Salicyloyl Cyclic Phosphate, a "Penicillin-Like" Inhibitor of β-Lactamases

R. F. Pratt* and Ned J. Hammar

Contribution from the Chemistry Department, Wesleyan University, Middletown, Connecticut 06459

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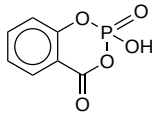
Abstract: Salicyloyl cyclic phosphate (1-hydroxy-4,5-benzo-2,6-dioxaphosphorinanone (3)-1-oxide) was designed as a "penicillin-like" inhibitor of β-lactamases. It was anticipated that, after nucleophilic attack on this molecule by the enzyme, the leaving group would remain tethered, and, as in the inhibition of DD-peptidases by penicillins, obstruct hydrolysis of the covalent intermediate back to free enzyme. The target molecule, hitherto only reported as a transient intermediate, was prepared by hydrolysis of the cognate cyclic phosphoryl chloride and isolated and characterized as the dicyclohexylammonium salt. It proved to transiently inhibit the class C β-lactamase of *Enterobacter cloacae* P99, the class A TEM β-lactamase, and also the DD-peptidase of *Streptomyces* R61. Most significantly, the half-lives of the complexes formed with these enzymes were 14, 140, and 340 min, respectively. Thus, this cyclic phosphate represents a new class of molecule leading to inert complexes of β-lactam-recognizing enzymes.

Introduction

The threat of bacterial infection to human health has increased in recent years as bacteria have become resistant to presently available antibiotics.[1] The latter is true of β-lactam antibiotics and has occurred to a considerable extent through the evolution and spread of β-lactamases, the bacterial enzymes that catalyze β-lactam hydrolysis.[2] New classes of molecule that inhibit these enzymes and/or the β-lactam targets, the cell wall synthesizing DD-peptidases, are therefore of considerable interest at present. We report here the structure and properties of such a molecule, salicyloyl cyclic phosphate (1-hydroxy-4,5-benz-2,6-dioxaphosphorinanone (3)-1-oxide, **1**). The genesis of the idea that led

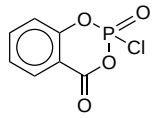


1

to this molecule lay in previous research in this laboratory that identified phosphonate monoester monoanions as β-lactamase inhibitors.[3] Structure−activity studies on this motif led to acyl phosph(on)ates.[3c,4] Cyclic variants of this theme, we surmised, where, as in penicillins themselves, the leaving group does not leave but remains in place to, potentially at least, obstruct incoming nucleophiles and thus slow the regeneration of free enzyme from covalent adducts, might be more effective inhibitors. This has proved to be the case as described below.

Results and Discussion

The cyclic phosphate **1** does not seem to have previously been isolated in stable form although it has been observed as a transient intermediate in various hydrolyses.[5,6] We examined the hydrolysis of the cyclic phosphoryl chloride **2**[8] in aqueous acetonitrile under the conditions described by Bruice et al.[5b] and found that a rapid hydrolysis product (3 min) had a ^{1}H NMR spectrum essentially identical to that reported for **1**.[9] After a



2

preparative scale hydrolysis (see the Experimental Section), **1** was isolated and characterized as a stable dicyclohexylamonium salt.

In aqueous buffer solution (20 mM MOPS, pH 7.5, 25 °C), **1** slowly hydrolyzed in two steps to salicylic acid. The intermediate has been previously characterized as salicyl phosphate **3** (Scheme 1).[5] The hydrolysis reactions could be conveniently monitored spectrophotometrically at the relevant isosbestic points (**1** to **3** was followed at 226 nm, an isosbestic point for conversion of **3** to salicyclic acid, and **3** to salicylic acid at 276 nm, an isosbestic point for conversion of **1** to **3**). This procedure yielded values of $k_1 = 1.7 \times 10^{-5}$ s^{-1} and $k_2 =$

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(2) Medeiros, A. A. *Clin. Infect. Dis.* **1997**, *24* (Suppl. 1), S19−S45.

(3) (a) Pratt, R. F. *Science* **1989**, *246*, 917−919. (b) Rahil, J. R.; Pratt, R. F. *Biochem. J.* **1991**, *275*, 793−795. (c) Rahil, J. R.; Pratt, R. F. *Biochemistry* **1992**, *31*, 5869−5878.

(4) (a) Rahil, J. R.; Pratt, R. F. *Biochem. J.* **1993**, *296*, 389−393. (b) Li, N.; Rahil, J. R.; Wright, M. E.; Pratt, R. F. *Biorg. Med. Chem.* **1997**, *5*, 1783−1788.

(5) (a) Bender, M. L.; Lawlor, J. M. *J. Am. Chem. Soc.* **1963**, *85*, 3010−3017. (b) Bruice, T. C.; Blasko, A.; Petyak, M. E. *J. Am. Chem. Soc.* **1995**, *117*, 12064−12069.

(6) Alkyl esters are however known, derived from alcoholysis of the cyclic phosphoryl chloride.[7]

(7) Cremlyn, R.; Ruddock, K.; Obisesan, O. *Phosphorus Sulfur* **1980**, *10*, 333−338.

(8) Montgomery, H. A. C.; Turnbull, J. H.; Wilson, W. *J. Chem. Soc.* **1956**, 4603−4606.

(9) Product observed after hydrolysis of the phosphoryl chloride **2** in [$^2H_3$]MeCN/^2H_2O 95:5: ^{1}H NMR δ ppm 7.25 (d, 1H, J = 8.0 Hz), 7.34 (t, 1H, J = 8.0 Hz), 7.75 (t, 1H, J = 8.0 Hz), 8.03 (d, 1H, J = 8.0 Hz) [lit.[5b] δ 7.249 (d, 1H), 7.335 (t, 1H), 7.75 (t, 1H), 8.027 (d, 1H)].

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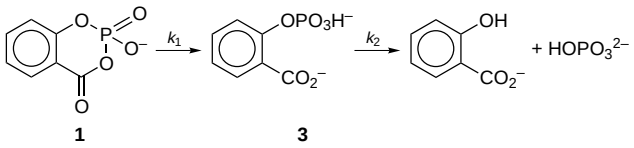
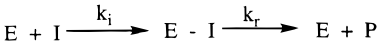
Scheme 1



1 **3**

Scheme 2



2.5×10^{-6} s^{-1}. These values are in good agreement with those from the literature: from the pH profiles provided by Bender and Lawlor,[5a] obtained in phosphate buffer, values of k_1 and k_2 of 3×10^{-5} s^{-1} and 0.75×10^{-6} s^{-1} can be estimated. Bruice et al.[5b] report a k_1 value of 1.2×10^{-4} s^{-1} in ^2H_2O (acetate buffer, pH 5.0) at 39 °C. For comparison, the rate constant for hydrolysis of benzylpenicillin (20 mM MOPS, pH 7.5, 25 °C) was found to be 1.5×10^{-5} s^{-1}. The cyclic acyl phosphate **1** is therefore of very similar lability to penicillin at neutral pH.

A representative class C and a class A β-lactamase were inhibited by **1** in a time-dependent fashion as described by Scheme 2. The inhibition reaction could be followed by loss of activity with time or by a concerted decrease in the fluorescence intensity of **1** with time (Figure 1A for the class C β-lactamase of *Enterobacter cloacae* P99). The rate constant k_r could be obtained from the return of activity observed in each case from data obtained on dilution of an aliquot of a reaction mixture into a substrate solution (e.g., Figure 1B for the TEM class A β-lactamase). Similarly, the DD-peptidase of *Streptomyces* R61 was inhibited, although more slowly. The relevant rate constants are presented in Table 1. Salicyl phosphate, (**3**), did not inhibit any of the above enzymes.

Despite the lack of structural similarity between **1** and the phosphonates previously examined,[3] its reactivity (k_i) with the class C β-lactamase is comparable to those of the former compounds, and this is true also of the other enzymes of Table 1. The order of reactivity between the β-lactamases, viz. class C > A, is also similar to that for acyclic ester substrates.[10] Perhaps of more interest than the k_i values are those of k_r. With both the class A and C β-lactamases and, interestingly, more so with the former, the E−I complex formed has considerable kinetic stability. In the former case, the half-life of E−I is ca. 2.3 h, signifying a rather seriously discommoded enzyme. In contrast to the above result, the TEM β-lactamase does not seem to form an accumulating intermediate with benzoyl phenyl phosphate, the acyclic analogue of **1** (N. Li and R. F. Pratt, unpublished observations). Thus, the rationale for the design of **1** appears confirmed. The R61 DD-peptidase also reacts with **1** to form a long-lived intermediate (as do β-lactams), although its rate of formation (k_i) is much smaller than with the β-lactamases.

Scheme 2 indicates that, in the presence of **1**, the enzyme activity/free enzyme concentration should decrease with time and then increase back to its original value as all of I is turned over. Under $I_o \gg E_o$ conditions, the minimum [E]/[E − I] ratio will be given by $k_r/k_i[I]$. At $I_o = 1$ mM, minimum [E]/[E − I] values for the P99 β-lactamase, the TEM β-lactamase, and the R61 DD-peptidase would thus be 1.5×10^{-4}, 6.0×10^{-3}, and 7.4×10^{-2}, respectively. The phosphate **1** is by this measure also a more effective inhibitor of the P99 β-lactamase than of the other two enzymes.

A major remaining question with respect to the reaction of these enzymes with **1** lies with the chemical nature of E−I.

(10) Govardhan, C. P.; Pratt, R. F. *Biochemistry* **1987**, *26*, 3385−3395.

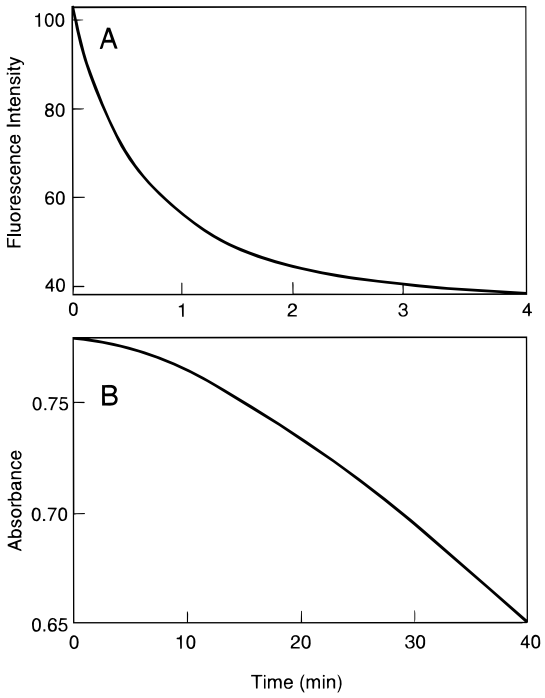


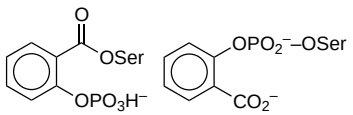
Figure 1. (A) Change in the fluorescence emission intensity of the phosphate **1** (2.0 μM, $\lambda_{ex} = 310$ nm, $\lambda_f = 370$ nm) as a function of time on reaction with the P99 β-lactamase (4.0 μM). (B) Return of β-lactamase activity on dilution (1/400) of a mixture of **1** (100 μM) and the TEM-2 β-lactamase (0.4 μM) into a solution of the substrate benzylpenicillin (0.94 mM). Consumption of substrate was followed spectrophotometrically at 238 nm as shown.

Table 1 Rate Constants for Inhibition and Recovery of Enzymes on Treatment with **1**

enzyme	k_i (s^{-1} M^{-1})	k_r (s^{-1})
P99 β-lactamase[a]	$(5.6 \pm 0.3) \times 10^3$ [d]	$(8.2 \pm 1.0) \times 10^{-4}$
	$(6.7 \pm 0.4) \times 10^3$ [e]	
TEM β-lactamase[b]	13.8 ± 2.4	$(8.3 \pm 1.0) \times 10^{-5}$
R61 DD-peptidase[c]	0.46 ± 0.07	$(3.4 \pm 0.4) \times 10^{-5}$

[a] The class C β-lactamase of *E. cloacae* P99. [b] The class A β-lactamase of the TEM-2 plasmid. [c] The DD-peptidase of *Streptomyces* R61. [d] From absorption measurements (see the Experimental Section). [e] From fluorescence measurements (see the Experimental Section).

Precedent suggests that a covalent species arising from reaction of **1** with the active site serine hydroxyl group is most likely.[3,11] Clearly, there is the potential for an acylenzyme (**4**) or a phosphoryl enzyme (**5**), where the actual structure might vary from enzyme to enzyme. Fluorescence spectra show that the product of slow turnover of **1** by the P99 β-lactamase is not salicyclic acid and therefore most likely salicyl phosphate **3**. This product could arise from either **4** or **5**.



4 **5**

The restoration of free enzyme from E−I for the P99 β-lactamase was not accelerated by the alternative nucleophile methanol. This is unusual if not unprecedented for acyl intermediates of this enzyme and suggests the structure **5** for

(11) Pratt, R. F. In *The Chemistry of β-Lactams*; Page, M. I., Ed.; Chapman & Hall: London, 1992; pp 229−271.

E−I in this case, but it is certainly also possible that the pendent erstwhile leaving group may prevent incursion of methanol into the active site.[12] More experiments are required to settle this issue.

Thus, the cyclic phosphate **1** represents the first member of a new class of molecules that can interact with β-lactam-recognizing molecules to generate inert complexes. As in the interaction of β-lactams with DD-peptidases, the latter property may result from its cyclic structure and tethered leaving group. Synthetic elaboration of **1** to give enzyme specificity is clearly conceivable.

Experimental Section

Materials. The β-lactamase of *Enterobacter cloacae* P99 and the TEM-2 β-lactamase from *Escherichia coli* W3310 were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. The *Streptomyces* R61 DD-peptidase was the generous gift of Dr. J.-M. Frère of the University of Liege, Belgium.

1-Chloro-4,5-benzo-2,6-dioxaphosphorinanone (3)-1-Oxide, (2). This compound was prepared from salicylic acid and phosphorus oxychloride essentially as described by Montgomery et al.[8] It was purified by short-path vacuum distillation followed by recrystallization from carbon tetrachloride. After purification, the air (water)-sensitive material had the following properties: mp 91−95 °C (lit.[8] mp 90−93 °C); FT-IR (KBr, cm^{-1}) 1792, 1611, 1286 (lit.[7] 1790, 1605, 1280 cm^{-1}); ^{1}H NMR (MeCN-d_3, 300 MHz) δ (ppm) 7.44 (1H, d, J = 8.0 Hz), 7.56 (1H, t, J = 8.0 Hz), 7.94 (1H, t, J = 8.0 Hz), 8.16 (1H, d, J = 8.0 Hz). It was stored in a sealed container at −20 °C.

1-Hydroxy-4,5-benzo-2,6-dioxaphosphorinanone (3)-1-Oxide, (1). The cyclic phosphoryl chloride **2** (500 mg, 2.3 mmol) was suspended in 60 mL of anhydrous acetonitrile (Aldrich, Sure/Seal) and water (41 μL, 2.3 mmol) added with magnetic stirring. The mixture was then gently warmed until the crystals of the starting material disappeared (ca. 5 min). Two molar equivalents of redistilled dicyclohexylamine (913 μL) were then added. The immediately precipitating solid (dicyclohexylamine hydrochloride) was removed by vacuum filtration, and the remaining solvent was removed by rotary evaporation. The residual colorless solid was recrystallized from acetonitrile/diethyl ether yielding 310 mg (35%) of the required product. Characterization: mp 160−163 °C; ^{1}H NMR (^2H_2O, 300 MHz) δ (aromatics, ppm) 7.27 (d, 1H, J = 8.0 Hz), 7.35 (t, 1H, J = 8.0 Hz), 7.78 (t, 1H, J = 8.0 Hz), 8.05 (d, 1H, J = 8.0 Hz); ^{31}P (^2H_2O) δ (ppm) −12.55; UV (20 mM MOPS buffer, pH 7.5), λ_{max} 239 nm ($\epsilon = 1.01 \times 10^4$ cm^{-1} M^{-1}), 295 nm ($\epsilon = 2.52 \times 10^3$ cm^{-1} M^{-1}); IR (KBr, cm^{-1}) 1737, 1613, 1286; ESMS (H_2O, m/z) 563.1 ($M + 2\ C_{12}H_{13}N + H^+$). Anal. Calcd for $C_{19}H_{28}NO_5P$: C, 59.80; H, 7.40; N, 3.67; P, 8.12. Found: C, 60.11; H, 7.44; N, 3.70; P, 7.91.

Analytical and Kinetic Methods. The concentrations of stock enzyme solutions were determined spectrophotometrically.[13] All enzyme kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5.

Second-order rate constants of inhibition of the P99 β-lactamase were determined from absorption traces (270 nm) showing turnover of the substrate cephalothin in the presence of enzyme and **1**. Initial rates decreased with time to a slow steady-state level when most of the free enzyme had been converted into the inert complex. Initial concentrations of enzyme, cephalothin and **1** were 1, 100, and 5−15 μM, respectively. Absorption versus time traces were fitted to Scheme 3 by means of the FITSIM program.[14] The K_m of cephalothin as a substrate of the P99 β-lactamase was taken to be 20 μM.[15] Over the concentration range of **1** studied, no enzyme saturation was required to fit the data.

Scheme 3

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

$$E + I \xrightarrow{k_i} E\text{-}I \xrightarrow{k_r} E + \underline{3}$$

$$I \xrightarrow{k_1} \underline{3}$$

The reaction of **1** with the P99 β-lactamase could also be monitored by the decrease in fluorescence emission as **1** (λ_{ex} = 310 nm, λ_f = 370 nm) was converted to **3**. The reaction was studied under second-order conditions (enzyme and **1** concentrations were 4.0 and 2.0 μM, respectively), and the data were fitted to eq 1 by means of a nonlinear least squares program.[16]

$$F = F_\infty + (F_o - F_\infty)\frac{(1 - I_o/E_o)\exp[-k_i(E_o - I_o)t]}{1 - (I_o/E_o)\exp[-k_i(E_o - I_o)t]} \qquad (1)$$

In eq 1, F is the fluorescence intensity at any time, F_o and F_∞ are the zero and infinite time intensities, respectively, and I_o and E_o are the initial concentrations of **1** and enzyme, respectively.

The rate of breakdown of the complex of **1** and the P99 β-lactamase to free enzyme (k_r, Scheme 2) was obtained by dilution of reaction mixtures containing enzyme (0.8 μM) and **1** (100 μM) into a saturating (0.94 mM) substrate (benzylpenicillin) solutions. The rate of turnover of benzylpenicillin (monitored spectrophotometrically at 240 nm) increased with time from close to zero to that characteristic of free enzyme. The absorption versus time traces were fitted to eq 2

$$A = A_o + V_\infty t - [(V_\infty - V_o)/k_r](1 - e^{-k_r t}) \qquad (2)$$

where A and A_o are the absorbance at any time and at zero time, respectively, and V_o and V_∞ are the observed initial and final rates of absorption change. The data were fitted to this equation by the nonlinear least-squares procedure.[16] Similar values of k_r were obtained as from the data fitted to Scheme 3.

Inactivation of the TEM enzyme was monitored by assaying the activity of a reaction mixture (enzyme and **1** concentrations 0.4 and 100 μM, respectively) against benzylpenicillin (0.94 mM) as a function of time. Initial rates decreased in a first-order fashion. The second-order rate constant was obtained by dividing the pseudo-first-order constant by the concentration of **1**.

The rate constant of reactivation of the TEM β-lactamase was determined in the same way as that of the P99 enzyme. The initial enzyme and **1** concentrations were 0.4 and 100 μM, respectively.

The rate constants for inactivation and reactivation of the R61 DD-peptidase were obtained by the general method employed for the TEM β-lactamase. Enzyme (2 μM) and **1** (2 mM) were incubated together and the concentration of the former with time determined by assay against the depsipeptide substrate *m*-carboxyphenyl phenaceturate[10] (3 mM). Reactivation was studied by means of the continuous monitoring of the rate of hydrolysis of the same substrate after dilution out of **1**.

Acknowledgment. This research was supported by National Institutes of Health grant AI 17986.

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(12) This phenomenon occurs for example with the class A PCl β-lactamase where the acyl enzymes derived from acyclic substrates are susceptible to methanolysis while those from penicillins are not.[10]

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