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BENZYLPENICILLIN METHYL PHOSPHATE. A PENICILLIN PRODRUG THAT INACTIVATES RTEM β-LACTAMASE

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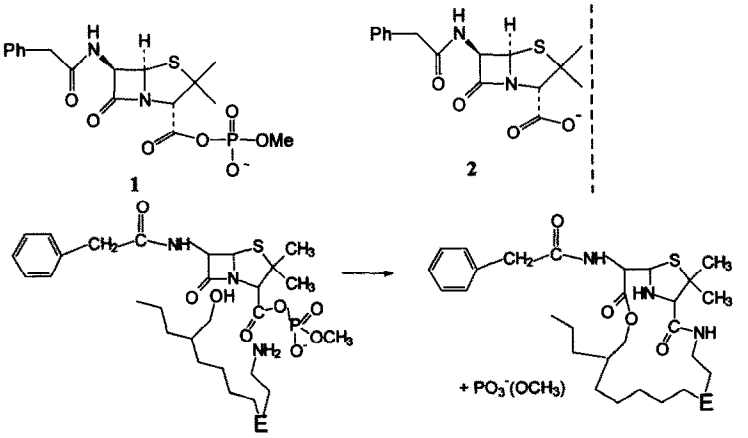
Abstract: Benzylpenicillin methyl phosphate (BPMP, **1**), hydrolyzes slowly to produce benzylpencillin and methyl phosphate. BPMP is a substrate and inactivator of *E. coli* RTEM β-lactamase. A competitive inhibitor of β-lactamase protects the enzyme from inactivation by BPMP. It is proposed that BPMP acylates the ε-amino group of Lys-234 of the enzyme.

Bacteria develop resistance to β-lactam antibiotics by producing β-lactamases that catalyze hydrolysis of the lactam ring.[1] Inactivators of β-lactamase provide a route to overcome this resistance. Most specific inactivators target the serine hydroxyl group of the enzyme which normally adds to the β-lactam of the antibiotic.[1-4] Lys-234 of the β-lactamases is another site essential for activity, possibly because the protonated ε-amino group provides electrostatic recognition and binding of the carboxylate group common to the antibiotics.[5-7] Pencillins with an isocyanate replacing the carboxylate have been reported to be affinity labels of β-lactamase.[8] It is probable that the isocyanate is attacked by the amino group of Lys-234. However, isocyanates are unstable in water and therefore these derivatives do not have medicinal potential. On the other hand, monoesters of acyl phosphates are stable carboxylate derivatives that hydrolyze slowly[9,10] but which rapidly acylate amino groups of proteins.[10-13] Thus, we reasoned that the acyl methyl phosphate of benzylpencillin (BPMP, **1**) will be a prodrug of benzylpencillin[14] (**2**) by hydrolysis and a potential inactivator

Scheme 1



of β-lactamase (Scheme 1).

We prepared BPMP by reacting potassium benzylpenicillin with $SOCl_2$ (1.0 equiv.) and pyridine (1.1 equiv., -23°C , 10 min) to give a solution of the acid chloride.[15] Bis(tetrabutylammonium) methylphosphate[16] was then added to produce BPMP in 83 % isolated yield.[17]

BPMP hydrolyzes slowly in neutral solution ($t_{1/2}$ = 90 h for hydrolysis of the methyl acyl phosphate, 50 mM NaH_2PO_4, pH 7.5, 25 °C), making the material a prodrug of benzylpencillin. Addition of BPMP to a solution of β-lactamase (3.0 μM, E. coli 205 TEMR+ (566)) leads to irreversible inactivation of the enzyme (assayed using 1.0 mM benzylpenicillin at 240 nm, pH 7.5, 50 mM NaH_2PO_4 25°C).[18] (Figure 1). BPMP reduces the β-lactamase activity to half its original level within 2.5 minutes. A solution containing methyl acetyl phosphate[10,19] in place of BPMP retained 85% activity over 40 minutes incubation, indicating that the penicillin moiety increases the affinity of the reagent for the enzyme. Prolonged dialysis of the inactivated enzyme against 50 mM NaH_2PO_4 buffer (pH 7.5) at 4°C restores none of the enzymic activity.

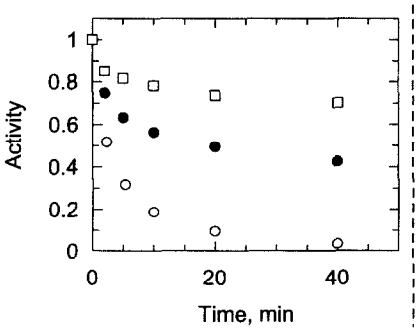


Figure 1. Inactivation of β-lactamase(3.0 μM) by BPMP at (❑) 2 mM, (●) 3.7 mM, (○) 7.4 mM.

To determine the affinity of the enzyme for BPMP, initial rates of inactivation were measured at five concentrations of BPMP (1.3 - 8.0 mM) . The dependence of the rates on the concentrations follows Michaelis-Menten kinetics with K_m = 2.0 mM. The observed rates give an apparent first order rate coefficient for inactivation, $k_{inact} = 0.0063\ s^{-1}$. As a test of whether reaction occurs at the active site, we utilized methyl benzylpenicilloate which is a reversible competitive inhibitor of β-lactamase.[20] It protects the enzyme from inactivation by BPMP (Figure 2), consistent with inactivation by BPMP taking place at the site to which BPMP binds.

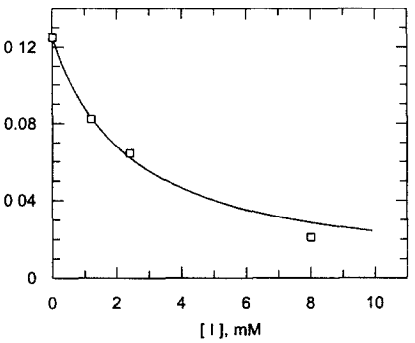
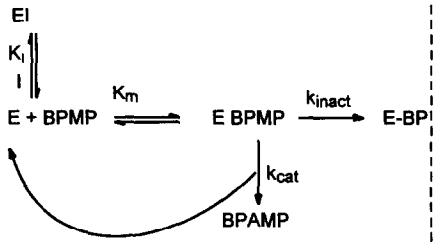


Figure 2. Effect on initial rates of inactivation of β-lactamase (3 μM) by BPMP (5.4 mM) from various concentrations of methyl benzylpenicilloate. The plot of $v = V_{max}S/(K_m (1+I/K_i) + S)$ uses calculated values with K_m= 2.0 mM for BPMP and K_i = 0.64 mM for methyl penicilloate.

BPMP is also a substrate for β-lactamase, giving the hydrolyzed lactam, BPAMP, (based on ^1H and ^{31}P NMR analysis of the final reaction solutions). If BPMP binds similarly to

benzylpencillin, its β-lactam ring should open upon reaction with the hydroxyl group of Ser-70. Subsequent hydrolysis of the acyl enzyme gives the product. Independent additon of the acyl phosphate ester of the hydrolyzed lactam does not inhibit the enzyme, consistent with the low affinity of hydrolyzed benzylpenicillin for the enzyme (K_m > 50 mM).[20] Thus, while BPMP inactivates the enzyme, the enzyme depletes BPMP via hydrolysis of its lactam ring (Scheme 2). (Although, the inactivation is shown as preceding from the Michaelis complex Scheme 2, the enzyme may add to the lactam of BPMP prior to the inactivation step, as shown in Scheme 1. Structural analysis of the inactivated enzyme is in progress to resolve this issue.)

Scheme 2



Because the competing lactam hydrolysis destroys the activity of BPMP, the inactivation of the enzyme (Figure 1) is not a zero-order process. The rate of inactivation decreases in parallel with lactam hydrolysis. From this, the rate constants for the two reaction routes from an E·BPMP complex can be estimated. Where (BPMP) = 2.0 mM, within 40 min, 31% of the enzyme (3 μM) is inactivated while 2.0 mM BPMP is consumed. Thus, BPMP turns over 2200 times per inactivation event (k_{cat}/k_{inact} = 2200).

These results demonstrate the utility of acyl phosphate monoesters as the basis of rational designs for leads in attacking medicinal targets. Preliminary bacteriological studies reveal that the compound provides synergistic effects with pencillins on resistant bacteria.

Acknowledgement. We thank the Protein Engineering Network of Centres of Excellence and the Natural Sciences and Engineering Research Council for support.

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(16) *Bis(tetrabutylammonum) methyl phosphate*: Methyl dichlorophosphate (5.0 g, 33.6 mmol) was added to 30 mL H_2O, 0°C. The solution was then kept at room temperature and stirred overnight. H_2O and HCl generated were removed in vacuo (<50°C). The resulting methyl phosphoric acid was titrated to pH 7.0 with tetrabutylammonium hydroxide solution (40%). The neutral solution was then vacuum dried at 60 °C.
(17) To a suspension of potassium benzylpenicillin (2.10 g, 5.64 mmol) and pyridine (0.55 mL, 6.80 mmol) in dry CH_2Cl_2 (30 mL) at -23°C, was added $SOCl_2$ (0.41 mL, 5.62 mmol) dropwise. After 10-15 min of stirring, during which time the suspension became clear, a solution of bis(tetrabutylammonium) methylphosphate (4.00 g, 6.73 mmol) in dry CH_2Cl_2 (30 mL) was added. The mixture was stirred at -23°C for 2-3 h. It was then warmed to room temperature, and stirred 2 h. The CH_2Cl_2 phase was washed with H_2O, satd $CuSO_4$, H_2O, 5% aq. $NaHCO_3$, H_2O and dried over Na_2SO_4. The organic layer was to give an amorphous solids (3.15 g, yield: 83%). ^1H NMR (200 MHz, $CDCl_3$) δ 7.45-7.20 (m, 5H, Ph), 6.13 (d, 1H, J=8.9Hz, N*H*), 5.60 (dd, 1H, J=4.5, 8.9Hz, HNC*H*CO), 4.49 (d, 1H, J=4.5Hz, NC*H*S), 4.36 (s, 1H, NC*H*COP), 3.66 (d, 3H, J=11Hz, OCH_3), 3.63 (s, 2H, PhCH_2), 3.45-3.15 (m, 8H, NCH_2CH$_2$), 1.80-1.25 (m, 16H, CH_2CH_2CH$_3$), 1.62 (s, 3H, CH$_3$CCH_3), 1.48 (s, 3H, CH_3CCH$_3$), 0.99 (t, 12H, J=8Hz, CH$_2$CH_3); ^{31}P NMR (120 MHz, $CDCl_3$) δ -6.95; ^{13}C NMR (50 MHz, CDCl3) δ 173.0, 170.2, 164.3 (d, J_{c-p}=8.5Hz), 133.8, 129.4, 128.9, 70.9, 67.6, 64.8, 58.6, 58.2 (d, J_{c-p} =8.1Hz), 53.3 (d, J_{c-p} =7.0Hz), 43.2, 31.9, 26.8, 23.9, 19.6, 13.6; FAB-MS (negative) 427; 427.07 calcd for $C_{17}H_{20}N_2O_7PS$; IR ($CHCl_3$) 3014, 1781, 1676, 1508, 1286 cm^{-1}.
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