced on dilution of an enzyme/inhibitor mixture into a substrate solution (the TEM β-lactamase with **6**; the substrate was cephalothin).

Background hydrolysis rate constants of **5**−**7** were determined spectrophotometrically at 288 nm ($\Delta\epsilon = -1.53 \times 10^4$ cm^{-1} M^{-1}), 260 nm ($\Delta\epsilon = 5700$ cm^{-1} M^{-1}), and 250 nm ($\Delta\epsilon = 2500$ cm^{-1} M^{-1}), respectively. Attempts to detect enzyme-catalyzed turnover were also made using these wavelengths.

^{31}P NMR Spectra. A sample of the P99 β-lactamase after inactivation by **6** was prepared for ^{31}P NMR spectroscopy as follows. A 1.25 mM solution of the β-lactamase in 20 mM MOPS buffer at pH 7.5 was inactivated at 0 °C to greater than 90% in 40 min by addition of a 50 μL aliquot of a 0.101 M stock solution of **6**; the final concentration of **6** in the reaction mixture was thus 7.8 mM. The reaction mixture was then passed through a 1 × 22 cm column of Biogel P-6DG equilibrated with MOPS buffer at 4 °C. The column fractions containing protein were then concentrated to 0.6 mL by means of a Centricon-10 concentrator, centrifuged at 5000 rpm. The sample at this stage had essentially no β-lactamase activity. An equal volume of ^2H_2O was then added, and the total volume was reduced to 0.4 mL by further centrifugation. Sufficient solid guanidinium hydrochoride was then added to make the solution 5 M in this solute. The proton-decoupled ^{31}P NMR spectrum at 20 °C was then obtained by means of a Varian Mercury 300 spectrometer operating at 121.5 MHz (29 000 transients, 35° pulse, and 1.0 s delay time). Chemical shifts were measured with respect to trimethyl phosphate as an external reference. For comparison, the chemical shifts of **11** and **21**, the hydrolysis product of **6**, were obtained from ^{31}P spectra obtained under identical conditions.

Lysinoalanine Analysis. Samples of the P99 β-lactamase (10 μM) were inactivated by **5** and **6** (20 μM each) by incubation together for 25 or 15 min, respectively. Similarly, the TEM β-lactamase (10 μM) was inactivated by reaction with **6** (200 μM) for 5 min. Amino acid analyses to determine the lysinoalanine content of these inhibited enzymes were carried out as previously described (*7*).

Molecular Modeling. The computations were set up essentially as previously described (*7*, *23*) and run on an SGI Octane 2 computer with Insight II 2000 (MSI, San Diego, CA). The starting point for the simulations of the P99 β-lactamase was the crystal structure with a phosphonate inhibitor covalently attached to the active site serine residue (PDB file 1bls, ref *4*). A variety of initial conformations of the ligand were explored by means of molecular dynamics, and a typical snapshot of the predominant conformation was selected for energy minimization. The protonation state of active site residues was as described previously (*7*) except as noted in the text below. The TEM simulations were set up similarly starting from the native crystal structure (PDB file 1blt, ref *24*). Ligand structures were taken from the P99 β-lactamase models. Lys 67 and Lys 234 were protonated, and Glu 166 was treated as described in the text below.

Other Enzymes. The susceptibility of **5**−**7** to acetylcholinesterase and phosphodiesterase I was determined by direct spectrophotometric observation at the wavelengths given above. For comparison, the fused ring compounds **3** and **4** were also examined, using 288 and 244 nm, respectively, as suitable wavelengths. Second-order rate constants (k_{cat}/K_m values) for the reaction were obtained from the total progress curves by Dynafit (*25*) analysis. No evidence of saturation was observed at the highest concentrations of **5**−**7** employed (100 μM).

RESULTS

The compounds **6** and **7** had similar kinetic stability to hydrolysis in 20 mM MOPS, pH 7.5; the rate constants of hydrolysis were 4.0×10^{-5} s^{-1} and 4.8×10^{-5} s^{-1}, respectively, at 25 °C. This is suggestive of hydrolysis by water/hydroxide attack at the acyl rather than the phosphyl group since attack at the latter should be significantly faster for the phosphonate. The hydrolysis of **4** under these conditions, for example, was some seven times faster than that of **3**, and hydrolysis of the former did involve phosphyl transfer (*9*). Solvolysis of **6** for 30 min at room temperature in aqueous alkaline methanol (80% methanol, 0.1 M NaOH) yielded a product with one methyl group singlet resonance at δ3.35 in the ^{1}H NMR spectrum. After the solution had stood for a further 2 h at room temperature, this singlet disappeared completely. These data suggest that under alkaline conditions at least, oxygen anion nucleophiles attack at the acyl carbon. Nucleophilic attack at the acyl group would yield a methyl singlet, whereas attack at phosphorus would yield a methyl doublet; a methyl carboxylate would also hydrolyze within the observed time frame under these conditions, whereas a methyl phosphate would not. The conclusion of attack at carbon has also been drawn for **3** (*9*). The rate constant for hydrolysis of **5** was significantly smaller than for **6** (viz. 2.3×10^{-6} s^{-1}), which may reflect the effect of the extended conjugated system of **6** on the carbonyl electrophilicity. For perspective, the comparable rate constant for benzylpenicillin is 1.5×10^{-5} s^{-1}.

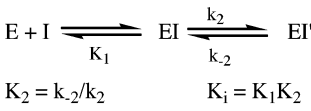
Preliminary testing showed that the cyclic phosph(on)ates **5**−**7** reacted in a time-dependent fashion with representative β-lactamases of classes A and C, the enzymes from the TEM-2 plasmid and *E. cloacae* P99, respectively. Close spectrophotometric examination, however, showed no detectable turnover of these compounds to an acyclic product. The interactions therefore were interpreted in terms of Scheme 5, where EI represents a rapidly reversible non-covalent

Table 1: Rate and Equilibrium Constants for the Inhibition of β-Lactamases by the Cyclic Phosph(on)ates **5**−**7** and the Kinetic Mechanism of Scheme 5

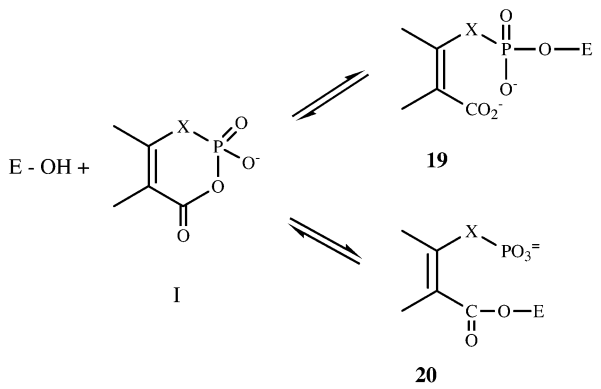
enzyme[a]	inhibitor	k_2[b] (s^{-1})	k_{-2}[c] (s^{-1})	k_2/K_1 ($s^{-1}M^{-1}$)	K_1[d] (μM)	K_i (μM)
P99	**5**	0.053	$\leq 10^{-5}$	$(1.61 \pm 0.14) \times 10^3$ [e]	33 ± 8	$\leq 6 \times 10^{-3}$ [f]
	6	0.26	$\leq 10^{-5}$	$(1.0 \pm 0.2) \times 10^4$ [e]	26 ± 2	$\leq 10^{-3}$ [f]
	7	>0.65	$(1.3 \pm 0.3) \times 10^{-2}$	$(1.1 \pm 0.1) \times 10^4$ [g]	≥ 60	1.2[f]
						2.2 ± 0.7[d]
TEM	**5**	$>5 \times 10^{-4}$	$\leq 10^{-5}$	(1.2 ± 0.2)[e]	≥ 400	<0.8
	6	1.0	$(1.0 \pm 0.1) \times 10^{-2}$	$(4.5 \pm 0.1) \times 10^3$[g]	100 ± 9	1.0 ± 0.1[d]
				10^4 [h]		
	7		$(2.3 \pm 0.6) \times 10^{-2}$	820[h]	n[j]	28 ± 3[d]

[a] The enzymes are the class C β-lactamase of *E. cloacae* P99 and the class A TEM-2 β-lactamase. [b] From k_2/K_1 and K_1 measurements [$k_2 = K_1(k_2/K_1)$]. [c] From return of activity experiments. [d] From rapid (stopped-flow where required) initial rate measurements. [e] From loss of activity on incubation of enzyme and inhibitor in the absence of substrate. [f] $K_i = K_1K_2 = k_{-2}/(k_2/K_1)$. [g] From loss of activity in competition with a substrate. [h] $k_2/K_1 = k_{-2}/K_i$. [i] $k_2 = k_{-2}K_1/K_i$. [j] Not determined.

Scheme 5



Scheme 6



complex, and EI′ represents a complex where the inhibitor is more tightly bound. Such inhibition could appear either reversible or irreversible, depending on the value of k_{-2}. In view of the very slow dissociation rates (k_{-2}) observed (no dissociation was observed in several cases), all precedent concerning the interaction of these enzymes with small molecule ligands, and the direct precedent of the cyclic phosphonate **4** (*9*), we assume that EI′ is a covalent complex where the active site nucleophile, a specific serine hydroxyl group, is either phosphylated or acylated (Scheme 6). The latter distinction will be discussed further below.

A variety of kinetics experiments, outlined in Experimental Procedures and specified in the footnotes to Table 1, were carried out to determine the rate and equilibrium constants of Scheme 5 for the two enzymes mentioned above. These constants are presented in Table 1.

The reactivity of these new compounds with the physiologically relevant enzymes phosphodiesterase I and acetylcholinesterase was also investigated. The results of these experiments and those performed with **3** and **4** for comparison are given in Table 2. Several of the compounds turned out to be poor substrates of these enzymes. No irreversible inhibition was observed except that of acetylcholinesterase by **7** ($k_i = 22\ s^{-1}\ M^{-1}$).

Table 2: Rate Constants for Turnover of Acyl Phosph(on)ates by Phosphodiesterase I and Acetylcholinesterase

	k_{cat}/K_m ($s^{-1}M^{-1}$)	
acyl phosph(on)ate	acetylcholinesterase	phosphodiesterase I
3	38	2.6×10^3
4	n[a]	3.4×10^3
5	27	61
6	460[b]	127
7	n	1650

[a] n: No turnover observed. [b] Biphasic kinetics were observed; this number corresponds to the faster phase.

DISCUSSION

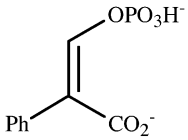
Inhibition of representative class C and class A β-lactamases by **5**−**7** can be accommodated by Scheme 5 with the parameters of Table 1. Inspection of these data suggests that the cyclic phosphate **6** is generally the most effective of the three compounds, both kinetically and thermodynamically. It is particularly striking that **6** is essentially as effective against the TEM β-lactamase as it is against the P99 enzyme. This breadth is unusual among phosph(on)ates, and indeed, among β-lactamase inhibitors in general (*1*, *26*).

The data also show that **6** is more effective than **5** against both enzymes. Compound **5** reacts more slowly with the P99 enzyme, but the difference is even greater for the TEM β-lactamase. The latter enzyme appears to be essentially unaffected by **5**. This order of potency may either be a result of the relative chemical reactivity of the two compounds, as indicated by the spontaneous hydrolysis rates discussed above, or because of unfavorable interaction of the phenyl group of **5** with the active sites of these enzymes. Inspection of the P99 β-lactamase models (see below), for example, suggested that in this case the phenyl group of **5** would directly impact upon the small helix 10a (*27*), which forms part of a loop that restricts access to the active site.

The chemical differences between phosphates and phosphonates is not evident in the rate constants in Table 1 for **6** and **7**. One might expect, if phosphylation were involved, that both on and off rates would be larger for **7** (*28*, *29*). This is not true in general here, although it is true that k_{-2} is much greater for the phosphonate than the phosphate for breakdown of the respective P99 complexes; this difference was also seen with **3**. On the other hand, this difference is not seen in the k_{-2} values of the complexes of **6** and **7** with

the TEM β-lactamase (Table 1). It is possible that the values given in Table 1 reflect the fact that, in some instances at least, the inhibition reaction involves acylation of the enzyme rather than phosphylation (see below).

Finally, with respect to Table 1, it is very striking that both **5** and **6** have quite significant noncovalent binding strengths (K_1 = 33 and 25 μM, respectively) to the active site of the P99 enzyme. Even with TEM, the K_1 of **6** is 100 μM. This is quite unusual for a small molecule and certainly contributes a lot to the effectiveness of **6** as an inhibitor. The more active-site directed phosphonates, **1**, bind noncovalently with dissociation constants higher than 1 mM (*30*). The product of hydrolysis of **6**, the acyclic phosphate **21**, acted as a noncovalent inhibitor with K_i for the P99 enzyme of 0.17 mM. The restricted conformation of **6** and/or its smaller negative charge must be important to its binding.



21

The question of **19** versus **20** as the product of reaction of **5** and **6** with the P99 and TEM β-lactamases was addressed, as previously (*9*), by means of amino acid analysis for lysinoalanine after treatment of the inactivated enzymes with sodium hydroxide. If the active site serine were phosphylated (**19**), phosph(on)ate would be eliminated by base treatment leading to dehydroalanine at position 64 (P99) or 70 (TEM). As previously demonstrated (*9*, *31*), such a residue would react stoichiometrically with the amine of Lys 67 (P99) or Lys 73 (TEM) to yield lysinoalanine. Alternatively, if the active site serine were acylated (**20**), alkaline hydrolysis of the ester would restore the active site serine and not yield lysinoalanine. Such analyses yielded 0.37 and 0.34 mol of lysinoalanine per mol of the P99 β-lactamase after inactivation with **5** and **6**, respectively. Similarly, 0.52 mol/mol of the TEM β-lactamase was obtained after reaction with **6**.

These results can be compared with the results from inactivation of the P99 β-lactamase with **1** and **3**, which yielded 1.0 and 1.2 mol/mol of enzyme, respectively, and which was interpreted in terms of preferential phosphorylation (*9*, *31*). The results in the present case of **5** and **6** suggest that a significant amount of acylation accompanied phosphorylation. To gain further insight into this question, a ^{31}P NMR spectrum of the β-lactamase after inactivation by **6** and denaturation in 5 M guanidinium hydrochloride was obtained as described in Experimental Procedures. This spectrum (Figure 1) contained a single peak with chemical shift 1.04 ppm (downfield of trimethyl phosphate). Under the same conditions the chemical shift of **11**, as a model of **19** (X = O), was either −5.60 or −5.81 ppm (*E* and *Z* isomers) and that of **21**, as a model of **20** (X = O), was −0.71 ppm. Inorganic phosphate under the same conditions had a chemical shift of −0.51 ppm. Thus, the protein-bound phosphate had a chemical shift much closer to that of the phosphate monoester **21** than the diester **11**. The NMR results suggest, therefore, first that only one inactivation product is obtained on reaction of the P99 β-lactamase with **6** and, second, that that product is more likely to be **20** than **19**. This may well also hold for the reaction of **6** with the TEM β-lactamase and of **5** with the P99 enzyme.

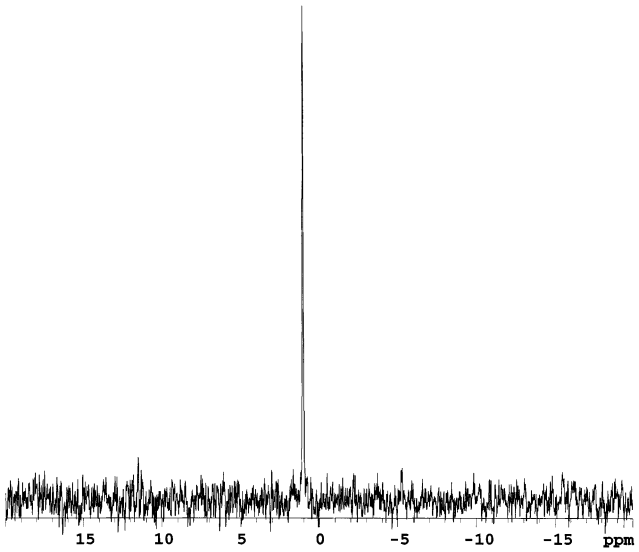


FIGURE 1: ^{31}P NMR spectrum of the covalent complex between the P99 β-lactamase and the acyl phosphate **6**.

An unresolved question is why, if inactivation of these enzymes were due to acylation, any lysinoalanine was detected in samples after base treatment. This must mean that **20**, unlike simple acyl serines, undergoes elimination from serine at a comparable rate to alkaline hydrolysis at the carbonyl group. This reactivity would presumably derive from the vinylogous carbonate nature of **20**. It is known, for example, that in the case of a vinylogous carbamate derivative, elimination is the preferred product in alkali (*31*).

Finally, to visualize the complex formed and perhaps to understand why recyclization and recovery of enzyme activity did not occur, a molecular model of the acyl-enzyme **20** (X = O) was constructed as described in Experimental Procedures. An important issue in constructing such a model is that of the state of dissociation of Tyr 150. One would expect on the basis of the commonly believed mechanism of catalysis by this enzyme (*4*, *32*) that the reactive form of the acyl enzyme would contain Tyr 150 as the oxyanion, poised for participation as a general base in deacylation. On the other hand, the presence of the negatively charged phosphate group on the inhibitor, which could, in principle, directly interact with Tyr 150, could substantially raise the pK_a of this residue, ensuring that in the acyl-enzyme it would not be dissociated at neutral pH. In view of this uncertainty as to the state of Tyr 150, both the neutral form and the anion were studied.

Molecular dynamics simulations of the structure containing neutral Tyr 150 [20 ps at 300 °K and 20 ps at 1000 °K (the protein structure fixed in the latter case)], suggested that the preferred conformation of the acyl-enzyme may be that shown in Figure 2. The structure shown here is an energy-minimized typical snapshot from the dynamics run at 300 °K. As anticipated, the acyl oxygen is hydrogen-bonded to the main chain NH of Ser 318 (one of the oxyanion hole components), and Tyr 150 is within hydrogen-bonding

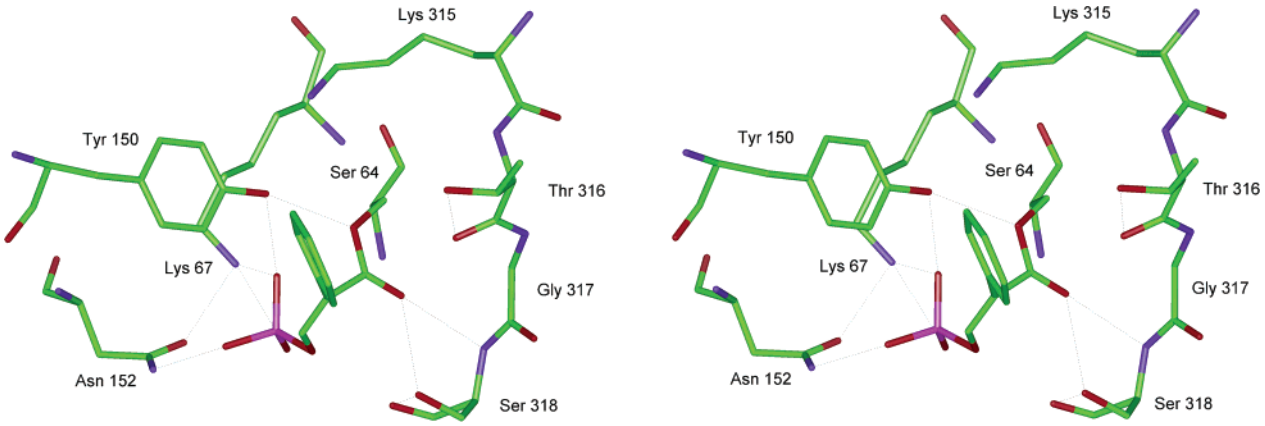


FIGURE 2: Stereoview of the energy-optimized acyl-enzyme formed on reaction of the P99 β-lactamase with the acyl phosphate **6**.

distance of Ser 64 O_γ. The general conformation of the bound inhibitor is very similar to that suggested by modeling of the interaction between the P99 β-lactamase and **3** (*9*). Of particular interest in the present case is the disposition of the phosphate group, which appears to be very firmly electrostatically held at the active site. Phosphoryl oxygen atoms are hydrogen-bonded to the hydroxyl of Tyr 150 [the distance of this hydroxyl oxygen to the phosphoryl oxygen is smaller (2.47 Å) than to Ser 64 O_γ (2.80 Å), and the C−O···H···O angle is more favorable], to the ammonium group of Lys 67, and to the side-chain NH_2 of Asn 152. Throughout the dynamics simulation, the distance from the closest phosphoryl oxygen to the acyl carbon remained at 3.2 ± 0.2 Å and the P−O−C angle at an unfavorable 88 ± 4°. It seems therefore that after ring-opening during acylation, the inhibitor may be held so firmly in a position unfavorable to recyclization that this reaction is not observed, even on a time scale of hours.

It is also of interest to reflect on why the acyl-enzyme derived from **6** is not hydrolyzed by the enzyme in the normal fashion. Two reasons for this are apparent from the structure of Figure 2. First, the Tyr 150 hydroxyl group is trapped by the phosphate moiety of the inhibitor in an unreactive (protonated) state on the wrong face of the acyl group for normal catalysis. In a class C β-lactamase, deacylation is thought to occur through general base catalysis by the anion of Tyr 150 promoting water attack from the Re (outer or solvent) face of the acyl group (*4*). Second, nucleophilic attack on the acyl carbonyl from the Si face is hindered sterically by the phenyl group of the inhibitor. Both of these circumstances resemble situations found in the inhibition of DD-peptidases and β-lactamases by β-lactams (*33*). For example, Crichlow et al. (*34*) have obtained the crystal structure of a covalent complex between a class C β-lactamase and a 7-alkylidene cephalosporin sulfone (*35*). In this structure, the sulfinate anion formed in the acylation reaction also intercepts Tyr 150 and is likely to thereby preclude its functioning as a deacylation catalyst. Further, steric inhibition of deacylation is a major source of the inhibitory properties of β-lactams against both DD-peptidases and β-lactamases (*33*); inhibition of the former groups of enzymes, of course, leads to the antibiotic activity of β-lactams.

A longer simulation of this model (200 ps) led to a situation where the carbonyl of the bound inhibitor rotated out of the oxyanion hole into free solution, while the phosphate group remained bound to Tyr 150 and Lys 67. In this conformation, the closest phosphoryl oxygen remains 4 Å from the carbonyl carbon and with a still unfavorable P−O−C angle of around 82°. This too appeared to be a conformation unfavorable to recyclization.

Finally, a 40 ps simulation of the modeled structure containing the Tyr 150 anion was conducted. In this sequence, the phosphate anion of the inhibitor moved away from Tyr 150 and Lys 67 out into solution and, with the inhibitor thus in an extended conformation, was also not in place for recyclization to occur.

Similar results were obtained in simulations with the class A TEM β-lactamase and irrespective of whether Glu 166 was protonated. The accessed conformations either held the phosphate group tightly with the carbonyl group directed out into solution or retained the carbonyl in the oxyanion hole but with the phosphate extending out into solution. In the latter case, the active site functional groups did not interact with the ligand and thus did not seem well-placed for catalysis. A water molecule, potentially available for hydrolysis, was held between the Glu 166 carboxylate and the Lys 73 ammonium ion but not in an optimal position for nucleophilic attack on the carbonyl group.

The experiments described above show that both class A and class C β-lactamases can be inhibited quite effectively by cyclic acyl phosph(on)ates such as **5**−**7**. Of these compounds, **6** was most effective against representatives of both classes of enzyme and particularly in the class A (TEM) case. In contrast to the situation with the bicyclic compound **3**, the reaction of the P99 enzyme with **5** and **6** is essentially irreversible. This may be due to the greater flexibility of **5** and **6** in the acyl-enzyme. This situation would then be reminiscent of that likely occurring in inhibition of bacterial DD-peptidases by β-lactams, where the opening of the β-lactam ring is followed by conformational motion of the now more flexible ligand that reduces the chance of recyclization. Inactivation of both enzymes by **5** and **6** most likely occurs through a process of active site (Ser OH) acylation rather than phosphorylation. Compound **6** is strikingly effective against the TEM enzyme and is unusual among β-lactamase inhibitors in having comparable activity against class A and class C enzymes. This is not true for the bicyclic analogue **3** (*8*), nor for the clinically used inhibitors clavulanic acid, sulbactam, and tazobactam. The difference between **6** and **3** with respect to TEM may also reflect the advantage of the more flexible **6** in the more restricted and specific class A active site. Finally, the noncovalent affinity

of **5** and **6** (dissociation constants ca. 30 μM) for the P99 active site and, to a lesser degree, of **6** for the TEM active site (100 μM) is unexpected and interesting. Previously studied, more active site directed, phosph(on)ates have much weaker noncovalent affinity for the β-lactamase active site. The cyclic acyl phosph(on)ates **5**−**7** retain only slight susceptibility to phosphodiesterase I and acetylcholinesterase catalyzed hydrolysis and thus may represent useful new lead compounds to further β-lactamase inhibitor development.

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