Penicillin 3-aldehyde is a good substrate and not an inhibitor of β-lactamases A and C

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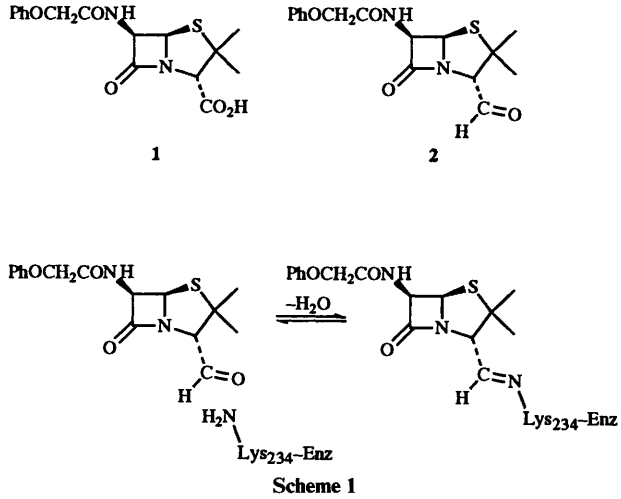
Replacement of the 3-carboxylate residue in phenoxymethylpenicillin by an aldehyde group gives a good substrate for *E. cloacae* P99 β-lactamase with k_{cat} = 34 s^{-1}, K_m = 7.5 × 10^{-5} mol dm^{-3} and k_{cat}/K_m = 4.5 × 10^5 dm^3 mol^{-1} s^{-1} at pH 7. With *B. cereus* 569/H β-lactamase I as a catalyst, k_{cat}/K_m = 1.87 × 10^4 dm^3 mol^{-1} s^{-1} at pH 7 and shows a bell-shaped dependence on pH with apparent pK_as of 4.76 and 9.72. Any close proximity between the penicillin 3-aldehyde and a lysine amino group on the protein does not result in iminine formation and inhibition of the enzyme.

β-Lactamase enzymes are largely responsible for bacterial resistance to penicillins, **1**, by catalysing the hydrolysis of the β-lactam to give an inactive product.[1] Despite several X-ray structures of the various enzymes and numerous kinetic studies the detailed mechanism and role of groups within the active site remains elusive.[1,2] In class A β-lactamases the lysine 234 residue is highly conserved and is thought to aid substrate binding by the interaction between its positively charged aminium ion and the negatively charged 3-carboxylate of the penicillin.[3] In the class C β-lactamases, the corresponding residue is lysine 315 or could even be an arginine residue (244).[4] Replacement of the anionic group in the β-lactam by a neutral residue may indicate the importance of this interaction. There are several examples known of enzyme inhibition which result from the close proximity of amine and carbonyl groups on the enzyme and inhibitor, respectively, giving rise to imine formation.[5] It therefore seemed appropriate to replace the penicillin 3-carboxylate, **1**, by an aldehyde, **2**, which could possibly generate a potential inhibitor of β-lactamase by forming a Schiff base with the enzyme which would then offer a method of exploring the active site of the enzyme, Scheme 1.

Phenoxymethylpenicillin 3-aldehyde, **2**, was synthesised by Moffat oxidation of the corresponding 3-hydroxymethyl derivative.[6] The penicillin 3-aldehyde, **2**, is, in fact, a reasonable substrate for *B. cereus* 569/H β-lactamase I (a class A enzyme) and shows a k_{cat}/K_m of 1.87 × 10^4 dm^3 mol^{-1} s^{-1} at pH 7, which is about 2 × 10^3 less than that observed for phenoxymethylpenicillin with a carboxylate at position 3. The enzyme rate enhancement factor[7] (the ratio of the second-order rate constant for the enzyme-catalysed reaction, k_{cat}/K_m, to that for the hydroxide-ion catalysed hydrolysis, k_{OH}) is 1.8 × 10^3, compared with a value of 2.6 × 10^8 for phenoxymethylpenicillin. Furthermore, the pH–rate profile for the β-lactamase catalysed hydrolysis of the 3-aldehyde, **2**, is the normal bell-shaped curve (Fig. 1). The data in Fig. 1 is fitted to eqn. (1)

$$k_{cat}/K_m = (k_{cat}/K_m)_{max}/([H^+]/K_a^1 + 1 + K_a^2/[H^+]) \quad (1)$$

with the following parameters $(k_{cat}/K_m)_{max}$ = 1.92 × 10^4 dm^3 mol^{-1} s^{-1}, pK_a^1 = 4.76 (±0.08) and pK_a^2 = 9.72 (±0.13). For comparison, the apparent pK_as observed with benzylpenicillin as substrate are 4.9 and 8.8.[6] The decrease in activity at high pH caused by the apparent ionisation of a group of pK_a 9.72 is not due to a residue which is interacting with the 3-carboxylate group on penicillins. Hydrolysis of the penicillin 3-aldehyde, **2**, using the RTEM-A β-lactamase as a catalyst yielded a k_{cat}/K_m of 2.28 × 10^4 dm^3 mol^{-1} s^{-1} which is about 500 times less than that for benzylpenicillin.



Scheme 1

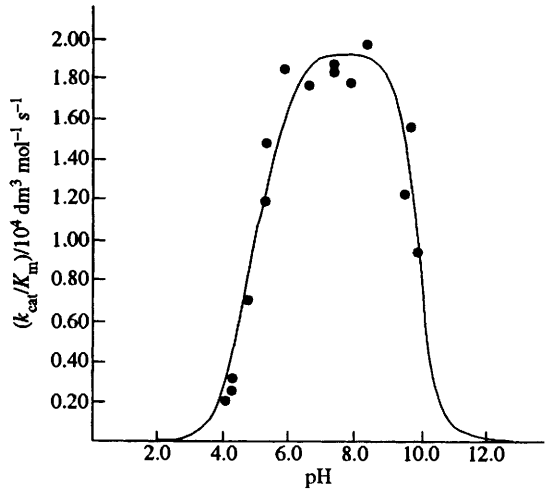


Fig. 1 A plot of k_{cat}/K_m against pH for the *B. cereus* β-lactamase catalysed hydrolysis of phenoxymethylpenicillin 3-aldehyde, **2**, at 30 °C, ionic strength 1.0 mol dm^{-3} (KCl). The solid line is calculated from eqn. (1).

Enterobacter cloacae P99 β-lactamase is a class C enzyme[1] and the penicillin 3-aldehyde, **2**, was found to be an excellent substrate for this enzyme despite lacking the 3-carboxylate group. In Table 1 are shown the kinetic parameters for the penicillin 3-aldehyde, **2**, and, for comparison, the corresponding data for benzylpenicillin, cephaloridine and cephalothin. The

Table 1 The kinetic parameters for the *E. cloacae* P99 β-lactamase catalysed hydrolysis of the penicillin C3 aldehyde, **2**, and β-lactam antibiotics at pH 7.0, 30 °C and I = 1.0 mol dm^{-3} (KCl)

Substrate	k_{cat}/s^{-1}	K_m/mol dm^{-3}	(k_{cat}/K_m)/dm^3 mol^{-1} s^{-1}
Phenoxymethylpenicillin 3-aldehyde **2**	34	7.5×10^{-5}	4.53×10^5
Benzylpenicillin	23	6.5×10^{-6}	3.54×10^6
Cephaloridine	320	1.8×10^{-4}	1.78×10^6
Cephalothin	350	7.2×10^{-5}	4.86×10^6

penicillin 3-aldehyde is a very good substrate for this enzyme and interestingly also shows saturation kinetics. Within an order of magnitude it is as good a substrate as benzylpenicillin for this enzyme. This is quite remarkable in view of the perceived importance of an anionic group at the 3-position which has traditionally been thought to be essential for activity.

To test for any inactivation of the enzyme, β-lactamase was incubated with an excess of the penicillin 3-aldehyde, **2**, at low (pH 3–4) and high (pH 8–9) pH to see if Schiff base formation was favoured under conditions of minimum hydrolytic activity. After various periods of incubation, the enzyme was assayed for activity at pH 7 using cephaloridine and benzyl penicillin as substrates. There was no decrease in activity above the background resulting from acid-catalysed deactivation of the enzyme.

Initial observations did in fact indicate that the penicillin aldehyde inactivated the enzyme at pH 3.5 but this was later shown to be due to the small amount of organic solvent (acetonitrile) used to dissolve the aldehyde, which dramatically increased the rate of the acid-catalysed deactivation of the enzyme. For example, at pH 3.5 and 30 °C and I = 1.0 mol dm^{-3} the background rate of deactivation of the P99 enzyme was increased tenfold in the presence of 0.45 mol dm^{-3} (*ca.* 2.3% v/v) of acetonitrile.

Based on the suggested mechanism of C–N fission in β-lactams which involves a rotational motion around C(5)–C(6) it is appropriate that there is not a strong inflexible binding site for the C3-carboxylate which could inhibit such motion.[8]

Experimental

Phenoxymethylpenicillin 3-aldehyde (2)

(3-Hydroxymethyl)phenoxymethylpenicillin[6] (0.5 g, 1.5 mmol) and dicyclohexylcarbodiimide (1.0 g, 4.9 mmol) were dissolved in dimethyl sulfoxide (6.9 cm^3) from a fresh Sureseal bottle. Freshly distilled dichloroacetic acid (100 μl) was added with stirring. At the onset the solution appeared pale yellow, however, after 5 min the solution had darkened to a bright orange colour, with the precipitation of a white solid (dicyclohexyl urea). The penicillin aldehyde was purified from the orange solution by preparative reversed-phase HPLC using a Dynamax 60-A reversed-phase column with 35% acetonitrile–65% distilled water as the eluent. The penicillin alcohol eluted after 19 min and the penicillin aldehyde after 14 min. The compound was isolated by extracting the HPLC fractions containing the aldehyde into dichloromethane and removing the solvent to yield a colourless solid (170 mg, 32%). δ_H(270 MHz; $CDCl_3$), 1.58 (3 H, s, 2-CH_3), 1.62 (3 H, s, 2-CH_3), 4.25 (1 H, d, J 2.3, H-3), 4.55 (2 H, s, $PhOCH_2$), 5.56 (1 H, d, J 4.4, H-5), 5.83 (1 H, dd, J 4.4, 9.04, H-6), 6.95 (2 H, m, Ph), 7.48 (1 H, d, J 9.04, CONH), 7.27–7.37 (3 H, m, Ph) and 9.62 (1 H, d, J 2.3, CHO); ν_{max}(Nujol)/cm^{-1} 1559, 1644, 1734, 1772 and 3442; m/z MH^+ = 335, mp 105–106 °C.

Kinetics

HPLC column: Merck Hibar Lichrocart mann-fix 250.4, C_{18} reversed-phase column with 20 μl loop interfaced to an IBM PC with the Beckman System Gold™ programme; detector: Beckmann analogue interface module 406 with diode array module 168; eluent: 30% acetonitrile, 70% distilled water, flow rate 1 cm^3 min^{-1}, λ 230 nm; the penicillin 3-aldehyde, **2**, eluted after 12 min. A calibration graph for the penicillin 3-aldehyde, **2**, was obtained and used to establish the concentration of aldehyde in the acetonitrile–water fractions from the preparative HPLC. The treatment of the kinetic data was as previously described.[9]

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