

Inhibition of Class D β -Lactamases by Acyl Phosphates and Phosphonates

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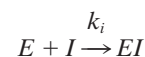
The susceptibility of typical class D β -lactamases to inhibition by acyl phosph(on)ates has been determined. To a large degree, these class D enzymes behaved very similarly to the class A TEM β -lactamase towards these reagents. Dibenzoyl phosphate stood out in both cases as a lead compound towards a new class of effective inhibitors.

The resistance of bacteria to β -lactam antibiotics is to a large extent due to β -lactamases (14). There is a wide range of β -lactamases, although from a structural standpoint, they can be divided into four classes, A, B, C, and D (11). Each class contains many variants of widely differing substrate specificities and thus clinical importance. Of the canonical four classes, it is perhaps class D that at present is least well studied at the molecular level.

The class D β -lactamases, also generally known as oxacillinases because of their general specificity for oxacillin and its derivatives, represent a diverse class of enzymes (1) that hydrolyze a broad spectrum of substrates (2). Like class A and C β -lactamases, the class D enzymes are serine hydrolases, catalyzing substrate hydrolysis by way of a covalent acyl-enzyme (acyl-serine) intermediate and thus by a double-displacement mechanism. Structural studies indicate that the class D β -lactamases are more closely related to class A than to class C, although there are distinct differences in active-site structures and thus, presumably, in chemical mechanisms (20). Although class D β -lactamases are clinically significant, there are no specific inhibitors known for them other than certain inhibitory β -lactams (8, 12) and a series of anthraquinone dyes (15). The classical mechanism-based inhibitors of class A β -lactamases are not generally effective against class D (16). A variety of phosph(on)ates have been found to be covalent inhibitors of class A and class C β -lactamases (4, 5, 9, 17–19). This paper describes the screening of a panel of these compounds, 1 to 9, against the class D OXA-1 β -lactamase. This enzyme is representative of one subclass of the D enzymes (1) and is clinically important in its own right; a crystal structure is also available (20). The more effective compounds of 1 to 9 (Fig. 1) were also tested against the OXA-10 enzyme, a representative of another major class D subgroup (1).

The OXA-1 and OXA-10 β -lactamases were prepared and purified as described previously (8, 20). The various phosph(on)ates were available from previous studies (4, 5, 9, 17–19). The phosphates 2 and 4 to 8 and the phosphonates 1, 3, and 9

were all irreversible or slowly reversible inhibitors of the OXA-1 β -lactamases, and the inactivation step could be described simply by scheme 1 as follows:



where E is the free enzyme and I is the inhibitor. Second-order rate constants of inactivation were obtained from measurements of the loss of enzyme activity as a function of time (18, 19). All kinetics studies were performed at 25°C in a buffer at pH 7.5 containing 20 mM MOPS (3-morpholinopropanesulfonic acid) and 50 mM sodium bicarbonate (3). The enzyme concentration in reaction mixtures was 1.4 μ M, and the inhibitor concentrations were 0 to 0.5 mM, depending on reactivity (Table 1). Aliquots of reaction mixtures were diluted 15-fold into 0.5 mM benzylpenicillin solution for assay. The values of k_i , obtained as described above, are reported in Table 1.

We see from these data that the “classical” substrate-like phosphonate 1 had little activity against the OXA-1 enzyme. Of the acyclic acyl phosphates 2 to 5, the diacyl phosphate 4 shows significant activity. Of the cyclic acyl phosph(on)ates 6 to 9, the 4-phenyl phosphate 7 and its phosphonate analogue 9 are most effective. It is interesting to note that this pattern of activity closely mimics that against the class A TEM β -lactamase (4, 9, 17, 18).

The reactivity of dibenzoyl phosphate, 4, with the OXA-1 β -lactamase is quite striking, and even without structural optimization, it is comparable to that of clavulanic acid ($k_i = 2.2 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$ under the same conditions). The inactivation by 4 was found to be slowly reversible (5, 7), corresponding to a directly measured turnover number (k_{cat}) of $(4.9 \pm 0.9) \times 10^{-3} \text{ s}^{-1}$ (the half-life of the EI complex was thus 2.4 min; cf. that of clavulanic acid [0.5 s]). The effective K_m of 4 as a substrate, or its K_i as an inhibitor, is given by k_{cat}/k_i , with a value of 94 nM (cf. that of clavulanic acid [6.4 μ M]). Inactivation of the enzyme by the cyclic phosphonate 9 was also reversible, with a rate constant of $1.5 \times 10^{-2} \text{ s}^{-1}$. In contrast, reactivation from treatment with 7 did not occur at a measurable rate, the difference perhaps indicating phosphorylation of the enzyme by 7 and 9 (6).

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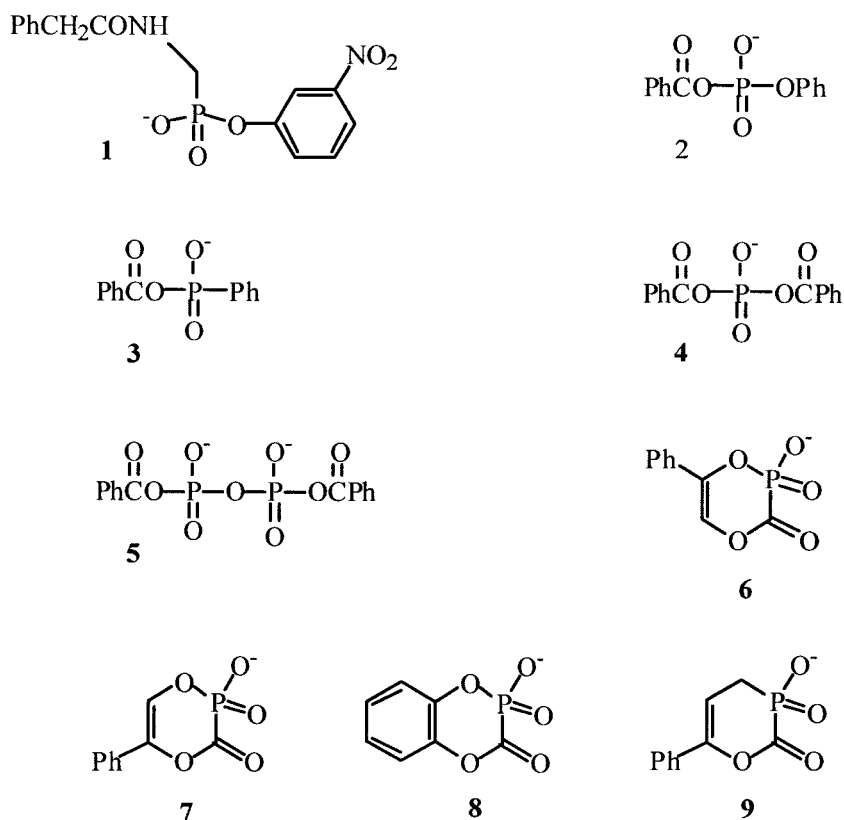


FIG. 1. Structures of phosph(on)ates 1 to 9.

The data of Table 1 also suggest that the pattern of reactivity established for the OXA-1 enzyme also applies to OXA-10. Dibenzoyl phosphate was again the most reactive. In this case, the reactivation rate constant (k_{cat}) was $(4.2 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$ and thus the effective K_i was $(0.24 \pm 0.02) \mu\text{M}$.

The slow reversibility of the reaction of these class D enzymes with 4 is suggestive of the formation of a covalent benzoyl-enzyme species, as is well established with a class C β -lactamase (5, 9) and is likely also with the class A TEM enzyme (9). It is notable, however, that no reversal of reaction with 2 and 3 was observed, despite the fact that these compounds

should generate the same benzoyl-enzyme as would 4. This difference may reflect nonspecific modification by 2 and 3 at the high concentrations of these species (cf. that of 4) required for inhibition. In the case of the class C β -lactamase of *Enterobacter cloacae* P99, a direct relationship, based on transition state analogy, was demonstrated between the reactivity of the enzyme with acyl phosphates and its inhibition by aryl boronates (5). In the present instance, we found that the OXA-1 β -lactamase was not strongly inhibited by benzenboronic acid ($K_i = 3.5 \text{ mM}$); the class A TEM β -lactamase is also not strongly inhibited by simple aryl boronic acids (10). The relatively high reactivity of 4 with both class A and D enzymes must therefore reflect the strong interaction of the PhCOOPO_2^- leaving group with the active sites of these enzymes.

In conclusion, this paper reports that typical phosph(on)ates inhibit typical class D β -lactamases to much the same degree that they do the class A TEM β -lactamase. It seems likely that this result reflects the overall similarity of these active sites despite the fact that details of catalysis must be different (13, 20). The reactivity of dibenzoyl phosphate 4, now evident with all classes of serine β -lactamases, suggests new avenues of inhibitor design for these enzymes. Further optimization of 4 should include an increase in the on rate constant (k_i) and some decrease in the off rate constant.

TABLE 1. Inhibition of class D β -lactamases by phosph(on)ates

Phosph(on)ate ^a	k_i ($\text{s}^{-1} \text{ M}^{-1}$) ^c	
	OXA-1	OXA-10
1	0.90 ± 0.25	2.9 ± 0.6
2	45 ± 10	ND
3	10 ± 3	ND
4	$(5.2 \pm 0.2) \times 10^{4b}$	$(1.7 \pm 0.4) \times 10^{5b}$
5	≤ 0.04	ND
6	0.6 ± 0.1	ND
7	$(2.8 \pm 0.2) \times 10^2$	$(1.0 \pm 0.3) \times 10^3$
8	27 ± 2	ND
9	$(3.3 \pm 0.6) \times 10^{2b}$	ND

^a The concentrations of 1 to 9 employed ranged up to 0.5 mM, 60 μM , 60 μM , 1.0 μM , 1.0 mM, 0.3 mM, 8.0 μM , 40 μM , and 12.6 μM , respectively.

^b Reversible (see the text).

^c ND, experiment not done.

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