Inhibition of Serine β-Lactamases by Acyl Phosph(on)ates: A New Source of Inert Acyl [and Phosphyl] Enzymes

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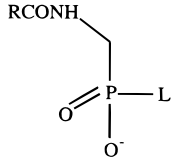
Abstract: Acyl phosph(on)ates are shown to inhibit serine β-lactamases and provide a new source of relatively stable complexes. Thus, benzoyl phenyl phosphate, benzoyl phenylphosphonate, and dibenzoyl phosphate react with the class C β-lactamase of *Enterobacter cloacae* P99 at micromolar concentrations to form an acyl enzyme of half-life about 40 s. The phosphonate reacts further more slowly to produce a much more inert complex. Dibenzoyl phosphate reacts with the class A TEM β-lactamase to form an acyl enzyme of half-life about 8 s and, more slowly, reaching completion after an average of about 80 turnovers, a more inert complex, of half-life about 2 h. The acyl phosphonates thus represent a new starting point for the design of β-lactamase inhibitors and perhaps of antibacterial agents.

Introduction

There has been a recent resurgence of the threat to human health by bacteria,[1] arising from their steadily improving ability to evade and resist presently available antibiotics and, in particular, the β-lactams.[2] Much of the bacterial resistance to the latter antibiotics still stems from the β-lactamases, enzymes produced by bacteria which catalyze β-lactam hydrolysis.[3] The processes of mutation and natural selection continue to give rise to extended-spectrum β-lactamases with greater specificity toward modern β-lactams.[4−6] Although several β-lactamase inhibitors are currently available to assist β-lactam antibiotics,[7,8] new classes of β-lactamase inhibitors and, indeed, antibiotics are clearly desirable.

Some years ago now, research in this laboratory led to a new class of serine β-lactamase inhibitors, the phosphonate monoester monoanions of structure **1** (where L is a leaving group).[9,10] These compounds inhibit β-lactamases by phosphonylation of the active site serine hydroxyl group[10−12] and thereby produce transition state analogue structures.[13] Structure−activity studies[10,14,15] have indicated that the leaving group ability of L is an important element in the inhibitory power of **1**. That notion has now led to a new group of β-lactamase inhibitors, the acyl phosph(on)ates **2**. The important distinction between



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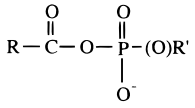

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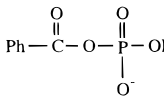
2 and the previously described **1** is that the former have the potential to form *both* acyl and phosphor(on)yl enzyme species. We describe here three prototypical examples of **2**, the compounds **3**−**5**, where slowly turned-over forms of both types



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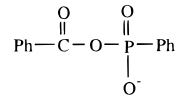

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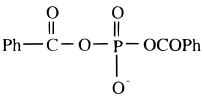

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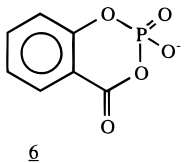
of intermediate can be found. The β-lactamases shown in this study to be affected by **3**−**5**, those of *Enterobacter cloacae* P99, a typical class C β-lactamase, and of the TEM-plasmid, a class A β-lactamase, are both of considerable clinical significance.[6] A recent report from this laboratory described the properties of **6**, a cyclic variant of this type of inhibitor.[16] The present paper extends and generalizes that result.



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The classical β-lactamase inhibitors inactivate β-lactamases by reacting with them to form inert acyl enzyme species.[7] A new source of relatively stable acyl (and/or phosphor(on)yl) enzymes, and one clearly susceptible to much structural elaboration, represents the novel finding of this paper.

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Experimental Section

Enzymes and Substrates. The β-lactamases of the TEM-2 plasmid and from *E. cloacae* P99 were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wilts, U.K.) and used as supplied. Cephalothin was a gift from Eli Lilly and Co. Benzylpenicillin was purchased from Sigma Chemical Co.

Synthesis of Acyl Phosph(on)ates. Sodium Benzoyl Phenyl Phosphate 3. This compound was prepared by a modification of the method employed by Jencks and Carriuolo for the synthesis of acetyl phenyl phosphate.[17] Thus, to a solution of 2.0 g (7.8 mmol) of disodium phenyl phosphate (Aldrich Chemical Co.) in 15 mL of water, cooled in an ice bath, was added 3.5 g (15.8 mmol) of benzoic anhydride (Acros Organics) dissolved in 10 mL of pyridine, dropwise with stirring. After 25 min, the reaction mixture was extracted three times with diethyl ether and the aqueous phase then freeze-dried. The solid residue was recrystallized twice from water and characterized by NMR spectra: ^{1}H (^2H_2O) δ 7.27 (t, $J = 7.5$ Hz, 1H), 7.29 (d, $J = 7.5$ Hz, 2H), 7.44 (t, $J = 7.5$ Hz, 2H), 7.60 (t, $J = 7.5$ Hz, 2H), 7.75 (t, $J = 7.5$ Hz, 1H), 8.10 (d, $J = 7.5$ Hz, 2H); ^{31}P (^2H_2O) δ −14.4. No resonances other than these were observed in the spectra. The compound was thus at least 95% pure, with respect to its organic and phosphorus content. The presence of impurities below this level however cannot give rise to the observations described below.

Sodium Benzoyl Phenylphosphonate 4. The procedure of Laird and Spence[18] appeared, in our hands, to yield the diester dibenzoyl phenylphosphonate, mp 104−106 °C (recrystallized from acetonitrile): ^{1}H NMR (C^2HCl_3) δ 7.50 (t, $J = 7.5$ Hz, 4H), 7.59 (t, $J = 7.5$ Hz, 2H), 7.68 (m, 3H), 8.13 (d, $J = 7.5$ Hz, 4H), 8.15 (m, 2H); ^{31}P NMR (C^2HCl_3) δ 7.20. This diester was dissolved in 1:1 acetone/water and titrated to a stable pH 7 endpoint over 30 min with sodium bicarbonate. Acetone was then removed by rotary evaporation and the residual aqueous solution freeze-dried. The required product was purified by elution with water from a Biorad P-2 column: ^{1}H NMR (^2H_2O) δ 7.54 (t, $J = 7.5$ Hz, 2H), 7.55−7.65 (m, 3H), 7.67 (t, $J = 7.5$ Hz, 1H), 7.87 (dd, $J = 7.5$, 13.8 Hz, 2H), 8.10 (d, $J = 7.5$ Hz, 2H); ^{31}P NMR (^2H_2O) δ 9.80. This compound was also pure to the same degree as **3**.

Sodium Dibenzoyl Phosphate 5. This compound was prepared analogously to **3**, beginning with disodium hydrogen phosphate and benzoic anhydride in a 1:2 molar ratio. The product was recrystallized twice from water, with a final melting point of 195−197 °C: ^{1}H NMR (^2H_2O) 7.55 (t, $J = 7.5$ Hz, 4H), 7.72 (t, $J = 7.5$ Hz, 2H), 8.09 (d, $J = 7.5$ Hz, 4H); ^{31}P NMR (^2H_2O) δ −17.9. Anal. Calcd for $C_{14}H_{10}NaO_6P$: C, 51.24; H, 3.07; P, 9.44. Found: C, 51.46; H, 2.80; P, 9.29.

Analytical and Kinetic Methods. The concentrations of stock enzyme solutions were determined spectrophotometrically.[19] Steady state kinetic parameters were directly obtained for **3** and **5** with the P99 β-lactamase and for **3** and **4** with TEM by the method of Wilkinson[20] from spectrophotometric initial velocity measurements. A Hewlett-Packard HP8452A spectrophotometer was routinely employed. The wavelengths employed were 244 nm ($\Delta\epsilon = 8720$ cm^{-1} M^{-1}), 252 nm ($\Delta\epsilon = 2375$ cm^{-1} M^{-1}), and 248 nm ($\Delta\epsilon = 14\ 440$ cm^{-1} M^{-1}) for **3**−**5**, respectively. All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5.

Second-order rate constants of irreversible inhibition were obtained from incubation mixtures of appropriate concentrations of enzyme and inhibitor where that of the latter much exceeded that of the former (pseudo-first-order conditions). Aliquots of these were diluted into assay mixtures containing the substrate cephalothin at saturating concentration (1 mM), and the residual enzyme activity was determined spectrophotometrically from the initial rates of substrate turnover. Rate constants were then obtained from eq 1 where v is the initial rate of cephalothin consumption in the assay at any time, v_0 the rate at time zero, k_i the second-order rate constant of inactivation, and I_o the inhibitor concentration.

$$v = v_0 e^{-k_i I_o t} \quad (1)$$

Essentially the same procedure was used to obtain rates of hydrolysis of transient intermediates in the cases of **3**−**5** with the P99 enzyme and of **5** with TEM. The velocity of cephalothin hydrolysis increased with time due to hydrolysis of the intermediate. Rate constants for this hydrolysis were obtained by the fitting of absorbance data to eq 2,

$$A = A_o - v_\infty t + (v_o/k_{return})(1 - e^{-k_{return} t}) \quad (2)$$

where A_o is the initial absorbance, v_o the initial rate, v_∞ the final rate after return of activity was complete, and k_{return} the rate constant for return of activity. In the cases of **4** with the P99 enzyme and **5** with TEM, a more complicated reaction scheme involving competing turnover and inhibition was required (see Scheme 1). To obtain rate constants (including k_{cat} and K_m) under these circumstances, the absorbance of the inhibitor with time in the presence of the enzyme was monitored and the data were analyzed by means of the FITSIM program.[21]

Results and Discussion

Acyl phosph(on)ates such as **3**−**5** can be readily prepared, e.g., see the Experimental Section, and form convenient stable salts. They are remarkably stable at neutral pH[22−24] (pseudo-first-order rate constants of hydrolysis of **3**−**5** in 20 mM MOPS, pH 7.5, were 1.6×10^{-7}, 8.3×10^{-6}, and 7.8×10^{-7} s^{-1}, respectively; the comparable value for benzylpenicillin is 1.5×10^{-5} s^{-1}) but more labile at alkaline pH, presumably through nucleophilic attack at the carbonyl group;[23] simple alkyl and aryl phosph(on)ate monoanions are extremely stable to nucleophilic cleavage in alkaline solution. Second-order rate constants of alkaline hydrolysis of **3**−**5** were 0.32, 0.090, and 1.5 s^{-1} M^{-1}, respectively. These rate constants can be compared with that for benzylpenicillin, 0.1 s^{-1} M^{-1}. The acyl phosph(on)ates are however quite labile to aminolysis by primary amines,[22] as are penicillins.[25]

Typical class A and C β-lactamases, the TEM-2 β-lactamase, and the β-lactamase of *E. cloacae* P99, respectively, were found to catalyze the hydrolysis of **3**−**5** (and of other analogous acyl phosph(on)ates) to benzoate and phosph(on)ate. The products could be readily identified in ^{1}H NMR spectra of reaction mixtures. It is clear however from the k_{cat} and K_m values presented in Table 1 that these compounds are poor substrates of these enzymes, especially with respect to k_{cat}, which, for good substrates, may exceed 1000 s^{-1} (see data for benzylpenicillin in Table 1). Nonetheless, it is a little surprising that compounds as nonspecific in structure as **3**−**5** are substrates at all. Previously studied ester substrates included β-lactamase-specific amido side chains in their structures.[19,26]

Important questions of course in each case are whether the enzyme is catalyzing an acyl or phosphor(on)yl transfer reaction and then whether a covalent acyl or phosphor(on)yl enzyme intermediate, respectively, is involved. These questions will be addressed below.

Of greater interest than the above-mentioned steady-state parameters was the observation that in several instances there was evidence that the intermediates involved only slowly (seconds to minutes) led back to free enzyme: when small aliquots of reaction mixtures were added to assay solutions

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Table 1. Rate Constants for Turnover of **3**−**5** by β-Lactamases and for Inhibition

	3	**4**	**5**	BP[f]
		P99 β-Lactamase		
k_{cat} (s^{-1})	0.017 ± 0.002[a]	0.020 ± 0.002[a]	0.017 ± 0.002[a]	54
k_{return} (s^{-1})	0.017 ± 0.003	0.015 ± 0.004	0.024 ± 0.003	*d*
K_m (μM)	2.8 ± 0.2	2.9 ± 1.6	4.3 ± 0.3	0.89
k_{cat}/K_m ($s^{-1}\ M^{-1}$)	6070	6900	3950	6.1×10^7
k_i ($s^{-1}\ M^{-1}$)	*b*	70 ± 26[c]	*b*	*d*
k_r (s^{-1})	*d*	very slow	*d*	*d*
		TEM β-Lactamase		
k_{cat} (s^{-1})	0.012 ± 0.002	$(7.8 \pm 0.8) \times 10^{-4}$	0.06 ± 0.001	2000
k_{return} (s^{-1})	*d*	*d*	0.09 ± 0.03	*d*
K_m (μM)	1200 ± 200	410 ± 60	50 ± 10	20
k_{cat}/K_m ($s^{-1}\ M^{-1}$)	10	1.9	1200	1×10^8
k_i ($s^{-1}\ M^{-1}$)	0.2 ± 0.1	1.6 ± 0.3	14.3 ± 2.6[e]	*d*
k_r (s^{-1})	$\ll 10^{-4}$	$\ll 10^{-4}$	$(1.0 \pm 0.1) \times 10^{-4}$	*d*

[a] The enzyme was assumed to be 60% active.[29] [b] Little or no inactivation. [c] Partition ratio [i.e., $(k_{cat}/K_m)/k_i$] = 99. [d] Not applicable. [e] Partition ratio 84. [f] Benzylpenicillin, a good substrate. Data taken from ref 29.

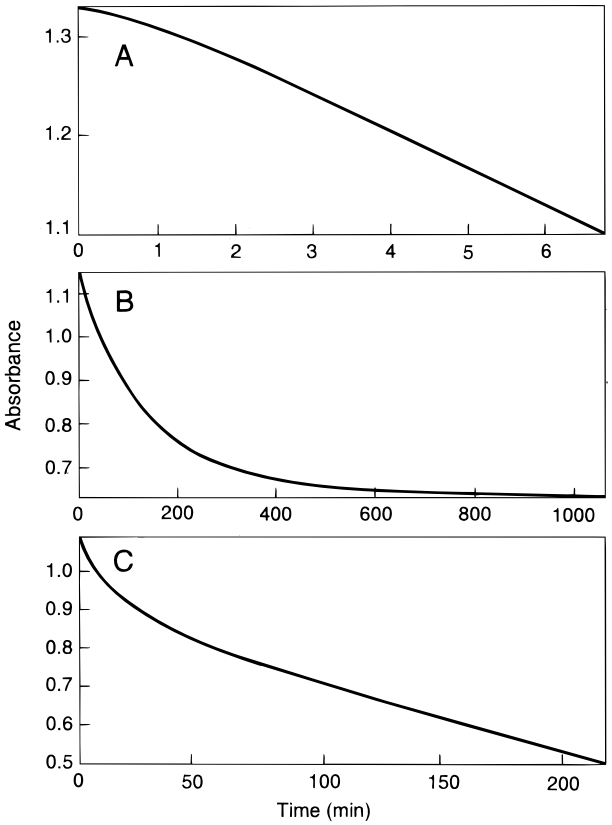
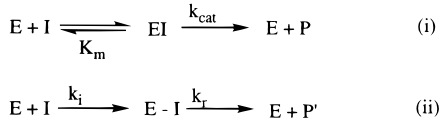


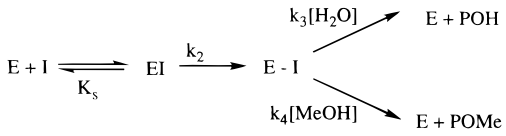
Figure 1. (A) Return of activity of the P99 β-lactamase (0.37 μM) after inactivation by incubation with **3** (1.5 mM) for 20 min. The absorbance of cephalothin at 288 nm as a function of time is shown after addition of an aliquot of the reaction mixture to a cuvette containing cephalothin (1 mM). The increase in slope with time reflects the increase in enzyme activity. (B) Burst of turnover observed on reaction of the P99 β-lactamase (1.3 μM) with **4** (240 μM). The absorbance of **4** at 250 nm as a function of time is shown. After ca. 100 turnovers the enzyme is converted into the inert E−I (Scheme 1). (C) Burst of turnover (ca. 80 per enzyme molecule) and subsequent steady-state reaction observed on reaction of the TEM β-lactamase (2.5 μM) with **5** (770 μM). The absorbance of **5** at 288 nm as a function of time is shown.

containing a good substrate, a delay was observed in return of full enzyme activity. For example, Figure 1A shows data from such an experiment involving **3** and the P99 β-lactamase. Similar results were obtained for **4** and **5** with the P99 enzyme. First-order rate constants k_{return} for this reaction could be obtained as described in the Experimental Section and are presented in Table 1. This result indicates the presence of a transiently stable intermediate species in these cases. All precedent with these enzymes[3,7] would suggest that a covalent acyl or phosphor(on)-yl enzyme intermediate is involved. The breakdown of this intermediate must be rate-determining to steady-state turnover, as shown by the coincidence of k_{cat} and k_{return} values. Acyl phosph(on)ates therefore may also act as covalent inhibitors of β-lactamases.

Scheme 1



Scheme 2

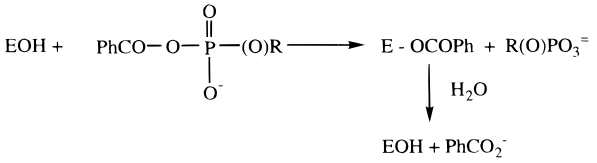


In two combinations, that of **4** with the P99 enzyme and **5** with TEM, a two-phased reaction, the second leading to a more inert enzyme−inhibitor (E−I) complex, was observed, suggestive of competing or branched pathways. Parts B and C of Figure 1 show for example bursts of turnover of **4** and **5**, reflecting around 100 and 80 turnovers per enzyme molecule, respectively, and leading to more slowly hydrolyzing species. Measurements of absorption vs time as the phosph(on)ates were converted to products were fitted to Scheme 1 by means of the FITSIM program.[21]

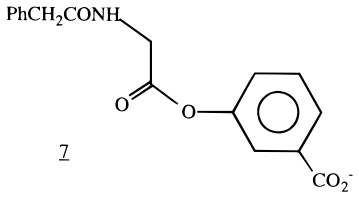
An alternative scheme wherein E−I is derived from branching of EI could not be distinguished on the basis of the kinetic data available at present. All of the rate constants described above are collected in Table 1.

Noticeable first in Table 1 is the similarity of k_{cat} and k_{return} for **3**−**5** with the P99 β-lactamase. This suggests that the transient intermediate discussed above, of half-life of about 40 s, is common to the three compounds **3**−**5**. A benzoyl enzyme is therefore the most likely possibility, rather than a phosphor-(on)yl enzyme which would be different for each compound and most likely hydrolyze to restore free enzyme at different rates. In accord with this proposition, the rate of reaction of **3** with the P99 enzyme under saturating conditions (0.37 mM) increased linearly with methanol concentration (data not shown) as would be expected[19] for an acyl enzyme (Scheme 2, where POH and POMe represent the hydrolysis and methanolysis

Scheme 3



products, respectively). From the slope of the line, the partition ratio k_4/k_3 was determined to be 23.6. This response to methanol is exactly as observed for more specific depsipeptide substrates such as **7**, where the k_4/k_3 ratio was found to be 28.1 ± 1.2.[19]



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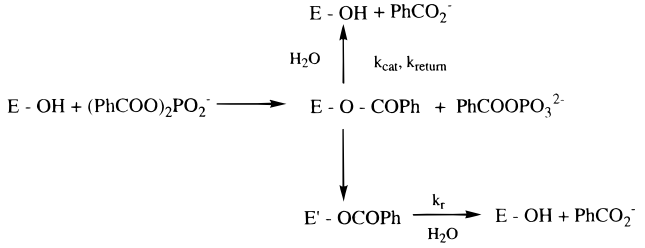
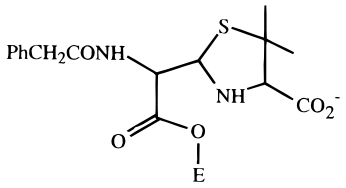
Further, a ^{1}H NMR study of reaction between **3** (5.6 mM) and the P99 β-lactamase (15 μM) in ^2H_2O containing 2.5 M [$^2H_4$]methanol showed the appearance of methyl benzoate (δ 7.52 (t, 2H), 7.65 (t, 1H), 8.01 (d, 2H)), benzoate (δ 7.45 (m, 3H), 7.82 (d, 2H)), and phenyl phosphate (δ 7.08 (t, 1H), 7.18 (d, 2H), 7.32 (t, 2H)) as products, where the ratio of methyl benzoate to benzoate was approximately 1.2:1, corresponding to a k_4/k_3 ratio of 27. These observations strongly indicate that turnover of **3**−**5** by the P99 β-lactamase involves an acyl-transfer reaction (Scheme 3) and an accumulating acyl enzyme intermediate. In terms of the rate constants of Scheme 2, therefore, $k_2 > k_3$ and $k_{cat} = k_3$ [H_2O].

The surprising ability of **3**−**5** to be substrates of this enzyme certainly must involve the negative charge on the phosph(on)-ate leaving group. This factor must be important for initial binding and for the acylation step. 4-Nitrophenyl benzoate, an ester with a leaving group of pK_a comparable to that of **3** or **4**, but uncharged, was turned over by the P99 β-lactamase much more slowly than **3**−**5** with a value of k_{cat}/K_m of 9.5 s^{-1} M^{-1} (K_m > 30 μM; measurements at higher concentrations were precluded by substrate insolubility). This would most likely reflect a much larger K_m value for 4-nitrophenyl benzoate since k_{cat} would probably be the same as for **3**−**5**; i.e., $k_{cat} = k_3 =$ 0.015 s^{-1}. If k_{cat} for 4-nitrophenyl benzoate were 0.015 s^{-1}, then K_m = 140 mM; cf. the micromolar values for **3**−**5**. The negative charge on the leaving group in **3**−**5** may well be important in interactions with the positively charged residues of the P99 β-lactamase active site during the acylation step.[12,27] Specific phosphonate monoester monoanions are thought to be inhibitors of this enzyme for similar reasons,[10] and natural β-lactam substrates also carry a carboxylate on the leaving group. On the other hand, it appears that although the presence of a negative charge appears to be important, other factors must also be involved. The hydrolysis of benzoyl (mono)phosphate was not catalyzed by the P99 β-lactamase to any detectable extent above background hydrolysis. This result suggests that the leaving group ability of the phosph(on)ate may also contribute to the acylation rate; the pK_a values of a protonated phosphate dianion and the trianion are around 7 and 12, respectively (the pK_a of benzoyl phosphate monoanion, the conjugate acid to the leaving group from **5**, is 4.8[28]).

Scheme 4



Benzylpenicillin is believed to form an acyl enzyme with the P99 β-lactamase of structure **8**. Acyclic depsipeptide substrates such as **7** most likely form acyl enzymes of similar structure, **9**.[19,26] The P99 enzyme catalyzes hydrolysis of these acyl



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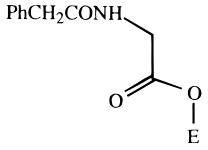

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enzymes, **8** and **9**, in the deacylation step of turnover, with rate constants (k_3[H_2O]) of 54 s^{-1} [29] and 125 s^{-1},[19] respectively. These values can be compared with that of 0.02 s^{-1} for the benzoyl enzyme derived from **3**−**5**. It is clear that the specific acylamido side chain of **7** and **8**, which is thought to be hydrogen-bonded into the active site,[12,30] contributes significantly to the deacylation rate, presumably with respect to the optimal orientation of the acyl group with respect to the catalytic machinery.

In contrast to the situation with the P99 β-lactamase, **3** and **4** do not form transiently inhibited species on turnover by the class A TEM β-lactamase despite the fact that there is slow turnover as with the former enzyme. Presumably with the latter enzyme, assuming an acyl-transfer mechanism, acylation is rate-determining, a situation more commonly observed with the TEM than the P99 β-lactamase.[31] In each case a very slow, essentially irreversible inactivation (k_i) is also observed. Such a reaction is also seen between **4** and the P99 enzyme. This may well reflect phosphor(on)ylation of the active site serine, a reaction more facile for phosphonates than phosphates.[14]

The interaction of the TEM β-lactamase with **5** however is much more interesting and unexpected. Both turnover and inhibition are more rapid than with **3** and **4**. A return of activity experiment with **5** indicates the presence of a slowly dissociating complex of half-life around 10 s; the low K_m is also suggestive of an accumulating intermediate. Transition to a more inert species occurs (Figure 1C), after about 80 turnovers on average. This complex, with a half-life of around 2 h, is however more labile than the final inert species slowly derived from **3** and **4**. A possible sequence of reactions to rationalize these observations is given in Scheme 4. ^{1}H NMR and UV spectral studies of the reaction of **5** with the TEM enzyme support the proposition that benzoate and benzoyl phosphate are the products of both phases of turnover. As with the P99 enzyme, hydrolysis of

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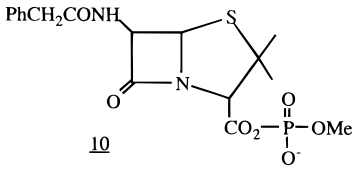
benzoyl monophosphate does not occur at any significant rate at the TEM β-lactamase active site.

Precipitation of the enzyme by trichloroacetic acid (5%) during the second and slower phase of turnover of **5** (Figure 1C) yielded a protein sample that contained little or no free phosphate[32] (nor did a sample of the native enzyme precipitated from phosphate buffer). Dissolution of the precipitated protein from the reaction mixture with **5** in 0.5 M NaOH followed by incubation at 37 °C for 15 h (conditions found to release phosphate from serine phosphate residues[33]) also yielded no phosphate. It seems unlikely therefore that the inert complex of the enzyme with **5** arises from active site phosphorylation; i.e., the slowly reactivating species is unlikely to be E−O−PO_2^-−OCOPh, and thus must also represent an acyl enzyme species (as implied by Scheme 4). It is well-known that long-lived acyl enzymes of class A β-lactamases undergo partitioning into more inert forms, probably by way of conformational transitions.[7] The present case may represent a further example of this phenomenon. Benzoyl transfer to another active site residue is also possible.

The striking result with regard to the TEM β-lactamase is the accumulation of slowly hydrolyzing acyl enzymes. Rate constants of breakdown for the faster and slower turning over species were 0.06 and 1.0×10^{-4} s^{-1}, respectively. For comparison, it may be noted that the rate constant for deacylation of the acyl enzyme derived from a good substrate benzylpenicillin is 1500 s^{-1},[34] from cefoxitin, a TEM resistant cephamycin, 4×10^{-3} s^{-1},[35] and from clavulanic acid, a β-lactamase inhibitor, 3.8×10^{-3} s^{-1}.[36] Thus, the stability of the more inert complex from reaction of the TEM β-lactamase with **5** is greater than those from these currently employed inhibitors. Breakdown of the complex of the cyclic phosphate **6** is somewhat slower (8.3×10^{-5} s^{-1}) for reasons discussed elsewhere.[16]

It should be noted that acyl phosphates have been employed previously for the inactivation of enzymes[37] and for protein modification.[38] Indeed, Song and Kluger[39] prepared the penicillin derivative **10** and found it to be an inactivator of the TEM



10

β-lactamase. The mechanism of action of this compound however appears to be quite different from that of the compounds reported in the present paper. The β-lactamase catalyzes β-lactam hydrolysis in **10** rather than acyl phosphate hydrolysis as demonstrated here for **3**−**5**. Inactivation by **10** was suggested to occur by acylation of enzyme amine groups during β-lactam turnover. Enzyme-catalyzed hydrolysis of an acyl phosphate appears unusual except, of course, by acyl phosphatase! This enzyme catalyzes a phosphoryl-transfer rather than acyl-transfer reaction, however.[40]

Acyl phosph(on)ates therefore have the potential to form tight, presumably covalent, and long-lived inhibitory complexes with serine β-lactamases. It will be interesting to find whether generally or specifically more potent analogues can be obtained by structural variation. The possibility of DD-peptidase inhibition (i.e., antibacterial agents) is also intriguing. Compounds **3** and **5**, for example, do inactivate the DD-peptidase of *Streptomyces R61*, although more slowly than the P99 β-lactamase. The central structural theme of these inhibitors seems well-suited for application of the methods of combinatorial chemistry.

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