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Phosphonate monoester inhibitors of class A β-lactamases

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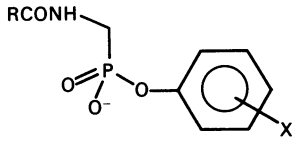
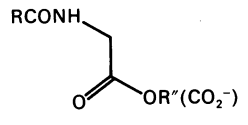
Phosphonate monoesters with the general structure:



are inhibitors of representative class A and class C β-lactamases. This result extends the range of this type of inhibitor to the class A enzymes. Compounds where X is an electron-withdrawing substituent are better inhibitors than the unsubstituted analogue (X = H), and enzyme inhibition is concerted with stoichiometric release of the substituted phenol. Slow turnover of the phosphonates also occurs. These observations support the proposition that the mechanism of action of these inhibitors involves phosphorylation of the β-lactamase active site. The inhibitory ability of these phosphonates suggests that the β-lactamase active site is very effective at stabilizing negatively charged transition states. One of the compounds described also inactivated the *Streptomyces* R61 D-alanyl-D-alanine carboxypeptidase/transpeptidase.

INTRODUCTION

The resistance of bacteria to β-lactam antibiotics is largely due to their ability to produce protective enzymes, the β-lactamases, which catalyse the hydrolysis of β-lactams [1,2]. The clinically important β-lactamases (of classes A, C and D, defined by amino-acid-sequence similarity [3]), are serine enzymes, i.e. they employ a serine hydroxy group as the primary active-site nucleophile, and catalyse β-lactam hydrolysis by a double-displacement mechanism involving an acyl-enzyme intermediate [4]. It has been shown in this laboratory that depsipeptides of structure **1** are also β-lactamase substrates [5,6] and, on this basis, the phosphonate monoester **2** was designed and found to be a β-lactamase inhibitor [7]. Compound **2**, however, appeared to have specificity limited to a representative class C β-lactamase and have no effect on class A β-lactamases. We show here how



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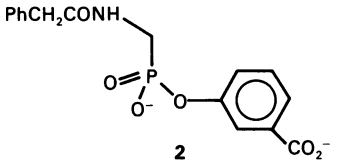

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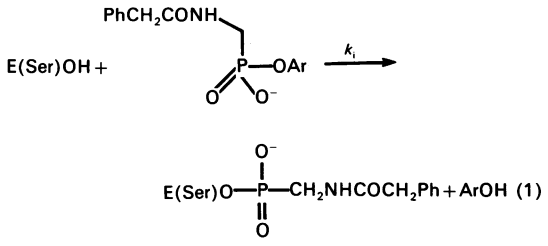
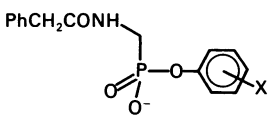
the specificity of the phosphonate inhibitors can be extended.

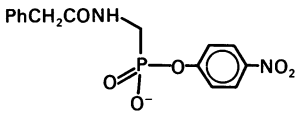
The phosphonate **2** appeared to inhibit the class C β-lactamase by phosphorylation of an active-site residue, most likely the serine-70 hydroxy group, according to eqn. (1) [7]:



Since an alkyl phosphonate analogous to **2** failed to inhibit either class A or class C enzymes [7], we surmised that the limited specificity of **2** might derive, in part at least, from too poor a leaving group (*m*-carboxyphenoxide) for the reaction of eqn. (1). Thus compounds **3** and **4** were prepared and tested for β-lactamase inhibitory activity.



3a X = H; **3b** X = m-NO_2;
3c, X = *p*-NO_2; **3d** X = *o*-NO_2



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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 [6]. The soluble D-alanyl-D-alanine transpeptidase/carboxypeptidase of *Streptomyces* R61 was generously provided by Dr. J.-M. Ghuysen and Dr. J.-M. Frère (University of Liège, Liège, Belgium). Benzylpenicillin was purchased from Sigma Chemical Co.

The phosphonates were prepared by trichloroacetonitrile-mediated condensation of phenols with phenylacetamidomethylphosphonic acid and chromatographically purified as described elsewhere [8].

Analytical and kinetic methods

Concentrations of the various enzymes were determined spectrophotometrically by employing absorption coefficients from the literature, as previously listed [6,9]. Concentrated stock solutions of the phosphonates were prepared in water. Since the phosphonate salts were hygroscopic and could not be accurately weighed out, phosphonate concentrations were determined from the absorption of the phenoxides after complete hydrolysis in

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hydroxide-ion solutions. Absorption coefficients of the relevant phenoxides were determined from the absorption of solutions prepared from purified phenols.

All inactivation kinetics were carried out in 20 mM-Mops buffer, pH 7.5, and 25 °C. The changes in β-lactamase activity against benzylpenicillin (determined spectrophotometrically at 232 nm [10]) with time after addition of a small portion of a phosphonate solution to a β-lactamase solution were determined. Final concentrations of phosphonates were much greater than those of the enzymes. Pseudo-first-order rate constants were obtained from semilogarithmic plots. The activity of the R61 transpeptidase/carboxypeptidase was determined against hippuryl-DL-phenyl-lactate [6].

Direct observations of the release of *p*-nitrophenoxide on reaction of the phosphonates **3c** and **4** was made spectrophotometrically at 400 nm. This data was fitted by a non-linear least-squares procedure [11] to eqn. (2):

$$A = A_0 + v_0 t + \pi(1 - e^{-\lambda t}) + v_s t \quad (2)$$

where A is the absorbance at any time, A_0 is the zero-time absorbance, v_0 is the initial rate of non-enzymic hydrolysis, v_s is the initial rate of the steady-state enzyme-catalysed reaction, π is the burst amplitude, and λ the burst pseudo-first-order rate constant (phosphonate concentrations were at least 10 times those of enzymes in these experiments). Values of v_0, that led to a small correction term in eqn. (2) were determined from control experiments in the absence of β-lactamase.

RESULTS AND DISCUSSION

Phosphonates **3** and **4** inhibited, in general, not only a representative class C β-lactamase (that of *Enterobacter cloacae* P99; J. Rahil & R. F. Pratt, unpublished work), but also three typical class A enzymes (*Bacillus cereus* β-lactamase I and the β-lactamases of *Staphylococcus aureus* PC1 and the TEM plasmid). The latter observation is the more interesting, since it expands the range of effectiveness of phosphonates against β-lactamases. The inhibition progressed to essentially complete inactivation of these enzymes and, under conditions where the inhibitor concentration was much greater than that of the enzyme, gave first-order kinetics in all cases. The inhibition appeared to be irreversible and presumably covalent, since it could not be alleviated by dilution of the inhibitor. The pseudo-first-order rate constants for inhibition of the TEM enzyme by **4** showed a linear relationship with the concentration of **4** up to 1.3 mM, suggesting that the initial binding of the phosphonates to these enzymes was weak, just as had been previously observed for **2** in the inhibition of the class C *Enterobacter cloacae* P99 β-lactamase [7]. On this basis, second-order rate constants, k_i, were obtained from the slopes of linear plots of the pseudo-first-order rate constants versus inhibitor concentration and are reported in Table 1.

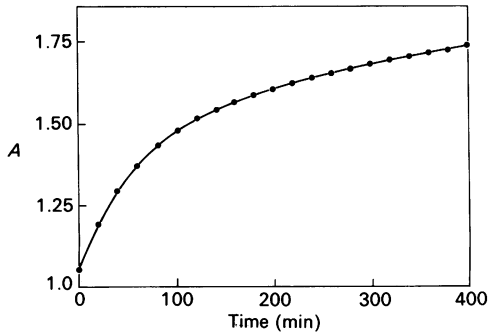


Fig. 1. Spectrophotometric (400 nm) observation of release of *p*-nitrophenoxide on inhibition of *B. cereus* β-lactamase I (48 μM) by 4 (680 μM)

The points are experimental, and the line was calculated as described in the text.

The general order of effectiveness of **3** and **4** was thereby **4 > 3d ⩾ 3c > 3b > 3a.** This result for the latter three compounds suggests that the leaving-group ability does correlate with inhibitor effectiveness, as might be anticipated for the phosphonylation mechanism of eqn. (1). It is also now clear that the carboxylate group of **2** is not essential for inhibition, although it may still facilitate binding in some cases and dictate specificity; the latter is indicated by the ineffectiveness of **2** against the class A β-lactamases, although its leaving group is comparable with that of **3a**. The absence of the carboxylate may change, and perhaps improve, the permeability of these compounds through the outer membrane of Gram-negative bacteria. These points need further investigation.

Direct evidence that eqn. (1) applied to these inhibition reactions was supplied by the observation that, as with **2** and P99 enzyme [7], a burst of phenol accompanied enzyme inactivation. Fig. 1 shows stoichiometric release of *p*-nitrophenoxide on reaction of **4** with *B. cereus* β-lactamase I. This data was fitted to eqn. (2) as described in the Experimental section. The rate constant for *p*-nitrophenoxide release ($k_i = \lambda/I_0 = 0.39\ s^{-1} \cdot M^{-1}$ from the data of Fig. 1, where I_0 is the initial phosphonate concentration) was closely similar to that for loss of β-lactamase activity ($0.32\ s^{-1} \cdot M^{-1}$ from Table 1), which also supports eqn. (1) as the mechanism of inhibition. Similar bursts were observed on reaction of **4** with the PC1 and TEM enzymes, and with the inhibitor **3c**. These compounds, or more strongly chromophoric or fluorophoric analogues, may be useful as active-site titrants for β-lactamases.

It is noticeable in Fig. 1 that slow *p*-nitrophenol release ($v_s/E_0 = 1.5 \times 10^{-5}\ s^{-1}$, where E_0 is the initial enzyme concentration) persists beyond the burst. This was observed with the TEM enzyme also. Note that the background hydrolysis rate, v_0, was considerably less than the steady-state rate observed (about 50-fold less in the example shown in Fig. 1). A weak phosphonatase activity of the P99 β-lactamase against **2** has previously been noted [7]. The β-lactamases in general, therefore, may be very weak phosphonate monoesterases.

Although **3** and **4** are not as effective as inhibitors of the class A β-lactamases, as is **2** of the class C P99 enzyme [7], the most interesting result here, as there, is that they are inhibitors at all, and particularly by a mechanism involving phosphonate cleavage. This suggests that the β-lactamase active site, in contrast

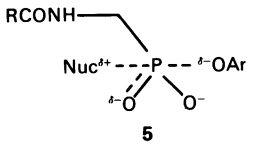
Table 1. Second-order rate constants of inhibition of class A β-lactamases by aryl phosphonates

Reactions were carried out in 20 mM-Mops buffer, pH 7.5, at 25 °C. Abbreviations for lactamases: TEM, TEM plasmid β-lactamase; PC1, β-lactamase of *Staphylococcus aureus* PC1; BC1, *Bacillus cereus* β-lactamase I.

	k_i ($s^{-1} \cdot M^{-1}$)		
Inhibitor	TEM	PC1	BC1
4	2.7	2.5	0.32
3c	0.87	4.0	0.020
3d	1.3	0.83	0.012
3b	0.038	0.11	-*
3a	-*	0.046	-*

* No inhibition was observed, which, in view of the conditions employed, implies a k_i value of significantly less than $0.01\ s^{-1} \cdot M^{-1}$.

with the serine-proteinase active site [12], is able to stabilize very effectively a transition state resembling **5**. The β-lactamase active site is characterized by the presence of much positive charge [13–15], which may be employed to stabilize the transition states of



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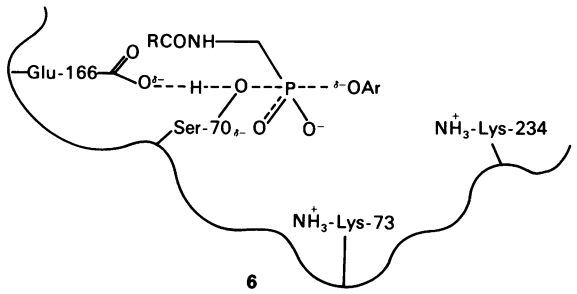
normal substrates [12] and of phosphonate monoesters (**6**); in **6**, the numbering system of Ambler [3] is employed, and the general-base role of Glu-166 is speculative [12]. Also present to stabilize the β-lactamase transition state, and presumably also **5**, are an oxyanion hole [9,13], the α2-helix macrodipole [13,15] and, perhaps, Arg-244 [15]. Second-order rate constants for cleavage of *p*-nitrophenyl methylphosphonate at 25 °C by water and by hydroxide ion can be estimated from the data provided by Edwards and co-workers [16] to be 2.8×10^{-11} $s^{-1} \cdot M^{-1}$ and 1.72×10^{-5} $s^{-1} \cdot M^{-1}$ respectively. To the extent that the transition states of these reactions are models for **6**, the PC1 β-lactamase has stabilized the transition state by 63.5 $kJ \cdot mol^{-1}$ (15.2 $kcal \cdot mol^{-1}$) or 30.5 $kJ \cdot mol^{-1}$ (7.3 $kcal \cdot mol^{-1}$) respectively.



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The phosphonate **4** was also a weak inhibitor ($k_i = 0.07$ $s^{-1} \cdot M^{-1}$) of the *Streptomyces* R61 D-alanyl-D-alanine carboxypeptidase/transpeptidase, a model enzyme for the bacterial cell-wall cross-linking enzymes [17]. Thus phosphonate antibiotics may be possible.

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