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Structure–Activity Studies of the Inhibition of Serine β-Lactamases by Phosphonate Monoesters

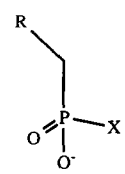
Naixin Li, Jubrail Rahil, Margaret E. Wright and R. F. Pratt*
Department of Chemistry, Wesleyan University, Middletown, CT 06459, U.S.A.

Abstract—A new series of phosphonyl derivatives has been prepared and tested for inhibition of serine (class A and C) β-lactamases. Variations of the leaving group in a series of methyl phosphonates showed that leaving groups better than the previously employed *p*-nitrophenoxide could give more effective inhibitors. Inclusion of a negative charge in the leaving group did not, *per se*, lead to better inhibitors. Aryl phosphonates appeared more effective than those with electronically comparable but smaller leaving groups. The combination of a good leaving group, 2,4-dinitrophenoxide, with an amido side-chain, phenylmethylsulfonamido—the latter rather than phenylacetamido in order to increase the stability of the compound with respect to intramolecular nucleophilic catalysis of hydrolysis by the amide group—did not yield overall a better inhibitor than previously employed *p*-nitrophenyl phosphonates. These results give the first indication of specific interactions between a β-lactamase and the leaving group of a phosphonate inhibitor. Only one enantiomer of a chiral thiophosphonate, presumably the R_p isomer, was an effective inhibitor. Addition of either a D- or a L-methyl group to the methylene group of a *p*-nitrophenyl amidomethylphosphonate did not enhance the inhibitory ability of the phosphonate. Class A β-lactamases remain refractory to phosphonates. © 1997 Elsevier Science Ltd.

Introduction

The β-lactamases are a group of bacterial enzymes that catalyze the hydrolysis of β-lactam antibiotics and thus contribute to the resistance of bacteria to these antibiotics. The extent of this type of resistance has continued to increase, most recently through the spread of mutant enzymes capable of catalyzing the hydrolysis of third generation cephalosporins and of carbapenems.[1,2] β-Lactamase inhibitors are therefore of considerable interest in the battle against bacteria and indeed some have already reached market.[3,4]

Phosphonate monoanions were introduced as a new motif in β-lactamase inhibitors through research in this laboratory.[5] Anionic phosphonic acid monoesters and monoamides have subsequently been shown to inhibit β-lactamases, both in this laboratory[6–9] and elsewhere.[10,11] Structural studies have demonstrated that these compounds act as inhibitors by phosphonylation of the nucleophilic serine hydroxyl group of the β-lactamase active site.[7,12,13] The compounds studied to date have been much more effective against class C than class A β-lactamases.[6–10] They also did not appear to be strongly reactive with bacterial DD-peptidases (ref 5 and J. Rahil and R. F. Pratt, unpublished observations) and thus are unlikely, as things stand at present, to be antibiotics in their own right.[10]



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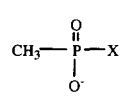
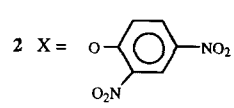
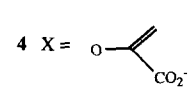
The phosphonate esters hitherto described were of structure **1**, where R is either H or RCONH and X is a leaving group. The better inhibitors had a good leaving group (e.g., *p*-nitrophenoxide) and an amido side-chain (e.g., $PhCH_2CONH$). Crystal structures showed the latter to fit into the usual binding site of a substrate side-chain.[12,13] The present paper describes the results obtained from a new series of structural variants on the phosphonate monoester theme. Compounds **2–5** were

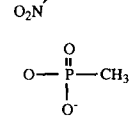




2 X =

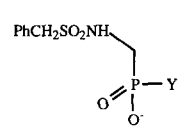


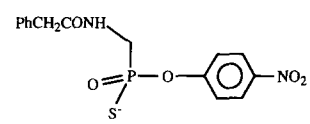
4 X =



3 X =

5 X = $OCOCH_3$





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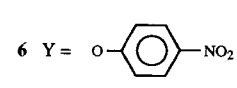
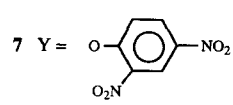
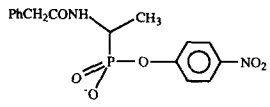


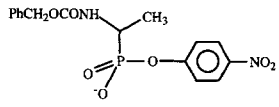
6 Y =



7 Y =



9 D - Me
10 L - Me



11 D - Me
12 L - Me

designed to explore further the effect on β-lactamase inhibition of leaving groups, compounds **6** and **7** the effect of leaving group with an amido side-chain, **8** the combination of a side-chain with a thiophosphonate, and **9–12** the effect of substitution and stereochemistry α to the phosphonyl group.

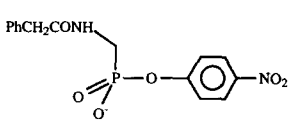
Results and Discussion

Inhibition of serine β-lactamases by phosphonate monoesters has been shown to involve phosphonylation of the active site serine hydroxyl group with concomitant displacement of a leaving group LO^- from phosphorus[7,12,13] (eq. 1). Purely second-order inactivation kinetics have been observed to date and in the present work involving concentrations up to several mM. The phosphonates so far examined therefore bind noncovalently only very weakly to β-lactamase active sites.

$$\text{E-OH} + \text{RP}(\text{O}_2^-)\text{OL} \xrightarrow{k_i} \text{E-OP}(\text{O}_2^-)\text{R} + \text{LOH} \quad (1)$$

As might be anticipated under such circumstances, the leaving group ability of LO^- is an important factor in determining the effectiveness of the inhibitor (i.e., the value of k_i).[6,7] Thus while monoaryl phosphonates have been found effective, their monoalkyl analogues are not.[5]

A problem that arises in derivatives of **1** with an amido side-chain and a good leaving group such as *p*-nitrophenoxide is instability of these compounds in solution. This derives from intramolecular nucleophilic catalysis of hydrolysis by the amido side-chain.[15] Replacement of the amido group with a carbamate such as $PhCH_2OCONH-$ greatly reduced the problem but we anticipated its resurgence when better leaving groups than *p*-nitrophenoxide were explored. We therefore examined the phenylmethylsulfonamido group $PhCH_2SO_2NH-$ as an alternative. The alkaline hydrolysis rates reported in Table 1 suggest that this strategy was successful. Previous data for **13–15** are included for comparison.



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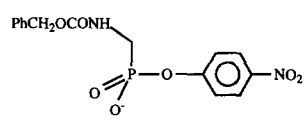

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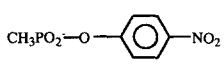

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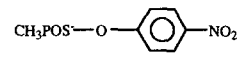

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Table 1. Second-order rate constants for alkaline hydrolysis of phosphonate monoesters at 25 °C

Phosphonate	$10^3 \times k_{OH}$ (s^{-1} M^{-1})
15	0.028[a]
2	2.1
13	2000[b]
14	15[b]
6	0.007
7	4.7
9	590
11	2.9

[a]Taken from ref 9.
[b]Taken from ref 15.

Comparison of the k_{OH} values of **15** and **2** and of **6** and **7** shows the effect of the better leaving group 2,4-dinitrophenoxide on reactivity. Comparison of the values for **13**, **14**, and **6** shows the effect of decreasing participation of the side-chain in the hydrolysis reaction. The values for **6** and **7**, in comparison with those of **15** and **2**, respectively, suggest that the sulfonamido group does not directly participate in the reaction at all. The k_{OH} values for **9** and **11**, in comparison with **13** and **14**, respectively, show that the α-methyl group slows down the hydrolysis reaction somewhat, presumably for largely steric reasons.

The mono-(2,4-dinitrophenyl) phosphonates **2** and **7** were generated in aqueous solution from the diesters **17** and **18** (see Experimental), respectively. Hydrolysis of the diesters, accompanied by release of one molar equivalent of 2,4-dinitrophenoxide, was effectively instantaneous in hydroxide ion solution and rapid in neutral buffer. For example, the pseudo first-order rate constant for generation of **2** from **17** in 20 mM MOPS

Table 2. Second-order rate constants for inhibition of the *Enterobacter cloacae* P99 β-lactamase by phosphonate monoesters

Inhibitor	k_i (s^{-1} M^{-1})
15 (7.1)[a]	0.64[b]
16 (7.1)	0.042[b,c]
4 (11.6)	<0.001
3 (7.1)	0.076
5 (4.8)	0.92
2 (4.1)	46
13	5.6×10^4[d]
14	3.3×10^4[d]
8	890[c]
6	1.28×10^3
7	1.20×10^3
9	<5
10	130
11	3.4
12	610

Reactions were carried out in 20 mM MOPS buffer, pH 7.5 at 25 °C.
[a]Values of the pK_a of the leaving group are given in parentheses; that for enol pyruvate in **5** is taken from Kresge.[29]
[b]Taken from ref 9.
[c]Rate constant for the reactive enantiomer,assuming only one to be active (see text).
[d]Taken from ref 7.

buffer, pH 7.5, was 0.16 s^{-1}. Under the same conditions the rate constant for hydrolysis of **2** was $1.8 \times 10^{-6} s^{-1}$.

The ability of **2–12** to inhibit the class C β-lactamase of *Enterobacter cloacae* P99 is seen in the k_i values of Table 2. Again, some previously obtained data are included for comparison.

The data for the relatively nonspecific methylphosphonates show a rough correlation between k_i and the leaving group ability as represented by the pK_a of the leaving group. It does seem however that the larger aryloxide leaving groups may be associated with larger k_i values. Apparently there is little virtue in incorporating a further negative charge into the leaving group as in **3** and **4** despite the overall positive electrical potential of the active site[13] and the beneficial effect of negative charges on substrate molecules. This point presumably further reflects the different geometry of acyl- and phosphonyl-transfer transition states.[7]

As would be expected,[7,9] the more specific phosphonates with amido side-chains are, for a given leaving group, more effective than the methylphosphonates. The comparison of the sulfonamide **6** with **13** and **14** is of particular interest. It seems that the sulfonamide must be less comfortably hydrogen-bonded into the active site[12,13] than is a comparable carboxamide. This difference presumably reflects the tetrahedral geometry at sulfur in the sulfonamide, and probably also the more effective competition by water for the more polar sulfonamide than the carboxamide, i.e., weaker noncovalent association with the enzyme by the former compound. Thus although **6** is hydrolytically more stable than **13** and **14**, it is significantly poorer as an inhibitor of the P99 β-lactamase. It might be noted that sulfonamido-β-lactams appear to be poorer antibiotics and poorer β-lactamase substrates than analogous carboxamides, presumably for similar reasons.[16] Their sulfones, however, may be more effective mechanism-based inhibitors.[17]

A more striking result is provided by the 2,4-dinitrophenyl phosphonate **7**. Despite its very good leaving group and impressive hydrolytic stability, this compound is no better an inhibitor of the P99 β-lactamase than the analogous 4-nitrophenyl ester **6**. This result might reflect a change in the rate-determining step of the inhibition reaction, or more likely, since more rapid inhibition is certainly possible (cf. **13** and **14**), direct, and unfavorable in this case, interaction between the leaving group and the enzyme in the phosphonylation transition state. This result, taken together with the data for the methylphosphonates described above, represents the first clear evidence for β-lactamase participation in the process of leaving group departure from a phosphonate inhibitor. Although the enzyme is unlikely to have specific functional groups to assist this departure,[7] there may be nonspecific interactions and perhaps yet-to-be-discovered specific interactions that promote or retard the reaction. Inspection of a model consisting of a phosphorane bound to the active site

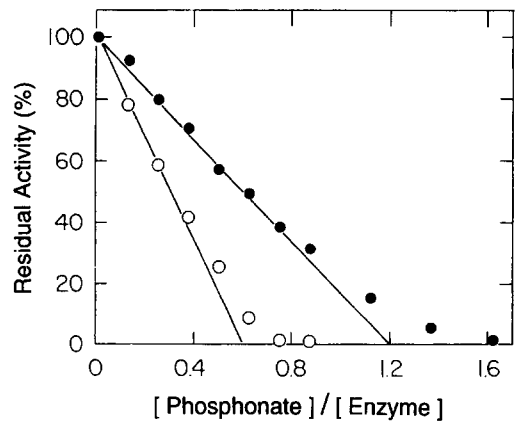
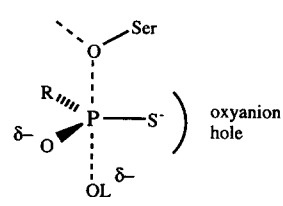


Figure 1. Residual activity of the P99 β-lactamase as a function of the ratio of phosphonate to enzyme concentration. The phosphonates represented are **8** (●) and **14** (○).

serine and constructed from the crystal structure of a P99–phosphonate complex,[13] suggested that interaction might occur between a bulky leaving group (the *o*-nitro substituent would be suspect in this regard) and the side-chain of the inhibitor itself, the residues of the adjacent β-strand, and/or Tyr 150 when in position to catalyze phosphonylation of Ser 64.[13]

Both of the thiophosphates **16** and **8** are considerably less effective than their oxo analogues, **15** and **14**, respectively. This correlates well with the relative effectiveness of thiono and oxo phosphonate diesters with serine proteinases and esterases.[18–20] Titration of the P99 β-lactamase with **8** and **14** yielded the results shown in Figure 1. It is clear that twice as much **8** as **14** is required to completely inactivate the enzyme. The inhibition by **14** has 1:1 stoichiometry (the enzyme employed has some 60% of its active sites functional[7]) as has been observed with the other phosphonates.[5,7] The result for **8** shown in Figure 1 suggests that only half of the sample of **8** is an inhibitor. This presumably means that one enantiomer (**8** is chiral at phosphorus) is active while the other is not. In view of the difficulty experienced by the P99 enzyme with thionosubstrates[21] it seems likely, as proposed previously,[9] that the unreactive enantiomer would have S-stereochemistry at phosphorus and thus, from the crystal structure of a phosphonate complex,[12] be required to pass through the transition state **19** during the phosphorylation reaction. The active enantiomer would thus be R_p, passing through the transition state **20** where occupation of the oxyanion hole by sulfur is not required. The possible sources of difficulty encountered by sulfur in the



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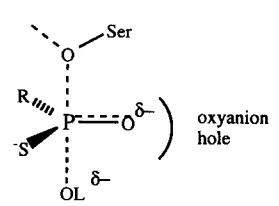

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oxyanion hole have been discussed in detail elsewhere.[9,21]

Finally, the data of Table 2 indicate that methyl substitution, either D or L, adjacent to phosphorus, slows the phosphorylation of the P99 β-lactamase active site. The D-methyl group in particular has a strongly negative effect. Inspection of the model referred to above suggested that a D-methyl substituent might interact with the side-chain amide oxygen of the inhibitor itself, thus possibly affecting side-chain binding, with the leaving group, and/or with Tyr 150 as it approaches Ser 64 O_γ. An L-methyl group may be less disruptive with possible interactions with the leaving group and the β-strand.

The general trends noted above with respect to the P99 β-lactamase also largely apply to the effect of **6–12** on representative class A β-lactamases and on the R61 DD-peptidase (Table 3). The 2,4-dinitrophenyl phosphonate **7**, however, is significantly more effective than the 4-nitrophenyl compounds **14** and **6** against the TEM-2 β-lactamase, although is less so against the PCl and the DD-peptidase. The latter enzyme is an example of a bacterial transpeptidase and is inhibited by β-lactams.[22] Methyl substitution, as present in **11** and **12**, depresses the activity of **14** against both the TEM-2 β-lactamase and the DD-peptidase. The latter result is of interest since analogous D-methyl substitution in specific amide substrates leads to greatly enhanced reactivity.[22] However, on the other hand, this methyl effect is not observed when the substrate is an analogous depsipeptide.[23] The phosphonates appear to follow the latter precedent—the specific interaction of the D-methyl group in amide hydrolysis is apparently not present or advantageous in the phosphonylation transition state.

The results of this study show that although a better leaving group may lead to a better phosphonate β-lactamase inhibitor,[6,7] the correlation is influenced by other factors. The side-chain is important, but a compromise must be struck between the ability of the leaving group (and concomitant hydrolytic stability) and enzyme specificity. Probably the most interesting finding is that of leaving group specificity beyond that dictated purely by pK_a and suggestive of leaving group interaction with the enzyme. This raises the possibility of specific leaving group design. Alkyl substitution adjacent to phosphorus does not seem immediately promising unless, perhaps, specific polar interactions were to be included.[24]

Table 3. Second-order rate constants for inhibition of other enzymes by phosphonate monoesters

	k_i (s^{-1} M^{-1})		
Inhibitor	**TEM**	**PCl**	**R61**
14	2.7[a]	2.5[a]	0.07[a]
6	0.36	0.43	—
7	46	9.4	0.06
8	0.073	0.52	—
11	0.10	—	≤0.01
12	0.08	—	≤0.01

Abbreviations: TEM, TEM-2 plasmid β-lactamase; PCl, β-lactamase of *S. aureus* PCl; R61, DD-peptidase of *Streptomyces* R61.
[a]Taken from ref 6.

Experimental

Materials

The β-lactamases were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. The Streptomyces R61 DD-peptidase was generously provided by Dr J.-M. Frére of the University of Liège, Liège, Belgium. Typical specific activities for these preparations have been reported previously.[14] Benzylpenicillin was purchased from Sigma Chemical Co. Cephalothin was a gift from Eli Lilly & Co. As previously observed,[7,15] the phosphonates were hygroscopic and unsuitable for combustion analysis and thus they were characterized by ^1H and ^{31}P NMR spectroscopy. Chemical shift standards were sodium 3-(trimethylsilyl)-1-propanesulfonate (internal) and 85% phosphoric acid (external), respectively.

Syntheses

Bis(2,4-dinitrophenyl)methylphosphonate (17). Under protection of a nitrogen atmosphere, a mixture of 2,4-dinitrophenol (2.0 g, 10.9 mmol) and methylphosphonic dichloride (1.2 mL, 13.3 mmol) was stirred, heated to 110–120 °C, and maintained at that temperature overnight. The reaction mixture was then allowed to cool to room temperature and the solid product recrystallized from methylene chloride, yielding 2.0 g (86%) of almost colorless crystals, mp 125–128 °C. NMR spectra confirmed the presence of the diester: ^1H (C^2HCl_3) δ 2.20 (d, 3H, J = 18 Hz, CH_3P), 7.71 (d, 2H, J = 8 Hz, ArH), 8.47 (d, 2H, J = 8 Hz, ArH), 8.89 (s, 2H, ArH); ^{31}P (C^2HCl_3) δ 25.8 (q, J = 18 Hz).

Sodium 4-nitrophenyl *N*-(phenylmethylsulfonyl)aminomethylphosphonate (6). Dimethyl aminomethylphosphonate was prepared as described by Jacobsen and Bartlett[25] and sulfonylated in chloroform by means of phenylmethylsulfonyl chloride in the presence of triethylamine. The phosphonic acid was then prepared by treatment of the dimethyl ester with trimethylsilyl bromide according to the procedure of Chakravarty et al.[26] A solution of the phosphonic acid (0.18 g, 0.68 mmol) in a mixture of thionyl chloride (1 mL), dichloromethane (2 mL), and dimethylformamide (50 μL) was stirred for ~4 h at room temperature. The volatiles were then removed by evaporation under vacuum and the residual dichloride taken up into dimethylformamide (1 mL). A solution of 4-nitrophenol

(95 mg, 0.68 mmol) and triethylamine (95 μL, 0.68 mmol) in dimethylformamide (1 mL) was added dropwise with stirring at room temperature. The reaction was allowed to proceed for 5 h at 37 °C after which it was cooled and 0.1 M aqueous sodium hydroxide (2 mL) added. The product was isolated by evaporation of the solvents under vacuum and then by elution with water from a Sephadex G-10 column. The purified product (10–20 mg) was characterized by its ^1H NMR spectrum: (^2H_2O) δ 3.40 (d, 2H, J = 12 Hz, CH_2P), 4.55 (s, 2H, CH_2S), 7.42 (d, 2H, J = 10 Hz, p-NO_2ArH), 7.47 (m, 5H, ArH), 8.25 (d, 2H, J = 10 Hz, p-NO_2ArH).

Bis(2,4-dinitrophenyl) *N*-(phenylmethylsulfonyl)aminomethylphosphonate (18). This compound was prepared from *N*-(phenylmethylsulfonyl)aminomethylphosphonic dichloride (see above) in the same way as **17**. ^1H NMR spectrum (C^2HCl_3) δ 3.85 (t, 2H, J = 7.5 Hz, CH_2P), 4.40 (s, 2H, CH_2S), 4.86 (br, 1H, NH), 7.43 (m, 5H, ArH), 7.82 (d, 2H, J = 10 Hz, NO_2ArH), 8.51 (d, 2H, J = 10 Hz, NO_2ArH), 8.90 (s, 2H, NO_2ArH).

Lithium acetyl methylphosphonate (5). A modification of the method of Lazarus et al.[27] was used for this synthesis. Thus, a solution of methylphosphonic dichloride (1 mL, 11.0 mmol) in dry diethyl ether (20 mL) was added dropwise to a stirred suspension of freshly prepared and dried silver acetate (4.6 g, 27.4 mmol) in diethyl ether (50 mL). The reaction was then allowed to proceed overnight at room temperature, shielded from light. Solids were then removed by filtration, the filtrate received into a flask containing lithium hydroxide monohydrate (0.45 g, 10.7 mmol) dissolved in water (2 mL) and kept cold in an ice–water bath. The resultant solution was stirred at 0 °C for about 20 min, most of the diethyl ether removed by rotary evaporation at 0 °C, and 50 mL acetone added. This mixture was stirred at 0 °C until precipitation of the product was complete at which time the precipitate was collected by filtration. The yield of the lithium salt was 0.58 g (37%). NMR spectra of the product agreed with those published:[27] ^1H (^2H_2O) δ 1.52 (d, 3H, J = 18 Hz, CH_3P), 2.19 (s, 3H, CH_3C); ^{31}P (^2H_2O) δ 21.6 (q, J = 18 Hz). The spectra also indicated the presence of methylphosphonate at a level of about 10 mol %—this too is comparable to that reported in the literature.[27]

P^1P^2-Dimethylpyrophosphonic acid (3). This compound was prepared by the method of Ruveda et al.[28] and purified by recrystallization from 1:1 acetic acid:benzene. The melting point was then 141–143 °C (literature value[28] 139.5–141.5 °C). NMR spectra: ^1H (^2H_2O) δ 1.30 (m); ^{31}P (^2H_2O) δ 18.3 (m).

Cyclohexylammonium hydrogen methylphosphonoenolpyruvate (4). The procedure of Lazarus et al.[27] was also used to prepare this compound. NMR spectra of the product were very similar to those published:[27] ^1H (^2H_2O) δ 1.1–2.1 (m, 10H, cyclohexyl CH_2), 1.45 (d, 3H, J = 18 Hz, CH_3P), 3.19 (m, 1H, cyclohexyl CH), 5.52 (t, 1H, J = 2 Hz, CH=), 5.90 (t, 1H, J = 2 Hz, CH=); ^{31}P (^2H_2O) δ 22.6 (q, J = 18 Hz).

Triethylammonium *p*-nitrophenyl *N*-(benzyloxycarbonyl)aminomethylthiophosphonate (8). *N*-(Benzyloxycarbonyl)aminomethylphosphonic dichloride was prepared from the phosphonic acid (400 mg, 1.6 mmol) and thionyl chloride as previously described.[8] This compound was dissolved in 10 mL of dry dichloromethane and stirred at room temperature with *p*-nitrophenol (222 mg, 1.6 mmol) and triethylamine (0.245 mL, 1.76 mmol) for 2 h. The resulting solution, diluted to 100 mL with dichloromethane, was then cooled to 0 °C, more triethylamine added (0.49 mL, 3.52 mmol), and H_2S gas bubbled through for 1 h. After the solution was stirred for a further 1 h at 0 °C, it was washed with water (2 × 50 mL) and the organic layer dried and evaporated. The product was then purified by Sephadex QAE-25 chromatography as described for the oxo analogue.[15] It was eluted by a linear triethylammonium bicarbonate gradient (0–1 M), which separated it from the oxo analogue—the oxo and thio compounds eluted at 0.56 and 0.90 M triethylammonium bicarbonate, respectively. The product was characterized by NMR spectroscopy: ^1H (^2H_2O) δ 3.75 (ABX, 2H, J = 15, 7.9, 4.4 Hz CH_2P), 5.15 (s, 2H CH_2O), 7.20, 8.20 (d, 2H, J = 10 Hz, p-NO_2ArH), 7.45 (br, 5H, ArH); ^{31}P (^2H_2O) δ 73.2.

The sodium salts of D-(9) and L-(10) *p*-nitrophenyl 1-(*N*-phenylacetylamino)-ethylphosphonate and D-(11) and L-(12) *p*-nitrophenyl 1-(*N*-benzyloxy-carbonyl-amino)ethyl-phosphonate. The acylaminophosphonic acids were prepared by reaction of D- and L-1-aminoethylphosphonic acids (Fluka) with the appropriate acid chloride as previously described.[15] These were coupled with *p*-nitrophenol in the presence of trichloroacetonitrile and the resulting esters purified by Biogel P2 chromatography.[15] The products were characterized by NMR spectroscopy: **11** and **12**: ^1H (^2H_2O) δ 1.40 (dd, 3H, J = 14.4, 7.0 Hz, CH_3), 4.00 (dq, 1H, J = 7.0, 13.2 Hz, CH), 5.01 (ABq, 2H, J = 12 Hz, CH_2), 7.17, 8.08 (d, 2H J = 10 Hz, p-NO_2ArH), 7.3 (m, 5H, ArH); ^{31}P (^2H_2O) δ 16.2. **9** and **10**: ^1H (^2H_2O) δ 1.41 (dd, 3H, J = 14.4, 7.0 Hz, CH_3), 3.56 (s, 2H, CH_2), 4.30 (dq, 1H, J = 7.0, 13.2 Hz, CH), 7.17, 8.15 (d, 2H, J = 10 Hz, p-NO_2ArH), 7.3 (m, 5H, ArH); ^{31}P (^2H_2O) δ 16.9.

Analytical and kinetic methods

Stock solutions of enzymes and phosphonates were prepared as previously described[6,7] except that those of the diesters **17** and **18** were prepared in dry acetonitrile. All kinetic measurements were made in solutions at 25 °C in 20 mM MOPS buffer, pH 7.5. Rates of reaction of phosphonates with the various enzymes were determined spectrophotometrically, either from measurements of enzyme activity against benzylpenicillin as a function of time or directly by monitoring of phenoxide release at either 400 nm (*p*-nitrophenoxide) or 360 nm (2,4-dinitrophenoxide). Both methods have been

employed previously[6,7] and, in any particular case, give the same rate constants for enzyme inactivation within experimental uncertainty. Titration of the P99 β-lactamase with **8** and **14** was carried out by incubation of appropriate concentrations of enzyme and phosphonate together at 25 °C for 5 min (the same result was obtained from a 30 min incubation) followed by β-lactamase assay against benzylpenicillin.

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