Structure–activity relationships in the inhibition of serine β -lactamases by phosphonic acid derivatives

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A new series of phosphonyl derivatives has been prepared and tested for inhibition of serine (classes A and C) β -lactamases. The results were compared with those previously acquired with aryl phosphonate monoesters and with alkaline hydrolysis rates. A methyl *p*-nitrophenyl phosphate monoanion was markedly poorer as an inhibitor of the class C β -lactamase of *Enterobacter cloacae* P99 than a comparable *p*-nitrophenyl phosphonate. Phosphonyl fluorides, thiophenyl esters, *N*-phenylphosphonamides and a *p*-nitrophenyl thionophosphonate were, in

general, comparable with *p*-nitrophenyl phosphonates in inhibitory power. The incorporation of a specific amido side chain led to an increase in the rates of inhibition of around 10^4 -fold. Apparently unresponsive to the addition of the side chain to the enzyme was *N*-phenyl methylphosphonamidate, where binding of the side chain may interfere with access of the leaving group to a proton which is necessary to active-site phosphorylation and inhibition. Typical class A β -lactamases were significantly more refractory than the class C enzyme to all of these reagents.

INTRODUCTION

In recent years we have demonstrated that phosphonate monoester monoanions of structure 1 are surprisingly effective inhibitors of the serine β -lactamases [1–3]. This discovery is of intrinsic interest, but beyond that it promises a new source of information on the much debated [4–8], but elusive, mechanism of β -lactamase-catalysed hydrolyses. The latter point derives from the fact that the inhibition of β -lactamases by 1 involves phosphorylation of the active-site serine hydroxy group, forming 2, which is likely to be an analogue of the transition-state or tetrahedral intermediate formed during either the acylation or deacylation step of turnover of depsipeptide substrates 3 [3]. Structural studies of these complexes ([9]; J. R. Knox, J. Rahil and R. F. Pratt, unpublished work) should reveal details of active-site functional group placement in the transition states of catalysis.

Previous studies [2,3] with aryl phosphonates, i.e. with aryloxy leaving groups, have indicated that better leaving groups give better inhibitors, and the mechanistic reasons for this have been discussed [3]. Beyond the aryloxy group, few variations in inhibitor structure have yet been explored. In the present paper the effects of structural variation at the phosphyl centre and, more broadly, at the leaving group, are described. The compounds prepared and examined for serine β -lactamase inhibitory activity include the methylphosphyl species 4–8 and the more β -lactamase specific, through the amido side chain, 9–11. The

inhibition results are compared with those previously obtained with the *p*-nitrophenyl phosphonates 12 and 13.

EXPERIMENTAL

Materials

The β -lactamases were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts, U.K.) and used as received. Typical specific activities for these preparations were as previously reported [10]. Benzylpenicillin was purchased from Sigma Chemical Co. The triethylammonium salts of *p*-nitrophenyl methylphosphonic acid (12), *p*-nitrophenyl *N*-(benzyloxycarbonyl)aminomethylphosphonic acid (13), *N*-(phenyl)methylphosphonamidate (7) and the potassium salt of *N*-phenyl *N'*-(benzyloxycarbonyl)aminomethylphosphonamidate (10) were available from previous and current studies [11,11a]. The other phosphonates were prepared as described below. Their salts were hygroscopic and unsuitable for combustion analysis and thus the identity and molecular purity of these compounds were established by ^1H and proton-decoupled ^{31}P n.m.r. spectroscopy, reported below [sodium 3-(trimethylsilyl)-1-propanesulphonate (internal) and 85 % phosphoric acid (external) respectively were used as chemical-shift standards].

Syntheses

Triethylammonium thiophenyl methylphosphonate (6)

Freshly distilled thiophenol (1.32 g, 12 mmol) together with 1.1 equiv. of triethylamine in 100 ml of dry diethyl ether was added dropwise to a stirred solution of methylphosphonic dichloride (Aldrich; 1.59 g, 12 mmol) in 100 ml of diethyl ether at 0 °C. The triethylammonium chloride by-product appeared instantaneously as a thick white solid. After the reaction mixture had been stirred for a further 2 h at 0 °C, the precipitate was removed by vacuum filtration. To the filtrate, 3 equiv. of aqueous KOH were added (50 ml of a 0.48 M solution) and the mixture was held on a rotary evaporator until all volatile compounds were lost. The remaining aqueous solution was freeze-dried. N.m.r. spectroscopy confirmed the presence of the desired compound in about 70 % yield. Purification was achieved by anion exchange chromatography on quaternary aminoethyl (QAE)-Sephadex, by elution with a triethylammonium bicarbonate gradient (0–1.0 M). The product showed the expected n.m.r. spectral characteristics: ^1H ($^2\text{H}_2\text{O}$) chemical shift (δ) 1.50 (d, J 15 Hz, 3H, CH_3P), 7.4–7.6 (m, 5H, ArH), 1.25 (t, J 7.5 Hz, 9H, CH_3), 3.20 (q, J 7.5 Hz, 6H, CH_2); ^{31}P (H_2O) δ 42.69.

Triethylammonium *p*-nitrophenyl methylthionophosphonate (5)

This compound was synthesized from methylthionophosphonic dichloride (Fluka) and purified essentially as described above for 6. The product exhibited the following characteristics: ^1H [$^2\text{H}_2\text{O}$] δ 1.9 (d, J 15 Hz, 3H, CH_3P), 7.4–8.3 (dd, 4H, ArH), 1.3 (t, J 7.5 Hz, 9H, CH_3), 3.2 (q, J 7.5 Hz, 6H, CH_2) and ^{31}P [(H_2O)] δ 78.78] n.m.r. spectra.

Triethylammonium *p*-nitrophenyl methyl phosphate (4)

This compound was also synthesized and purified by the general procedures described above from *p*-nitrophenyl phosphorodichloridate (Aldrich). ^1H n.m.r. spectrum: ($^2\text{H}_2\text{O}$) δ 3.70 (d, 3H, J 10 Hz, CH_3OP), 7.35 and 8.30 (dd, 4H, ArH), 1.3 (t, J 7.5 Hz, 9H, CH_3), 3.2 (q, J 7.5 Hz, 6H, CH_2); ^{31}P n.m.r. spectrum (H_2O) δ -2.69.

Potassium methylphosphonofluoridate (8)

Aq. KOH (10 mmol; 10 ml of 1 M KOH) was added dropwise to a stirred solution of methylphosphonic difluoride (Johnson Matthey Electronics) in acetonitrile (5 mmol; 0.5 g in 5 ml). The pH of the solution was monitored with a pH-meter and was kept at about 7. After the addition was complete, the solution was freeze-dried. N.m.r. spectra showed only the required compound: ^1H ($^2\text{H}_2\text{O}$) δ 1.45 (dd, $J_{\text{P-CH}_3}$ 17.6 Hz, $J_{\text{F-P-CH}_3}$ 5.6 Hz, CH_3P); ^{31}P (H_2O) δ 29.12 (d, J_{PF} 934 Hz).

Triethylammonium *N*-(benzyloxycarbonyl)aminomethylphosphonofluoridate (11)

N-(Benzyloxycarbonyl)aminomethylphosphonic dichloride was prepared from the phosphonic acid (200 mg, 0.8 mmol) on stirring with thionyl chloride (1 ml) for 1 h at room temperature, followed by removal of the volatile compounds under vacuum. Water was removed from tetrabutylammonium fluoride hydrate (Aldrich) by an azeotropic distillation with benzene followed by vacuum dessication overnight at room temperature. The dried fluoride (0.42 g, 1.6 mmol) in dichloromethane (2 ml) was added to the phosphonic dichloride dissolved in dichloromethane

(2 ml) at 0 °C. After the addition was completed, the reaction mixture was stirred for 0.5 h at 0 °C and another 0.5 h at room temperature. Aq. NaOH (16 ml of a 0.1 M solution) was then added at room temperature. Dichloromethane was removed by rotary evaporation and the remaining aqueous solution was freeze-dried. ^1H -n.m.r. spectroscopy of the crude product showed the presence of the desired product in about 45 % yield. After purification by QAE-Sephadex chromatography, n.m.r. spectra of the product were in accord with the required compound: ^1H ($^2\text{H}_2\text{O}$) δ 3.55 (dd, 2H, $J_{\text{P-CH}}$ 12 Hz, $J_{\text{F-P-CH}_2}$ 6 Hz, CH_2P) 5.20 (s, 2H, CH_2O), 7.45 (m, 5H, Ar), 1.35 (t, J 7.5 Hz, 9H, CH_3), 3.20 (q, J 7.5 Hz, 6H, CH_2); ^{31}P (H_2O) δ 22.01 (d, J_{PF} 1000 Hz).

Triethylammonium thiophenyl *N*-(benzyloxycarbonyl)-aminomethylphosphonate (9)

The synthesis of this compound largely followed the example for 11 given above, but with the following variation, required because of the lability of the product. Instead of adding aq. NaOH to the dichloromethane solution of the intermediate monochloridate monoester ($\text{PhCH}_2\text{OCONHCH}_2\text{POClSPh}$), 2 ml of aq. 2 M triethylammonium bicarbonate was added and the resulting mixture stirred for 0.5 h at 0 °C. Rotary evaporation then removed all volatile material. Some precipitate, probably diphenyl disulphide, appeared in the remaining aqueous solution and was discarded after filtration. The filtrate was then freeze-dried. The resulting solid was re-dissolved in 1–2 ml of water and the desired product was extracted into dichloromethane. The solvent was evaporated and the solid residue kept *in vacuo* overnight. N.m.r. spectra confirmed the presence of the desired compound in pure form: ^1H ($^2\text{H}_2\text{O}$) δ 3.5 (d, 2H, J 15 Hz, CH_2P), 5.05 (s, 2H, CH_2O), 7.35 (m, 5H, ArH), 1.20 (t, J 7.5 Hz, 9H, CH_3), 3.1 (q, J 7.5 Hz, 6H, CH_2); ^{31}P (H_2O) δ 39.78.

Analytical and kinetic methods

Methods of preparation of enzyme and phosphonate solutions and of their employment in the determination of second-order rate constants of inhibition by measurements of enzyme activity as a function of time were essentially as described in previous studies [2,3]. All kinetic measurements were made at 25 °C in 20 mM Mops buffer, pH 7.5. The concentration of 8 in stock solutions was estimated by assuming that the solid product of the preparative reaction described above was a 1:1 mixture of 8 and KF. Alkaline hydrolysis rate constants of phosphonate esters were obtained from spectrophotometric rate measurements, monitoring *p*-nitrophenoxide production at 400 nm and thiophenoxide at 263 nm in KOH solutions. The alkaline hydrolysis rate constant of 8 was obtained from ^1H -n.m.r. measurements of rates in NaO^2H solutions in $^2\text{H}_2\text{O}$.

RESULTS AND DISCUSSION

Compounds 4–11 were synthesized in order to further explore structure–activity relationships in the inhibition of serine β -lactamases by phosphyl species. The methyl compounds 4–8 were the initial targets since their syntheses are more straightforward than those with amido side chains, and compound 12 had shown inhibitory activity against the class C β -lactamase of *Enterobacter cloacae* P99 [3]. Subsequently, when 4–8 had revealed inhibitory activity, 9–11 were prepared in order to assess the effect of the amido side chain. Benzyloxycarbonyl rather than phenylacetyl derivatives were prepared because of their greater stability in aqueous solution [11]; this side-chain difference has

Table 1 Second-order rate constants for inhibition of the *Enterobacter cloacae* P99 β -lactamase by phosphonate monoanions

Reactions were carried out in 20 mM Mops buffer, pH 7.5, at 25 °C.

Inhibitor	k_i ($s^{-1} \cdot M^{-1}$)
12	0.64*
4	0.0018
5	0.021
6	10.5
7	1.6
8	0.79
13	3.3×10^4 *
9	2.6×10^4
10	0.5
11	2.6×10^4

* Taken from [3].

previously been shown to have little effect on inhibitory activity [2,3].

The inhibitory activities of 4–11 against the P99 β -lactamase were determined from measurements of enzyme activity versus time; second-order rate constants for the inhibition reaction are given in Table 1. Similarly, the activities of 9–11 against class A β -lactamases of *Staphylococcus aureus* (PC1), the TEM plasmid, and *Bacillus cereus* (β -lactamase I) were determined, with the results shown in Table 2; the methyl compounds 4–8 showed little activity against the class A enzymes. Alkaline hydrolysis rates for compounds 4–6, 8 and 12 were determined (Table 3) in order to assess relative susceptibilities to nucleophilic displacement. The absolute and, more particularly, comparative significance of these results are discussed below. Much can be directly interpreted in terms of a simple associative mechanism of displacement at phosphorus.

First, the smaller inhibitory activity of the phosphate diester (4) than the phosphonate (5) is readily understandable on chemical grounds: phosphonates are considerably more susceptible to nucleophilic cleavage than analogous phosphates in any mechanism with significant associative character [12–14]. The latter would likely be true for nucleophilic displacement at phosphate diester and phosphonate monoester monoanions [15,16]. This difference in reactivity is clear in the alkaline hydrolysis rates of 12 and 4 (Table 3).

Phosphoryl fluorides generally have a considerably higher reactivity towards nucleophiles than do the analogous *p*-nitrophenyl esters [17–19] and might thus be expected to be better inhibitors of serine hydrolases. The latter seems to be often [19–21], but not always [17,18,20], true. It should be noted, however, that the quoted precedents here involve neutral rather than anionic compounds. In the present instance, 8 is certainly more reactive towards hydroxide ion than 12, but the two compounds are comparably effective as P99 β -lactamase inhibitors. Similarly, the fluoride 11 is comparably active with 13 against the P99 β -lactamase (Table 1) and against the class A TEM and PC1 β -lactamases (Table 2). Strikingly, the *p*-nitrophenyl derivative 13 is more effective against the class A PC1 enzyme than is the analogous fluoride. This variability of relative effectiveness seems to indicate the existence of specific interactions with the leaving group in at least some of these enzymes, despite its likely position in the transition state at a site normally unoccupied by substrate [3].

The thionophosphonyl ester (5) hydrolysed in alkali at a very

Table 2 Second-order rate constants for inhibition of class A β -lactamases by phosphonate monoanionsReactions were carried out in 20 mM Mops buffer pH 7.5, at 25 °C. Abbreviations for β -lactamases: TEM, TEM plasmid β -lactamase; PC1, β -lactamase of *S. aureus* PC1; BCI, *B. cereus* β -lactamase I.

Inhibitor	k_i ($s^{-1} \cdot M^{-1}$)		
	TEM	PC1	BCI
13	2.7*	2.5*	0.32*
9	2.0	0.066	0.30
10	0.022	0.0028	0.12
11	2.1	0.34	0.31

* Taken from [2].

Table 3 Second-order rate constants for alkaline hydrolysis of phosphonate monoanions at 25 °C

Phosphonate	$10^5 \times k_{OH}$ ($s^{-1} \cdot M^{-1}$)
12	2.8
4	0.097
5	3.5
6	9.1
8	53

similar rate to its oxo analogue. This is not unprecedented, at least in neutral compounds where data are available, although oxo derivatives are usually somewhat more reactive [22,23]. In general also, neutral phosphonyl compounds are significantly better inhibitors of serine hydrolases than are their thiono analogues [17,20,21]. The latter result is in fact seen in the comparison of 5 and 12 as inhibitors of the P99 β -lactamase, where the latter, the oxo derivative, is some 30-fold more effective. The two cases, neutral versus anionic phosphonates, should not, however, be directly compared, since in the transition state for phosphorylation of the active-site serine hydroxy group by a neutral thionophosphonate 14, the $S^{\delta-}$ would occupy the oxyanion hole. The oxyanion hole of β -lactamases is known to resist the sulphur of thiocarbonyl substrates [24] and so may well also reject that of a thionophosphonyl inhibitor. The thionophosphonate (5) is presumably a 1:1 mixture of two enantiomers, *S* and *R*, at phosphorus. Reaction of one of these, the *S_p* isomer, as deduced from the crystal structure of a phosphonate complex [9], with the active site should generate the transition state 15, analogous to 14, where the sulphur occupies the oxyanion hole. This enantiomer should be a poor inhibitor. On the other hand the other, probably *R_p*, isomer should react

via the transition state **16**, where the oxygen more comfortably occupies the oxyanion hole.

This enantiomer should be the better inhibitor but, from the data of Table 1, it must still be at least 15 times poorer than **12**. This would not seem likely to be a result of the bulk of sulphur in this instance, since its position in **16** is probably that of the leaving group of a normal substrate [9]. It may reflect a less favourable interaction of the negative charge on S in **16**, which is more distant from the central phosphorus than is the analogous O in **15**, with the positive electrostatic field which is thought to promote the phosphorylation reaction [1–3].

The phosphonothiolate (**6**) hydrolysed in base at a rate comparable with that of the *p*-nitrophenyl ester, **12**. Although this observation seems reasonable in terms of the similar pK_a values of thiophenol and *p*-nitrophenol, it does give a clear indication of a strongly associative mechanism of the displacement reaction at phosphorus. The relative reactivity of phosphyl esters and thiol esters is a strong function of the transition-state structure, with highly dissociative transition states favouring the thiol ester by many orders of magnitude, probably because of the weakness of P–S versus P–O bonds [25]. The significantly greater effectiveness (18-fold) of **6** than **12** as an inhibitor of the P99 β -lactamase may reflect a slightly more dissociative transition state in the inhibition reaction than in alkaline hydrolysis.

The phosphoramidate (**7**) is of interest, since all precedent [26] suggests that the nitrogen atom of the leaving group must be protonated prior to, or concomitant with, departure. The fact that **7** is an inhibitor of the P99 β -lactamase at pH 7.5 shows that a proton must be obtainable under these conditions, although its source is not immediately evident. Indeed, the active-site functional groups are probably poorly placed to deliver such a proton [3]. Further insight into the source of the required proton can be achieved by careful analysis of the pH–rate profile of the inhibition reaction. Such profiles for **7**, **10** and related compounds are analysed in detail [11a]. In some phosphoramidates the dominant pathway involves a proton brought directly from solution with the inhibitor (as the zwitterion), while in others a less obvious route is needed to explain the results.

Addition of the amido side chain to enhance β -lactamase specificity led to greatly improved inhibitors in the cases of compounds **9** and **11** with the thiophenoxide and fluoride leaving groups, just as it had previously in the *p*-nitrophenyl compounds **13** versus **12** [3]. It is noticeable, however, that this change did not enhance the activity of **6** as much as it did **8** and **12**. The reason for this is not clear, but it may involve the greater restrictions imposed on the position of the phosphonyl group by specific binding of the amido side chain, i.e. the orientation of **6** and **12** may be slightly different in the binding mode optimal for their respective phosphorylations of the active-site serine, but **9** and **11** have less freedom to achieve such an optimal orientation: the steric requirements of the bipyramidal phosphorylation transition state are of course different for those of the tetrahedral transition states of acyl transfer.

Very noticeably, the reactivity of **7** is apparently not increased by incorporation of the amido side chain in **10**. Again the crucial issue is access to protons. Apparently at pH 7.5 that access is more limited in the more specific compound **10**. That suggests again limitations to the phosphorylation mechanism imposed by specific side-chain binding.

The *p*-nitrophenyl phosphonate (**13**) has been shown to be a weak inhibitor of class A β -lactamases. The new analogues, **9–12**, show comparable activity (Table 2). The most striking result here is perhaps the comparable inhibitory activity of the phosphoramidate (**10**) with that of the compounds with better leaving groups against *B. cereus* β -lactamase I. Apparently better access to protons is available in that active site.

In summary, this paper presents a substantial expansion of the range of phosphonates examined for β -lactamase-inhibitory potential. The results can be understood in terms of an associative phosphorylation mechanism where a pentaco-ordinated phosphorus intermediate/transition state, with the active-site serine hydroxy group and the leaving group occupying the apical positions, is involved. There is little evidence of strong interaction between the leaving group and the enzyme. This is perhaps not unexpected, since the leaving group at the apical position of a trigonal bipyramid should occupy a different position in space to that of a leaving group from the tetrahedral carbon of the normal acyl-transfer transition state. These results support those from the model-building of a pentaco-ordinated transition state into the active site of the class A PCI β -lactamase as revealed by a crystal structure. This modelling suggested that a conformation of the transition state involving little direct interaction between the enzyme and a nitrophenol leaving group was readily achievable [9]. This would seem to leave considerable scope for leaving group modulation and optimization in inhibitor/drug design. More elaborate leaving groups could presumably be devised to provide specific interactions.

The β -lactamase-specific amido side chain, as would be expected, generally improved the effectiveness of the phosphonate inhibitors. This again agrees with the crystal structure of the PCI- β -lactamase–phosphonate complex [9] from which strong hydrogen-bonding between the amido side chain on the enzyme, just as is believed to occur with substrates, was inferred. There is some indication from the present work, however, particularly with the phosphoramidates, that this interaction can be, in some circumstances, self-defeating, perhaps because the greater rigidity of the complex thereby formed can reduce the ability of the unnatural pentaco-ordinated transition state to optimize any positive contacts with the enzyme or minimize negative ones. Modulation of this effect by elaboration of the acyl group of the amido side chain may, however, be possible.

The phosphonates are, as previously noted [2], far more effective against the class C β -lactamase than against the class A enzymes. This no doubt correlates with the fact that the class C enzyme is more effective against the analogous acyclic substrates (**3**). The structural reasons for this difference are not known at present, but may involve the shape required of substrates for productive binding and reaction [27]. This will perhaps become evident when details of the class C β -lactamase crystal structures become available [28,29].

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