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Inverse Acyl Phosph(on)ates: Substrates or Inhibitors of β-Lactam-Recognizing Enzymes?

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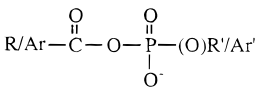
Acyl phosph(on)ates represent a new class of inhibitors of β-lactam—recognizing enzymes. Previously described members of this class were aroyl phosph(on)ates. These compounds have been shown to acylate and/or phosphylate the active site serine residue, leading to either transient or essentially irreversible inhibition [Li, N., and Pratt, R. F. (1998) *J. Am. Chem. Soc.* **120**, 4264–4268]. The present paper describes the synthesis and evaluation as inhibitors of an inverse pair of acyl phosph(on)ates that incorporate the amido side chain that represents a major substrate specificity determinant of these enzymes. Thus, *N*-(phenylacetyl)glycyl phenyl phosphate and benzoyl *N*-(benzyloxycarbonyl)aminomethyl phosphonate were prepared. The former of these compounds was found to be a substrate of typical class A and C β-lactamases and of the DD-peptidase of *Streptomyces* R61; it thus acylates the active site serine. In contrast, the latter compound was an irreversible inhibitor of the above enzymes, probably by phosphonylation of the active site serine. With each of these enzymes therefore, the amido side chain rather than the acyl group dictates the orientation of the bound phosph(on)ate and thus the mode of reaction.

Key Words: β-lactamase; DD-peptidase; acyl phosph(on)ate; enzyme inhibition; enzyme substrates.

INTRODUCTION

Acyl phosph(on)ates **1** have been shown in this laboratory to be novel substrates/covalent inhibitors of β-lactamases of classes A and C (*1*). The nucleophilic hydroxyl



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group of the active site serine can, in principle, attack either the carbonyl or the phosphyl center. The former yields an acyl-enzyme and the latter a phosphyl-enzyme species. Precedent would suggest that the enzyme could catalyze hydrolysis of the

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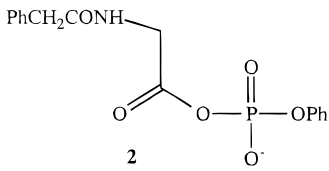
acyl-enzyme to regenerate free enzyme but is less likely to be able to do this to a phosphyl-enzyme (*2,3*). Acting in the former mode, **1** would appear to be a substrate, while in the latter, it would be seen as an irreversible covalent inhibitor. Depending on the choice of R/Ar, deacylation of the acyl-enzyme may be slow, and thus transiently inhibited enzymes can be generated by the acyl transfer route (*1*). To date, our work with these compounds has involved only simple alky/aryl substituents on **1** (*1*).

In the present paper, we describe the synthesis and evaluation of compounds **2** and **3** as substrates/inhibitors of β-lactamases and a bacterial DD-peptidase. The latter is



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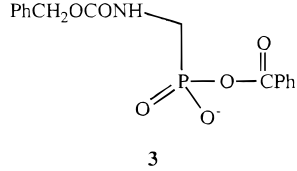

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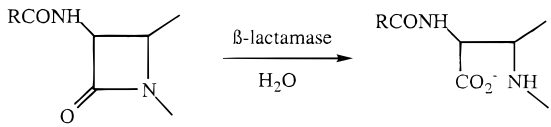
a representative of a group of enzymes that catalyze the hydrolysis and transpeptidation of D-Ala-D-Ala peptides, essential reactions in bacterial cell wall biosynthesis. These enzymes are also the targets of the β-lactam antibiotics. The β-lactamases, which catalyze the hydrolysis of β-lactams (Scheme 1), are a major source of bacterial resistance to β-lactams. In these new phosph(on)ates, the specific amido side chain found in good β-lactamase substrates has been incorporated either in the acyl (**2**) or in the phosphyl (**3**) moiety. We were interested in the issue of whether, assuming that these molecules interacted covalently with the β-lactamase active site, the amido side chain or the acyl group dictated the nature of the reaction observed. Studies of other, chemically very different, ligands have shown that covalent adducts can be formed with a variety of chemical moieties in either the amido side chain or leaving group areas of the active site (*4,5*). The results of the present research show that, in this case, the side chain has a dominant effect on the reactivity of **2** and **3** with the enzymes.

MATERIALS AND METHODS

Materials

The β-lactamases were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. Typical specific activities for these preparations were as previously reported (*6*). The *Streptomyces* R61 DD-peptidase was kindly provided by Professor J.-M. Frère (University of Liège, Liège, Belgium). Benzylpenicillin was purchased from Sigma Chemical Co.,



SCHEME 1.

cephalothin was a gift from Eli Lilly and Co., and m-carboxyphenyl phenaceturate was available from previous studies in this laboratory (*7*).

*Tetraethylammonium N-(phenylacetyl)glycyl phenyl phosphate (**2**).* This compound was prepared by means of a method described by Kluger *et al.* (*8*). First, bis(tetraethylammonium) phenyl phosphate was prepared by the addition, with stirring over 10 min, of phenyl dichlorophosphate (7.86 g, 50 mmol; Aldrich Chemical Co.) to 20 ml of water in an ice bath. The mixture was stirred for a further 1 h and the water removed by rotary evaporation. Two equivalents of tetraethylammonium hydroxide (35% in water, 41.1 ml; Aldrich) were then added, the pH adjusted to 7.0 with hydrochloric acid, and the resulting solution feeeze–dried. The residue (16 g) was dissolved in 80 ml dichloromethane and dried overnight over 4 Å molecular sieves.

Dicyclohexylcarbodiimide (1.2 g, 5.8 mmol) was added to a stirred solution of phenylacetylglycine (1.16 g, 6 mmol) in dry dichloromethane (125 ml). The resulting mixture was stirred for 10 min and then bis(tetraethylammonium) phenyl phosphate (5 mmol) in dichloromethane added. After a further hour, dicyclohexyl urea was removed by filtration and the dichloromethane solution extracted twice with 50-ml portions of water. The aqueous solution was freeze–dried to yield the product which was then purified by Sephadex LH-20 chromatography in dichloromethane. The final oily product was characterized as the tetraethylammonium salt by its ^{1}H NMR spectrum [(D_2O)δ 1.24 (t, 12H, NEt_4), 3.23 (q, 8H, NEt_4), 3.65 (s, 2H, PhCH_2), 4.08 (s, 2H, NHCH_2), 7.3 (m, 10H, ArH)] and ESMS [*m*/*e* 608.7; bis(tetraethylammonium cation)].

*N,N′-Dibenzylethylenediammonium bis[benzoyl N-(benzyloxycarbonyl)aminomethylphosphonate] (**3**).* *N*-(Benzyloxycarbonyl)aminomethylphosphonic acid was prepared as previously described (*9*). The required acyl phosphate was then obtained by the method of Jencks and Carriuolo (*10*), where the ratio of starting materials, the time of mixing, and the mode of removal of pyridine, which catalyzes hydrolysis of the product as well as its formation, were varied to optimize the yield. Thus, a solution of benzoic anhydride (0.94 g, 4.2 mmol; Acros Organics) in pyridine (0.5 ml) was added dropwise with stirring to an ice-cooled solution of *N*-(benzyloxycarbonyl)aminomethylphosphonic acid (100 mg, 0.41 mmol) and sodium hydroxide (0.85 mmol) in water (1.8 ml). After 15 min, the reaction mixture was rapidly extracted three times with diethyl ether, where suction by an aspirator was used to remove the ether phase. The pH of the aqueous phase was lowered to 3.5 by addition of 1 M HCl and the resulting solution freeze–dried. This procedure yielded a mixture of product and starting phosphonate in a ratio of ca. 5:1. The product could be isolated by precipitation as a *N*,*N′*-dibenzylethylenediamine salt. Thus, the above product was dissolved in a minimum volume of water and to it was added an equal volume of a 14 mM aqueous solution of *N*,*N′*-dibenzylethylenediamine diacetate. The resulting mixture was stirred in an ice bath for 10 min and the precipitated product removed by filtration, washed with water, and dried *in vacuo*. The product, a colorless solid was characterized by its melting point (133–135°C), ^{1}H NMR spectrum [($^2H_6$-DMSO) δ 3.41 (s, 4H, CH_2CH_2), 3.60 (dd, J = 6, 12 Hz, CH_2P), 4.15 (s, 4H, CH_2Ph), 5.11 (s, 2H, CH_2O), 7.10 (brt, 1H, NH), 7.5 (m, 20H, ArH)], ^{31}P NMR spectrum [($^2H_6$-DMSO) δ 10.5], IR spectrum [(KBr) $\gamma_{C=0}$ = 1709 cm^{-1}], and ESMS (*m*/*e* 348.1; M + H$^+$).

Attempts to prepare the *N*-phenylacetyl analog of **3** by the same method failed, probably because of the lability of this compound (*9,11*).

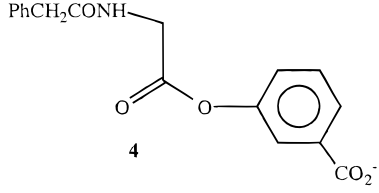
Analytical and Kinetic Methods

Fresh stock solutions of enzymes and phosph(on)ates were prepared in 20 mM Mops buffer, pH 7.5, and kinetics experiments were conducted in this buffer at 25°C. Rate constants of spontaneous hydrolysis and enzyme steady-state rate parameters were determined spectrophotometrically. The analytical wavelengths employed were 270 nm ($\Delta\varepsilon$ = 1120 M^{-1} cm^{-1}) and 275 nm ($\Delta\varepsilon$ = 440 M^{-1} cm^{-1}) for **2** and **3**, respectively. The steady-state parameters were obtained from initial rate measurements by the method of Wilkinson (*12*). Irreversible inactivation rates were determined by incubation together of suitable concentrations of enzyme and inhibitor. Small aliquots were taken at appropriate times and the enzyme activity assayed against either cephalothin or benzylpenicillin. Second-order rate constants, k_i, for inactivation were then obtained by employment of Scheme 2 and the program Dynafit (*13*). In Scheme 2, k_o is the pseudo-first-order rate constant for spontaneous hydrolysis of I under the conditions employed, and Q the hydrolysis product of I. Phosphorus analyses on trichloroacetic acid precipitated enzyme samples (*1*) were carried out by a combination of the methods of Hess and Derr (*14*) and Kapoulas *et al.* (*15*), where phenylphosphonic acid was used to construct a standard curve.

RESULTS AND DISCUSSION

^{1}H NMR experiments demonstrated that **2** and **3** hydrolyzed to the expected carboxylate and phosph(on)ate products in aqueous buffer at pH 7.5. The pseudo-first-order rate constants for this process, obtained spectrophotometrically, were 6.4×10^{-5} and 1.2×10^{-4} s^{-1}, respectively. The substantial rates of hydrolysis strongly suggested that C–O bond cleavage had occurred; i.e., the hydrolyses were acyl transfer reactions, as is generally found with acyl phosphates (*16*).

The hydrolysis of **2** was strongly catalyzed by the P99 β-lactamase. Steady-state parameters are given in Table 1. The value of k_{cat}/K_m identifies **2** as the most specific acyclic substrate of this β-lactamase yet discovered (*7*). It seems likely that the negatively charged phosphate leaving group interacts favorably with the electropositive active site (*17*) during reaction of **2** with the enzyme (*18*). The k_{cat} value is very similar to that obtained for various aryl phenaceturates such as **4** (*7*), where the same



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$$E + I \xrightarrow{k_i} EI$$

$$I \xrightarrow{k_o} Q$$

SCHEME 2.

TABLE 1

Kinetic Parameters for Interactions of **2** and **3** with the Enzymes

Enzyme/Parameter		Compound **2**	Compound **3**
P99	k_{cat} (s^{-1})	90 ± 7	*a*
	K_m (mM)	0.09 ± 0.01	
	k_{cat}/K_m (s^{-1} M^{-1})	1.0×10^6	
	k_i (s^{-1} M^{-1})	*b*	$(5.9 \pm 0.3) \times 10^4$
TEM	k_{cat} (s^{-1})	30 ± 6	*a*
	K_m (mM)	16 ± 4	
	k_{cat}/K_m (s^{-1} M^{-1})	1880	
	k_i (s^{-1} M^{-1})	*b*	130 ± 8
PCl	k_{cat} (s^{-1})	0.105 ± 0.002	*a*
	K_m (mM)	0.26 ± 0.02	
	k_{cat}/K_m (s^{-1} M^{-1})	400	
	k_i (s^{-1} M^{-1})	*b*	14.7 ± 0.7
R61	k_{cat} (s^{-1})	4.1 ± 0.01	$(3.5 \pm 0.2) \times 10^{-4}$
	K_m (mM)	0.20 ± 0.02	0.21
	k_{cat}/K_m (s^{-1} M^{-1})	2.05×10^4	1.65 ± 0.13
	k_i (s^{-1} M^{-1})	*b*	5×10^{-3}

Note. P99, β-lactamase of *Enterobacter cloacae* P99; TEM, TEM-2 plasmid β-lactamase; PCl, β-lactamase of *Staphylococcus aureus* PCl; R61, DD-peptidase of *Streptomyces* R61.

[a] No turnover observed.

[b] No inhibition observed.

acyl-enzyme would be generated and where the hydrolysis of this intermediate is rate-determining (*6,7*). It seems likely then that turnover of **2** by this enzyme involves an intermediate acyl-enzyme (Scheme 3A). Further support for this proposition was obtained from experiments with the alternative nucleophiles, methanol and D-α-aminobutyrate. Methanol (0–2.5 M) increases the rate of reaction of **2** (0.98 mM) with the P99 β-lactamase (Fig. 1) in a fashion observed for most other substrates of this enzyme (*6,7,19*). A ^1H NMR experiment where **2** was reacted with the enzyme in a solution of 2M $^2H_4$-methanol in ^2H_2O, also containing 50 mM $NaHCO_3$ as buffer, showed that the methanolysis product, methyl phenaceturate, characterized by its glycyl methylene peak at δ4.00 (*6*), accompanied the hydrolysis product after the reaction was complete. The conclusion is that deacylation is rate-determining but can be accelerated by addition of an alternative nucleophile to intercept the acyl-enzyme (Scheme 3A). The NMR experiment showed that 50% of **1** methanolyzed under the conditions employed which corresponds to a k_4/k_3 ratio (Scheme 3A) of 25.5. Treatment of the kinetic data with the appropriate equation (*7*) yielded a value for the partition ratio k_4/k_3 of 29. These estimates of k_4/k_3 agree very well with the value of 28 obtained by Xu *et al.* (*7*) for what is presumably the same acyl-enzyme generated from **4**.

The behavior of **2** with the P99 enzyme in the presence of D-amino acids also strongly resembles that previously observed with the above-mentioned depsipeptides. Typical behavior is shown in Fig. 1 where the amino acid employed was D-2-aminobutyric acid. The substrate inhibition observed was interpreted, as previously, in terms