Kinetics and Mechanism of β-Lactamase Inhibition by Phosphonamidates: The Quest for a Proton†

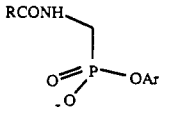
Jubrail Rahil and R. F. Pratt*

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

Received April 27, 1993; Revised Manuscript Received July 12, 1993®

ABSTRACT: Four phosphonamidates were synthesized as potential β-lactamase inhibitors. Three were methanephosphonamidates [$CH_3PO_2^-NHR$/Ar, where R/Ar = 4-methoxybenzyl (**3a**), phenyl (**3b**), and *m*-nitrophenyl (**3c**)], while the fourth, $PhCH_2OCONHCH_2PO_2^-NHPh$ (**2a**), also contained a β-lactamase active site-directed amido side chain. The pH–rate profiles for the hydrolyses of these compounds in the absence of enzyme demonstrated the necessity of nitrogen protonation in the transition state; the reactive neutral form was the zwitterion, IH. The four phosphonamidates irreversibly inhibited the class C β-lactamase of *Enterobacter cloacae* P99 by phosphonylation of the active-site serine hydroxyl group, but they displayed strikingly different inhibition pH–rate profiles. The pH profile and inhibition rates of the *N*-alkyl derivative **3a** could be understood in terms of a direct reaction between IH and EH, the form of the enzyme reactive with substrates and phosphonate monoester inhibitors. The pH profile for **2a** also indicated that EH was the reactive enzyme form, but its direct reaction with IH is unlikely because of the low concentration of the latter, stemming from its low nitrogen pK_a. In this case, proton uptake from solution subsequent to phosphonamidate anion binding probably accounts for the observed rates. The anilides **3b** and **3c** were weak inhibitors with respect to **2a** and **3a**. Their major inhibitory activity, observed at above neutral pH in contrast to that of **2a** and **3a**, probably involves modes of binding not typical of substrate analogs but which allow access to protons. Inhibition by **3c** was interpreted to involve rate-determining protonation at high pH. At and above neutral pH, phosphonamidates will generally be less effective inhibitors than phosphonate *p*-nitrophenyl monoesters. Below pH 7, enzyme-specific phosphonamidates, especially *N*-alkyl derivatives, will become more effective than the esters. The results are consistent with the view that, because of the specific geometry of the phosphonyl-transfer transition state, the effectiveness of phosphonic acid derivatives as β-lactamase inhibitors is limited by the absence of a suitably positioned general acid catalyst at the active site.

The β-lactamases are bacterial enzymes that provide much of the resistance offered by bacteria to β-lactam antibiotics (Donowitz & Mandell, 1988; Jacoby & Archer, 1991). Inhibitors of these enzymes are therefore of both academic and practical interest (Pratt, 1989b, 1992). Recently, a new class of molecules, phosphonate monoester monoanions **1**, were discovered to be β-lactamase inhibitors (Pratt, 1989a; Rahil & Pratt, 1991a, 1992). Rather surprisingly from a chemical

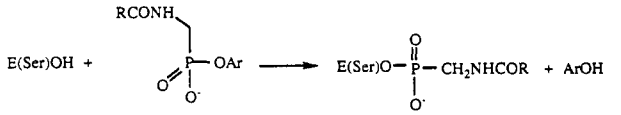


1

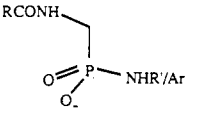
point of view, these compounds phosphonylate the serine hydroxyl group of the β-lactamase active site (Scheme I), giving rise to hydrolytically refractory phosphonyl derivatives and thereby inactivating the enzyme.

One clear result from mechanistic studies with these inhibitors was that the inhibitory activity of **1** increased with the leaving group ability of ArO^- (Rahil & Pratt, 1991a, 1992). It was suggested (Rahil & Pratt, 1992) that, because of the different stereoelectronic requirements of acyl- and phosphonyl-transfer reactions, only compounds with good leaving groups that did not need general acid catalysis to assist departure, i.e., protonation in the transition state, would be effective inhibitors. It follows that an interesting class of compounds with which to further investigate these ideas would

Scheme I



be phosphonamidates of structure **2**. All precedent (Chanley



2

2a R = $PhCH_2O$, Ar = Ph

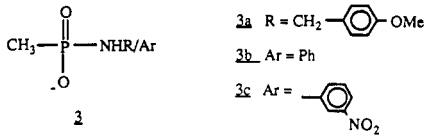
& Feageson, 1958, 1963; Jencks & Gilchrist, 1964, 1965; Benkovic & Benkovic, 1967; Oney & Caplow, 1967; Benkovic & Sampson, 1971; Rahil & Haake, 1981) suggests that phosphonylation of the active site by these reagents could occur only if the leaving group were protonated, either before it reached the active site or subsequently, but necessarily prior to or concerted with leaving group departure. Therefore, an investigation of the inhibitory properties of **2** might well provide information on the availability of protons at the active site in a position to facilitate departure of the leaving group from phosphorus.

For synthetic expediency, we chose to carry out preliminary experiments with the methanephosphonates **3**; previous results with phosphonate monoesters (Rahil & Pratt, 1992) led us to believe that these would also be inhibitory if **2** were, although more weakly so. Subsequently, because of the intriguing

† This research was supported by the National Institutes of Health.
® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

0006-2960/93/0432-10763$04.00/0

results obtained with **3**, compound **2a** was prepared. In this article we present an account of the β-lactamase inhibitory capability of **3a–c** and **2a** and, more particularly, because of the focus on leaving group protonation, a study of the pH dependence of the inhibition. To assist interpretation of the data, the hydrolytic behavior of **3a–c** and **2a** in the absence of enzyme was studied. The latter results are also briefly reported here since there are few mechanistic accounts of phosphonamidate hydrolysis in the literature.



EXPERIMENTAL PROCEDURES

Materials. The β-lactamase of *Enterobacter cloacae* P99 was obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts, U.K.) and used as received. Reagents for synthesis were generally from Aldrich Chemical Co. Standardized hydrochloric acid and sodium hydroxide solutions were obtained from VWR Scientific. Deionized water was used throughout, and all buffer materials were of reagent grade. Dansylcephalothin and triethylammonium *p*-nitrophenyl methanephosphonate were available from previous studies in this laboratory.

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were obtained by means of a Cary 219 or a Perkin-Elmer Lambda 4B spectrophotometer. β-Lactamase activity was determined against benzylpenicillin according to the spectrophotometric method of Waley (1974). Concentrations of the *Ent. cloacae* P99 β-lactamase were determined spectrophotometrically, employing a published extinction coefficient at 280 nm, 7.1×10^4 M^{-1} cm^{-1} (Joris et al., 1985). Concentrated stock solutions of the phosphonamidates were prepared in 20 mM MOPS buffer at pH 7.5. The salts of these phosphonamidates were hygroscopic and unsuitable for combustion analysis, and thus solutions of accurately known concentration could not be prepared by direct weighing. Therefore, the concentrations of phosphonamidates in the stock solutions were determined spectrophotometrically after complete hydrolysis in acid. The required extinction coefficients were determined from absorption measurements on solutions prepared from the purified amines.

For the kinetic measurements required for the pH–rate profiles of enzyme inactivation, a mixed buffer system was employed, consisting of 20 mM each of pyridine, MES, MOPS, HEPES, and AMPSO. The pH–rate profiles of spontaneous hydrolysis were determined in hydrochloric acid solutions or in 20 mM acetate, formate, or chloroacetate buffers. In all cases, an ionic strength of 1.0, adjusted with NaCl, was maintained. All kinetics measurements were made at 25 °C. Rates of enzyme inactivation in the presence of *p*-nitrophenyl methanephosphonate and the phosphonamidates were determined from measurements of enzyme activity against benzylpenicillin as a function of time. According to the general procedure, enzyme (ca. 5 μM) and the inhibitor (20 μM to 20 mM) were incubated together in buffer at the required pH. Aliquots of the reaction mixture were withdrawn at suitable times and immediately assayed for enzyme activity. The activity of control samples of enzyme 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 plots of the first-order rate constants *vs* inhibitor concentration; these plots were linear for all inhibitors at the concentrations employed. The pH–rate profiles were fitted to theoretical equations by a nonlinear least-squares procedure.

The pK_a of the neutral form of **3a** was determined spectrophotometrically as follows. Aliquots of a concentrated stock solution of **3a** were added to buffers at appropriate pH values. Measurements of absorbance at 271 nm *vs* time were recorded and extrapolated back to obtain the zero time absorbances. The latter values were then fitted to a titration curve by a nonlinear least-squares procedure.

The hydrolysis of dansylcephalothin was monitored directly fluorimetrically by means of a Perkin-Elmer MPF-44A spectrofluorimeter (λ_{ex} = 330 nm, λ_f = 575 nm). Reaction mixtures in the buffers described above contained enzyme (0.25 nM) and dansylcephalothin (0.46 μM). Since this substrate concentration was at least 10-fold lower than K_m, pseudo-first-order kinetics were observed. Pseudo-first-order rate constants ($k_{cat}E_0/K_m$) were obtained from nonlinear least-squares curve fitting.

The generation and determination of lysinoalanine from β-lactamase that had been inactivated by *N*-(*m*-nitrophenyl)-methanephosphonamidate (**3c**) was carried out exactly as previously described (Rahil & Pratt, 1992). Amino acid analyses were performed by the staff of the Yale Protein and Nucleic Acid Facility, New Haven, CT.

NMR spectra were obtained from a Varian XL-400 spectrometer. ^1H and ^{31}P chemical shifts are reported as ppm downfield of sodium 3-(trimethylsilyl)-1-propanesulfonate and 85% H_3PO_4, respectively. FAB mass spectra were obtained by Mr. Dan Pentek of the Instrument Center, Chemistry Department, Yale University, New Haven, CT.

Syntheses

Triethylammonium N-Phenylmethanephosphonamidate (***3b***). Aniline (0.86 g, 9.25 mmol) and triethylamine (1.03 g, 10.2 mmol), both freshly distilled from calcium hydride, in 100 mL of dry diethyl ether were added dropwise to a stirred solution of methanephosphonic dichloride (1.23 g, 9.25 mmol) in the same solvent at 0 °C. After the addition, the reaction mixture was allowed to stir for about 2 h at the same temperature and 2 more h at room temperature. Triethylammonium chloride was then removed by filtration, and potassium hydroxide solution (50 mL, 0.4 M) was added to the ether solution. The ether was removed by rotary evaporation. The remaining aqueous alkaline solution was freeze-dried. NMR spectroscopy confirmed the presence of the desired compound in about 50% yield (*vs* methanephosphonate). The compound was purified by anion-exchange chromatography on a QAE-Sephadex column (30 × 2 cm) and eluted with a triethylammonium bicarbonate gradient (500 mL, 0–0.5 M). Triethylammonium bicarbonate was removed by repeated lyophilization. The product was stored dry at −20 °C and was stable under these conditions for several months at least. The structure of the pure product was confirmed by its ^1H [(^2H_2O) δ 1.30 (t, J = 7.5 Hz, 9 H, CH_3CH_2), 1.4 (d, J = 15 Hz, 3 H, CH_3P), 3.20 (q, J = 7.5 Hz, 6 H, CH_3CH_2), 6.95–7.3 (m, 5 H, ArH)] and ^{31}P [(^2H_2O) δ 21.82] NMR spectra and its FAB mass spectrum [*m/e* 273 ($M + H^+$), 374 ($M + Et_3NH^+$)].

Triethylammonium N-(m-Nitrophenyl)methanephosphonamidate (***3c***). *m*-Nitroaniline (1.31 g, 9.48 mmol), purified by recrystallization from water, and triethylamine (1.05 g, 10.4 mmol) in 50 mL of dry THF (freshly distilled from Na/benzophenone) were added at room temperature to a stirred solution of methanephosphonic dichloride (1.26 g, 9.48 mmol)

in THF (50 mL). After the addition, the reaction mixture was heated under reflux for 24 h. The solution became dark yellow, and a precipitate appeared which was removed by filtration at the end of the reaction. Potassium hydroxide solution (20 mL, 1 M) was added to the reaction mixture. All volatiles were then removed by rotary evaporation. The remaining aqueous solution was extracted three times with 20 mL of diethyl ether to remove unreacted nitroaniline and then freeze-dried. A ^{1}H NMR spectrum of the dry residue demonstrated the presence of the desired product; the yield was ca. 80%. The anilide was purified by anion-exchange chromatography and stored as described above. The identity and purity of the final product was confirmed by ^{1}H [(^2H_2O) δ 1.30 (t, J = 7.5 Hz, 9 H, CH_3CH_2), 1.45 (d, J = 15 Hz, 3 H, CH_3P), 3.20 (q, J = 7.5 Hz, 6 H, CH_3CH_2), 7.45–7.92 (m, 4 H, ArH)] and ^{31}P [(^2H_2O) δ 21.39] NMR spectroscopy.

Potassium N-(p-Methoxybenzyl)methanephosphonamidate (**3a**). Essentially the same procedure was employed as for the synthesis of the *N*-phenyl compound described above. However, the attempted purification by anion-exchange chromatography with buffer elution (triethylammonium bicarbonate at pH 9.0) resulted in extensive hydrolysis, even at 5 °C. Successful purification was achieved by gel filtration chromatography at 5 °C using Sephadex G-10 and water as eluent. The product was isolated by lyophilization and stored as described above. The identity and purity of the desired compound was confirmed by ^{1}H [(^2H_2O) δ 1.22 (d, J = 15 Hz, 3 H, CH_3P), 3.85 (s, 3 H, CH_3O), 3.97 (d, J = 10 Hz, 2 H, Ar-CH_2-N), 7.0–7.4 (m, 4, ArH)] and ^{31}P [(^2H_2O) δ 28.06] NMR spectroscopy.

Potassium N-Phenyl-N'-[(benzyloxycarbonyl)amino]-methanephosphonamidate (**2a**). This compound was also prepared by reaction of aniline with the phosphonic acid chloride. *N*-[(Benzyloxycarbonyl)amino]methanephosphonic dichloride was prepared by stirring the *N*-[(benzyloxycarbonyl)amino]methanephosphonic acid (200 mg, 0.8 mmol) with 1 mL of thionyl chloride for 1 h at room temperature. Excess thionyl chloride was then removed by rotary evaporation. The residue, a clear glassy material, was dissolved in 1 mL of CH_2Cl_2 (distilled from BaO) and evaporated to dryness again. The product was left overnight to dry under vacuum. The dichloride was dissolved in 2 mL of CH_2Cl_2. To this solution, freshly distilled aniline (74.6 mg, 0.8 mmol) and triethylamine (89 mg, 0.82 mmol) in 2 mL of CH_2Cl_2 were added dropwise at 0 °C. The solution was then allowed to stir for 1 h at room temperature. Three equivalents of potassium hydroxide (2.4 mL, 1 M KOH) were added, and the volatiles were removed by rotary evaporation. The remaining aqueous solution was freeze-dried. An NMR spectrum of the crude product showed the presence of the desired compound in about 80% yield. The final pure compound was obtained by chromatography on a Sephadex G-10 gel filtration column (45 × 1 cm) at 5 °C with water as eluent. ^{1}H NMR: (^2H_2O) δ 3.50 (d, J = 15 Hz, 2 H, CH_2P), 5.2 (s, 2 H, CH_2O), 6.95–7.55 (m, 10 H, ArH). ^{31}P NMR: (^2H_2O) δ 15.30. FAB mass spectrum: m/e 343 (M + H$^+$), 365 (M + Na$^+$).

RESULTS

Preliminary observations suggested that **3a–c** *did* irreversibly inhibit the β-lactamase of *Ent. cloacae* P99, presumably by the mechanism of Scheme I, but it soon became clear that even semiquantitative comparisons among the three compounds would require careful consideration of the pH dependence of the inhibition in each case. The P99 β-lactamase is susceptible to inhibition by buffer anions, so a careful choice of conditions to study the pH variation of the inhibition by **3a–c** was important. Eventually a mixture of buffer species, each at low concentration, was settled on, where the effects of individual buffer species were blanketed by a high ionic strength (1 M) provided by sodium chloride. Under these conditions, small changes in the concentrations of buffer species (5–20 mM) had no measurable effect on inhibition constants. No buffer catalysis was observed from the low buffer concentrations employed in the measurements of nonenzymic hydrolysis rates either.

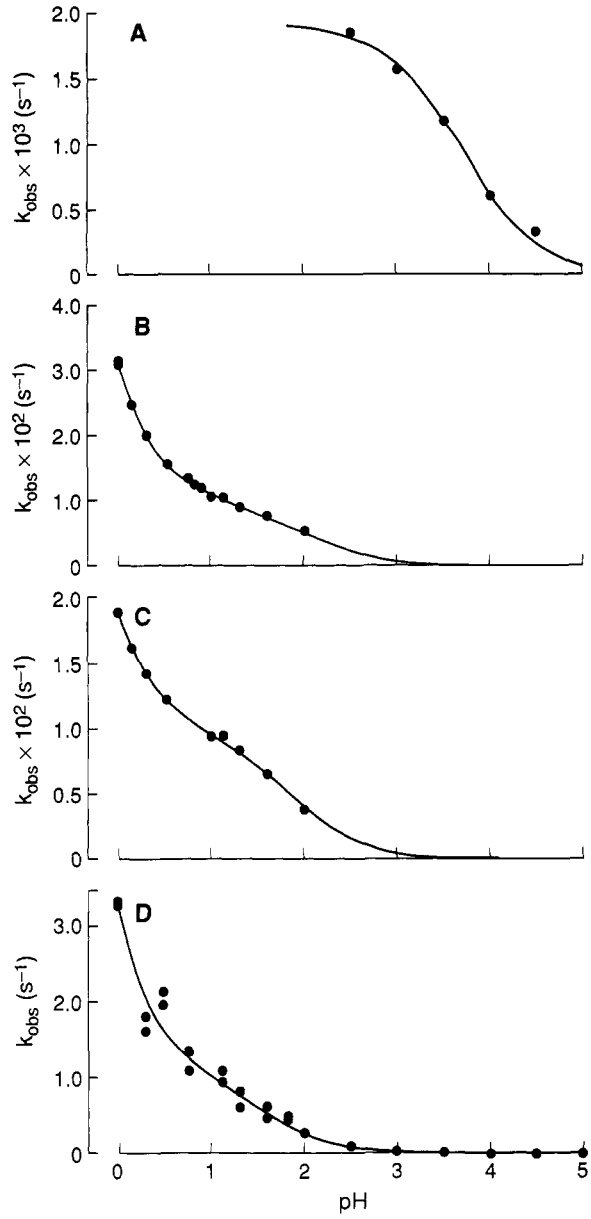


FIGURE 1: pH–rate profiles for the spontaneous hydrolysis of the phosphonamidates **3a** (A), **3b** (B), **3c** (C), and **2a** (D) in aqueous solution. The points are from experiment, and the lines were calculated as described in the text.

In aqueous solution, **3a–c** and **2a** hydrolyze to the expected phosphonate and amine, as was shown by ^{1}H NMR spectra subsequent to the reaction. These hydrolyses have the pH–rate dependences shown in Figure 1. Theoretical fits to these data were achieved through eq 1, which is derived from Scheme

$$k_{obs} = \frac{k_1^I a_{H^+}^2 + k_2^I K_{a1}^I a_{H^+} + k_3^I K_{a1}^I K_{a2}^I}{a_{H^+}^2 + K_{a1}^I a_{H^+} + K_{a1}^I K_{a2}^I} \quad (1)$$

Scheme II

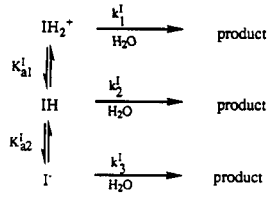


Table I: Rate and Equilibrium Constants[a] for the Hydrolysis of the Phosphonamidates in Aqueous Solution

compd	$10^2 k_2^I$ (s^{-1})	k_2^N (s^{-1})	k_3^I (s^{-1})	pK_{a1}^I	pK_{a2}^I
3a	0.20 ± 0.02	0.002	$<10^{-5}$	<2	3.71 ± 0.08
3b	0.97 ± 0.03	34	$<10^{-5}$	<0	2.04 ± 0.09
3c	1.02 ± 0.05	1940	$<10^{-5}$	<0	1.78 ± 0.08
2a	110 ± 17	1230	$<10^{-5}$	<0	1.55 ± 0.31

[a] These parameters relate to Scheme II and eqs 1 and 5.

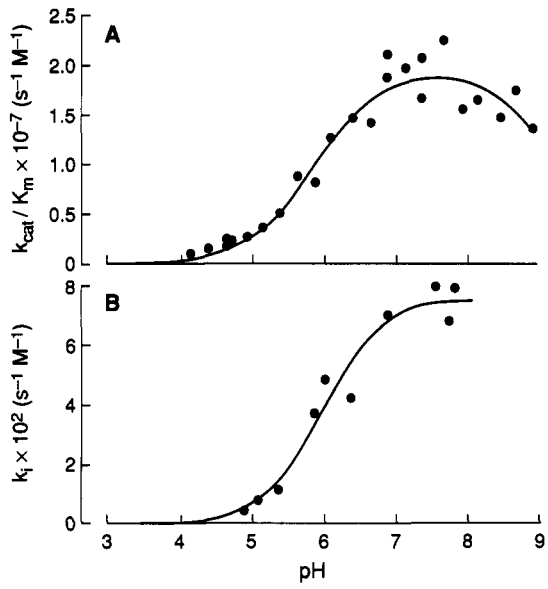
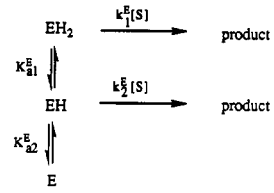


FIGURE 2: pH–rate profiles for the reaction of the *Ent. cloacae* P99 β-lactamase with a substrate, dansylcephalothin (A) and for inhibition by *p*-nitrophenyl methanephosphonate (B). The points are from experiment, and the lines were calculated as described in the text.

II. The latter is assumed to apply from precedent, as discussed below. In Scheme II, IH_2^+ represents the cationic form of the phosphonamidate, IH the neutral form, and I^- the anion; k_1^I, k_2^I, and k_3^I represent the pseudo-first-order rate constants for hydrolysis of IH^+, IH, and I^-, respectively, and K_{a1}^I and K_{a2}^I the acid dissociation constants of IH_2^+ and IH, respectively. In eq 1, k_{obs} represents the observed pseudo-first-order rate constant at a particular pH. The rate and equilibrium constants derived from the fitting of the data of Figure 1 are given in Table I. Since measurements were not made at pH values as low as pK_{a1}^I, values for this constant and thus for k_1^I were not obtained; for compounds **3b**, **3c**, and **2a**, k_1^I/K_{a1}^I values could be obtained from the increase in rate at low pH. The *N*-alkylphosphonamidate **3a** was sufficiently stable to allow a spectrophotometric titration to be carried out (data not shown), yielding a pK_a (corresponding to pK_{a2}^I) of 3.46 ± 0.12, a value in reasonable agreement with that kinetically derived and reported in Table I.

The pH dependence of the reaction of the *Ent. cloacae* P99 β-lactamase with a substrate (dansylcephalothin) is given in Figure 2A. The parameter reported is k_{cat}/K_m, and hence the pK_a values revealed are likely to those of the free enzyme (Alberty & Massey, 1954). The pK_a of the dimethylammonium ion of N-protonated dansylcephalothin is around 4, and hence this dissociation probably does not significantly affect the data of Figure 2A. The N-protonated species was a poorer substrate of a class A β-lactamase through weaker binding (Anderson & Pratt, 1983). The data of Figure 2A were fitted to eq 2, which was derived from Scheme III. In Scheme III,

Scheme III



$$k_{cat}/K_M = \frac{k_1^E a_{H^+}^2 + k_2^E K_{a1}^E a_{H^+}}{a_{H^+}^2 + K_{a1}^E a_{H^+} + K_{a1}^E K_{a2}^E} \quad (2)$$

the rate constants represent second-order rate constants for reaction of free enzyme with substrate, i.e., k_{cat}/K_m values. It was not necessary to include a contribution from E to fit the data, indicating that E was considerably less reactive than EH. The theoretical fit shown in Figure 2A was generated using the constants shown in Table II. Also shown in Figure 2 (part B) is the low-pH region of the rate profile for inactivation of the β-lactamase by *p*-nitrophenyl methanephosphonate. The fit of these data to eq 2 (the experimental parameter here is k_i, the second-order rate constant for enzyme inactivation) gave the values reported in Table II for k_1^E and k_2^E (which should be second-order rate constants for the inactivation reaction between free enzyme and inhibitor) and pK_{a1}^E.

Finally, in Figure 3, pH–rate profiles are presented for inactivation of the enzyme by **3a–c** and **2a**. These data were also fitted to a classical bell-shaped curve by means of the empirical eq 3, where k_i represents the second-order rate

$$k_i = \frac{a a_{H^+}^2 + b K_a a_{H^+}}{a_{H^+}^2 + K_a a_{H^+} + K_a K_b} \quad (3)$$

constant for inactivation of the enzyme by these compounds. The values of the empirical parameters (a, b, K_a, and K_b) giving the best fits to the data (solid lines of Figure 3) are also reported in Table II and will be interpreted below.

In view of the anomalous pH dependence of k_i for **3c** (discussed below), the identity of the modified active-site residue was checked. Previously, the phosphonates **1** had been shown, by a procedure involving base treatment followed by acid hydrolysis, to modify the primary active-site nucleophile, Ser-64. This yielded 1 equiv of lysinoalanine, probably obtained from the cross-linking of the dehydroalanine, derived from the phosphonylated serine by base treatment, to the adjacent conserved active-site residue Lys-67 (Rahil & Pratt, 1992). The application of this procedure to the P99 β-lactamase after inactivation by **3c** yielded 1.0 equiv of lysinoalanine.

DISCUSSION

Nonenzymatic Phosphonamidate Hydrolysis. On considering the pH–rate profiles for hydrolysis of the phosphonamidates (Figure 1), the first issue that must be dealt with is that of the ground-state structure as a function of pH. At high pH, phosphonamidates will obviously exist as anions, but on decreasing the pH, either N- or O-protonation may, in principle, occur (Scheme IV), yielding a zwitterion (**4**) or

Table II: Kinetic and Equilibrium Constants[a] from pH Profiles of the β-Lactamase Reactions

compound	exptl param	k_1^E (s^{-1} M^{-1})	k_2^E (s^{-1} M^{-1})	pK_{a1}^E	pK_{a2}^E
dansylcephalothin	k_{cat}/K_m	0	$(1.95 \pm 0.20) \times 10^7$	5.92 ± 0.16	9.26 ± 0.35
p-nitrophenyl methylphosphonate	k_i	0	$(3.6 \pm 0.3) \times 10^{-2}$	6.05 ± 0.2	*b*

compd	exptl param	*a* (s^{-1} M^{-1})	*b* (s^{-1} M^{-1})	pK_a	pK_b
3a	k_i	0	46 ± 4	3.95 ± 0.12	6.46 ± 0.15
3b	k_i	$(2.6 \pm 0.6) \times 10^{-2}$	$(3.31 \pm 0.01) \times 10^{-1}$	5.96 ± 0.05	9.62 ± 0.15
3c	k_i	$\leq 3 \times 10^{-4}$	$(3.09 \pm 0.15) \times 10^{-2}$	6.96 ± 0.10	>9.5
2a	k_i	0	4.85 ± 0.14	<4	6.38 ± 0.06

[a] These parameters relate to Scheme III and eqs 2 and 3. [b] Not determined; a preliminary experiment performed in 20 mM bicine buffer without NaCl gave a value of 9.26.

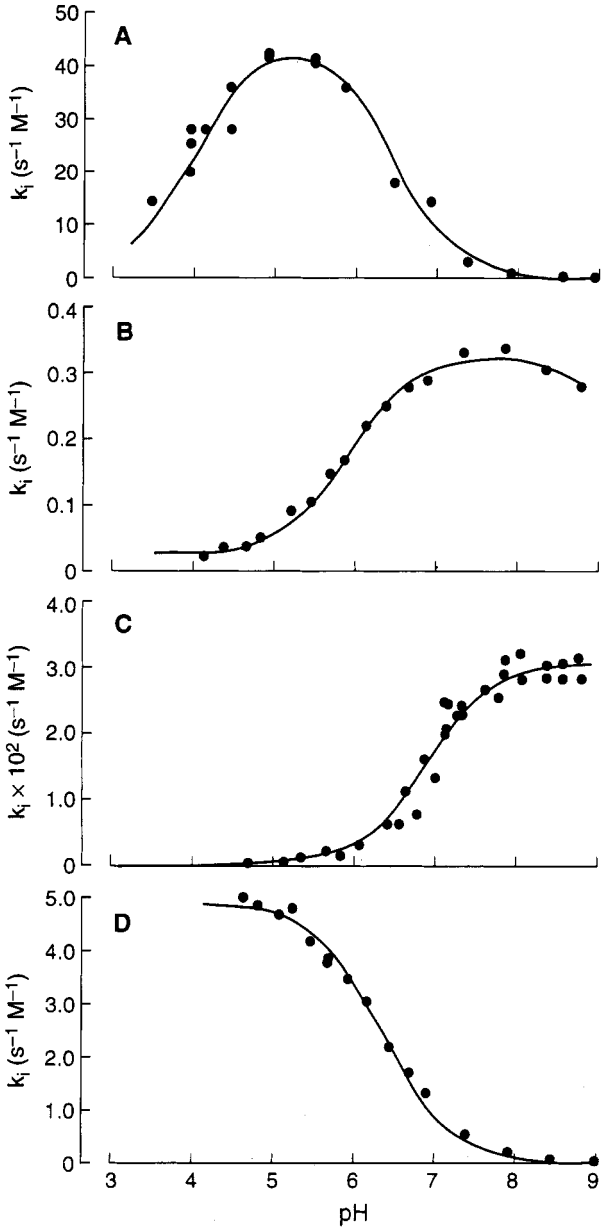
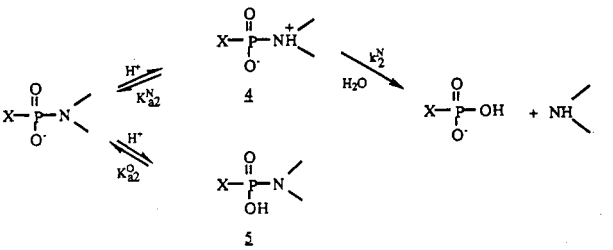


FIGURE 3: pH–rate profiles for inhibition of the *Ent. cloacae* P99 β-lactamase by the phosphonamidates **3a** (A), **3b** (B), **3c** (C), and **2a** (D). The points are from experiment, and the lines were calculated as described in the text.

an uncharged species (**5**), respectively. The observed dissociation constant of a neutral phosphonamidate, K_{a2}^I, will therefore be given by eq 4. The pK_a of **5**, pK_{a2}^O, would

$$1/K_{a2}^I = 1/K_{a2}^O + 1/K_{a2}^N \quad (4)$$

Scheme IV



presumably be similar to the first pK_a of methylphosphonic acid, *viz.* 2.4 (Oney & Caplow, 1967). An earlier study of *N*-alkylphosphonamidates suggested that the pK_a of the ammonium ion of **4** is some 6 units lower than that of the parent amine (Rahil & Haake, 1981). For such compounds, pK_{a2}^N is therefore greater than pK_{a2}^O, and the predominant neutral form of the phosphonamidate in solution would be the zwitterion; this should be true for **3a**. On the other hand, for phosphonyl derivatives of arylamines, such as **3b**, **3c**, and **2a**, pK_{a2}^N will be less than pK_{a2}^O, the uncharged species **5** will predominate in solution, and pK_{a2}^I will approximate pK_{a2}^O.

The available evidence for compounds **3a–c** and **2a** supports these conclusions. The pK_{a2}^I value of **3a**, 3.6, is greater than the first pK_a of methanephosphonic acid and 5.7 units less than that of the parent amine, suggesting N-protonation. Furthermore, the absorption spectral change on protonation, observed in the spectrophotometric titration, was very similar to that observed on titration of the parent amine. Thus, the hydrolysis of **3a** could be readily followed spectrophotometrically at pH values between pK_{a2}^I and the pK_a of the parent amine, where neutral nitrogen in the phosphonamidate was converted into a protonated nitrogen in the amine cation, but less readily, because of the much smaller spectral change, outside this region where the state of protonation of nitrogen did not change on phosphonamidate hydrolysis. Conversely, the pK_{a2}^I values of **3b**, **3c**, and **2a** are similar to the pK_a of methanephosphonic acid, suggesting that O-protonation obtains. The decrease in pK_{a2}^I on *m*-nitro substitution of **3b** (0.26 units) is much smaller than that produced in the parent anilines (ca. 2 units), also indicating protonation further from the phosphonamidate nitrogen. Finally, absorption spectral changes on hydrolysis of **3b**, **3c**, and **2a** at pH values between 0 and the pK_a of the parent aniline are very similar to those of protonation of the aniline, while they are negligible above that pH region. These spectral observations suggest that there is little electronic interaction other than inductive between the phosphonyl group and the nitrogen atom of phosphonamidates [see Rahil and Haake (1981) and references therein for a discussion of this point].

The discussion above therefore makes it seem likely that $pK_{a2}^I \approx pK_{a2}^N$ for **3a**, while $pK_{a2}^I \approx pK_{a2}^O$ for **3b**, **3c**, and **2a**; the

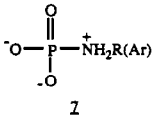
estimated pK_{a2}^N values for **3b**, **3c**, and **2a** would be −1.5, −3.5, and −1.5, respectively, 6 pK_a units below that of the parent amine (Jencks & Regenstein, 1976) in each case.

The pH–rate profiles for **3a–c** in Figure 1 indicate that the phosphonamidate anion is inert to hydrolysis and that at least one proton is required for facile hydrolysis. The rate constants associated with the pathway requiring one proton (k_2^I of Scheme II and Table I) are remarkably similar for compounds **3a–c** despite the very different amine leaving groups present, indicating the influence of some kind of compensation phenomenon. This could arise, for example, if the N-protonated species **4** were the actual reacting species, such that the greater reactivity of **4** from **3b** and **3c** was masked by their lower pK_as for N-protonation. Compensation of this sort has been previously observed in a series of phosphoranilides by Chanley and Feageson (1958) and also interpreted in terms of a reactive zwitterion. Indeed, as mentioned in the introduction, all precedent suggests that facile hydrolysis of phosph(or/on)amidates proceeds in this way, i.e., N-protonation precedes nucleophilic attack by water. On assuming this to be true in the present case (Scheme IV), k_2^I in eq 1 should be replaced with the expression of eq 5, where k_2^N is

$$k_2^I = k_2^N(K_{a2}^I/K_{a2}^N) \quad (5)$$

the pseudo-first-order rate constant for hydrolysis of the zwitterion **4**. Values of k_2^N, calculated using the estimates of K_{a2}^N given above, are also given in Table I.

A Brönsted plot of log k_2^N *vs* the pK_a of the ammonium ion of the leaving group for compounds **3a–c** (with the assumption that the pK_as of *p*-methoxybenzylamine, aniline, and *m*-nitroaniline are 9.47, 4.58, and 2.45 (Jencks & Regenstein, 1976), respectively) is linear ($R = 0.99$), of slope $\beta_{lg} = -0.86$, which can be interpreted in terms of the change in formal charge on nitrogen on achieving the transition state (Williams, 1992). This is suggestive of considerable bond breaking in the transition state. If the initial formal charge on nitrogen were 1.74 (estimated from the data of Williams (1992) for phosphate esters) and the final charge 0 (in aniline), then the extent of bond breaking would be approximately 50%. A similar value of β_{lg} (−1.0), presumably derived from a similar transition state, was obtained for *N*-alkylphosphonamidates by Rahil and Haake (1981). Interestingly, very similar β_{lg} values, and hence changes in charge as the leaving group, are obtained for the hydrolysis of monoanionic phosphoramidate zwitterions **7** (Chanley & Feageson, 1958; Benkovic & Sampson, 1971). The extent of bond breaking in these latter



7

transition states will be greater, however, since the initial formal charge on nitrogen will be less because of the smaller electronegativity of $-PO_3^{2-}$ than that of $-P(Me)O_2^-$ (Williams, 1992). The greater bond breaking in the phosphoramidates probably correlates with the greater stability of the corresponding metaphosphate, which is relevant to the highly dissociative transition states found in phosphoramidate reactions (Jencks & Gilchrist, 1964, 1965; Benkovic & Sampson, 1971; Jameson & Lawlor, 1970). The transition state for hydrolysis of phosphonamidate zwitterions may be similar to that for phosphate diester anions, where bond making and breaking appear to be comparable (Ba-Saif et al., 1989; Cleland, 1990; Hengge & Cleland, 1991).

The hydrolytic reactivity of the zwitterion of anilide **2a** is some 30 times that of **3b** (Table I). Some of the difference may involve the more electron-withdrawing (amido) side chain of **2a**, but this explanation alone is probably insufficient (Rahil & Pratt, 1991b). The remaining enhanced reactivity probably reflects some degree of intramolecular nucleophilic catalysis by the carbamate group, as observed in the alkaline hydrolysis of *p*-nitrophenyl phosphonates (Rahil & Pratt, 1991b); the extent of this participation must be greater in the latter case, however.

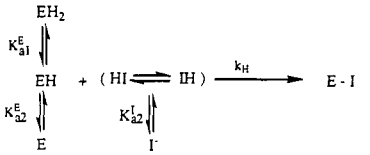
*β-Lactamase Inhibition by **2a** and **3a**.* The most striking feature of the above results relevant to the potential of phosphonamidates as β-lactamase inhibitors is the unreactive nature of these compounds in their most prevalent form at neutral pH, the anion. It seems likely that any phosphonyl-transfer reaction of **3a–c** and **2a**, such as would occur in covalent enzyme inhibition, would require protonation of the leaving group nitrogen atom prior to or concerted with any significant P–N bond cleavage. It has been argued that the currently available structure–reactivity results for inhibition of β-lactamases by phosphonates imply the absence of an enzymic general acid to catalyze departure of the leaving group, and there are good reasons why this should be so for an acyl-transfer enzyme such as catalyzed by a β-lactamase (Rahil & Pratt, 1992). In any covalent inhibition of β-lactamases by phosphonamidates, therefore, such as shown in Scheme I for phosphonate monoesters, perhaps the mechanistically most interesting question is that of the source of the required proton.

In subsequent discussion it will be assumed that the active form of the *Ent. cloacae* P99 β-lactamase, with respect to normal substrates such as dansylcephalothin, is defined by two functional groups, one required in the basic form, of pK_a around 6, and the other, of pK_a around 9.3, in the acidic form, i.e., it will be assumed that these pK_as, derived from the pH dependence of k_{cat}/K_m (Figure 2A, Table II), are not kinetic pK_as or composite quantities, functions of the substrate, but instead relate to dissociations of enzymic functional groups that control the catalytic structure of the active site. It is not necessary for this discussion to assume that these pK_as belong to functional groups actually in the active site, let alone to assume anything of the chemical nature of such groups. Indeed, these matters are still very much problematic (Ellerby et al., 1990; Knap & Pratt, 1991; Herzberg & Moult, 1991; Strynadka et al., 1992). For perspective, however, it might be noted that Waley and co-workers have found a pH dependence of k_{cat}/K_m similar to that observed here for another class C β-lactamase, that of *Pseudomonas aeruginosa*, with cephalosporin C as substrate; the pK_as in that case were 5.2 and 9.7 at 30 °C in an environment of 0.1 M NaCl (Bicknell et al., 1983).

It is important to the matter at hand, however, to also note that the reactivity of the P99 β-lactamase with *p*-nitrophenyl methanephosphonate is controlled, on the acid side, by a functional group of pK_a around 6, which is likely to be, and will subsequently be assumed to be, the same group that controls reactions with normal substrates. It thus seems likely that the catalytically operative active-site structure is common to both substrates and phosphonate monoesters. This proposition would be in accord with the notion of the phosphonate monoesters being transition-state analogs/mechanism-based inhibitors (Rahil & Pratt, 1992).

The pH dependence of the inhibition by the phosphonamidates **3a** and **2a** (Figure 3A,D) can be readily understood

Scheme V



as deriving from the intuitively reasonable, in terms of the above discussion, Scheme V, where HI represents the O-protonated species **5** and IH the N-protonated **4**. It can be assumed that the inhibition derives from phosphonylation of the active-site serine, as previously demonstrated for phosphonate monoesters (Scheme I) (Rahil & Pratt, 1992) and confirmed here for **3c** (see below). One kinetic equivalent of Scheme V, where the inhibition reaction proceeds by reaction of I^- with EH_2, will be, for the moment, set aside on the grounds that since EH_2 shows no reactivity with dansylcephalothin and *p*-nitrophenyl methanephosphonate, there seems little reason to believe that it would react with **3a** and **2a**, directly at least; the latter point will be returned to below. The other uncertainty arises from the kinetic equivalent of HI and IH. It will be assumed, again for the moment, that the reactive species of the inhibitor is the zwitterion IH. With these assumptions, eq 6 can be derived for k_i,

$$k_i = \left[\frac{a_{H^+}}{(1 + [K^N_{a2}/K^O_{a2}])(a_{H^+} + K^I_{a2})}\right]\left[\frac{K_H K^E_{a1} a_{H^+}}{a^2_{H^+} + K^E_{a1} a_{H^+} + K^E_{a1}K^E_{a2}}\right] \quad (6)$$

the observed second-order rate constant for inhibition at any pH. For **3a**, $K^O_{a2} \gg K^N_{a2}$, and thus (from eq 4) $K^I_{a2} = K^N_{a2}$, $K^I_{a2} \gg K^E_{a1}$, and, at pH < 8, $a_{H^+} \gg K^E_{a2}$. Thus eq 6 becomes eq 7, which describes a classical bell curve with associated

$$k_i = \frac{k_H K^E_{a1} a_{H^+}}{a^2_{H^+} + K^I_{a2} a_{H^+} + K^I_{a2}K^E_{a1}} \quad (7)$$

pK_as, pK^I_{a2}, and pK^E_{a1}. A comparison of pK^I_{a2} (Table I) and pK^E_{a1} (Table II) with the empirical pK_as for inhibition by **3a**, pK_a, and pK_b (Table II) shows agreement sufficient to support this model. Further, from inspection of eqs 3 and 7, $a = 0$, meaning no significant acid-catalyzed reaction occurs, and $b = k_H K^E_{a1}/K^I_{a2}$. Thus k_H, the second-order rate constant for reaction of the active form of the enzyme EH with the zwitterion of **3a**, would be 9.0×10^3 s^{-1} M^{-1}.

For the anilide **2a**, where the dominant neutral species is **5** or HI, $K^N_{a2} \gg K^O_{a2}$, and, at the pH values represented in the pH–rate profile of Figure 3D, $a_{H^+} \gg K^E_{a2}$ and $K^I_{a2} \gg a_{H^+}$. Thus eq 6 reduces to eq 8, which describes a sigmoid curve,

$$k_i = \frac{(k_H K^E_{a1}/K^N_{a2}) a_{H^+}}{a_{H^+} + K^E_{a1}} \quad (8)$$

descending with pH increase, and where the observed pK_a should be pK^E_{a1}. This is clearly so for **2a** from inspection of Figure 3D and comparison of pK^E_{a1} with the empirical pK_b of Table II. In this case, k_H will be also given by bK^N_{a2}/K^E_{a1}. Thus, on the assumption of a pK^N_{a2} value of −1.5 (see above), k_H will be 1.5×10^8 s^{-1} M^{-1}. Since this value may be higher than that for the diffusion-controlled productive combination of EH and IH (Hiromi, 1979), some further discussion of kinetically equivalent mechanisms is probably necessary, but this will be postponed until the results for **3b** and **3c** have been considered.

β-Lactamase Inhibition by 3b. The bell-shaped pH profile for the anilide **3b** is qualitatively different from those of **2a** and **3a** in that the ascending limb, from low pH, is not described by a pK_a of the phosphonamidate, nor is the pK_a of the descending limb pK^E_{a1}. The profile does compare well with that of Figure 2A for dansylcephalothin hydrolysis, however, where the pK_as are those of the enzyme, marking the limits of the functional active site. Noticeable also at lower pH in the rate profile for **3b** is a low plateau. This could correspond to the reactivity observed with **2a** and **3a** between EH and IH. If one continues to assume that the only reactive form of a phosphonamidate is the zwitterion, then the rate equation for inactivation of the P99 β-lactamase, including activity at pH > 7, might be that of eq 9, where k, not shown in Scheme V,

$$\text{rate} = k_H[EH][IH] + k[E][IH] \quad (9)$$

represents the second-order rate constant for reaction of IH with the deprotonated from of the enzyme E. From eq 9, the expression for k_i given in eq 10 can be derived, assuming $K^N_{a2} \gg K^O_{a2}$ and $K^I_{a2} \gg a_{H^+}$. This equation is of the same form

$$k_i = \frac{(k_H K^E_{a1}/K^N_{a2}) a^2_{H^+} + (kK^E_{a1}K^E_{a2}/K^N_{a2}) a_{H^+}}{a^2_{H^+} + K^E_{a1} a_{H^+} + K^E_{a1}K^E_{a2}} \quad (10)$$

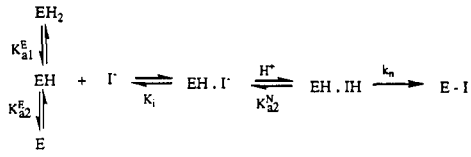
as the empirical eq 3 and provides a good fit to the pH–rate profile for **3b** shown in Figure 3B. The pK_as should be those of the free enzyme, which is as observed (compare pK_a and pK_b of Table II with pK^E_{a1} and pK^E_{a2}, respectively). At low pH (but above pK^I_{a2},) a plateau should be observed (as seen for **2a**) where $k_i = (k_H K^E_{a1}/K^N_{a2})$. Hence, from eq 3, $k_H = (aK^N_{a2}/K^E_{a1})$, and thus, assuming again that for an anilide pK^N_{a2} is −1.5, $k_H = 8 \times 10^5$ s^{-1} M^{-1}; the rate of this reaction might also be approaching a diffusion limit, depending on the actual value of pK^N_{a2}.

From the fit to the bell, comparison with eq 3 yields $b = (kK^E_{a2}/K^N_{a2})$, and hence k, the second-order rate constant, formally describing reaction of E with IH, has a value of 2×10^{10} s^{-1} M^{-1}. This rate constant clearly exceeds that of a diffusion-limited reaction, and thus the putative mechanism, reaction of E with IH, cannot be responsible for the observed rate; the reason for this is basically the very low concentration of IH at the pH values where reaction is observed.

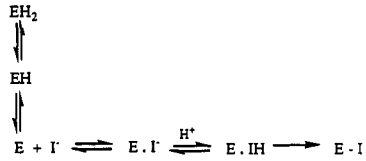
It is clear now that alternative kinetically equivalent mechanisms of inactivation must be considered for the major inhibitory reactivity of the anilides **2a** and **3b**. These may apply to some extent to **3a**, as well, although they are not required by the data in that case. The existence of reasonable alternatives arises from the fact that the process described by the second-order inactivation rate constant k_i is not a simple second-order reaction with a single bimolecular step but must include an initial binding to the enzyme active site. Between the binding and nucleophilic attack by the active-site serine hydroxyl group on phosphorus, there is clearly scope for proton-transfer steps. There seem to be two general alternatives: the required proton could come either directly from the external solution or indirectly by intramolecular transfer at the active site. The implications of these two mechanisms are considered below.

(1) Direct Proton Transfer from Solution. Since the phosphonamidate anion is by far the dominant form of **2a** and **3b** in solution at the pHs of greatest inhibitory activity, it seems likely that this form would be bound by the enzyme; phosphate monoester monoanions of closely similar structure to the phosphonamidate anions presumably bind prior to their

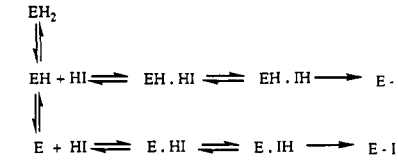
Scheme VI



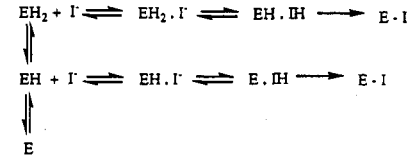
Scheme VII



Scheme VIII



Scheme IX



inactivation reaction (Pratt, 1989a, Rahil & Pratt, 1991a, 1992). Subsequent to binding of the anion, proton transfer to the phosphonamidate nitrogen must then occur prior to (or perhaps concerted with) nucleophilic attack (Scheme VI). If it is assumed that all proton-transfer reactions are at equilibrium, and if the dissociation constant of IH in EH·IH is the same that of IH in solution, *viz.*, $10^{1.5}$, then k_i at low pH ($\leq pK_{a1}^{E}$) is given by eq 11, from which, for **2a**, (k_n/K_i) =

$$k_i = \frac{(k_n K_{a1}^{E}/K_{a2}^{N}K_i)a_{H+}}{a_{H+} + K_{a1}^{E}} \qquad (11)$$

1.5×10^8 s^{-1} M^{-1} can be derived. Assuming $K_i \geq 10^{-3}$ M, as seen with phosphate monoester monoanions (Pratt, 1989a; Rahil & Pratt, 1991a, 1992), $k_n \geq 1.5 \times 10^5$ s^{-1}. In view of the fact that the reaction with rate constant k_n is effectively intramolecular, involving a very reactive zwitterion, such a value for k_n is not unreasonable. In free solution, water at 55 M achieves a rate of *ca.* 30 s^{-1} with the zwitterion of **3b**, a value that could probably be significantly increased by an intramolecular nucleophile and general base catalyst such as are probably present at the β-lactamase active site. In the nonenzymic hydrolysis of **2a**, the carbamate group, normally a poor nucleophile, achieves a rate constant for intramolecular attack at the phosphonamidate zwitterion of *ca.* 10^3 s^{-1}. A smaller value of k_n than 1.5×10^5 s^{-1} might also be sufficient if the enzyme active site happened to preferentially stabilize the phosphonamidate zwitterion, i.e., if the dissociation constant of EH·IH were smaller than K_{a2}^{N}. This possibility is difficult to judge at present.

A kinetic point relating to Scheme VI is whether proton transfer from solution is fast enough to ensure that Scheme VI is kinetically competent. Since the pK_as of H_3O^+ and IH in EH·IH are comparable, proton transfer between H_3O^+ and EH·I^- will occur at approximately the diffusion-controlled rate, 10^9 s^{-1} M^{-1} (Eigen, 1964). At pH 7, therefore, an observed rate constant of around 100 s^{-1} should be observed, which would be sufficient, in the absence of complications introduced by the protein, to accommodate the observed rate of reaction of **2a** with the enzyme.

Thus, preassociation of EH and I^- prior to protonation of the phosphonamidate nitrogen probably explains the reactivity of **2a** with the β-lactamase. It may also occur as the preferential pathway in the inhibition by **3b** at low pH.

The reactivity of **3b** at higher pH, its major reactivity, cannot be explained in exactly the same way since the transition state for this reaction contains 1 fewer proton. Scheme VII would be the direct analog of Scheme VI that would, in principle, fit the data. This seems to require, however, that a form of the enzyme, E, not reactive with normal substrates, attacks the bound phosphonamidate zwitterion. Such a scenario is not impossible. The inactivation of the TEM β-lactamase by methanesulfonyl fluoride, a nonspecific reagent as, it could be argued, is **3b**, included a significant contribution from the reactivity of E (Knap & Pratt, 1991). It does seem unlikely, however, that the reactivity of E would greatly exceed that of EH, the reactive form of the enzyme with substrates and phosphonate monoesters.

(*2*) *Intramolecular Proton Transfer.* Two possibilities come to mind here. First (Scheme VIII), the enzyme might bind the uncharged neutral forms of **2a** and **3b**, which are at significantly higher concentrations (*ca.* $10^{3.5}$-fold) than the zwitterion at all pH values. The neutral diester methyl *p*-nitrophenyl methanephosphonate inactivates the P99 β-lactamase and thus presumably must bind to some degree (Rahil & Pratt, 1992). After the binding of the neutral form, a facile intramolecular proton shift to form the zwitterion might be possible at the active site. The zwitterion might be more stable with respect to the uncharged form at the active site than in free solution. It would still be necessary, however, to explain the major reactivity of **2a** and **3b** differently, as involving the binding of HI to EH and E, respectively. As in Scheme V, the required proton in Scheme VIII comes from solution with I.

Alternatively, Scheme IX, where intramolecular proton transfer from an enzymic functional group occurs, might be considered. In this case, the data for **2a** would require initial binding of I^- to EH_2 while those for **3b** require EH; again, the final reaction of **3b** would be formally with E. An attractive feature of this scheme is that the initial binding involves the dominant species of both enzyme and inhibitor at the pH region where major reactivity is observed. Its problem lies with the previous argument that no proton donor that could assist leaving group departure is suitably placed at the enzyme active site (Rahil & Pratt, 1992).

An important question which must relate to the above discussion is that of why the pH profiles of the anilides **2a** and **3b** are so different, in that the major reactivity of **3b** is observed to employ 1 fewer proton than that of **2a**. The difference presumably must lie with the structures of **2a** and **3b**. The compound **2a**, with its amido side chain, is much more substrate-like than **3b** and would be expected to be more likely to employ the binding and catalytic features of the active enzyme from EH. Certainly, irrespective of the exact mechanism, the observed rate constants for the specific structure **2a** are much greater than those for **3b**. This effect of the amido side chain is seen also in phosphonate monoesters (Rahil & Pratt, 1992). The difference in observed rate constants at pH 4.0, 400-fold in favor of **2a**, is less than the observed with the phosphonate monoesters, but this may reflect the change in mechanism enforced on **2a** by the diffusion-

Scheme X

$$EH + I^- \rightleftharpoons EH.I^- \longrightarrow E\text{–}I$$

controlled limit. The anomalous reactivity, if it can be so called, seems that of **3b**, which appears to require the enzyme form E.

A variant of Scheme IX which might explain the reactivity of **3b** derives from the proposition that, because of the absence a β-lactamase-specific side chain in this compound, it might be able to bind in such a way that proton transfer from an active-site functional group in EH *is* possible. The reaction could then be represented simply as in Scheme X, where the irreversible rate process would include nucleophilic attack with proton transfer to the phosphonamidate nitrogen, catalyzed by the normal general base/general acid of the active site, in a concerted fashion or otherwise—a pentacoordinated intermediate and pseudorotation may be involved. This mode of reactivity would not be seen in the specifically bound **2a** nor in **3a**, where the reaction of the plentiful zwitterion would mask it.

β-Lactamase Inhibition by 3c. There remains the nitroanilide **3c** to be considered. Again, counterintuitively, the major reactivity has moved to higher pH. This must result from the acid-catalyzed reactions at lower pH becoming effectively less favorable, because of the further decrease in the phosphonamidate nitrogen pK_a. Despite the lower observed rate constant, *b*, for **3c** than for **3b**, the second-order rate constants for reaction of EH with the respective zwitterions may be comparable—for **3c**, k_H ($= aK_{a2}^N/K_{a1}^E$) must be less than 10^6 s^{-1} M^{-1}; that is not larger suggests a reaction rate-limited by factors other than nucleophilic displacement at phosphorus, e.g., noncovalent association with the enzyme. These rate-limiting phenomena apparently leave for **3c**, by default, a mode of reaction not observable with the more effective inhibitors **2a**, **3a**, and **3b**. The striking feature of the reactivity of **3c** is its sigmoid pH profile (Figure 3C), with an apparent pK_a of 7.0. This does not correspond to a dissociation of the inhibitor or to any of the enzyme yet observed. In view of this anomalous pH–rate profile, it seemed important to establish that the modified functional group leading to inactivation was in fact the active-site serine hydroxyl group, as had been demonstrated for the phosphate monoesters (Rahil & Pratt, 1992) and assumed to be true here for the phosphonamidates. The isolation of 1 equiv of lysinoalanine from the enzyme inhibited by **3c** seemed to prove the point. The observed pK_a at 7.0, therefore, does not appear to be that of a nonspecific nucleophile.

There appear to be two possibilities for the reactivity of **3c**. (a) The pK_a reflects dissociation of an enzymic functional group that does not affect the normal reactivity of the active site with substrates and phosphonates but whose dissociation permits the binding of **3c** in such a way as to gain access to an enzymic proton source. (b) The apparent pK_a is kinetic rather than thermodynamic in nature, reflecting a change in rate-determining step, likely to one that requires a proton. Scheme XI represents the minimal scheme for the pH region of inhibitory activity, $pK_{a1}^E < pH < pK_{a2}^E$. In this scheme, the second step must represent loss of a proton from the enzyme to yield an intermediate which then reacts to form products. Presumably there must be a fast step, omitted from Scheme XI and subequent to the third step, involving proton transfer to the phosphonamidate nitrogen. Equation 12 can be derived from this scheme, assuming $E^*{\cdot}I^-$ to be a steady-state intermediate and that $K_i \gg [I^-]$. This equation can generate the required sigmoid form: at high pH, $k_i \rightarrow k_2/K_i$, and the

Scheme XI

$$EH + I^- \underset{K_i}{\rightleftharpoons} EH.I^- \underset{k_{-2}a_{H^+}}{\overset{k_2}{\rightleftharpoons}} E^*.I^- \xrightarrow{k_3} E\text{–}I$$

Scheme XII

$$EH + I^- \underset{K_i}{\rightleftharpoons} EH{\cdot}I^- \underset{H^+}{\overset{K_2}{\rightleftharpoons}} E^*.I^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X \xrightarrow{k_4a_{H^+}} E\text{–}I$$

$$k_i = \frac{k_2k_3}{(k_{-2}a_{H^+} + k_3)K_i} \qquad (12)$$

apparent pK_a, achieved when $k_3 = k_{-2}a_{H^+}$, is given by $-\log_{10}(k_3/k_{-2})$. The next-simplest scheme (Scheme XII) explicitly includes protonation of the phosphonamidate nitrogen. Under the same pH conditions that led to eq 12 from Scheme XI, Scheme XII yields eq 13, which is also of the

$$k_i = \frac{k_3k_4K_2}{(k_4a_{H^+} + k_{-3})K_i} \qquad (13)$$

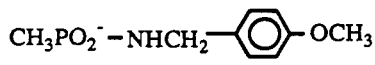
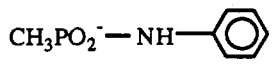
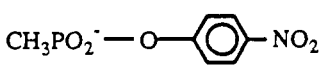
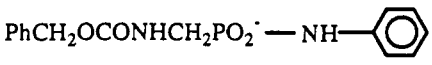
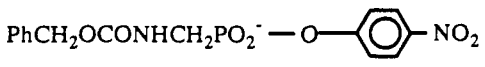
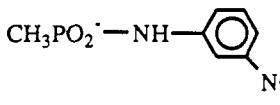
correct form; at high pH, $k_i \rightarrow k_3K_4K_2/k_{-3}K_i$, and the apparent pK_a, when $k_{-3} = k_4a_{H^+}$, is given by $-\log_{10}(k_{-3}/k_4)$. In Schemes XI and XII, E* may represent the major high-pH form of the enzyme, E, with I^- bound in some novel and reactive orientation, or it could represent the basic form of the enzyme after dissociation of a higher pK_a functional group. A likely possibility would be the active-site serine hydroxyl, whose dissociation would presumably yield a more powerful nucleophile. In Scheme XII, X could represent an intermediate containing pentacoordinated phosphorus, whose breakdown to products requires protonation of the amine leaving group. The higher pK_a of the nitrogen in this intermediate would facilitate its protonation. In passing, it should be noted that the rate of β-lactamase inactivation by **3c** was not affected by the concentration of buffer in the solution. An external proton donor is thus apparently not a participant.

General Conclusions. In summary, the major inhibitory reactivity of phosphonamidates **2a** and **3a** seems to involve pathways where the proton needed to assist leaving group departure comes directly from solution, either with the substrate or after the substrate is bound. In the former case, the reactivity of the inhibitor is enhanced by specific binding. These pathways are inefficient with **3b** and **3c** because of their lack of specific binding and low zwitterion pK_as. In these cases, other, normally even less efficient, inhibition mechanisms are observed which probably involve modes of inhibitor binding and active-site functional group involvement not related to inhibition by specific phosphonate monoesters or to normal catalysis.

Irrespective of mechanism, the phosphonamidates **2a** and **3a–c** are all inhibitors of the P99 β-lactamase. Their relative effectiveness varies with pH and is a strong function of the pK_a of the parent amine. At pH 7.5, their relative effectiveness is seen in the k_i values of Table III, with those of two analogous *p*-nitrophenyl methanephosphonates for comparison; clearly the esters are superior inhibitors at that pH. At lower pH, however, *N*-alkylphosphonamidates would become more effective than the esters, although this gain would be offset, in practice, to some extent by the hydrolytic instability of the phosphonamidates. Any assessment of the relative effectiveness of phosphonamidates as covalent enzyme inhibitors must therefore include a careful study of the influence of pH.

With respect to the β-lactamase active site and the mechanism of inactivation of β-lactamases by phosphonate

Table III: Second-Order Rate Constants for the Inhibition of the *Ent. cloacae* P99 β-Lactamase by Methylphosphonate Amides and *p*-Nitrophenyl Esters at pH 7.5

compd	k_i (s^{-1} M^{-1})
	2.90
	1.56
	0.64
	0.5
	3.3×10^4
	0.083

monoanions, the current results are informative. They show clearly that the β-lactamase active site is unable to efficiently protonate the leaving group of a phosphonamidate inhibitor. It seems likely that effective inhibition by phosphonamidates requires a proton from solution either before or after their binding to the enzyme active site. There also seem to be inefficient intramolecular pathways of protonation at the active site. This conclusion is in accord with previous thought on the inactivation of serine proteinases and β-lactamases by phosph(or/on)yl derivatives and reflects the different stereoelectronic requirements of the transition states of acyl- and phosph(or/on)yl-transfer reactions (Rahil & Pratt, 1992). A corollary of these conclusions would be that it is unncessary and possibly counterproductive to prepare inhibitors of this type with substrate-like leaving groups.

ACKNOWLEDGMENT

We thank Dr. Igor Goljer for assistance with the ^{31}P NMR spectra.

REFERENCES

Alberty, R. A., & Massey, V. (1954) *Biochim. Biophys. Acta 13*, 347–353.

Anderson, E. G., & Pratt, R. F. (1983) *J. Biol. Chem. 258*, 13120–13126.

Ba-Saif, S. A., Davis, A. M., & Williams, A. (1989) *J. Org. Chem. 54*, 5483–5486.

Benkovic, S. J., & Benkovic, P. A. (1967) *J. Am. Chem. Soc. 89*, 4714–4722.

Benkovic, S. J., & Sampson, E. J. (1971) *J. Am. Chem. Soc. 93*, 4009–4016.

Bicknell, R., Knott-Hunziker, V., & Waley, S. G. (1983) *Biochem. J. 213*, 61–66.

Chanley, J. D., & Feageson, E. (1958) *J. Am. Chem. Soc. 80*, 2686–2691.

Chanley, J. D., & Feageson, E. (1963) *J. Am. Chem. Soc. 85*, 1181–1190.

Cleland, W. W. (1990) *FASEB J. 4*, 2899–2905.

Donowitz, G. R., & Mandell, G. L. (1988) *N. Engl. J. Med. 318*, 419–426.

Eigen, M. (1964) *Angew. Chem., Int. Ed. Engl. 3*, 1–19.

Ellerby, L. M., Escobar, W. A., Fink, A. L., Mitchinson, C., & Wells, J. A. (1990) *Biochemistry 29*, 5797–5806.

Hengge, A. C., & Cleland, W. W. (1991) *J. Am. Chem. Soc. 113*, 5835–5841.

Herzberg, O., & Moult, J. (1991) *Curr. Opin. Struct. Biol. 1*, 946–953.

Hiromi, K. (1979) in *Kinetics of Fast Enzyme Reactions*, pp 256–261, Halsted Press, New York.

Jacoby, G. A., & Archer, G. L. (1991) *N. Engl. J. Med. 324*, 601–612.

Jameson, G. W., & Lawlor, J. M. (1970) *J. Chem. Soc. B*, 53–57.

Jencks, W. P., & Gilchrist, M. (1964) *J. Am. Chem. Soc. 86*, 1410–1417.

Jencks, W. P., & Gilchrist, M. (1965) *J. Am. Chem. Soc. 87*, 3199–3209.

Jencks, W. P., & Regenstein, J. (1976) in *Handbook of Biochemistry and Biology* (Fasman, G. D., Ed.) 3rd ed., Physical and Chemical Data, Vol. 1, pp 305–351, CRC Press, Cleveland.

Joris, B., De Meester, F., Galleni, M., Reckinger, G., Coyette, J., Frère, J.-M., & Van Beeumen, J. (1985) *Biochem. J. 228*, 241–248.

Knap, A. K., & Pratt, R. F. (1991) *Biochem. J. 273*, 85–91.

Oney, I. & Caplow, M. (1967) *J. Am. Chem. Soc. 89*, 6972–6980.

Pratt, R. F. (1989a) *Science 246*, 917–919.

Pratt, R. F. (1989b) in *Design of Enzyme Inhibitors as Drugs* (Sandler, M., & Smith, H. J., Eds.) pp 178–205, Oxford University Press, Oxford.

Pratt, R. F. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) Blackie, Glasgow.

Rahil, J., & Haake, P. (1981) *J. Am. Chem. Soc. 103*, 1723–1734.

Rahil, J., & Pratt, R. F. (1991a) *Biochem. J. 275*, 793–795.

Rahil, J., & Pratt, R. F. (1991b) *J. Chem. Soc., Perkin Trans. 2*, 947–950.

Rahil, J., & Pratt, R. F. (1992) *Biochemistry 31*, 5869–5878.

Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., & James, M. N. G. (1992) *Nature 359*, 700–705.

Waley, S. G. (1974) *Biochem. J. 139*, 789–790.

Williams, A. (1992) *Adv. Phys. Org. Chem. 27*, 1–55.