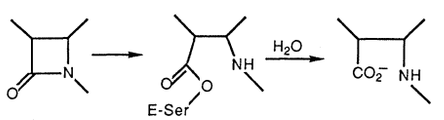
Reports

Inhibition of a Class C β-Lactamase by a Specific Phosphonate Monoester

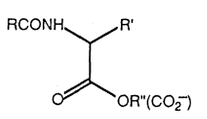
R. F. PRATT

A phosphonate monoester, *m*-carboxyphenyl phenylacetamidomethylphosphonate, has been found to be a specific inhibitor of the class C β-lactamase of *Enterobacter cloacae* P99. Inactivation is rapid (10^3 per second per molar concentration) and reactivation very slow (2.2×10^{-6} per second). Apparently concerted with the inactivation, one equivalent (with respect to the enzyme) of *m*-hydroxybenzoate is released. Reactivation is accelerated by hydroxylamine and benzohydroxamate. This suggests that the loss of enzyme activity is due to phosphonylation of an active site functional group. This discovery holds the promise of a new general class of β-lactamase inhibitors and, perhaps, antibiotics.

THE β-LACTAMASE ENZYMES provide much of the bacterial resistance toward β-lactam antibiotics (*1*). The majority of these enzymes, and in particular those of clinical importance, catalyze hydrolysis of β-lactams by a double displacement mechanism (scheme 1), where the acyl-enzyme intermediate is an acyl-serine (*2*). Most of the effective inhibitors of these enzymes, including those currently used clinically, are of the mechanism-based variety and are themselves β-lactams (*2, 3*). Research in this laboratory has shown that the serine β-lactamases also catalyze the hydrolysis of a variety of acyclic depsipeptides of general structure **1** (*4*). This result suggested that new classes of acyclic inhibitors of these enzymes may be devised, and, in particular, transition state–analog inhibitors. Such inhibitors of acyl-transfer enzymes usually have a tetrahedral and negatively charged group in place of the acyl carbonyl group of the substrate, which is thought to mimic the transition state for nucleophilic attack at the acyl group (*5*). Boronic acids, for example, which form tetrahedral adducts with active site nucleophiles, are well known as inhibitors of serine proteinases (*6*) and β-lactamases (*7*). In this paper, I describe a phosphonate monoester analog of **1**, which is a novel and very effective inhibitor of the class C β-lactamase of *Enterobacter cloacae* P99.



Scheme 1.



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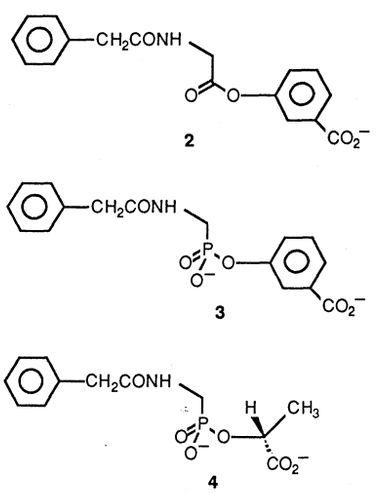
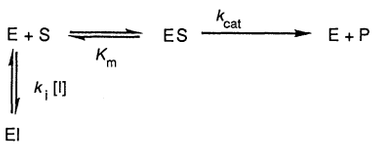
The depsipeptide **2** is the best (k_{cat}/K_M) depsipeptide substrate that we have yet found for β-lactamases (*4*) (where k_{cat} is the catalytic rate and K_m is the Michaelis constant). Therefore *m*-carboxyphenyl phenylacetamidomethylphosphonate **3** was a rational target for a transition state–analog inhibitor (*8, 9*); in addition, the phosphonate **4** was synthesized (*10*).



Compound **3** strongly inhibited the class C β-lactamase of *Enterobacter cloacae* P99 in a time-dependent fashion. For example, on incubation of the enzyme (2 μ*M*) with **3** (10 μ*M*), the activity of the enzyme against benzylpenicillin or **2**, fell to zero after about 10 min. The kinetics of the inhibition (*11*) could be determined directly from measurements of β-lactamase activity as a function of time (*12*). These data demonstrated that the inactivation followed second-order kinetics, and yielded a rate constant (k_i) of (1120 ± 150) $s^{-1}\ M^{-1}$ (all error limits are ± SE) (*13*). Kinetic data could also be obtained, and at higher inhibitor concentrations, by following the absorbance of a competing substrate, such as cephalothin, as a function of time in the presence of **3** (*14*). The reaction sequence of scheme 2 applied at concentrations of **3** up to 2.5 m*M*, which means that there was no evidence for tight binding of **3** to the enzyme before the inhibition reaction. The inhibited enzyme, isolated from any excess **3** by gel-exclusion chromatography, slowly reactivated on standing in buffer, with a rate constant of $(2.32 \pm 0.22) \times 10^{-6}\ s^{-1}$. Thus the lifetime of the enzyme-inhibitor (EI) complex under these conditions was ~86 hours.



Scheme 2.

Since **3** reacts rapidly and effectively irreversibly on a time scale of minutes with the enzyme at micromolar concentrations, it could be used as an active-site titrant at such concentrations. Compound **3** was titrated against 13.9 μ*M* enzyme (Fig. 1). The *x* intercept of the plot indicates that (0.84 ± 0.03) mole of **3** per mole of enzyme were required for inactivation; this is presumably a direct measure of the proportion of the enzyme sample with a functional active site.

Another important feature of the inhibition, and an unexpected one, was the release

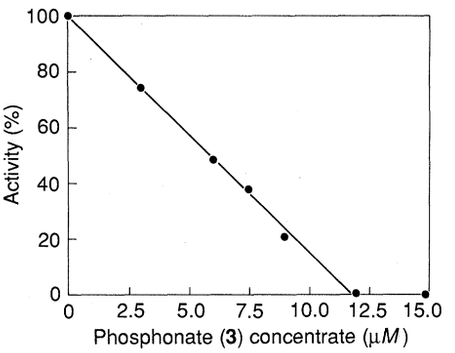


Fig. 1. Activity of the *E. cloacae* P99 β-lactamase (13.9 μ*M*) as a function of added **3** concentration. Enzyme and inhibitor were incubated together at 25°C and assayed against benzylpenicillin after 5 hours.

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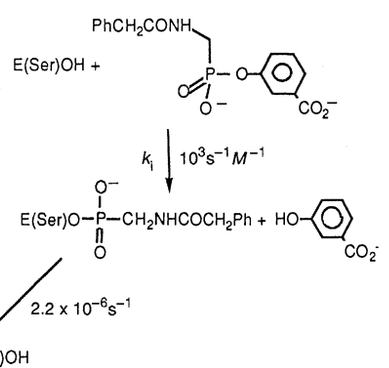
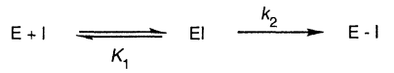
of *m*-hydroxybenzoate in amounts stoichiometric with the enzyme. *m*-Hydroxybenzoate was detected and determined by its characteristic fluorescence [excitation wavelength of 294 nm and a fluorescence wavelength of 410 nm (*15*)]. A sample of enzyme (18.0 μM) was treated with an equimolar amount of **3**. After incubation of the mixture for 30 min at 25°C, at which time essentially no enzyme activity remained, the enzyme was separated from small molecules in solution by ultrafiltration (Amicon Centricon). The filtrate contained a compound with a fluorescence emission and excitation spectrum identical to that of *m*-hydroxybenzoate and present to the extent of 0.75 ± 0.05 mole per mole of protein, or 0.89 ± 0.06 mole per mole of active sites; the data from Fig. 1 was used to estimate the latter. Rates of *m*-hydroxybenzoate release could also be measured by the increase in fluorescence of reaction mixtures with time. Under pseudo–first-order conditions, the rate constants determined from measurements of *m*-hydroxybenzoate release were linear with the concentration of **3** up to 25 μM. The slope of this plot yielded a second-order rate constant of (950 ± 60) s^{-1} M^{-1}. The close agreement between this rate constant and that obtained from activity measurements suggested that loss of enzyme activity was concerted with release of one molar equivalent (with respect to the amount of enzyme) of *m*-hydroxybenzoate.

The above observations strongly suggest that **3** inhibits the P99 β-lactamase by phosphonylation of a component of the active site; the phosphonyl-enzyme thus produced hydrolyzes to regenerate free enzyme only very slowly. This idea was supported by the observation that reactivation of the enzyme was accelerated by hydroxylamine (0.1M) and benzohydroxamic acid (0.02M) (*16*). Since **3** is a close analog of the substrate **2**, which is thought to form a covalent intermediate during turnover by acylation of the active site Ser hydroxy group (*4*), it appears likely that **3** inhibits by phosphonylation of the same residue (scheme 3). If **3**, as is likely



Scheme 3.

for a substrate analog, binds specifically and noncovalently to the enzyme before the phosphonylation reaction (scheme 4, where



Scheme 4.

E − I is the phosphonyl-enzyme of scheme 3), the above data show $K_I \geq 2.5$ mM and thus k_2 ($= k_i K_I$) ≥ 2.5 s^{-1}.

The reaction depicted in scheme 3, if correct, is of considerable interest for several reasons. First, it represents an unusually facile nucleophilic displacement at the phosphorus atom of a phosphonate monoester (*17*). This indicates a strong and specific interaction of **3** with the components of the β-lactamase active site in the transition state. This reactivity can be contrasted with that of the serine proteinases. Although the latter enzymes are rapidly and specifically inactivated by phosphate triesters and other neutral phosphoryl derivatives (*18*), there seems to be no example where any such enzyme is so rapidly modified and inhibited by a negatively charged phosphate diester or phosphonate monoester. This contrast indicates the existence of a significant difference between the active sites, and the details, at least, of the catalytic mechanisms of β-lactamases and serine proteinases. Reflection on the ribonuclease mechanism and the mechanisms of phosphoryl transfer reactions suggests that the negatively charged phosphonate must be activated toward nucleophilic attack by interaction with a positively charged enzyme functional group. The β-lactamase active site, of course, contains two conserved and essential Lys residues, one of which, Lys^{73}, appears to lie close to the active site Ser in crystal structures (*19*). The Lys ammonium ion may activate the phosphonate to an extent not possible by the histidinium ion that develops during the reaction of serine proteinases. This ion may similarly act to stabilize tetrahedral intermediates in normal catalysis (*20*). Although the putative phosphonyl enzyme reacts spontaneously to regenerate the free enzyme only very slowly [as does the "aged" product of reaction between chymotrypsin and diisopropylfluorophosphate (*21*)], the reaction is actually fast with respect to normal phosphonate monoester hydrolysis (*17*), and therefore must also be enzyme-catalyzed; the β-lactamase is thus a modestly effective phosphonate monoesterase.

The importance of specific interaction between **3** and the P99 β-lactamase was evident from the observations that **3** (at 0.5 to 1 mM concentration) had little effect on class A β-lactamases (the TEM β-lactamase, *Bacillus cereus* β-lactamase I, and the *Staphylococcus aureus* PC1 β-lactamase), the class B *B. cereus* β-lactamase II, and the D-alanyl-D-alanine transpeptidase/carboxypeptidase of *Streptomyces* R61; this would correlate with the lesser ability of **2** as a substrate of these enzymes (*4*). Furthermore, **3** had no significant antibacterial activity against a variety of Gram-positive and Gram-negative strains. The D-lactate **4** (at 0.5 to 1 mM concentration) is also not an inhibitor of any of the above enzymes, even the P99 β-lactamase, nor does it demonstrate antibiotic activity. The much poorer leaving group in **4** than in **3** may contribute to this difference. Despite this specificity, this result suggests the feasibility of design of a new general class of inhibitors (phosphonate, phosphate, phosphonamidate, and so forth) of β-lactamases, and therefore perhaps also of the related (*4, 22*) D-alanyl-D-alanine transpeptidases (that is, of antibiotics). Optimization of negative charge separation in the phosphonate may well be an essential feature of this process.

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10. Compounds **3** and **4** were prepared by standard methods. Thus, reaction of phenylacetyl chloride with aminomethylphosphonic acid in aqueous carbonate buffer yielded phenylacetylamidomethylphosphonic acid [melting point (mp) 165° to 166°C], characterized by ^1H nuclear magnetic resonance (NMR) spectroscopy. This compound was condensed under the influence of trichloroacetonitrile [C. Wasielewski, M. Hoffman, E. Witowska, J. Rachon, *Rocz. Chem.* **50**, 1613 (1976)] with benzyl *m*-hydroxybenzoate (*4*) to give the benzyl (carboxyl) ester of **3**, which was isolated as the sodium salt in about 50% yield. The ester was hydrogenated in methanol over 10% Pd/C to give a sodium salt of **3**, characterized by ^1H and ^{31}P NMR spectroscopy, as a hygroscopic powder. This material was used for the inhibition studies. Exact concentrations of **3** were determined from the absorption of *m*-hydroxybenzoic acid after acid-catalyzed hydrolysis. The compound was stable indefinitely in neutral or basic solution, as determined by ^1H NMR spectroscopy, and hydrolyzed slowly in acid ($k_{H^+} = 2.3 \times 10^{-4}$ s^{-1} M^{-1} at 25°C). The free diacid of **3** was generated by treatment of a solution of the sodium salt in ethanol with Dowex 50W-X4 (H^+) resin, and precipitated with diethyl ether as the dibenzylethylenediamine salt after amine addition. This salt, mp 139° to 140°C, was characterized by combustion analysis and ^1H NMR spectroscopy. The phosphonate **4** was similarly prepared with the substitution of benzyl D-lactate (*4*) for benzyl *m*-hydroxybenzoate. Details of these syntheses will be published elsewhere.
11. All kinetic measurements were made in 0.02M 2-(*N*-morpholino)propanesulfonic acid (MOPS) buffer at *p*H 7.5 and 25°C. The β-lactamases were purchased

from the P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, United Kingdom.
12. The enzyme (~2.2 μ*M*) and various concentrations of inhibitor (0.5 to 5.0 μ*M*) were incubated together. Small samples were withdrawn at suitable times and assayed spectrophotometrically (232 nm) against benzylpenicillin for β-lactamase activity.
13. Under second-order conditions, the fraction X of residual activity at time t is given by $X = (I_0 - E_0)/(I_0 \exp[(I_0 - E_0)k_i t] - E_0)$, where E_0 and I_0 are the initial concentrations of enzyme and inhibitor, respectively, k_i the second-order rate constant for the inactivation. Experimental data was fitted to this equation by a nonlinear least-squares procedure.
14. The hydrolysis of cephalothin (2.46 m*M*), catalyzed by the *E. cloacae* P99 β-lactamase (2.3 n*M*), was followed spectrophotometrically at 292 nm in the presence of **3** (0.5 to 2.5 m*M*).
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23. I am grateful to B. A. Pelak, H. Kropp, and B. G. Christensen of Merck, Sharp & Dohme Research Laboratories for the antibacterial tests. The DD-peptidase of *Streptomyces* R61 was a gift from J.-M. Ghuysen, University of Liege, Belgium. I also thank P. C. Moews and J. R. Knox for access to their paper (*19*) before publication. This research was supported by NIH.

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Molecular Mechanisms and Forces Involved in the Adhesion and Fusion of Amphiphilic Bilayers

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The surface forces apparatus technique was used for measuring the adhesion, deformation, and fusion of bilayers supported on mica surfaces in aqueous solutions. The most important force leading to the direct fusion of bilayers is the hydrophobic interaction, although the occurrence of fusion is not simply related to the force law between bilayers. Bilayers do not need to "overcome" some repulsive force barrier, such as hydration, before they can fuse. Instead, once bilayer surfaces come within about 1 nanometer of each other, local deformations and molecular rearrangements allow them to "bypass" these forces.

THE FUSION OF AMPHIPHILIC (surfactant and lipid) monolayers or bilayers arises in both colloidal and biological systems (*1*). However, an understanding of interbilayer forces, the mechanisms of fusion, and the relation between the two is still far from clear. Some of the forces between amphiphilic surfaces have only recently been discovered. Thus, in addition to the expected attractive van der Waals forces and repulsive electrostatic double-layer forces (*1–3*), the existence and importance of repulsive hydration and attractive hydrophobic forces (*2–6*), short-range attractive ion-correlation forces (*7*), and medium- to short-range repulsive undulation (or fluctuation) forces (*8*) are only now being recognized. Their role in fusion has not yet been investigated. The origin of the so-called "hydration" and "hydrophobic" forces is still far from clear. Second, various models of the fusion process have been suggested, mainly based on electron micrographs of small vesicles or membranes (*1, 9, 10*) or of optical, capacitance, and conductance measurements of large fusing vesicles or "black lipid membranes" (BLMs) (*1, 11*). However, the precise molecular events and rearrangements accompanying the fusion process are still unknown. We present results of measurements of the interactions between various adsorbed bilayers in aqueous electrolyte solutions using the surface forces apparatus (SFA) technique.

The SFA technique allows forces and pressures to be measured between two curved or flattened molecularly smooth mica surfaces (*12*), or between monolayers or bilayers deposited on such surfaces (*5, 6, 13*), with a distance resolution at the angstrom level. Surface deformations accompanying adhesion (*14*) and fusion (*15*) can be directly visualized in real time. We note the good agreement in the measured forces between lecithin bilayers using the SFA and osmotic pressure technique (*16*).

We used two methods for coating mica surfaces with bilayers: (i) adsorption from solution and (ii) for insoluble lipids, controlled deposition using a Langmuir-Blodgett (LB) trough. Single-chained bilayers of CTAB (recrystallized in 9:1 ethanol:ether) were adsorbed onto mica surfaces at concentrations at or above their critical micelle

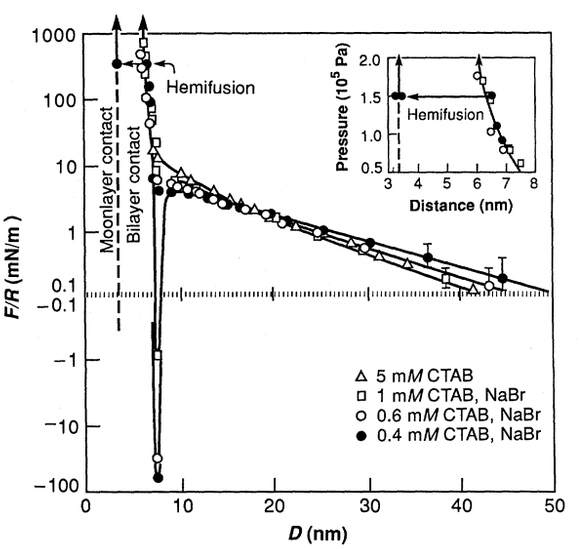

Fig. 1. Forces (F/R, force to radius) as a function of distance D between CTAB bilayers 3.2 to 3.6 nm thick adsorbed onto mica surfaces from CTAB solution; $D = 0$ corresponds to mica-mica contact in water, in contrast to Fig. 4, where it corresponds to bilayer contact. The concentration of CTAB + NaBr of the solution in the chamber was progressively diluted below the CMC (about 1 m*M*), until hemifusion occurred at and below 0.4 m*M*. The long-range forces are repulsive electrostatic "double-layer" interactions. The measured Debye lengths were 11 nm at 0.4 m*M*, 9 nm at 0.6 m*M*, and 8 nm at both 1 and 5 m*M*. [The similarity of the latter two Debye lengths arises because above the CMC the micelles and their bound counterions no longer contribute to the Debye length (*17*).] The adhesion forces at the minima are for bilayers that had been in contact for 1 hour. At high values of F/R, the surfaces flatten elastically, and the pressure can be measured, as shown in the inset. Hemifusion (see Fig. 2) occurred at 0.4 m*M* at a pressure of 1.5 atm.

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