Inhibition of Class D β-Lactamases by Diaroyl Phosphates†

Sudipta Majumdar,‡ S. A. Adediran,‡ Michiyoshi Nukaga,§ and R. F. Pratt*,‡

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459, and Faculty of Pharmaceutical Sciences, Jyosai International University, 1 Gumyo, Togane-shi, Chiba 283-8555, Japan

Received August 26, 2005; Revised Manuscript Received October 17, 2005

ABSTRACT: The production of β-lactamases is an important component of bacterial resistance to β-lactam antibiotics. These enzymes catalyze the hydrolytic destruction of β-lactams. The class D serine β-lactamases have, in recent years, been expanding in sequence space and substrate spectrum under the challenge of currently dispensed β-lactams. Further, the β-lactamase inhibitors now employed in medicine are not generally effective against class D enzymes. In this paper, we show that diaroyl phosphates are very effective inhibitory substrates of these enzymes. Reaction of the OXA-1 β-lactamase, a typical class D enzyme, with diaroyl phosphates involves acylation of the active site with departure of an aroyl phosphate leaving group. The interaction of the latter with polar active-site residues is most likely responsible for the general reactivity of these molecules with the enzyme. The rate of acylation of the OXA-1 β-lactamase by diaroyl phosphates is not greatly affected by the electronic effects of substituents, probably because of compensation phenomena, but is greatly enhanced by hydrophobic substituents; the second-order rate constant for acylation of the OXA-1 β-lactamase by bis(4-phenylbenzoyl) phosphate, for example, is 1.1×10^7 s^{-1} M^{-1}. This acylation reactivity correlates with the hydrophobic nature of the β-lactam side-chain binding site of class D β-lactamases. Deacylation of the enzyme is slow, e.g., 1.24×10^{-3} s^{-1} for the above-mentioned phosphate and directly influenced by the electronic effects of substituents. The effective steady-state inhibition constants, K_i, are nanomolar, e.g., 0.11 nM for the above-mentioned phosphate. The diaroyl phosphates, which have now been shown to be inhibitory substrates of all serine β-lactamases, represent an intriguing new platform for the design of β-lactamase inhibitors.

The β-lactamases provide bacteria with an important source of resistance to β-lactam antibiotics. These enzymes have, in general, broad specificity for β-lactams, and this specificity is further refined in mutants that are rapidly selected for under β-lactam pressure. Studies of amino acid sequence homology have identified four classes of β-lactamase, classes A−D (*1*). Three of these classes, A, C, and D are serine nucleophile-based enzymes, while the fourth, class B, contains zinc metallo-β-lactamases. Of the serine β-lactamases, classes A and C are currently the most important in human disease and have been most intensely studied. In recent years, however, extended spectrum class D enzymes have been growing in clinical importance (*2*). Class D β-lactamases were first identified as enzymes capable of rapidly hydrolyzing second generation penicillins such as oxacillin and cloxacillin, but as further study has shown, they clearly have greater potential. At least 80 variants of these enzymes have now been described, some of which have been identified as producing β-lactam, including carbapenem, resistance in pathogens such as *Acinetobacter baumannii* (*3*), *Klebsiella pneumoniae* (*4*), and *Pseudomonas aeruginosa* (*5*).

The active site of class D β-lactamases superficially resembles that of class A enzymes (*6*−*8*). For example, in the OXA-1 enzyme, the S (67) XYK (70) motif of all serine β-lactamases is present, K (212) T(S) G(214) is present, but SXN is replaced by S(110)VV. The latter is an indication of the replacement of side-chain amide specificity with general hydrophobicity. Finally, and most dramatically, Glu 166 of class A β-lactamases is not present. Instead, it is likely that Lys 70 of class D β-lactamases is carboxylated by carbon dioxide to form a carbamate (*6*, *8*), and it has been proposed that the carbamate anion is an important general acid/base catalyst in substrate turnover (*6*, *8*).

The class D β-lactamases are, in general, not especially susceptible to the classical, commercial β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam, although the latter is more effective (*9*). Further, there have been essentially no reports of specific class D inhibitors. Recently (*10*), we examined the reactivity of two class D β-lactamases, OXA-1 and OXA-10, with a variety of phosph(on)ates that we had previously found to have inhibitory activity against class A and C β-lactamases (*11*). Although a classical phosphonate inhibitor such as **1** and an acyl phosphate **2** were ineffective against these enzymes, a diacyl phosphate, dibenzoyl phosphate, **3**, was strikingly more reactive (*10*). In view of the latter result, we looked further into the reactivity of such compounds and report here on the effectiveness of **3**−**8** and on their mechanism of inhibition of the OXA-1 β-lactamase.

MATERIALS AND METHODS

Synthesis of Diaroyl Phosphates: Sodium Bis(4-biphenylcarbonyl) Phosphate (7). This compound was prepared

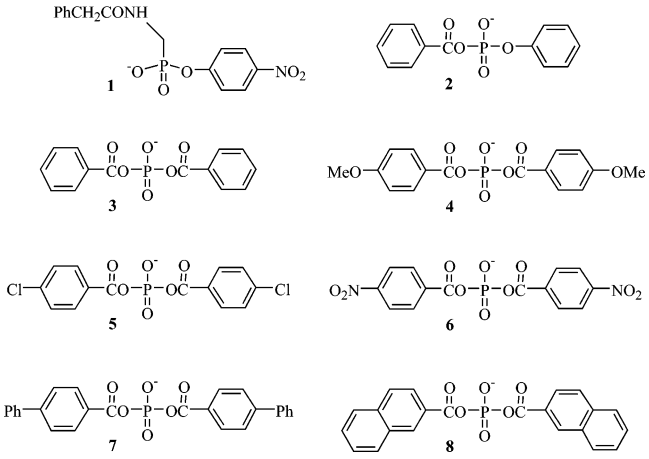
† This research was supported by National Institutes of Health Grant AI-17986 (to R.F.P.).

* To whom correspondence should be addressed. Telephone: 860-685-2629. Fax: 860-685-2211. E-mail: rpratt@wesleyan.edu.

‡ Wesleyan University.

§ Jyosai International University.

10.1021/bi051719s CCC: $30.25
Published on Web 11/12/2005



following the general method of Chantrenne (*12*). The entire procedure was carried out in a cold room at 4 °C. Thus, a 5 g (0.012 mmol) sample of dry silver phosphate and 3 g (0.03 mmol) of 85% orthophosphoric acid were mixed and triturated for ca. 10 min until the yellow paste became gray. It was then dispersed in 7 mL of tetrahydrofuran (THF)[1] and a solution of 5.2 g (0.024 mmol) of 4-biphenylcarbonyl chloride in 40 mL of THF, added dropwise to the suspension with continuous and gentle stirring. After the mixture was allowed to react for 90 min, the silver salts were removed by centrifugation and washed with THF. The supernatant and wash were combined, and an equal volume of cold petroleum ether was added. Separation of the mixture into two liquid phases was observed. The lighter phase was discarded, and 20 mL of ice-cold water was added to the heavy viscous phase. Cold 20% aq. NaOH was then added to the latter until the pH reached 4.5 when a white precipitate appeared. This was removed by filtration and recrystallized from 4:1 EtOH/H_2O. The product, in 46% yield, was characterized by nuclear magnetic resonance (NMR) and IR spectra. ^{1}H NMR [dimethyl sulfoxide (DMSO)-d_6, 300 MHz] δ: 7.42 (t, J = 6.6 Hz, 1H), 7.5 (t, J = 8.1 Hz, 2H), 7.75 (d, J = 8.4 Hz, 2H), 7.84 (d, J = 6.9 Hz, 2H), 8.03 (d, J = 7.5 Hz, 2H). ^{31}P NMR (DMSO-d_6, 300 MHz) δ: −20.6. Fourier transform infrared (FTIR) (KBr, cm^{-1}): 1719. Anal. Calcd for $C_{26}H_{18}O_6PNa$: C, 65.01; H, 3.79; P, 6.45. Found: C, 65.01; H, 3.75; P, 6.15.

*Sodium Bis(2-naphthoyl) Phosphate (**8**).* This compound was prepared analogously to **7** from 2-naphthoyl chloride and silver phosphate; the optimal reaction time was 45 min. The product was recrystallized from 1:1 EtOH/H_2O and characterized by NMR and IR spectra. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 7.61 (t, J = 7.2 Hz, 1H), 7.67 (t, J = 6.9 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 8.01 (d, J = 8.4 Hz, 1H), 8.04 (d, J = 8.7 Hz, 1H), 8.15 (d, J = 8.1 Hz, 1H), 8.64 (s, 1H). ^{31}P NMR (DMSO-d_6, 300 MHz) δ: −20.58. FTIR (KBr, cm^{-1}): 1725.

*Sodium Bis(4-methoxybenzoyl) Phosphate (**4**).* This compound was also prepared analogously to **7**, beginning with 4-methoxybenzoyl chloride dissolved in 11 mL of diethyl ether and silver phosphate dispersed in 8 mL of diethyl ether; the reaction time was 15 min. The product was recrystallized from EtOH and characterized by NMR and IR spectra. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 3.82 (s, 3H), 7.03 (d, J = 7.8 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H). ^{31}P NMR (DMSO-d_6, 300 MHz) δ: −20.42. FTIR (KBr, cm^{-1}): 1713.

*Sodium Bis(4-chlorobenzoyl) Phosphate (**5**).* This compound was prepared analogously to **4**, beginning with 4-chlorobenzoyl chloride dissolved in 20 mL of diethyl ether and with a reaction time of 60 min. The product was recrystallized from EtOH and characterized by NMR and IR spectra. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 7.59 (d, J = 8.4 Hz, 2H), 7.93 (d, J = 8.4 Hz, 2H). ^{31}P NMR (DMSO-d_6, 300 MHz) δ: −20.88. FTIR (KBr, cm^{-1}): 1743.

*Sodium Bis(4-nitrobenzoyl) Phosphate (**6**).* This compound was prepared analogously to **7**, beginning with 4-nitrobenzoyl chloride dissolved in 16 mL of THF, and the reaction time was 45 min. The product was recrystallized from EtOH and characterized by NMR and IR spectra. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.18 (d, J = 9 Hz, 2H), 8.35 (d, J = 9 Hz, 2H). ^{31}P NMR (DMSO-d_6, 300 MHz) δ: −21.26. FTIR (KBr, cm^{-1}): 1737.

*Sodium Dibenzoyl Phosphate (**3**).* Sodium dibenzoyl phosphate was prepared in this laboratory by Dr. Naixin Li (*13*). The OXA-1 β-lactamase was prepared and purified as previously described (*8*). The OXA-10 enzyme was a generous gift from Professor J. -M. Frère of the University of Liège, Belgium. Phosphodiesterase I (bovine intestine) was purchased form Sigma. Cephalothin was a gift of the Eli Lilly Co., and 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid (Centa) was prepared in this laboratory by a literature method (*14*).

Analytical and Kinetics Methods. Absorption spectra and spectrophotometric reaction rates were measured with Hewlett−Packard 8452A and 8453E spectrophotometers. All kinetics experiments were performed at 25 °C in a buffer containing 20 mM 3-morpholinopropanesulfonic acid (MOPS) and 50 mM $NaHCO_3$ at pH 7.5. Steady-state kinetics parameters were obtained as described below. First, k_{cat} values for compounds **3**−**8** were obtained spectrophotometrically, at the wavelengths listed in Table 1, from initial rates. In these experiments, the concentration of the enzyme was 0.2 μM, the initial concentrations of compounds **7** and **8** were 1−4 μM, and those of compound **6** were 5−30 μM. In the case of compounds **3**, **4**, and **5**, the enzyme concentration was 1.0 μM and the concentrations of the compounds were 4−20 μM.

Determination of k_i Values. Cephalothin was used as the substrate in competition experiments to determine k_i (Scheme 1) values for **3**−**8**. In these experiments, enzyme (final concentration of 0.1 μM) was added to a mixture of

Table 1: Absorption Spectral Data and Background Hydrolysis Rates for the Diaroyl Phosphates **3**−**8**

diaroyl phosphate	λ_{max} (nm)	$\Delta\epsilon \times 10^{-4}$ (cm^{-1} M^{-1})	$k_0 \times 10^6$ (s^{-1})
3	248	1.18	1.20
4	240	1.24	0.65
5	248	2.00	6.52
6	260	0.93	63.8
7	300	2.00	1.56
8	254	1.46	1.73

[1] Abbreviations: Centa, 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FTIR, Fourier transform infrared; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; THF, tetrahydrofuran.

Scheme 1

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

$$E + I \xrightarrow{k_i} EI \xrightarrow{k_2} E + P'$$

cephalothin (200 μM) and the inhibitor (0.1−4.0 μM, except in the case of **6**, where 10−30 μM was used) in buffer also containing 0.1% gelatin; in the absence of this additive, a slow irreversible inactivation was also observed with certain compounds, especially **6**. To obtain k_i values, the total progress curve was fitted to Scheme 1 by means of the Dynafit program (*15*). Experimental values of k_{cat}, obtained as described above, were fixed during the fitting procedure.

Active-Site Titration with 7. The enzyme (0.12 μM) was incubated with various concentrations of **7** (0−0.1 μM). After 5 min, it was assayed against Centa (75 μM). The initial rates were then plotted against the inhibitor concentration.

Methanolysis. The effect of methanol on the initial rates of solvolysis of **3** and **7** in the presence of the enzyme was determined. Concentrations of **3** and the enzyme were 40 and 0.2 μM, respectively, and the methanol concentration was varied from 0 to 2.5 M. Concentrations of **7** and the enzyme were 2.0 and 1.0 μM, respectively, and the same methanol concentrations were employed as with **3**.

Product Inhibition. Inhibition by 0−1 mM 4-biphenyl carboxylic acid (a product of OXA-1 catalyzed hydrolysis of **7**) was assessed from initial rate measurements (spectrophotometrically at 410 nm) with Centa (75 μM) and an enzyme concentration of 0.02 μM. These measurements were also made with 2-naphthoic acid, the hydrolysis product of **8**. Similar experiments were also performed with 0−10 mM sodium dibenzyl phosphate; in this case, cephalothin (100 μM) was used as the substrate.

Mass Spectroscopy. The OXA-1 β-lactamase (20 μL, 28.7 μM) was inactivated by incubating it with the phosphate **3** (5 μL, 2.5 mM) for 10 min. Protein was then precipitated from the solution by the addition of trichloroacetic acid to a final concentration of 5%, and the resultant mixture was allowed to stand in an ice bath for 30 min. The mixture was then centrifuged, and the solid precipitate was washed once with 5% aqueous trichloroacetic acid and 3 times with acetone. The acetone was removed by evaporation under vacuum, and the dry protein was submitted for electrospray ionization (ESI) mass spectrometry (Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois).

Phosphodiesterase Susceptibility. The susceptibility of **3** and **7** to phosphodiesterase I was determined by direct spectrophotometric observation at the wavelengths 316 and 254 nm, respectively. Initial rates were determined of the reactions between **3** and **7** (10−250 μM) with 0.2 μM enzyme, and steady-state parameters were obtained from nonlinear least-squares fits to the Michaelis−Menten equation.

Molecular Modeling. The computations were performed by means of an sgi octane2 workstation with the InsightII 2000 suite of molecular-modeling programs (Accelrys, San Diego, CA). The starting point for the structures was the crystal structure of the OXA-1 β-lactamase, including the crystallographic water molecules [PDB entry 1M6K (*8*)]. A tetrahedral intermediate structure, **10**, derived from **3**, covalently bound to the active-site serine nucleophile, was built onto this structure by means of the Builder module of InsightII. The MOPAC module was used to calculate MNDO charges for the ligand. The charges on the protein were assigned by the Builder module in InsightII. The carbamoylated Lys 70 side chain was in the anionic form, and the side chain of Lys 212 was cationic. The ligand was initially oriented with the oxyanion in the oxyanion hole and with the phenyl and phosphate groups placed much as in our previous models of complexes of acyl phosphates with a class C β-lactamase (*16*). Interactions with the enzyme then directed the leaving-group phenyl group back out into solution. A 15 Å sphere of water molecules centered at Ser62 Oγ was added to each structure. Molecular dynamics simulations, followed by energy minimization procedures on representative snapshots, lead to several largely similar structures (*16*). The structure with the lowest interaction energy was adopted as the most likely structure (*16*).

RESULTS AND DISCUSSION

Dibenzoyl phosphate could be prepared from the reaction of benzoic anhydride with disodium hydrogen phosphate in aqueous pyridine (*13*, *17*). The biphenyl and naphthyl analogues could not be prepared in this way because of the low solubility of the corresponding anhydrides in the reaction medium. Consequently, the other diaroyl phosphates used in this study were prepared from the reaction of aryl chlorides with silver hydrogen phosphate as described by Chantrenne (*12*, *18*). The products could be conveniently handled as the crystalline sodium salts.

Compounds **3**−**8** hydrolyzed slowly in the buffer employed for the enzyme studies [20 mM MOPS and 50 mM $NaHCO_3$ at pH 7.5; preliminary experiments showed that the OXA-1 β-lactamase activity was lower in the absence of the $NaHCO_3$; this result is presumably in accordance with the findings of Golemi et al. that class D β-lactamases require carboxylation of Lys 70 for activity (*19*)]. ^{1}H and ^{31}P NMR studies showed that the disappearance of **3** correlated with the appearance of benzoate and phosphate. Only a steady-state trace of benzoyl phosphate was observed; an independent experiment showed that benzoyl phosphate hydrolyzed faster than **3** under these conditions. Pseudo-first-order rate constants for hydrolysis of **3**−**8** are presented in Table 1. These display the order of reactivity expected on the basis of electronic substituent effects, viz. *p*-OMe < H, biphenyl, naphthyl < *p*-Cl < *p*-NO_2. The compounds, surprisingly perhaps, because they are bis(carboxylic)phosphoric anhydrides, are generally (except for **6**) more stable in solution than benzylpenicillin, which hydrolyzes with a pseudo-first-order rate constant of 1.5×10^{-5} s^{-1} under similar conditions (*20*). The negatively charged phosphate moiety is therefore very effective at repulsing nucleophiles, not only from phosphorus but also from the adjacent carbonyl groups.

The diaroyl phosphates acted as poor substrates and transient inhibitors of the OXA-1 β-lactamase. When they were incubated with the enzyme, the activity of the latter, assayed against a good substrate, fell rapidly but later returned. This suggests the rapid formation of an enzyme−inhibitor (EI) complex, which slowly breaks down to free enzyme. The turnover rate constant could best be determined

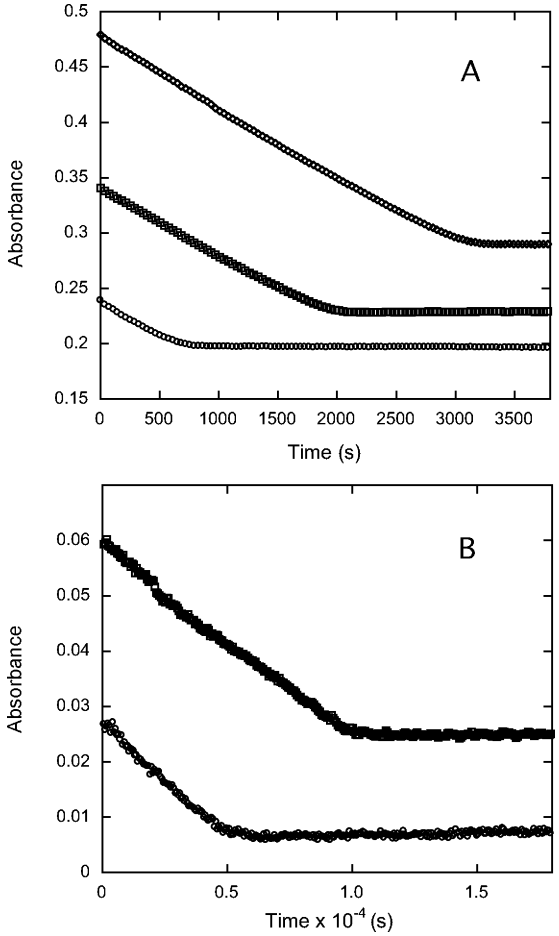


FIGURE 1: (A) Total progress curves for turnover of **3** (4.0 μM, ○; 12.0 μM, □; 20.0 μM, ◇) by the OXA-1 β-lactamase (1.0 μM). The absorbance changes at 248 nm are shown. (B) Total progress curves for turnover of **7** (1.0 μM, ○; 2.0 μM, □) by the OXA-1 β-lactamase (0.2 μM). The absorbance changes at 300 nm are shown.

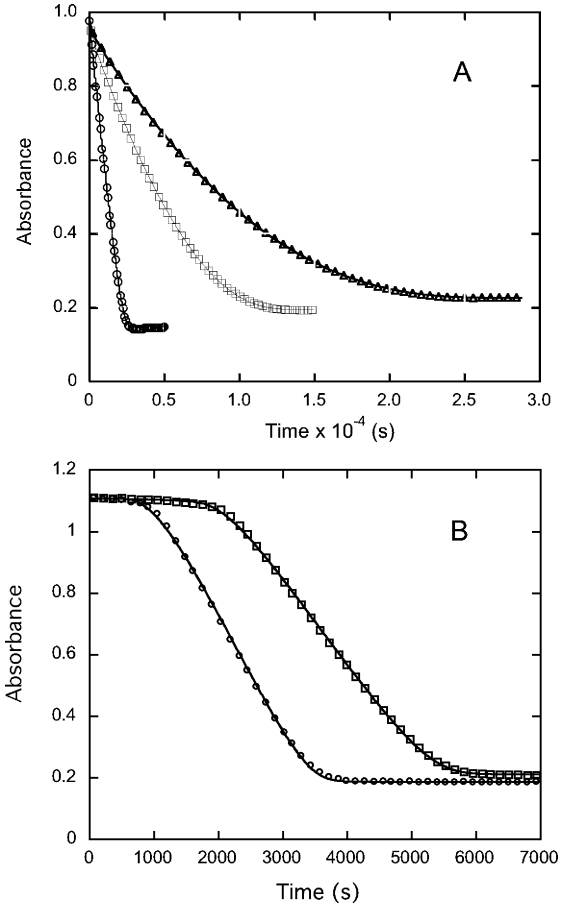
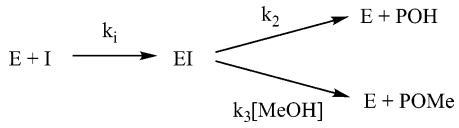


FIGURE 2: (A) Total progress curves showing the effect of **3** (0 μM, ○; 2.0 μM, □; 4.0 μM, △) on the turnover of cephalothin (200 μM) by the OXA-1 β-lactamase (0.1 μM). The absorbance changes at 278 nm are shown. The points are experimental, and the lines are calculated from the fit of the data to Scheme 1. (B) Total progress curves showing the effect of **7** (0.2 μM, ○; 0.4 μM, □) on the turnover of cephalothin (200 μM) by the OXA-1 β-lactamase (0.1 μM). The absorbance changes at 278 nm are shown. The curve in the absence of **7** is shown in A. The points are experimental, and the lines are calculated from the fit of the data to Scheme 1.

directly. For example, Figure 1A shows, spectrophotometrically, the turnover of **3**, at three initial concentrations, by the OXA-1 β-lactamase. The initial rates at these concentrations were essentially the same showing that $K_m \leq 1$ μM and $k_{cat} = (5.5 \pm 0.1) \times 10^{-3}$ s^{-1}. The intermediate EI complex thus had a half-life of 2.1 min.

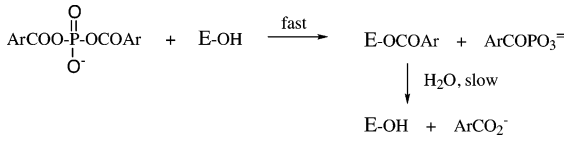
The rate of formation of EI was best established by a competition experiment. The disappearance of cephalothin from a mixture of cephalothin and **3** could be followed spectrophotometrically after the enzyme had been added. Turnover of cephalothin was slower than in the absence of **3** (Figure 2A). From the fitting of these progress curves to Scheme 1, where S is cephalothin, I is the inhibitory substrate **3**, and EI is the slowly breaking down complex of E and I of a half-life of 2.1 min, and k_2, therefore, is 5.5×10^{-3} s^{-1}, a value of the second-order rate constant k_i, the inhibition rate constant, could be obtained; the value of k_i for **3** was thus found to be $(2.7 \pm 0.1) \times 10^4$ s^{-1} M^{-1}. The effective value of K_m of **3** as a substrate or, equivalently, of K_i as a competitive inhibitor would therefore be $k_2/k_i = 0.2$ μM. Noncovalent binding of **3** to the enzyme is probably weak because no fast pre-equilibrium binding step was required to fit the kinetics data (Scheme 1) at the inhibitor concentrations employed ($\leq$20 μM); further, the dissociation constant (K_i) of dibenzyl phosphate was found to be ca. 1.8 mM.

Scheme 2



The regeneration of free E from the EI complex was accelerated by the alternative nucleophile methanol. Thus, the initial rates of disappearance of **3** and **7** under saturating concentration conditions were found to increase linearly with the methanol concentration (data not shown). This result may be interpreted in terms of Scheme 2. A ^{1}H NMR experiment with **3** showed that this enzyme-catalyzed reaction yielded methyl benzoate (POMe), characterized by an aromatic doublet at δ 8.03, which is readily distinguished from that of the hydrolysis products, benzoate, at δ 7.85, and, transiently, benzoyl phosphate, at δ 8.08. The ratio of methanolysis to hydrolysis products yielded a value of $k_3/k_{2'}$ of 17 (where $k_{2'} = k_2/[H_2O]$ is the second-order rate constant for hydrolysis of EI and $[H_2O] = 55.5$ M). This experiment strongly suggests that turnover of **3** by the OXA-1 β-lactamase involves transient formation of an acyl-

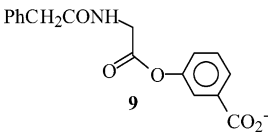
Scheme 3



(benzoyl)−enzyme intermediate (rather than a phosphoryl enzyme species). This is true also for the turnover of **3** by the class C β-lactamase of *Enterobacter cloacae* P99 and, most likely, by the class A TEM-2 enzyme (*13*). Quantitative analysis of the kinetics data for the OXA-1 enzyme (*21*) yielded a value of $k_3/k_{2'}$ of 18.2 ± 1.0, in good agreement with that derived from product analysis. Thus, the rate constant k_i represents enzyme acylation and k_2 deacylation (Scheme 3).

Acylation of the active site by **3** was confirmed by electrospray mass spectrometry. A reaction mixture comprising OXA-1 (28.7 μM) and **3** (500 μM) was quenched, and the protein precipitated by trichloroacetic acid after 10 min of the reaction. A mass spectrum of the native enzyme showed peaks at 28 133 and 28 230 amu; the former may derive from the loss of the leucine residue from the C terminus in the original sample. The enzyme sample treated with **3** had peaks at 28 235 and 28 336 amu. Thus, a fragment of mass 102 or 106 amu, corresponding well with benzoyl (net increase of 104 amu), is present in the enzyme treated with **3**. Again, acylation is indicated, and thus, the accumulating EI complex is an acyl−enzyme.

The alternative nucleophile experiment also suggested that the OXA-1 enzyme catalyzed hydrolysis of the depsipeptide **9** by way of an acyl−enzyme intermediate whose hydrolysis was rate-determining.



9

Steady-state parameters for turnover of **9** by the OXA-1 enzyme were $k_{cat} = 3.6 \pm 0.3\ s^{-1}$, $K_m = 5.1 \pm 0.5$ mM, and $k_{cat}/K_m = 706\ s^{-1}\ M^{-1}$. At a concentration of **9** of 5.0 mM, methanol (0−2.5 M) accelerated the overall reaction (data not shown) to an extent giving rise to a value of the partition ratio, analogous to $k_3/k_{2'}$ of Scheme 2, of 11.5 ± 1.3. A ^{1}H NMR experiment showed the presence of both hydrolysis and methanolysis products, with the latter distinguished by its glycyl resonance at δ 4.00 (*21*). The access of methanol to the acyl−enzymes derived from **3** and **9** distinguishes the OXA-1 β-lactamase from the class A TEM-1 and TEM-2 enzymes where enzyme-catalyzed methanolysis does not occur (*22*). The rate of hydrolysis of benzylpenicillin by the OXA-1 enzyme was not accelerated by methanol, however, and no methanolysis product was observed by ^{1}H NMR spectroscopy. This is also the result with the class A TEM-2 and *Staphylococcus aureus* PCI enzymes (*22*). The nature of the rate-determining step in these cases cannot, therefore, be deduced from the methanolysis experiment. Finally, it might be noted that a typical class C β-lactamase catalyzes methanolysis of both **9** and benzylpenicillin (*22*) and also of a benzoyl−enzyme intermediate (*13*). Thus, access of methanol to the acyl−enzymes formed between substrates and the OXA-1 enzyme seems possible to an extent intermediate between that of class C and class A enzymes. Rate-determining deacylation may also be more prevalent with class D enzymes than with the classical class A β-lactamases from *Bacillus cereus* and the TEM plasmid. The presence of branched pathway steady-state kinetics with the OXA-1 enzyme (*23*) is also suggestive of accumulating acyl−enzymes. The OXA-1 enzyme is monomeric in solution, it might be noted (*8*); therefore, complex kinetics with this enzyme cannot be attributed to a monomer−dimer equilibrium (*24*).

Table 2: Rate Constants for Reactions between the OXA-1 β-Lactamase and Diaroyl Phosphates **3**−**8**

diaroyl phosphate	$k_i \times 10^{-6}$ ($s^{-1}\ M^{-1}$)	$k_2 \times 10^3$ (s^{-1})	K_i (nM)[a]
3	0.027 ± 0.001	5.5 ± 0.1	200
4	0.049 ± 0.001	0.87 ± 0.10	17.8
5	0.25 ± 0.01	33 ± 2	130
6	0.073 ± 0.002	174 ± 19	2380
7	10.8 ± 0.9	1.24 ± 0.03	0.11
8	1.8 ± 0.1	8.2 ± 1.0	4.6

[a] $K_i = k_2/k_i$.

The reactions between the OXA-1 enzyme and the substituted benzoyl derivatives **4**−**6** were studied in the same way as described above for **3**, and the kinetics results are reported in Table 2. The acylation rate constants, k_i, do not reflect the electronic effects of the substituents on **3**−**6**; indeed, the substituents have little effect. This may indicate the presence of specific steric interactions between the leaving groups and the enzyme or, more likely, of general acid/hydrogen-bonding catalysis, which offsets the effects of electron-withdrawing groups (see below). The somewhat larger k_i value of **5** probably reflects the hydrophobic contribution (see below) of the chlorine substituent.

Electronic substituent effects are, however, seen in the deacylation rate constant, k_2, where the leaving group is not present and the effect of electron-withdrawing groups on the acyl group on nucleophilic attack by water is observed. Hammett plots (four points, not shown) from the data of Tables 1 and 2 yielded ρ values of 1.97 and 2.16 for the background hydrolysis rate constants and the deacylation rate constants, k_2, respectively. These values agree quite well with the value (2.5) for alkaline hydrolysis of alkyl benzoates (*25*) and indicate that direct nucleophilic attack on the carbonyl group of the acyl enzyme is probably rate-determining in deacylation (k_2). Because of the expression of substituent effects described above, K_i, the effective steady-state inhibition constant, given by the ratio k_2/k_i (see above), will increase with electron-withdrawing substituents ($\rho = 1.86$); i.e., steady-state competitive inhibition will become weaker, and **4** is thus, in a steady-state experiment, the most effective inhibitor of **3**−**6**.

The hydrophobic biphenyl (**7**) and 2-naphthyl (**8**) derivatives proved more difficult to study. At low concentrations ($\leq$20 μM), they behaved essentially as **3**−**6** (Figures 1B and 2B) and k_i and k_2 values could be obtained in the same way as in the latter cases (Table 2). At higher inhibitor concentrations, however, substrate inhibition and slow, essentially irreversible, inhibition were also observed. The substrate inhibition indicates the presence of at least one other substrate-binding site. It may be that multiple interactions occur between the enzyme and these very hydrophobic

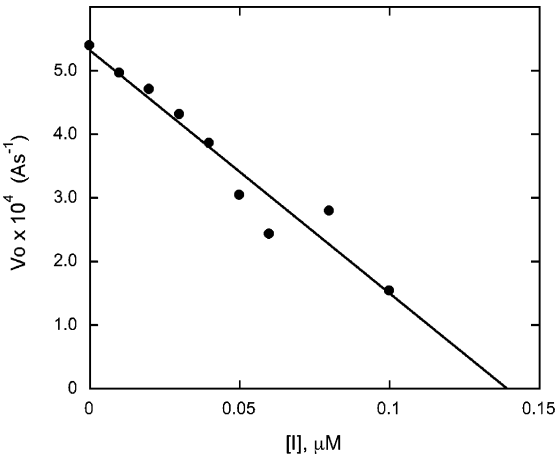


FIGURE 3: Measurements of the activity of the OXA-1 β-lactamase (0.12 μM) against Centa (75 μM) after incubation of the enzyme with various concentrations of **7** (0−0.1 μM) for 5 min. A linear least-squares line through the experimental points is shown.

molecules when the latter are at high concentrations. The complex kinetics occurring under these conditions were not analyzed quantitatively. It might be noted that the absorption intensity of **7** at its absorption maximum was linear with the concentration until 100 μM, and thus, the kinetic complexity observed at concentrations above 30 μM was probably not due to aggregation of **7** in solution. The absorption spectral change on the addition of enzyme to **7** at a low concentration, where the reaction went to completion, was exactly the same as for hydrolysis in the absence of the enzyme; thus, no complications from the presence of different chemical reactions seemed likely.

To gain evidence that the hydrolysis of **7** did occur at the active site, a sample of the enzyme was inactivated by 6-β-iodopenicillanic acid (*26*) (0.93 mM, 60 min) and the residual reagent was removed by dialysis. The resulting enzyme was essentially inactive both with respect to cephalothin and also to **7**.

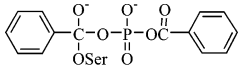
As the results for **7** and **8** provided in Table 2 indicate, these compounds, especially **7**, are much better inhibitors of the OXA-1 β-lactamase than are **3**−**6** (compare parts A and B of Figure 2, for example, noting the different concentrations of **3** and **7** employed and the almost complete absence of the turnover of cephalothin in the presence of **7**). This difference is clearly evident in the high acylation rates k_i, where that for **7**, ca. 10^7 s^{-1} M^{-1}, may approach the diffusion-controlled limit. In contrast, the deacylation rate constants are similar to that of **3**, presumably simply reflecting the electronic nature of the acyl group. It seems likely then that the large k_i values of **7** and **8** derive from the presence of the hydrophobic leaving group in the acylation step and its interaction with the enzyme active site. A result of the high k_i values is the correspondingly low K_i (k_2/k_i) values, where the effective dissociation constant of **7**, for example, is 0.11 nM. This tight binding permitted an active-site titration of the enzyme by **7** (Figure 3). With the assumption of the formation of a 1:1 complex, the data indicate an enzyme concentration of 0.14 ± 0.01 μM; the measured enzyme concentration was 0.12 μM.

Compound **7** was also a strong inhibitor of the OXA-10 enzyme: values of k_i, k_2, and K_i were (5.4 ± 0.7) × 10^7 s^{-1} M^{-1}, (4.6 ± 0.2) × 10^{-3} s^{-1}, and 0.085 nM, respectively.

The marked affinity of **7** and **8** for the OXA-1 active site dramatically demonstrates the hydrophobicity of this site. This hydrophobicity has been noted previously, of course, with respect to the side chains of the preferred substrates of class D β-lactamases, viz. oxacillins, and the affinity of the enzyme for hydrophobic dyes (*27*). With respect to the present work, it might be noted that biphenyl 4-carboxylate, a product of hydrolysis of **7**, is an inhibitor of the OXA-1 β-lactamase with a K_i value of 90 μM; the dissociation constant of 2-naphthoate, from hydrolysis of **8**, however, is >1 mM. Also, although benzeneboronic acid is an unremarkable inhibitor [K_i = 3.5 mM (*10*)], biphenyl-4-boronic acid is considerably more powerful (K_i = 7.3 μM). It is likely, therefore that specific aromatic/heterocyclic boronic acids would be as powerful inhibitors of class D β-lactamases as they are of classes A and C (*28*). The crystal structures of the OXA-1 and OXA-10 enzymes (*6*, *8*) illustrate the molecular basis for the hydrophobicity of the class D active site (see below).

Because **3**−**8** are phosphate diesters, it was of interest to test the reactivity of these compounds with a typical phosphodiesterase. The enzyme chosen was the phosphodiesterase I of the bovine intestine. Compounds **3** and **7** were tested and both were found to be substrates of this enzyme. Steady-state parameters were, for **3**, k_{cat} = 0.28 ± 0.04 s^{-1}, K_m = 0.13 ± 0.03 mM, k_{cat}/K_m = 2.15 × 10^3 s^{-1} M^{-1} and, for **7**, k_{cat} = 3.1 ± 0.7 s^{-1}, K_m = 0.11 ± 0.04 mM, k_{cat}/K_m = 2.95 × 10^4 s^{-1} M^{-1}. The phosphate diesters **3** and **7** are thus moderately good substrates of phosphodiesterase I, and the reactivity of **3** is enhanced somewhat by the hydrophobic substituents present in **7**.

Molecular Modeling. To understand how diaroyl phosphates might interact with the OXA-1 β-lactamase active site, models of the acylation tetrahedral intermediate **10** were constructed. Intuitively, it seemed likely and supported by previous modeling of aroyl phosphates at the class C β-lactamase active site (*16*) and by many crystal structures of β-lactamase−substrate complexes that the polar elements of the leaving group would interact with the polar residues of the general leaving-group binding area of the active site. This area in the OXA-1 active site is marked by the side chains of Lys 212, Thr 213, and Ser 115. The phenyl group of the benzoyl moiety attacked by the enzyme (see **10**) would then lie in the general β-lactam side-chain binding area of the β-lactamase. Exploration by a combination of molecular dynamics and energy minimization of structures, where the leaving group (−OPO_2^-−OCOPh) was initially placed in the general orientation described above, led to the structure shown in Figure 4 as the most favorable conformation of the tetrahedral intermediate. It should be noted, however, that the energetic comparisons between various conformations do not include explicit contributions from the classical hydrophobic effect.



10

The model predicts, first, that the tetrahedral oxyanion oxygen is firmly hydrogen-bonded into the oxyanion hole (backbone NH groups of Ser 67 and Ser 215), as would be expected. The acyl side chain (phenyl group) lies in the very

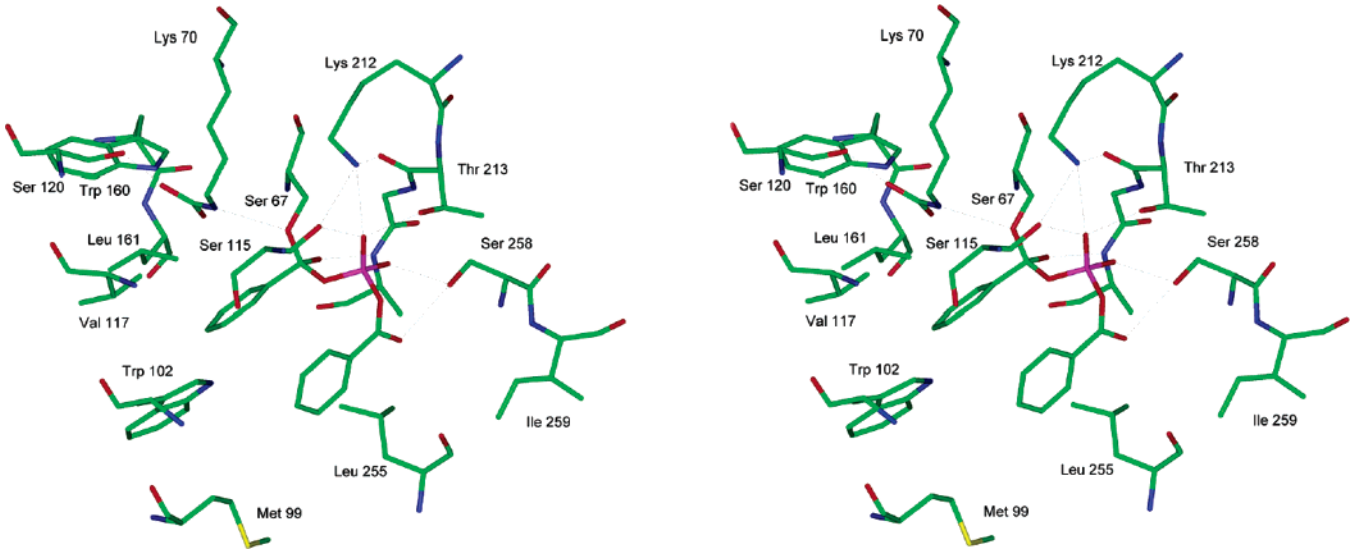


FIGURE 4: Stereoview of an energy-minimized tetrahedral intermediate structure, **10**, formed on the reaction of the OXA-1 β-lactamase with the diaroyl phosphate **3**. Only heavy atoms are shown.

hydrophobic side-chain site of this β-lactamase, with likely hydrophobic interactions with the side chains of Leu 161 (closest heavy atom approach of 4.0 Å), Val 117 (3.5 Å), and also with the leaving-group phenyl moiety of the ligand itself (4.0 Å); the latter may compensate for the hydrophobic interaction between the two phenyl rings of **3** in aqueous solution.

The leaving group ($-OPO_2^- - OCOPh$) is directed out of the active site but bending back over the side-chain site. The two anionic phosphoryl oxygens appear to be strongly hydrogen-bonded to protein residues, one to the side chains of Ser 115, Lys 212, and Thr 213 and the other to Ser 258. The latter residue may functionally replace Arg 250 of OXA-10 and OXA-13 (for which there is no analogue in OXA-1) that probably interacts directly with β-lactam substrate carboxylate groups (*29*); conversely, Ser 258 is not conserved among class D enzymes (*8*). These residues presumably represent analogues of Arg 349/Asn 346/Asn 343 of class C and Arg 244 of class A β-lactamases, which are found to interact, directly or indirectly, with the carboxylates of β-lactam and other substrates (*30*−*32*). Ser 115 is the analogue of Ser 130 in class A β-lactamases, where it is believed to be involved in leaving-group protonation (*33*). The carbonyl oxygen of the leaving group of **3** is also within hydrogen-bonding distance of the Ser 258 side-chain hydroxyl group. Finally, the phenyl moiety of the leaving group also seems comfortable in a hydrophobic cavity comprising the acyl donor phenyl group, as mentioned above, and the side chains of Trp 102 (closest heavy atom approach 4.0 Å), Met 99 (4.5 Å), Leu 255 (5.0 Å), and Ile 259 (6.0 Å).

The position of Lys 70 in the energy-minimized structure of Figure 4 is of some significance with respect to the issue of catalytic mechanism. We have assumed, following the best crystallographic and NMR evidence for the structure of class D β-lactamases under pH conditions of maximal enzyme activity (*6*, *8*), that the ϵ-amine of Lys 70 is carboxylated and, as has been proposed (*6*, *8*), that the carboxylate of this carbamate is a general acid/base catalyst for substrate turnover. In the tetrahedral intermediate structure of Figure 4, the carboxyl of the Lys 70 carbamate is therefore protonated, as it would be after accepting a proton from Ser 67 Oγ during nucleophilic attack of the latter on the acyl group of **3**. Under the influence of the force field employed, under the energy minimization protocols, the carboxyl group has moved away from a position where it would be hydrogen-bonded to Ser 67 Oγ, but the carbamate NH of Lys 70, rather than the carboxyl group, is hydrogen-bonded to Ser 67 Oγ in the energy-minimized structure. It is less likely that the nitrogen of a carbamate would act as a general base rather than the carboxylate; although not completely unambiguous, the best evidence seems to suggest that the most basic center of a carbamate is the carboxylate oxygen (*34*−*39*). An N-protonated carbamate would also have a great tendency to decarboxylate. In terms of the proposed mechanism (*6*, *8*), therefore, the structure of Figure 4, with respect to the position of Lys 70, presumably reflects the point that the structure represents an energy-minimized tetrahedral intermediate rather than a transition state. Whether a tetrahedral intermediate of the actual chemical reaction would be long-lived enough to relax to the structure of Figure 4 is not known.

Departure of the leaving group from the tetrahedral intermediate **10** would probably not need direct general acid catalysis; the pK_a of the benzoyl phosphate monoanion is 4.8 (*40*). Its departure would, however, be promoted by the interaction with the hydrogen-bond donors described above. It is these interactions, perhaps, that lead to the absence of strong electronic substituent effects on k_i (Table 2), as discussed above. Models also indicated that the elaboration of the phenyl groups of **3** with further hydrophobic substituents, as in the biphenyl derivative **7**, would lead to a closer interaction of the aroyl groups, both in the acyl donor moiety of the inhibitor and in the leaving group, with the hydrophobic residues of the β-lactam side-chain site of the OXA-1 β-lactamase mentioned above (see Figure S1 in Supporting Information). Hydrophobic interactions with the active site must strongly promote acylation.

CONCLUSIONS

We have shown previously that dibenzoyl phosphate, **3**, is an inhibitory substrate of typical class A and C β-lactamases (*13*). This molecule reacts rapidly with an enzyme

but forms an intermediate that only slowly regenerates free enzyme. In this paper, we provide evidence that **3** and several of its aroyl analogues are probably even more effective against typical class D enzymes (OXA-1 and OXA-10); second-order rate constants for the reaction of **3** with the TEM-2 β-lactamase, the *Enterobacter cloacae* P99 β-lactamase, and the OXA-1 enzyme are 3950, 1200, and 2.7×10^4 s^{-1} M^{-1}, respectively. As with the earlier-studied enzymes, reactions of OXA-1 with **3**−**8** apparently involve acylation of the active site (serine) with the departure of an aroyl phosphate leaving group. The interaction of the latter with polar active-site residues is likely to be responsible for the general reactivity of diaroyl phosphates with serine β-lactamases (*16*). The rate of acylation of the OXA-1 β-lactamase by **3**−**8** is not greatly affected by the electronic effects of substituents, probably because of compensation phenomena, but is greatly enhanced by hydrophobic substituents such as are present in **7** and **8**. The acylation reactivity correlates with the hydrophobic nature of the β-lactam side-chain binding site in class D β-lactamases. Deacylation of the enzyme is slow and directly influenced by electronic substituent effects. The combination of facile acylation and slow deacylation leads to nanomolar steady-state K_i values, subnanomolar for **7** and **8**. The diaroyl phosphates, where elaboration of substituents should be readily achieved, represent an intriguing new platform for the design of β-lactamase inhibitors. Compounds **7** and **8** are already considerably more effective against the OXA-1 β-lactamase than is clavulanic acid (*10*). The threat of phosphodiesterases would, however, have to be overcome.

SUPPORTING INFORMATION AVAILABLE

Stereoview of a tetrahedral intermediate structure formed on the reaction of the OXA-1 β-lactamase with **7** (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Ambler, R. P. (1980) The structure of β-lactamases, *Philos. Trans. R. Soc. London, Ser. B 289*, 155−165.
2. Naas, T., and Nordmann, P. (1999) OXA-type β-lactamases, *Curr. Pharm. Des. 5*, 865−879.
3. Brown, S., and Amyes, S. G. B. (2005) The sequences of seven class D β-lactamases isolated from carbapenem-resistant *Acinetobacter baumannii* from four continents, *Clin. Microbiol. Infect. 11*, 326−329.
4. Poirel, L., Héritier, C., Tolun, V., and Nordmann, P. (2004) Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*, *Antimicrob. Agents Chemother. 48*, 15−22.
5. Girlich, D., Naas, T., and Nordmann, P. (2004) Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother. 48*, 2043−2048.
6. Maveyraud, L., Golemi, D., Kotra, L. P., Tranier, S., Vakulenko, S., Mobashery, S., and Samama, J.-P. (2000) Insights into class D β-lactamases are revealed by the crystal structure of the OXA10 enzyme from *Pseudomonas aeruginosa*, *Structure 8*, 1289−1298.
7. Paetzel, M., Danel, F., De Castro, L., Mosimann, S. C., Page, M. G. P., and Strynadka, N. C. J. (2000) Crystal structure of the class D β-lactamase OXA-10, *Nat. Struct. Biol. 7*, 918−925.
8. Sun, T., Nukaga, M., Mayama, K., Braswell, E. H., and Knox, J. R. (2003) Comparison of β-lactamases of classes A and D: 1.5-Å crystallographic structure of the class D OXA-1 oxacillinase, *Protein Sci. 12*, 82−91.
9. Page, M. G. P. (2000) β-Lactamase inhibitors, *Drug Resist. Updates 3*, 109−125.
10. Adediran, S. A., Nukaga, M., Baurin, S., Frère, J.-M., and Pratt, R. F. (2005) Inhibition of class D β-lactamases by acyl phosphates and phosphonates, *Antimicrob. Agents Chemother. 49*, 4410−4412.
11. Pratt, R. F. (2002) Functional evolution of the serine β-lactamase active site, *J. Chem. Soc., Perkin Trans. 2*, 851−861.
12. Chantrenne, H. (1948) Mixed anhydrides of benzoic and phosphoric acids, *C. R. Trav. Lab. Carlsberg, Ser. Chim. 26*, 297−314.
13. Li, N., and Pratt, R. F. (1998) Inhibition of serine β-lactamases by acyl phosph(on)ates: A new source of inert acyl [and phosphyl] enzymes, *J. Am. Chem. Soc. 120*, 4264−4268.
14. Bebrone, C., Moall, C., Mahy, F., Rival, S., Docqier, J. D., Rossolini, G. M., Fastrez, J., Pratt, R. F., Frère, J.-M., and Galleni, M. (2001) Centa as a chromogenic substrate for studying β-lactamases, *Antimicrob. Agents Chemother. 45*, 1868−1871.
15. Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV proteinase, *Anal. Biochem. 237*, 260−273.
16. Kaur, K., and Pratt, R. F. (2001) Mechanism of reaction of acyl phosph(on)ates with the β-lactamase of *Enterobacter cloacae* P99, *Biochemistry 40*, 4610−4621.
17. Jencks, W. P., and Carriuolo, J. (1959) Imidazole catalysis. 2. Acyl transfer and the reactions of acetyl imidazole with water and oxygen anions, *J. Biol. Chem. 234*, 1272−1279.
18. Chantrenne, H. (1947) Hippuric acid formation from glycine and dibenzoyl phosphate, *Nature 160*, 603−604.
19. Golemi, D., Maveyraud, L., Vakulenko, S., Samama, J.-P., and Mobashery, S. (2001) Critical involvement of a carbamylated lysine in catalytic function of class D β-lactamases, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14280−14285.
20. Cabaret, D., Adediran, S. A., Garcia Gonzalez, M. J., Pratt, R. F., and Wakselman, M. (1999) Synthesis and reactivity with β-lactamases of "penicillin-like" cyclic depsipeptides, *J. Org. Chem. 64*, 713−720.
21. Xu, Y., Soto, G., Hirsch, K. R., and Pratt, R. F. (1996) Kinetics and mechanism of the hydrolysis of depsipeptides catalyzed by the β-lactamase of *Enterobacter cloacae* P99, *Biochemistry 35*, 3595−3603.
22. Govardhan, C. P., and Pratt, R. F. (1987) Kinetics and mechanism of the serine β-lactamase catalyzed hydrolysis of depsipeptides, *Biochemistry 26*, 3385−3395.
23. Ledent, P., Raquet, X., Joris, B., van Beeumen, J., and Frère, J.-M. (1993) A comparative study of class-D β-lactamases, *Biochem. J. 292*, 555−562.
24. Danel, F., Frère, J.-M., and Livermore, D. M. (2001) Evidence of dimerisation among class D β-lactamases: Kinetics of OXA-14 β-lactamase, *Biochim. Biophys. Acta 1546*, 132−142.
25. Taft, R. W., Jr. (1952) Polar and steric substituent constants for aliphatic and *o*-benzoate groups from rates of esterification and hydrolysis of esters, *J. Am. Chem. Soc. 74*, 3120−3128.
26. Neu, H. C. (1983) β-Lactamase inhibitory activity of iodopenicillanate and bromopenicillanate, *Antimicrob. Agents Chemother. 23*, 63−66.
27. Monaghan, C., Holland, S., and Dale, J. W. (1982) The interaction of anthraquinone dyes with the plasmid-mediated OXA-2 β-lactamase, *Biochem. J. 205*, 413−417.
28. Tondi, D., Powers, R. A., Caselli, E., Negri, M.-C., Blázquez, J., Costi, M. P., and Shoichet, B. K. (2001) Structure-based design and in-parallel synthesis of inhibitors of AmpC β-lactamase, *Chem. Biol. 8*, 593−610.
29. Pernot, L., Frénois, F., Rybkine, T., L'Hermite, G., Petrella, S., Delettre, J., Jarlier, V., Collatz, E., and Sougakoff, W. (2001) Crystal structures of the class D β-lactamase OXA-13 in the native form and in complex with meropenem, *J. Mol. Biol. 310*, 859−874.
30. Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C. A., Sutoh, K., and James, M. N. G. (1992) Molecular structure of the acyl−enzyme intermediate in β-lactam hydrolysis at 1.7 Å resolution, *Nature 359*, 700−705.
31. Patera, A., Blaszczak, L. C., and Shoichet, B. K. (2000) Crystal structures of substrate and inhibitor complexes with AmpC β-lactamase: Possible implications for substrate-assisted catalysis, *J. Am. Chem. Soc. 122*, 10504−10512.
32. Ahn, Y.-M., and Pratt, R. F. (2004) Kinetic and structural consequences of the leaving group in substrates of a class C β-lactamase, *Bioorg. Med. Chem. 12*, 1537−1542.
33. Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M., and Ghuysen, J.-M. (1991) Mechanism of acyl transfer by

the class A serine β-lactamase of *Streptomyces* albus G, *Biochem. J. 279*, 213−221.
34. Caplow, M. (1968) Kinetics of carbamate formation and breakdown, *J. Am. Chem. Soc. 90*, 6795−6803.
35. Johnson, S. L., and Morrison, D. L (1972) Kinetics and mechanism of decarboxylation of *N*-arylcarbamates: Evidence for kinetically important zwitterionic carbamic acid species of short lifetime, *J. Am. Chem. Soc. 94*, 1323−1334.
36. Danckwerts, P. V. (1979) The reaction of carbon dioxide with ethanolamines, *Chem. Eng. Sci. 34*, 443−446.
37. Wen, N., and Brooker, M. H. (1995) Ammonium carbonate, bicarbonate, and carbamate equilibria: A Raman study, *J. Phys. Chem. 99*, 359−368.
38. Aresta, M., Ballivet-Tkatchenko, D., Dell'Amico, D. B., Bonnet, M. C., Boschi, D., Calderazzo, F., Faure, R., Labella, L., and Marchetti, F. (2000) *Chem. Commun.* 1099−1100.
39. da Silva, E. F., and Svendsen, H. F. (2004) *Ab initio* study of the reaction of carbamate formation from CO_2 and alkanolamines, *Ind. Eng. Chem. Res. 43*, 3413−3418.
40. Satchell, D. P. N., Spencer, N., and White, G. F. (1972) Kinetic studies with muscle acylphosphatase, *Biochim. Biophys. Acta 268*, 233−248.

BI051719S