Mechanism of Inhibition of the Class C β-Lactamase of *Enterobacter cloacae* P99 by Phosphonate Monoesters†

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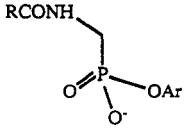
ABSTRACT: The class C serine β-lactamase of *Enterobacter cloacae* P99 was inhibited by a series of aryl methylphosphonate monoester monoanions. The effectiveness of these inhibitors was promoted by an acylamido substituent on the methyl group and a good leaving group at phosphorus. The former preference suggests that noncovalent interaction of these inhibitors with the enzyme resembles that of substrates, while the latter suggests that nucleophilic displacement at phosphorus occurs as part of the inhibition mechanism. The truth of the latter proposition was confirmed by observation of release of 1 equiv of phenol concomitant with inhibition and of the presence of an equivalent amount of ^{14}C-label on the enzyme after inhibition by a ^{14}C-labeled phosphonate. The hydrolytically inert nature of the enzyme–inhibitor adduct, and its ^{31}P chemical shift, suggested that O-phosphonylation of the enzyme had occurred. Although, by analogy with substrates, one might expect that the hydroxyl of the active site serine residue would be covalently modified by these inhibitors, successive alkali and acid treatment of the enzyme–inhibitor adduct generated no pyruvate. Instead, 1 equiv of lysinoalanine was found. This product was rationalized to arise through intramolecular capture by an adjacent lysine amine group of the dehydroalanine residue produced by alkali treatment of an O-phosphonylated serine residue. One equivalent of lysinoalanine was also produced by alkali treatment of the enzyme that had been inhibited by 6β-bromopenicillanic acid, a mechanism-based inhibitor known to acylate the hydroxyl group of the active site serine residue. It is therefore likely that the aryl phosphonates phosphonylate this residue. These compounds should be useful as β-lactamase active site titrants and as sources of fresh insight into the chemical properties of the active site. The significant mechanistic features of the inhibition, in particular its strong leaving group dependence and the distinctive ability of the β-lactamase active site to stabilize a dianionic transition state containing a pentacoordinated phosphorus, are discussed with respect to the active site structure. The comparison with phosph(or/on)yl inhibitors of serine proteinases is made, and the mechanism-based features of inhibition of serine hydrolases by phosph(on)ates are noted.

β-Lactamases are still the most important cause of bacterial resistance to β-lactam antibiotics (Donowitz & Mandell, 1988; Jacoby & Archer, 1991). Indeed, new variants of these enzymes, with specificities seeming to adapt to the latest antibiotics, continue to emerge (Bush, 1989; Gutmann et al., 1990; Jacoby & Medeiros, 1991). β-Lactamase inhibitors represent one obvious method of combating the threat posed by these enzymes and one which has now been demonstrated to be successful in vitro, in vivo, and commercially (Pratt, 1989b, 1992). The most effective β-lactamase inhibitors described, and employed clinically to date, are of the mechanism-based variety and are themselves β-lactams.

New classes of β-lactamase inhibitors are of interest not only because of their promise of direct practical application but also as a source of chemical information about the β-lactamase active site. Such information is, of course, invaluable to further inhibitor design. We have recently demonstrated that acyclic phosphonate monoesters of general structure **1** are inhibitors



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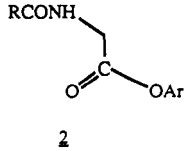

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of typical serine β-lactamases of classes A and C (Pratt, 1989a; Rahil & Pratt, 1991a). These phosphonates were designed as transition-state analog inhibitors because of their probable resemblance to the transition states involved in turnover of the depsipeptide substrates **2** (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987). In this paper, we describe the essential features of the inhibition of the class C β-lactamase of *Enterobacter cloacae* by **1**; several structural variants of **1** are reported and compared in effectiveness as inhibitors, and the mechanism of the inhibition is explored. A comparison of this mechanism with that of phosph(on)ates as inhibitors of other serine hydrolases is made.

Experimental Procedures

Materials. The β-lactamase of *E. cloacae* P99 and *Bacillus cereus* β-lactamase I were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. α-Chymotrypsin (type VII), acetylcholinesterase (type III), L-lactate dehydrogenase (type II), and other biochemical reagents, including lysinoalanine and phenyl[1-^{14}C]acetic acid, were obtained from Sigma Chemical Co. Chemical reagents for synthesis were generally purchased from Aldrich Chemical Co.; the hydroxyphthalic acids, however, were obtained from Kodak Laboratory Chemicals.

The aryl phosphonates **4d–g**, **5**, and **7** were available from our previous studies (Rahil & Pratt, 1991a,b). The carboxyphenyl esters **3** and **4a–c** were prepared from [[*N*-(phenylacetyl)amino]methyl]phosphonic acid by the general method previously described (Pratt, 1989a; Rahil & Pratt, 1991b). A specific example is given below.

Benzyl esters of the carboxyphenols were prepared by the method of Cavallito and Buck (1943) (*m*- and *p*-carboxy-

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phenol) or, in the case of the dicarboxy compounds, through reaction of their bis(cesium) salts with benzyl chloride in dimethylformamide (Wang et al., 1977).

Triethylammonium p-Carboxyphenyl [[N-(Phenylacetyl)amino]methyl]phosphonate (**4a**). Benzyl *p*-hydroxybenzoate (0.2 g, 0.8 mmol) was condensed with [[*N*-(phenylacetyl)amino]methyl]phosphonic acid (0.2 g, 0.8 mmol) in pyridine (3.0 mL, distilled from CaH_2) in the presence of trichloroacetonitrile (0.88 mL, 8 mmol) (Wasielewski et al., 1976). The reaction mixture was stirred for 3 h in an oil bath at 100 °C. Volatiles were then removed by rotary evaporation, and the oily residue was partitioned between saturated aqueous sodium bicarbonate and ethyl acetate (3 mL each). The aqueous layer was further extracted with ethyl acetate (3 × 3 mL), and the combined organic extracts were evaporated to dryness. Trituration of the residue with diethyl ether yielded the required product, sodium *p*-(benzyloxycarbonyl)phenyl [[*N*-(phenylacetyl)amino]methyl]phosphonate (0.21 g, 57%), as a pale brown solid [^{1}H NMR (^2H_2O) δ 3.55 (s, 2, PhCH_2CO), 3.65 (d, J = 12 Hz, 2, CH_2P), 5.40 (s, 2, CO_2CH_2Ph), 7.1–7.9 (m, 14, Ar H)]. The benzyl protecting group was removed by hydrogenation for 3 h in methanol (50 mL) in the presence of a 10% Pd/C catalyst (50 mg) under 30 psi of hydrogen pressure. The product was purified by anion-exchange chromatography [eluted with a linear 0–2 M gradient of triethylammonium bicarbonate (pH 8.0) from a Sephadex QAE A-25 column]. The final product, a colorless hygroscopic solid, was unsuitable for combustion analysis but yielded a monotriethylammonium parent ion in a FAB mass spectrum (*m/e* 451, M + H$^+$) and ^{1}H NMR spectrum [(^2H_2O) δ 1.3 (t, J = 7.5 Hz, 18, CH_3), 3.2 (q, J = 7.5 Hz, 12, CH_3CH_2), 3.62 (s, 2, PhCH_2), 3.65 (d, J = 12 Hz, 2, CH_2P), 7.1–7.85 (m, 9, Ar H)].

The two dicarboxy derivatives that follow were similarly prepared:

Triethylammonium 3,4-dicarboxyphenyl [[N-(phenylacetyl)amino]methyl]phosphonate (**4b**): ^{1}H NMR (^2H_2O) δ 1.3 (t, J = 7.5 Hz, 18, CH_3), 3.2 (q, J = 7.5 Hz, 12, CH_3CH_2), 3.60 (s, 2, PhCH_2), 3.70 (d, J = 12 Hz, 2, CH_2P), 6.95–7.75 (m, 8, Ar H).

Triethylammonium 3,5-dicarboxyphenyl [[N-(phenylacetyl)amino]methyl]phosphonate (**4c**): ^{1}H NMR (^2H_2O) δ 1.3 (t, J = 7.5 Hz, 18, CH_3), 3.2 (q, J = 7.5 Hz, 12, CH_3CH_2), 3.60 (s, 2, PhCH_2), 3.70 (d, J = 12 Hz, 2, CH_2P), 7.2–8.1 (m, 8, Ar H).

Methyl p-Nitrophenyl Methylphosphonate (**8**). A solution of *p*-nitrophenol (2.8 g, 20 mmol) and triethylamine (2.2 g, 22 mmol, distilled from CaH_2) in 100 mL of dry diethyl ether was added dropwise to a stirred solution of methylphosphonic dichloride (2.7 g, 20 mmol) in 100 mL of dry ether at 0 °C. Stirring was continued for 1 h after completion of the addition, and then the white precipitate of triethylammonium chloride was removed by filtration. To the resulting solution was added a solution of absolute methanol (0.64 g, 20 mmol) and triethylamine (2.2 g, 20 mmol) in 50 mL of dry diethyl ether with stirring at 0 °C. After 0.5 h, triethylammonium chloride was again removed by filtration and the remaining solution evaporated to dryness under vacuum. The crude product thus obtained was purified by low-pressure distillation in a short-path apparatus (bp 120 °C, 0.02 Torr). The product, a pale yellow liquid, yielded the following ^{1}H NMR spectrum: (C^2HCl_3) δ 1.75 (d, J = 20 Hz, 3, CH_3P), 3.85 (d, J = 10 Hz, 3, CH_3OP), 7.45 (d, J = 10 Hz, 2, Ar H), 8.27 (d, J = 10 Hz, 2 Ar H).

Sodium m-Carboxyphenyl (Aminomethyl)phosphonate (**6**). Sodium *m*-(benzyloxycarbonyl)phenyl [[*N*-(benzyloxycarbonyl)amino]methyl]phosphonate [^{1}H NMR (^2H_2O) δ 3.55 (d, J = 12 Hz, 2, CH_2P), 4.95 (s, 2, PhCH_2OCONH), 5.35 (s, 2, PhCH_2OCOAr), 7.2–7.8 (m, 14, Ar H)] was prepared from [[*N*-(benzyloxycarbonyl)amino]methyl]phosphonic acid (Rahil & Pratt, 1991b) and *m*-(benzyloxycarbonyl)phenol in the manner for **4a** described above. Catalytic hydrogenation (30 mg, 10% Pd/C, 35 psi of H_2) of a solution of this compound (0.1 g) in methanol (30 mL) yielded, after removal of the catalyst and evaporation of the methanol, a colorless solid, the required product [^{1}H NMR (^2H_2O) δ 3.22 (d, J = 12 Hz, 2, CH_2P), 7.35–7.7 (m, 4, Ar H)], which was used without further purification as described below.

Triethylammonium m-Carboxyphenyl [[N-(Phenyl[1-^{14}C]acetyl)amino]methyl]phosphonate ([^{14}C]-**3**). Phenyl[1-^{14}C]acetic acid (10 mg, 3.4 mCi/mmol) and freshly distilled thionyl chloride (0.5 mL) were stirred together at room temperature for 24 h. Excess thionyl chloride was then removed under vacuum and the residual phenyl[1-^{14}C]acetyl chloride dissolved in methylene chloride (0.5 mL). A solution of *m*-carboxyphenyl (aminomethyl)phosphonate (30 mg, 0.12 mmol), prepared as described above, and triethylamine (0.2 mL, 1.4 mmol) in 0.5 mL of absolute methanol was added dropwise to the solution of the acid chloride, stirred at 0 °C. The mixture was stirred to room temperature over 1 h, and the solvents were removed by evaporation under vacuum. The solid residue was extracted with methylene chloride to remove any methyl phenylacetate and purified by QAE-Sephadex anion-exchange chromatography as described above, employing a 0.2–0.7 M linear gradient of triethylammonium bicarbonate. The identity and purity of the product were established from the known ^{1}H NMR spectrum (Pratt, 1989a). The specific radioactivity of the compound was determined to be 3.44 mCi/mmol. Scintillation counting was carried out in a LKB 1214 Rackbeta scintillation counter. The sample was diluted with cold **3** for the labeling studies described below.

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were obtained by means of either a Cary 219 or a Perkin-Elmer Lambda 4B spectrophotometer. β-Lactamase activity was routinely determined against benzylpenicillin by the spectrophotometric method (Waley, 1974). Concentrations of the *E. cloacae* P99 β-lactamase were determined spectrophotometrically by employing a published extinction coefficient, 7.1×10^4 M^{-1} cm^{-1} (Joris et al., 1985). Concentrated stock solutions of the phosphonate monoesters were prepared in 20 mM MOPS buffer at pH 7.5. The salts of these phosphonates were, as previously described (Rahil & Pratt, 1991b), hygroscopic, and thus solutions of precisely known concentration could not be obtained by weighing. Thus the concentrations of their solutions were determined from the absorption at an appropriate wavelength of the phenoxides produced from complete hydrolysis in alkaline solution; the extinction coefficients of the relevant phenoxides were determined from the absorption of solutions of the purified phenols. Stock solutions of the phosphonate diester **8** were prepared in acetonitrile.

All kinetic measurements were carried out in 20 mM MOPS buffer at pH 7.5 and at 25 °C. Rates of enzyme inactivation in the presence of the phosphonates were determined either from enzyme activity measurements or by direct spectrophotometry. The former method was employed with compounds **3**, **4a–d**, **6**, and **8**. Thus, the enzyme (ca. 0.1 μM) and various concentrations of the inhibitor (1.0 μM–5 mM) were incubated together. Aliquots were withdrawn at suitable times, and the

enzyme was assayed spectrophotometrically against benzylpenicillin. The activity of control samples, without inhibitor, was also routinely monitored. Pseudo-first-order rate constants of inactivation were determined from semilogarithmic plots. Second-order rate constants were obtained from the slopes of linear plots of the psuedo-first-order rate constants against inhibitor concentration.

With the nitrophenyl phosphonates **4e–g**, **5**, and **7**, direct observation of phenol/phenoxide release was carried out at 400, 330, and 410 nm for the *p*-, *m*-, and *o*-nitrophenyl compounds, respectively. A Durrum D110 stopped-flow spectrophotometer was employed for the faster reactions. The data were fitted by a nonlinear least squares procedure (Johnson et al., 1976) to eq 1, where A is the absorbance at any time,

$$A = A_0 + v_0 t + \pi(1 - e^{-\lambda t}) + v_s t \quad (1)$$

A_0 is the zero-time absorbance, v_0 is the initial rate of nonenzymic hydrolysis (determined from measurements in the absence of enzyme), v_s is the initial rate of the (slow) steady-state enzyme-catalyzed reaction (Pratt, 1989a; Rahil & Pratt, 1991a), π is the amplitude of the phenol burst accompanying inactivation, and λ is the burst pseudo-first-order rate constant ($\lambda = k_i[I]$, where k_i is the second-order rate constant for reaction between the enzyme and inhibitor and [I] is the inhibitor concentration).

Compounds **3** and **5** were also tested as inhibitors of α-chymotrypsin and acetylcholinesterase. α-Chymotrypsin (8.9 μM) was incubated separately with **3** (1.26 mM) and **5** (1.06 mM) in 20 mM MOPS buffer, pH 7.5, at 25 °C. The enzyme was assayed spectrophotometrically (256 nm) against *N*-benzoyl-L-tyrosine ethyl ester as a function of time. Similarly, acetylcholinesterase (0.38 μM) was also tested with **3** and **5**. The residual activity in this case was determined by assay against thiocholine in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (Whittaker, 1984). Controls without inhibitor were also followed in each case.

Characterization of the β-Lactamase–Inhibitor Complex. (*1*) *Stoichiometry.* A solution of the P99 β-lactamase (45 μM) in MOPS buffer, pH 7.5 (0.5 mL), was titrated with aliquots of a standardized solution of [^{14}C]-**3** (4.68 mM). After each addition (10 μL) of inhibitor solution, the mixture was incubated at 25 °C for 5 min and the residual β-lactamase activity assayed against benzylpenicillin. After complete inhibition of the enzyme was achieved, the enzyme was separated from a small excess of inhibitor by means of a Bio-Gel P6DG exclusion column. The fractions were assayed for radioactivity, absorbance at 280 nm, and β-lactamase activity.

(*2*) *Lability.* The lability of the inactive complex formed between the P99 β-lactamase and **3** was examined by measurement of the rate of loss of radioactivity from the labeled complex, prepared as described above, as a function of pH. Buffers containing 6 M guanidine hydrochloride were prepared at pH 5.0 (0.25 M acetate), 6.0, 7.0, and 8.0 (0.05 M phosphate), 9.0 (0.05 M Bicine), and 13.0 (0.1 M KOH). Aliquots (50 μL) of the labeled enzyme (94 μCi/mmol) were added to 1-mL samples of these buffers (final protein concentration 1.86 μM) and the mixtures incubated at 25 °C. At appropriate times, 120-μL aliquots were withdrawn, and the protein was separated by ultrafiltration with an Amicon Centricon-10 microconcentrator. Portions of the filtrate (50 μL) were scintillation counted. As a control, sufficient [^{14}C]-**3** was added to an enzyme sample to achieve 50% inhibition. The protein, diluted into the pH 7.0 buffer, was then immediately subjected to ultrafiltration as described above. All of the radioactivity was found in the protein concentrate, none above background in the filtrate. The loss of the label at pH 7.0 in the absence of guanidine hydrochloride was also measured in this way.

(*3*) ^{31}*P NMR Spectra.* The P99 β-lactamase (1.0 mL, 1.0 mM) in 20 mM MOPS, pH 7.5, was inactivated by **3** (7.6 mM). The inactivated enzyme was separated from excess **3** by Bio-Gel chromatography as described above. The protein fractions were concentrated by ultrafiltration, employing an Amicon Centricon-10 microconcentrator. The final solution, ca. 1 mL, included 10% v/v ^2H_2O and 1 mM EDTA. ^{31}P NMR spectra at 15 °C (162 MHz, 20 000 transients, 50° pulse, 1.5-s delay time) were obtained by means of a Varian XL-400 spectrometer. The low temperature was employed in order to decrease the dephosphonylation rate. After the spectrum of the inactivated enzyme was obtained, sufficient guanidine hydrochloride was added to give a 6 M solution and the sample reconcentrated to 1.0 mL. The ^{31}P NMR spectrum in this medium was also recorded. The reported chemical shifts are referenced to external 85% H_3PO_4.

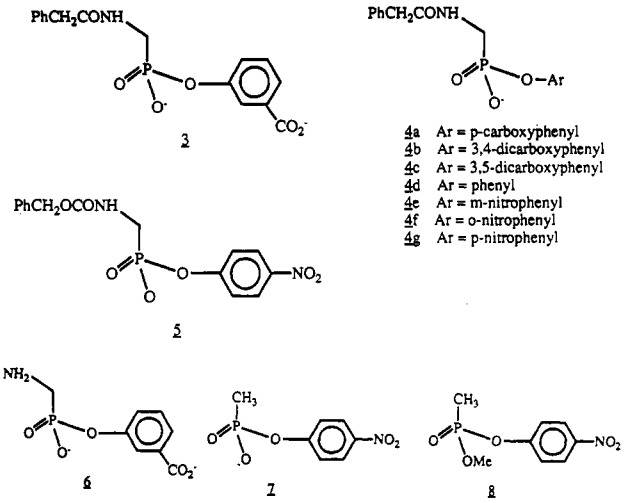
(*4*) *Pyruvate Assays.* The inhibited enzyme was treated with alkali and then acid, much as described by Weiner et al. (1966), in order to produce pyruvate from any modified serine residues. Thus, in a typical experiment, the enzyme (ca. 0.5 mM in 0.45 mL of 20 mM MOPS buffer, pH 7.5) was inactivated with 1 equiv of **3**. The solution was then made 0.1 M in hydroxide ion by addition of a calculated amount of concentrated (5 M) sodium hydroxide and allowed to stand at room temperature for 1 h. The calculated amount of 6 M HCl was then added to give a final acid concentration of 3 M, and the mixture was heated in a sealed ampule in an oil bath at 100 °C for 3 h. After the hydrolysis mixture had cooled, its pH was raised to 7.4 with concentrated sodium hydroxide solution and the neutralized solution freeze-dried. The solid residue was dissolved quantitatively in 1.0 mL of phosphate buffer (30 mM, pH 7.4) and analyzed for pyruvate with L-lactate dehydrogenase (1.62 μM) and NADH (0.1 mM). The pyruvate assay was checked with standard pyruvate solutions. Attempts to detect pyruvate were also made in samples of the inactivated β-lactamase that were treated with guanidine hydrochloride (6 M), urea (6 M), and heat (100 °C for 10 min) prior to alkali treatment.

In control experiments, the above procedure was carried out with native enzyme and with 2-acetamidoacrylic acid. The procedure was also applied to samples of the P99 β-lactamase that had been inactivated by **5**, **7**, and phenylmethanesulfonyl fluoride and by 6β-bromopenicillanic acid.

(*5*) *Lysinoalanine Analysis.* The P99 β-lactamase, 1.3 mg in 1.0 mL of 20 mM MOPS, pH 7.5, was completely inactivated by 1 equiv of **3** (or, in a separate experiment, 80 equiv of 6β-bromopenicillanic acid). Then, as above, the solution was made 0.1 M in sodium hydroxide and held at room temperature for 1.5 h. It was then dialyzed at room temperature against 0.01 M HCl for 24 h (two changes). Aliquots (10 μL) were then freeze-dried in acid-washed tubes and submitted for amino acid analysis to the Yale Protein and Nucleic Acid Chemistry Facility. Control samples of native, active enzyme, treated as above, were also prepared. Standard solutions of lysinoalanine were prepared for quantitation. Lysinoalanine was identified by its retention time (41.7 min) on the amino acid analysis column by comparison with that of an authentic sample; it emerged after phenylalanine (35.6 min) and the buffer change but before histidine (42.8 min).

Results

The phosphonate monoesters **3–7**, at concentrations of up to a few millimolar, inactivate the P99 β-lactamase in a pseudo-first-order fashion. This inactivation was accompanied by release of the phenol/phenoxide leaving group, as shown,



for example, in Figure 1 for the inhibition of the P99 β-lactamase by **5**. The amplitude of the burst of phenol/phenoxide, determined through fitting eq 1 to these data, corresponded to 0.64 molar equiv per mole of enzyme. The pseudo-first-order rate constant, λ (eq 1), varied in a linear fashion with the concentration of **5** (Figure 1, inset), yielding a second-order rate constant (k_i) for the inactivation reaction. The second-order rate constants for compounds **3**–**7**, determined by spectrophotometric means as described above, or from enzyme activity measurements, are given in Table I. Also shown there is the rate constant for inactivation of the P99 β-lactamase by the phosphonate diester **8**. α-Chymotrypsin was not affected by either **3** or **5** at ca. 1 mM concentrations. Acetylcholinesterase was not affected by **3**, but slow inactivation (k_i = 2.7 s^{-1} M^{-1}) in the presence of **5** was observed.

In order to obtain further information on the stoichiometry and chemistry of the inhibition, the P99 enzyme was titrated with [^{14}C]-**3** (data not shown). From the concentration of [^{14}C]-**3** necessary to achieve complete inactivation, obtained from linear extrapolation, a stoichiometry of 0.66 mol of [^{14}C]-**3**/mol of enzyme could be calculated. This number agrees well with that obtained spectrophotometrically as described above. It is less than unity, presumably because the enzyme employed was not completely active (Pratt, 1989a). The inhibited enzyme, isolated by Bio-Gel P6DG chromatography at room temperature, contained 0.52 molar equiv of [^{14}C]-**3**/mol of protein. The lower content of label after isolation was presumably due to some dephosphonylation during chromatography since it was accompanied by a corresponding increase in enzyme activity.

The enzyme-bound radiolabel was very stably bound to the protein. In 6 M guanidine hydrochloride, essentially no label was lost in 48 h at 25 °C at pH values between and including 5.0 and 9.0. At pH 7.0 in 0.05 M phosphate buffer and in the absence of guanidine, the label was lost more rapidly, but still very slowly compared to the inactivation reaction—the first-order rate constant of release under these conditions was 5.8×10^{-6} s^{-1}. This is similar to the rate constant for enzyme reactivation, 2.2×10^{-6} s^{-1}, previously measured (Pratt, 1989a) under somewhat different conditions (20 mM MOPS, pH 7.5). In 0.1 M NaOH, however, the label was lost from the enzyme in less than 1 min. In 0.1 M HCl, in the presence of 6 M guanidine hydrochloride, the label was lost over a period of 6 h.

In view of the stability of the inhibitor–enzyme complex, an attempt was made to obtain its ^{31}P NMR spectrum. The ^{31}P NMR spectrum of Figure 2A, taken at 15 °C, shows peaks at 14.09, 20.39, and 26.01 ppm downfield from phosphoric acid. The most upfield of these peaks increased in intensity with respect to the other two with time and more rapidly at

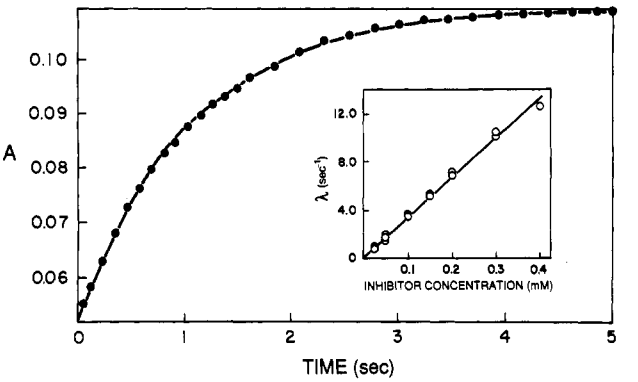


FIGURE 1: Absorption change at 400 nm on reaction of the *E. cloacae* P99 β-lactamase (4.2 μM) with the phosphonate inhibitor **5** (25 μM). In the inset is shown the variation of the pseudo-first-order rate constant for the reaction (λ, eq 1) with the concentration of **5**.

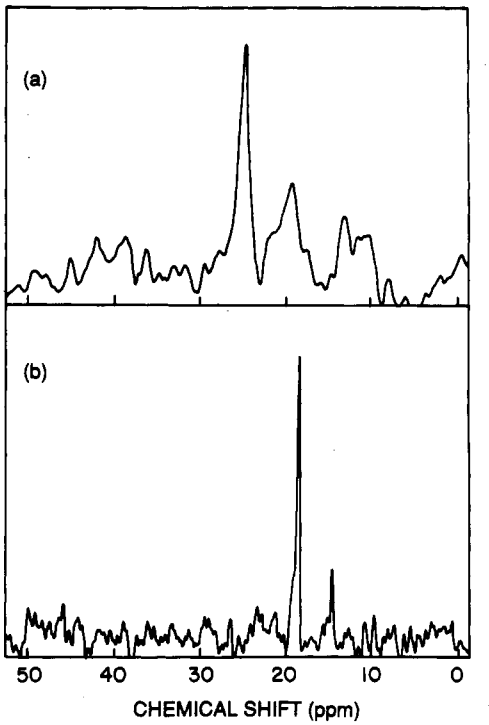


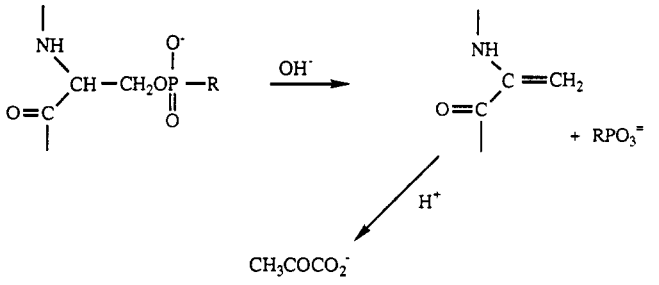
FIGURE 2: ^{31}P NMR spectra of the inert complex obtained on reaction of the *E. cloacae* P99 β-lactamase with the phosphonate inhibitor **3** in the absence (a) and presence (b) of 6 M guanidine hydrochloride. The spectrum in (a) includes 130-Hz line broadening.

Table I: Second-Order Rate Constants for Inactivation of the P99 β-Lactamase by Aryl Phosphonates

	inhibitor	k_i (s^{-1} M^{-1})[a]
3	PAG(P)[b]–O–C_6H_4-3-CO_2^-	817
4a	PAG(P)–O–C_6H_4-4-CO_2^-	352
4b	PAG(P)–O–C_6H_3-3,4-$(CO_2^-)_2$	493
4c	PAG(P)–O–C_6H_3-3,5-$(CO_2^-)_2$	5.3
4d	PAG(P)–O–C_6H_5	258
4e	PAG(P)–O–C_6H_4-3-NO_2	2.27×10^3
4f	PAG(P)–O–C_6H_4-2-NO_2	5.45×10^3
4g	PAG(P)–O–C_6H_4-4-NO_2	5.62×10^4
5	ZG(P)[c]–O–C_6H_4-4-NO_2	3.27×10^4
6	$NH_2CH_2PO_2^-$–O–C_6H_4-3-CO_2^-	0.46
7	$CH_3PO_2^-$–O–C_6H_4-4-NO_2	0.64
8	$CH_3PO(OMe)$–O–OC_6H_4-4-NO_2	0.69

[a] 20 mM MOPS buffer, pH 7.5. [b] [[*N*-(Phenylacetyl)amino]-methyl]phosphonyl. [c] [[*N*-(Benzyloxycarbonyl)amino]methyl]-phosphonyl.

Scheme I



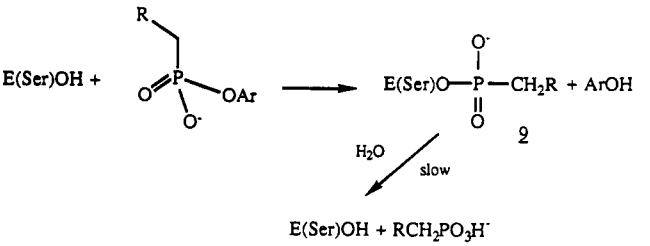
25 °C. Upon addition of guanidine hydrochloride to 6 M final concentration, the spectrum of Figure 2B was obtained. This showed only two peaks, at 14.55 and 18.62 ppm. Addition of authentic [[*N*-(phenylacetyl)amino]methyl]phosphonic acid to the sample showed that the upfield peak belonged to this species. Thus it seems likely that, in both of the spectra of Figure 2, the upfield peak corresponds to the phosphonate hydrolysis product of **3**, which could arise from the hydrolysis of a phosphonyl-enzyme since the *m*-carboxyphenol leaving group was lost in the inactivation reaction. The most intense resonance of Figure 2A is some 100 Hz wide, not unreasonable for a covalent phosphonyl derivative of a 39-kDa protein (Vogel, 1984). The reason for the breadth of the highest field peak, identified as the hydrolysis product, is not clear but could perhaps reflect dynamic broadening from noncovalent binding of this species to the enzyme. For comparison with the above chemical shifts, the position of the ^{31}P resonance of an alkyl phosphonate monoanion, D-lactyl [[*N*-(phenylacetyl)amino]-methyl]phosphonate (Pratt, 1989a), was determined to be 17.54 ppm in 20 mM MOPS, pH 7.5, and 17.95 ppm on addition of guanidine hydrochloride to 6 M; the chemical shift of the dianion of **3** was 15.49 ppm.

Since it seemed likely that any phosphonylation of the enzyme would take place at the hydroxyl group of Ser-64, the primary active site nucleophile (Pratt, 1989a; Rahil & Pratt, 1991a), an attempt was made to support this idea by means of a well-known elimination reaction of serine derivatives (Scheme I). O-Phosphorylated or sulfonylated serine peptides are well-known to undergo this type of reaction in base, leading to dehydroalanine (Photaki, 1963; Samuel & Silver, 1963). It has also been demonstrated in naturally occurring phosphoproteins where serine is phosphorylated (Anderson & Kelly, 1959) and in cases where a specific serine residue, such as that in a serine proteinase active site, has been modified (Weiner et al., 1966, Ako et al., 1974). Acid hydrolysis of the dehydroalanine should quantitatively yield pyruvate which can be estimated through L-lactate dehydrogenase (Weiner et al., 1966).

Rather surprisingly, no pyruvate was detected from the P99 β-lactamase that had been inhibited by **3** on base and then acid treatment as described under Experimental Procedures. Denaturation of the inhibited enzyme by guanidine hydrochloride, urea, or heat prior to base treatment did not change this result. Hydrolysis of the phosphonyl derivative via intramolecular amide participation (Rahil & Pratt, 1991b) did not convincingly explain this finding since no pyruvate was obtained from the β-lactamase inhibited by **7**, phenylmethanesulfonyl fluoride, or 6β-bromopenicillanic acid either. On the other hand, identical treatment of 2-acetamidoacrylic acid yielded pyruvate quantitatively, and chymotrypsin, inactivated with phenylmethanesulfonyl fluoride, produced 1 equiv of pyruvate.

Significantly, however, amino acid analysis of the P99 β-lactamase after inactivation by **3** and alkali treatment yielded

Scheme II



1 mol of lysinoalanine/mol of protein [1.05 and 1.03 mol on the basis of 44 alanine and 29 leucine residues/mol, respectively (Galleni et al., 1988)]. A control sample containing uninhibited enzyme yielded no lysinoalanine. Similarly, enzyme inactivated by 6β-bromopenicillanic acid gave 1 mol of lysinoalanine/mol of enzyme (0.97 and 0.94 mol with respect to alanine and leucine, respectively). The class A *B. cereus* 569H β-lactamase I, inhibited by 6β-bromopenicillanic acid and then alkali-treated, also yielded 1 molar equiv of lysinoalanine [1.14 and 1.04 mol with respect to alanine and leucine content (Madonna et al., 1987, and references therein), respectively].

Discussion

The data of Table I show that a number of phosphonate monoesters inactivate the class C *E. cloacae* P99 β-lactamase and that their effectiveness is a function of their structure. With respect to the latter point, it is clear, first, that although the carboxylate substituent in the prototypic inhibitor **3** is useful, it is far from essential—compare k_i values of **3** and the unsubstituted analog **4d**, for example. The position of the carboxylate, if present, has moderate influence—the *p*-carboxylate **4a** is a significantly poorer inhibitor than the meta isomer **3**. We were unable to prepare the *o*-carboxy compound since it appeared to undergo a facile intramolecular reaction; the analogous *o*-carboxydepsipeptide was not a better substrate than the meta isomer, however (Govardhan & Pratt, 1987). An additional carboxylate, which we thought might have been useful in view of the positive charges in the vicinity of the active site (Moews & Knox, 1990; Oefner et al., 1990; Knap & Pratt, 1991), did not improve **3** as an inhibitor, either when the second carboxylate was adjacent to the first, as in **4b**, or, and more noticeably, when the second carboxylate was meta to it, as in **4c**. The less than essential nature of the carboxylate has also been noted in the effectiveness of these phosphonate inhibitors against class A β-lactamases (Rahil & Pratt, 1991a) and, indeed, in β-lactamase substrates in general (Laws & Page, 1989; Varetto et al., 1991).

A second feature of the present results, also in common with those obtained with the class A β-lactamases, is the improvement of the inhibitor when a better leaving group is present—**4g** is more effective than **4e**, which is significantly better than **4d**. Thus the *p*-nitrophenyl derivative **4g** is a much better inactivator of the P99 enzyme than is the prototype **3**. The nature of the phosphonic acid is also important. Although **5** is comparable to **4g**, compounds lacking the amido side chain are much less effective—compare **6** with **3** or **7** with **4g**. This specificity also exists in substrates of course (Matagne et al., 1990), which suggests that the phosphonates may share part at least of the substrate binding site. Finally, the negatively charged phosphonate monoester **7** is a comparable inhibitor to the neutral diester **8**. These results will be interpreted further below in terms of a mechanism of inhibition.

Scheme II has been suggested as a likely framework within which to consider the inhibition of β-lactamases by phospho-

nates (Pratt, 1989a; Rahil & Pratt, 1991a). Inhibition is thereby supposed to occur through phosphonylation of the active site serine hydroxyl group [Ser-64 (Galleni et al., 1988)] to form the hydrolytically refractory covalent species **9**. The results obtained in the present work provide strong evidence for Scheme II, as discussed in detail below.

First, the nitrophenyl phosphonates **4e–g** and **5** reacted with the P99 β-lactamase with stoichiometric release of nitrophenol/phenoxide as shown, for example, in Figure 1. Concerted release of *m*-hydroxybenzoate with inhibition by **3** has previously been demonstrated (Pratt, 1989a). Second, as shown by the results of the experiments with radioactively labeled **3** and by the ^{31}P NMR spectrum (Figure 2), the inert complex contained both the phenylacetyl carbonyl carbon and a phosphorus atom, supporting structure **9**. This reaction pathway was proposed to follow that of the analogous depsipeptide substrate, whose turnover proceeds by way of an acyl-enzyme intermediate (Govardhan & Pratt, 1987). That the radioactivity is not immediately dissociated on treatment of the inhibited enzyme with 6 M guanidine hydrochloride is indicative of a covalent linkage, again as in **9**. Thus, a phosphonyl-enzyme seemed likely.

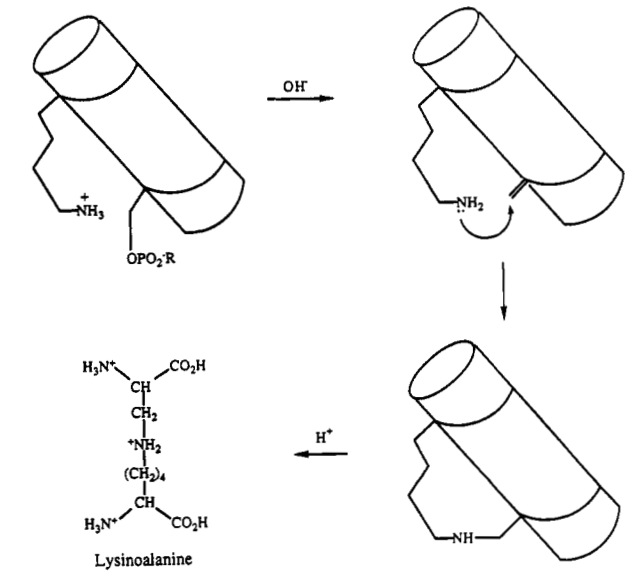
The chemical nature of the linkage between the inhibitor and the enzyme in **9** is indicated by its hydrolytic stability. This is strongly suggestive of that of a phosphonate monoester monoanion—extremely slow hydrolysis in the pH 5–9 region and more rapid hydrolysis in acid (Behrman et al., 1970a). These experiments were carried out in 6 M guanidine hydrochloride, and thus the observed rates are presumably little affected by the enzyme active site or adjacent protein structure. The absence of an acid-catalyzed reaction in the pH 5–7 region suggests that an *O*-phosphonyl rather than a *N*-phosphonyl derivative is present (Rahil & Haake, 1981).

The ^{31}P NMR spectrum (Figure 2) exhibited two peaks other than the phosphonate hydrolysis product. The smaller of these has a chemical shift appropriate to a phosphonate monoalkyl ester monoanion, as seen, for example, in the model lactate referred to under Results and in phosphonolipids (Henderson et al., 1971), while the more intense has an unusually high chemical shift for such a species. The position of the latter resonance may reflect the existence of the phosphonate group in a protonated form (Appleton et al., 1984; Glowacki et al., 1991) or, more likely, adjacent to a hydrogen bond donor and/or positive charge (Appleton et al., 1984).

In 6 M guanidine hydrochloride, the two peaks collapsed to a singlet with chemical shift at a position eminently suitable to a phosphonate monoalkyl ester monoanion. This observation suggests that, prior to denaturation, two conformers of the inert complex exist, each having a different environment adjacent to the phosphorus atom. The major conformer may include phosphonate occupancy of the oxyanion hole and the immediacy of a positively charged hydrogen bond donor such as a lysine ammonium group (see below). It is also possible that the two peaks arise from two different points of covalent attachment of the phosphonyl group to the enzyme; i.e., inhibition occurs through modification of one of two alternative functional groups, such as the serine hydroxyl group and an adjacently lysine amine, and on denaturation, phosphonyl transfer to one, most likely the hydroxyl (see below), occurs. There was, however, no sign of a two-phased process, either in the inactivation reaction or in release of the labeled inhibitor from the enzyme in acid or base.

Any structural interpretation of the NMR spectrum of Figure 2 can, of course, be only speculative since ^{31}P chemical shifts appear susceptible to modulation by many factors involving the geometry, bonding, and environment of the phosphorus atom (Gorenstein, 1984, 1989); ^{31}P chemical shifts of enzyme-bound phosphonates in particular have not in general been usefully analyzed (Copié et al., 1990; Bone et al., 1991). It might be noted, however, that Bone et al. (1991) record a chemical shift of 20.42 ppm for a phosphonate monoester of the hydroxyl group of Ser-195 of α-lytic protease.

Scheme III



Further strong evidence as to the point of attachment of the phosphonyl group to the enzyme came from the observation of its significantly lability in alkaline solution. Although this would certainly be in accord with elimination from a serine residue (Scheme I), none of the pyruvate expected from subsequent acid hydrolysis could be detected. Although other possibilities existed, e.g., attachment to threonine rather than to serine, it was also conceivable that the absence of dehydroalanine and hence pyruvate could result from intramolecular trapping of the former in base (Scheme III). This phenomenon has been previously observed in the trapping by lysine of dehydroalanine from base treatment of modified serine or cysteine residues (Bohak, 1964; Patchornik & Sokolovsky, 1964; Ziegler, 1964; Creamer et al., 1977).

In the present case, 1 equiv of lysinoalanine was obtained after base treatment of the inhibited enzyme. This demonstrates not only that serine is the phosphonylated residue but also that a lysine amine group is probably closely adjacent to that residue in the enzyme. This, of course, is true of the active site serine side chain of a β-lactamase, of both class A and class C, as revealed by both structural and functional studies (Knap & Pratt, 1991). Crystal structures (Herzberg & Moult, 1987; Moews et al., 1990; Oefner et al., 1990) show the ammonium ion of lysine-73 (class A)/lysine-315 (P99) directly adjacent and probably hydrogen-bonded to the active site serine hydroxyl group. This stems from the adjacent positioning of the lysine and serine residues on the α-2 helix (Scheme III). It seems likely that this structure is maintained, transiently at least, on base treatment, so that the free amine can trap the adjacent dehydroalanine.

This result also explains why no dehydroalanine (pyruvate) was obtained from the enzyme inhibited by **7**, phenylmethanesulfonyl fluoride, and 6β-bromopenicillanic acid. It also explains why previous attempts to prepare anhydro-β-lactamase from enzymes inhibited by the latter two reagents were unsuccessful (A. Knap, S. Pazhanisamy, and R. F. Pratt, unpublished experiments). Indeed, amino acid analysis of

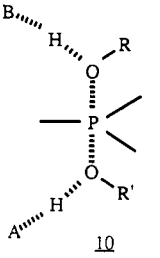
samples of both the P99 β-lactamase and the class A *B. cereus* β-lactamase I, after 6β-bromopenicillanic acid inhibition and base treatment—the latter is known to discharge the rearrangement product of 6β-bromopenicillanic acid (Cohen & Pratt, 1980)—also revealed the presence of 1 equiv of lysinoalanine. This result, in turn, provides strong circumstantial evidence that it is Ser-64 of the P99 β-lactamase that becomes phosphonylated by the present group of inhibitors, since it is the homologous Ser-70 of class A β-lactamases (Ambler, 1980) that becomes acylated by 6β-bromopenicillanic acid (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980).

Given that phosphonylation of the active site serine hydroxyl group of β-lactamases by the phosphonate monoesters **3–7** is responsible for the inactivation of the enzymes by these reagents (Scheme II), it is appropriate to discuss the mechanism of the inactivation reaction. In this discussion it is useful to compare and contrast the present results with the wide array of previous data on the inhibition of serine proteinases, peptidases, and esterases by phosphoryl and phosphonyl derivatives. This inhibition also derives from phosph(or/on)ylation of the active site serine residue (Kraut, 1977). The major difference between the response of β-lactamases and serine proteinases to these reagents is that we find, with the former enzymes, that anionic phosphonate monoesters are effective inhibitors, apparently as effective as neutral phosphonate diesters—compare the effectiveness of **7** with that of **8** (Table I)—whereas with the serine proteinases, neutral reagents are orders of magnitude more effective.

Neutral phosph(on)ates differ markedly from monoanions such as **3–7** not only in charge but also in susceptibility of their phosphorus atoms to nucleophilic attack—ligands in the neutral species are some 10^4 times more susceptible to nucleophilic displacement by H_2O and OH^-, for example than in comparable monoanions (Behrman et al., 1970b; Kovach & Bennet, 1990). This difference could clearly be invoked to explain the relative effectiveness of neutral and anionic phosph(on)ates as inhibitors of serine proteinases. The latter reagents appear to be poor at best as inhibitors of these enzymes. Aharoni and O'Brien (1968) have reported, for example, that, in general, neutral phosphate triesters are 10^4–10^6 times more effective as inhibitors of acetylcholinesterase than are comparable diester monoanions; this difference seems in good agreement with the difference in susceptibility to nucleophilic displacement at phosphorus. The comparable reactivity of **7** and **8** toward the P99 β-lactamase is thus quite striking. It might also be noted that diisopropyl phosphofluoridate and methanesulfonyl fluoride are also unimpressively reactive with β-lactamases (Pain & Virden, 1979; Bush et al., 1982; Knap & Pratt, 1991), an observation that prevented their early identification as serine enzymes. In general accord with these results, we found that one of our most reactive phosphonate monoanions, **5**, had no effect on α-chymotrypsin but was a weak inhibitor, even compared with the monoanions employed by Aharoni and O'Brien, of acetylcholinesterase. The latter result does, however, indicate that any potential phosphonate antibiotic would have to be designed with care.

We have suggested previously that the surprising reactivity of phosphonate monoanions at the β-lactamase active site might reflect a greater positive electrostatic potential than at the serine proteinase site (Pratt, 1989a; Rahil & Pratt, 1991a). In addition to an oxyanion hole (Herzberg & Moult, 1987; Murphy & Pratt, 1988) and the α-2 helix macrodipole, class A β-lactamases have the terminal ammonium ions of Lys-73, directly adjacent to the Ser-70 hydroxyl group, and Lys-234 at the active site (Herzberg & Moult, 1987). Lys-73 has been suggested to electrostatically stabilize the anionic tetrahedral intermediate of β-lactamase catalysis (Ellerby et al., 1990; Knap & Pratt, 1991), and Lys-234 might also stabilize transition states (Ellerby et al., 1990; Brannigan et al., 1991). The class C β-lactamase active site is thought to include homologous residues (Joris et al., 1988; Oefner et al., 1990). Class A β-lactamases, at least, also appear to have an arginine residue close to the active site, which may be involved in binding and catalysis (Moews et al., 1990; Jacob-Dubuisson et al., 1991).

Another feature of the phosphonate inhibitors **3–7** is the presence of a good leaving group, and in general, the better the leaving group, the better the inhibitor—compare, from Table I, the rate constants for **4d**, **4e**, and **4g**, for example. This progression suggests significant negative charge localization on the leaving group in the transition state of the inactivation reaction. A similar leaving group effect has been seen in the inactivation of class A β-lactamases by phosphonate monoesters (Rahil & Pratt, 1991a). Further to the point, a monoalkyl ester of [[*N*-(phenylacetyl)amino]methyl]-phosphonic acid did not inhibit β-lactamases (Pratt, 1989a). A good leaving group is also required for effective inhibition of serine proteinases by neutral phosph(on)ates. This has been persuasively argued as deriving from the significant difference in the geometry of the transition states for acyl- and phosph(or/on)yl-transfer reactions (Järv, 1984; Kovach et al., 1986; Kovach, 1988). With a tetrahedral transition state/intermediate such as found in acyl-transfer reactions, a single functional group, for example, the histidine imidazole group of the catalytic triad of serine proteinases, can swivel between adjacent positions, playing the role of a general base in the formation of the tetrahedral intermediate and a general acid in the breakdown of the intermediate, catalyzing departure of the leaving group. In displacement reactions at tetrahedral phosphorus V, the departing and leaving groups typically add to and leave from the opposite apices of a trigonal bipyramid (Westheimer, 1968; Thatcher & Kluger, 1989). A single catalytic group cannot, of course, span these positions and catalyze both steps of the reaction. Thus, phosphoryl-transfer enzymes, ribonuclease, for example, typically have two functional groups, situated adjacent to the apices of the pentacoordinate intermediate/transition state **10** (Fersht, 1985). It



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might be noted in passing that recent proposals for the mechanism of action of ribonuclease initially employ the general acid catalyst to ensure a phosphorane monoanion intermediate; subsequently, it is also employed to facilitate departure of the leaving group, and overall, therefore, two catalytic groups are still required (Breslow, 1991). The absence of a second catalytic group, HA, not needed to catalyze departure of the leaving group in acyl-transfer enzymes, such as the serine proteinases and β-lactamases, leads to the requirement in phosph(on)ate inhibitors for good leaving groups (halide, aryloxy) which do not require significant protonation prior to their departure.

Phosph(or/on)yl derivatives have often been considered to be transition-state analog inhibitors of serine proteinases

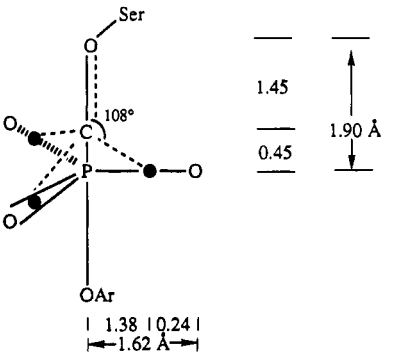
(Bernhard & Orgel, 1959; Kraut, 1977), although it is the phosph(or/on)ylated enzyme that really mimics the acylation or deacylation transition state (Ashani & Green, 1982). They differ from classical transition-state analog inhibitors, however (Wolfenden, 1972; Lienhard, 1973), in that the primary reasons for their effectiveness are kinetic rather than thermodynamic, i.e., not necessarily related to the thermodynamic stability of an enzyme–inhibitor complex. The inert complex is generated in an essentially irreversible reaction whose transition state is strongly stabilized by the enzyme (see below), yielding a product that interacts with the enzyme active site in a way similar to that of the tetrahedral intermediate of acyl-transfer reactions. These same interactions, however, can catalyze dephosph(or/on)ylation—it is not clear, because of the geometry at phosphorus, whether the tetrahedral phosph(on)ate itself or the trigonal bipyramidal transition states leading to it or from it are better stabilized by the active site—and this reaction occurs at an enhanced rate (compared to that of a chemically comparable compound in free solution) in both serine proteinases and β-lactamases. The dephosphonylation of **9** at neutral pH, for example, occurs much more rapidly than the hydrolysis of a simple phosphonate monoalkyl ester. Nevertheless, **9** is sufficiently *kinetically* stable to cause inhibition of the enzyme because, as described above, there is no catalytic group, A^-, in optimal position to catalyze dephosph(or/on)ylation although the reaction is thermodynamically favored.

This situation should be distinguished from that with aldehyde and boronate inhibitors of these enzymes which rapidly and reversibly form *thermodynamically* stable and clearly enzyme-stabilized tetrahedral complexes with the active site serine hydroxyl groups (Kraut, 1977). This distinction has also been pointed out by Berman and Leonard (1989) with respect to the inhibition of acetylcholinesterase by phosphonate diesters.

In some ways, therefore, phosph(on)ates are perhaps better seen as a class of mechanism-based inhibitors of serine hydrolases. The active site nucleophile attacks the phosph(or/on)yl group using, or "recruiting" (Kovach et al., 1986), the normal catalytic machinery. Then, because of the stereoelectronic properties of displacements at phosphorus, and because of the presence of a good leaving group whose departure needs no catalysis, an in-line rather than the expected (by the enzyme) adjacent ligand departs, yielding a kinetically inert complex, inert because the enzyme active site is not designed to efficiently catalyze its hydrolysis. This closely resembles the mode of action of a passive-covalent mechanism-based inhibitor (Pratt, 1989b).

Apart from the kinetic stability of the phosph(or/on)yl enzyme, the most striking feature of these inhibitors is the high rate of the phosph(or/on)ylation itself—seen in serine β-lactamases with phosphonate monoanions particularly and in serine proteinases with neutral reagents. This indicates that these active sites must be significantly, and perhaps rather surprisingly, effective at stabilizing pentacoordinate species. Convincing reasons for such impressive stabilization do not seem to have been found. Although the transition state for phosph(or/on)ylation by a monoanion might tend to be dissociative in nature and that by a neutral species associative (Cleland, 1990), both should contain an at least loosely pentacoordinated phosphorus.

In terms of molecular recognition, perhaps the best superimposition of tetrahedral and pentacoordinate species is given by **11**, where, with the position of the serine hydroxyl oxygen fixed, the other ligands of carbon (filled O) are placed in the



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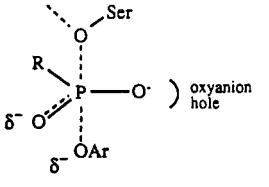
equatorial plane of the trigonal bipyramidal ligand of phosphorus (O). The bonds to phosphorus are represented by the solid lines and those to carbon by dashed lines. This arrangement would give an axial P–O Ser bond length of about 1.90 Å, which is somewhat large for phosphoranes [bond lengths of 1.45 and 1.62 Å have been chosen for C–O and P–O (equatorial), respectively; the corresponding P–O (axial) distance should be 1.69 Å (Allen et al., 1987)] and thus should be appropriate perhaps for a transition state. In a transition-state structure the C–O Ser bond length would also increase and the carbon atom move a little closer to the equatorial plane of the trigonal bipyramid. Obviously, because of the P–O vs C–O bond lengths, it is not possible to superimpose the carbon and phosphorus ligands in the equatorial plane, but the difference is small (ca. 0.24 Å) in comparison to O···HN hydrogen bond distances (1.9 Å) such that phosphoryl oxygens in inactivated serine proteinases appear to fit into the oxyanion hole (Stroud et al., 1974; Kossiakoff & Spencer, 1980). As drawn in **11**, the phosphoryl oxygen will be slightly out of the H···O···H plane of the oxyanion hole, which may help accommodate the greater P–O bond length. Some movement on the part of the protein is of course also likely. The closest correspondence of ligands occurs between the phosphonate transition state **12**, and the tetrahedral intermediate in dea-



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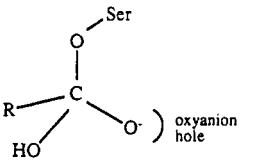

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cylation of the acyl-enzyme intermediate, **13**. It might be noted here, in passing, that the binding of the tetrahedral phosphonate starting material is weak (Figure 1). This could reflect less than optimal binding of the important phosphonate side chain in concert with that of the phosphonyl oxygens.

Thus the phosph(on)ates include in their mode of action an intriguing admixture of transition-state analog and mechanism-based inhibitor characteristics. They are able to recruit the functionality of the active site to stabilize the transition state for phosph(or/on)ylation in the same way as it stabilizes the homostructural, in the sense of **11**, tetrahedral intermediate derived from substrates. Then, because of the unexpected (by the enzyme) departure of the axial ligand from phosphorus, a kinetically inert phosph(or/on)yl enzyme is attained, causing enzyme inhibition. This scenario applies equally well to serine proteinases as to β-lactamases.

Phosphonate monoanions such as **3**–**7** represent a new class of β-lactamase inhibitors. They should be useful as active site titrants, as sources of fresh insight into the chemical properties

of the β-lactamase active site, and possibly as lead compounds to new antibiotics.

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Role of Asp222 in the Catalytic Mechanism of *Escherichia coli* Aspartate Aminotransferase: The Amino Acid Residue Which Enhances the Function of the Enzyme-Bound Coenzyme Pyridoxal 5'-Phosphate†

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ABSTRACT: Asp222 is an invariant residue in all known sequences of aspartate aminotransferases from a variety of sources and is located within a distance of strong ionic interaction with N(1) of the coenzyme, pyridoxal 5'-phosphate (PLP), or pyridoxamine 5'-phosphate (PMP). This residue of *Escherichia coli* aspartate aminotransferase was replaced by Ala, Asn, or Glu by site-directed mutagenesis. The PLP form of the mutant enzyme D222E showed pH-dependent spectral changes with a pK_a value of 6.44 for the protonation of the internal aldimine bond, slightly lower than that (6.7) for the wild-type enzyme. In contrast, the internal aldimine bond in the D222A or D222N enzyme did not titrate over the pH range 5.3-9.5, and a 430-nm band attributed to the protonated aldimine persisted even at high pH. The binding affinity of the D222A and D222N enzymes for PMP decreased by 3 orders of magnitude as compared to that of the wild-type enzyme. Pre-steady-state half-transamination reactions of all the mutant enzymes with substrates exhibited anomalous progress curves comprising multiphasic exponential processes, which were accounted for by postulating several kinetically different enzyme species for both the PLP and PMP forms of each mutant enzyme. While the replacement of Asp222 by Glu yielded fairly active enzyme species, the replacement by Ala and Asn resulted in 8600- and 20 000-fold decreases, respectively, in the catalytic efficiency (k_{max}/K_d value for the most active species of each mutant enzyme) in the reactions of the PLP form with aspartate. In contrast, the catalytic efficiency of the PMP form of the D222A or D222N enzyme with 2-oxoglutarate was still retained at a level as high as 2-10% of that of the wild-type enzyme. The pre-steady-state reactions of these two mutant enzymes with [2-^{2}H]aspartate revealed a deuterium isotope effect (k^H/k^D = 6.0) greater than that [k^H/k^D = 2.2; Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., & Kagamiyama, H. (1990) *Biochemistry 29*, 5469-5476] for the wild-type enzyme. These findings indicate that the presence of a negatively charged residue at position 222 is particularly critical for the withdrawal of the α-proton of the amino acid substrate and accelerates this rate-determining step by about 5 kcal·mol^{-1}. Thus it is concluded that Asp222 serves as a protein ligand tethering the coenzyme in a productive mode within the active site and stabilizes the protonated N(1) of the coenzyme to strengthen the electron-withdrawing capacity of the coenzyme.

The catalytic mechanism for the AspAT[1]-catalyzed reaction proposed by Ivanov and Karpeisky (Karpeisky & Ivanov, 1966; Ivanov & Karpeisky, 1969) was in principle supported by the X-ray crystallographic studies of animal AspATs and their complexes with substrate analogues (Jansonius et al., 1985; Arnone et al., 1985b; Borisov et al., 1985; Harutyunyan et al., 1985; Jansonius & Vincent, 1987). The recent X-ray crystallographic studies on *Escherichia coli* AspAT (Kamitori et al., 1988, 1990; Smith et al., 1989) indicated that the positions of the active-site residues of *E. coli* AspAT were virtually identical with those of the animal AspATs. In the active site of all these AspATs, Asp222[2] is situated within a salt-bridge formation and/or hydrogen-bonding distance to N(1) of the coenzyme PLP or PMP (Figure 1). Hence it has been postulated that the negative charge of its side chain would stabilize the positive charge at N(1) of the coenzyme and would thus

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[1] Abbreviations: AspAT, aspartate aminotransferase; D222E, D222N, or D222A, AspAT in which Asp222 is replaced by Glu, Asn, or Ala, respectively; CD, circular dichroism; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

[2] The amino acid residue is numbered according to the sequence of cytosolic AspAT from pig (Ovchinnikov et al., 1973).

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