Alcohol-catalysed Hydrolysis of Benzylpenicillin

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The hydrolysis of benzylpenicillin is catalysed by alkoxide ions and other oxygen bases. Catalysis occurs by a nucleophilic pathway and the intermediate ester can be detected in some cases. The Brønsted β_{nuc} for alkoxide ions is 0.97, and is compatible with rate-limiting ring opening of the β-lactam. A solvent isotope effect of 3.2 for the trifluoroethanol-catalysed reaction suggests protonation by water occurs to the departing β-lactam nitrogen. Penicillin is not a particularly effective acylating agent of alcohols.

The alcoholysis of penicillin is thought to be the first chemical step in the reaction of β-lactam antibiotics with transpeptidase and β-lactamase enzymes.[1] The first of these enzymes is the primary killing site for the lethal action of β-lactam antibiotics against bacteria.[2] The second is the primary method of defence used by bacteria to resist this bactericide.[3] Both enzymes share a common method of opening the β-lactam ring which involves the attack of a serine hydroxy group on the carbonyl carbon to give a penicilloyl enzyme intermediate, an ester of penicilloic acid (Scheme 1).[1–4] The difference between the two enzymes is that, with transpeptidase, the acyl enzyme is thought either to be relatively stable or to undergo another chemical step to generate the actual inhibited enzyme complex[2,5] whereas, with β-lactamase, the acyl enzyme is rapidly hydrolysed to regenerate the enzyme and penicilloic acid[4,6] (Scheme 1).

The reaction of alcohols and other oxygen nucleophiles with penicillin is therefore of obvious interest. In the absence of enzymes, do penicilloyl esters undergo reactions at a rate which is competitive with or faster than hydrolysis? In this paper we describe the kinetics and mechanism of the alcohol catalysed hydrolysis of penicillins. Since this process occurs by nucleophilic catalysis it represents a model for the first step of the enzyme catalysed reactions (Scheme 1) and also for the second step in the case of β-lactamase. In the following two papers[7,8] we describe reactions of penicilloic acid derivatives other than hydrolysis.

Experimental

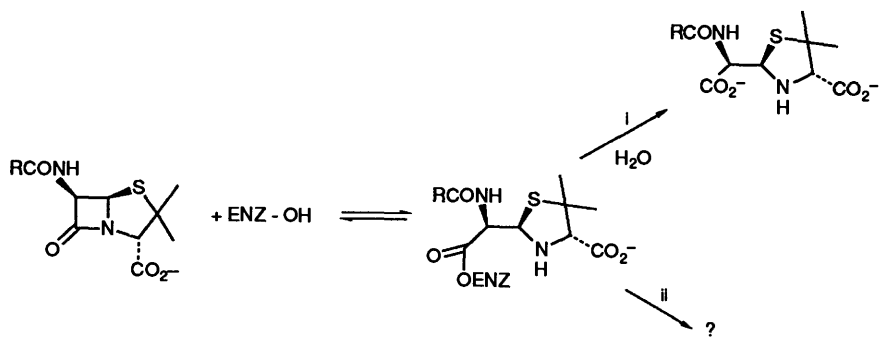
Materials.—Benzylpenicillin was a gift from Glaxo Group and other materials were of AnalaR grade. The alcohols were purified by distillation. Freshly boiled deionised water was used throughout and the ionic strength maintained at 1.0 mol dm^{-3} with potassium chloride unless otherwise stated. The alcohol buffers were prepared by partial neutralisation just prior to the kinetic run.

(3S,5R,6R)-*Methylbenzylpenicilloate.* Benzylpenicillin (6 g) was dissolved in methanol (100 cm^3) and triethylamine (4 cm^3) and kept at room temperature, under nitrogen, for 72 h. The solvent was removed under reduced pressure to give a residue which was taken up in water (75 cm^3) and diethyl ether (75 cm^3) and then acidified to pH 3.5 with 1 mol dm^{-3} hydrochloric acid. The diethyl ether layer was separated and the aqueous phase further extracted with diethyl ether. The combined organic fractions were dried and evaporated to give a white solid which was recrystallised from methanol–diethyl ether (1:9). M.p. 125–127 °C; $\delta_H(D_2O)$ 1.22 (s, 2-α-CH_3), 1.52 (s, 2-β-CH_3), 3.59 (s, 1 H, 3-H), 3.79 (s, 2 H, PhCH_2), 3.84 (s, 3 H, OMe), 4.70 (d, 1 H, $J_{6,5}$ 8, 6-H), 5.20 (d, 1 H, $J_{6,5}$ 8, 5-H) and 7.37 (s, 5 H, Ph). (Coupling constants are in Hz.)

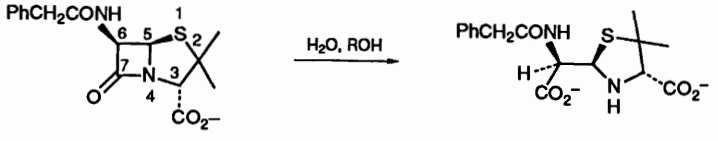
(3S,5R,6R)-2,2,2-*Trifluoroethylbenzylpenicilloate.* Benzylpenicillin (1 g) was dissolved in trifluoroethanol (5 cm^3) to which was added 1 mol dm^{-3} sodium hydroxide (75 mm^3) in 25 mm^3 aliquots over 100 min. The reaction was followed by HPLC and found to be complete after 2.5 h. $\delta_H([^2H_6]DMSO)$ 1.14 (s, 2-α-CH_3), 1.52 (s, 2-β-CH_3), 2.51 (s, 1 H, 3-H), 3.53 (s, 2 H, PhCH_2), 4.33 (t, 1 H, 6-H, $J_{6,5}$ 8), 4.74 (q, 2 H, CH_2CF_3, $J_{H,F}$ 12), 4.88 (d, 1 H, 5-H, $J_{5,6}$ 8), 7.28 (s, 5 H, Ph) and 8.57 (d, 1 H, NH, $J_{6,N}$ 8); ν_{max}(Nujol)/cm^{-1} 1600, 1660 and 1755.

HPLC Conditions.—Using an 8 μm polystyrene–divinylbenzene polymer reversed-phase column and eluting with a 30% (v/v) acetonitrile–70% aqueous solution of 0.1 mol dm^{-3} phosphoric acid containing 4.8 × 10^{-3} mol dm^{-3} hexanesulphonic acid the following retention times were observed: benzylpenicilloic acid, 4.6 min; methyl benzylpenicilloate, 8.0 min and benzylpenicillin, 10.4 min.

Kinetics.—The kinetics of the reactions were studied by monitoring the change in UV absorption or HPLC peak areas with time. The reactions were initiated by the addition and



Scheme 1 *Reagents:* i, ENZ-OH = β-lactamase; ii, ENZ-OH = transpeptidase



(1)

1 3

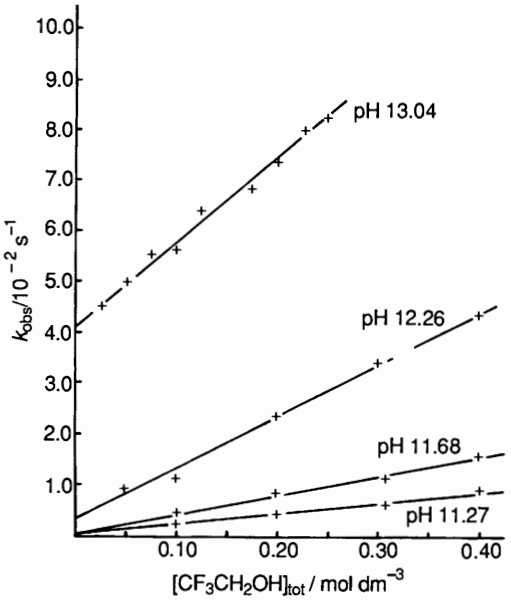


Fig. 1 A plot of the observed pseudo-first-order rate constants (k_{obs}/s^{-1}) for the hydrolysis of benzylpenicillin in aqueous buffers of trifluoroethanol at 30 °C against the total concentration of alcohol, $[ROH]_{tot}$, at various pH values

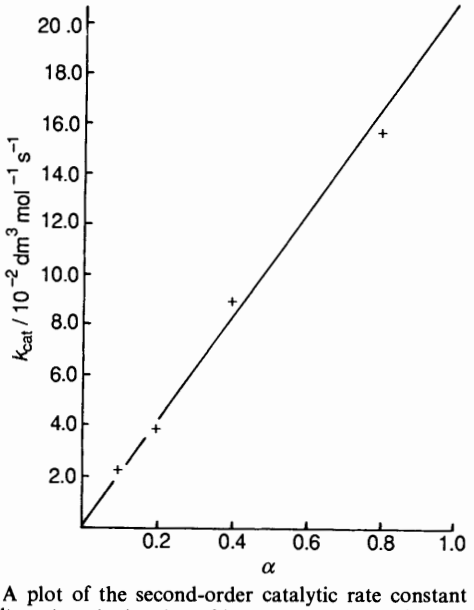


Fig. 2 A plot of the second-order catalytic rate constant (k_{cat}/dm^3 mol^{-1} s^{-1}) against the fraction of free base (α) present for the hydrolysis of benzylpenicillin at 30 °C in aqueous buffers of trifluoroethanol

thorough mixing of 25 mm^3 and of a stock solution (0.1 mmol dm^{-3}) of benzylpenicillin to 2.5 cm^3 of the aqueous buffer or sodium hydroxide solution containing the alcohol, preincubated at 30 ± 0.05 °C. The disappearance of the substrate was followed spectrophotometrically using a Gilford 2600 spectrophotometer at 235 nm. The data from the spectrophotometer was fed directly into a BBC microcomputer from which the rate constants were calculated using an iterative non-linear least-squares program which treated the first-order rate constants, and the absorbances at time zero and infinity, as adjustable parameters.[9]

Results

The first product of the reaction of benzylpenicillin in aqueous solutions of acidic alcohols which can be isolated is penicilloic acid with the same stereochemistry as the parent penicillin *i.e.* 3*S*,5*R*,6*R* [eqn. (1)]. As reported in the following paper this product subsequently slowly epimerises at C-5. The kinetics of the reaction were studied in aqueous solutions of the alcohol, which was used as the buffer. The alcohols catalyse the rate of hydrolysis of benzylpenicillin as shown in Fig. 1 for 2,2,2-trifluoroethanol. The observed pseudo-first-order rate constant, k_{obs}, increases linearly with total alcohol concentration, $[ROH]_{tot}$, which indicates that there is no term in the rate law which is second order in alcohol. The slopes of these plots, designated k_{cat}, increase with increasing pH, and plots of k_{cat} against the fraction of alcohol present as the free base in the buffer solution, α, give a positive intercept at $\alpha = 1$ and an intercept at $\alpha = 0$ which is indistinguishable from zero (Fig. 2). This indicates that the catalytically reactive form of the alcohol is the alkoxide ion, and that there is no catalysis by the neutral, undissociated alcohol. The second-order rate constant, k_{RO^-}, for the alkoxide-ion-catalysed hydrolysis is given by the value of the intercept at $\alpha = 1$. The rate law for the hydrolysis of benzylpenicillin in aqueous buffers of alcohol is therefore given by eqn. (2). The intercept, k_{int}, of the plot of k_{obs} against total

$$\begin{aligned} k_{obs} &= k_{int} + k_{cat}[ROH]_{tot} \\ &= k_{OH}[OH^-] + k_{RO^-}\alpha[ROH]_{tot} \\ &= k_{OH}[OH^-] + k_{RO^-}[RO^-] \end{aligned} \quad (2)$$

alcohol concentration (Fig. 1) corresponds to the calculated first-order rate constant for the hydroxide-ion-catalysed hydrolysis.

With less acidic alcohols such as 2-chloroethanol, the kinetics were studied in aqueous carbonate buffers containing the alcohol. The concentration of alkoxide ion was calculated from the pK_a of the alcohol and the measured pH. The second-order rate constant was deduced from measurements of the observed pseudo-first-order rate constant as a function of increasing alkoxide ion concentration. Some measurements were also conducted in aqueous solutions of sodium hydroxide containing the alcohol, *e.g.* methanol. The observed pseudo-first-order rate constant increases with increasing concentration of alcohol and hydroxide ion. The alkoxide ion concentration was calculated from eqn. (3) where K_w is the dissociation

$$[RO^-] = \frac{K_a}{K_w}[ROH][OH^-] \quad (3)$$

constant of water at 30 °C, $10^{-13.84}$,[10] and K_a the dissociation constant of the alcohol. With these less acidic alcohols the initial product was not penicilloic acid but the corresponding penicilloyl ester. This was evident from HPLC studies which showed the intermediate ester build-up before decaying to penicilloic acid. The intermediacy of the ester was also indi-

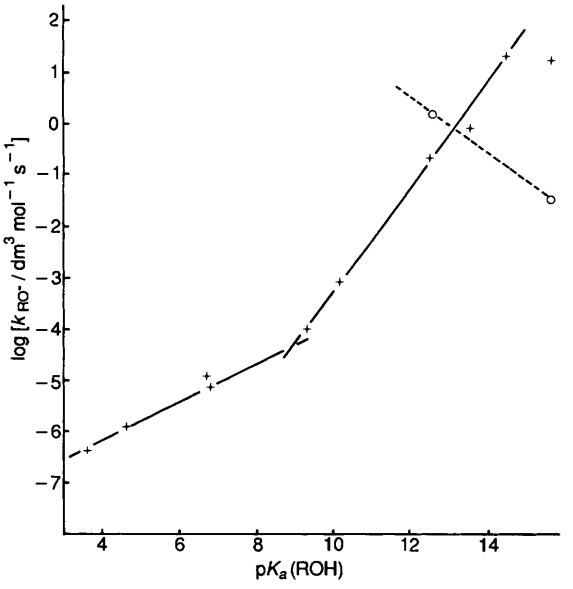


Fig. 3 A Brønsted plot of the logarithm of the second-order rate constants for the reaction of oxygen bases with benzylpenicillin at 30 °C in water against the pK_a of the conjugate acid (+). Also shown are second-order rate constants for the alkaline hydrolysis of penicilloyl esters (○).

cated by an increase in the UV absorbance at 280 nm, followed by its decay. This is interpreted as conversion of the intermediate ester into a penamaldate derivative by base-catalysed elimination across C5–C6. This reaction is described in further detail in the third paper of this series.[8] The intermediacy of the ester was also suggested by the penamaldate assay used to distinguish penicilloic acid from its derivatives.[11] Finally, these inferences were confirmed by synthesising the penicilloyl esters, subjecting them to alkaline hydrolysis and observing the same increase in absorbance at 280 nm before the formation of penicilloic acid.

The rates of alkaline hydrolysis of the methyl and trifluoroethyl penicilloates were determined, and the second-order rate constants are 0.324 and 16.3 dm^3 mol^{-1} s^{-1}, respectively. These rate constants are plotted against the pK_a of the appropriate alcohol in Fig. 3. There was no increase in UV absorption at 280 nm during the hydrolysis of the trifluoroethyl ester and it is therefore concluded that hydrolysis of the ester is faster than formation of penamaldate.

Table 1 The second-order rate constants for the hydrolysis of benzylpenicillin in aqueous solution catalysed by oxygen bases and nucleophiles at 30 °C and I = 1.0 mol dm^{-3} (KCl)

Oxygen base/nucleophile	pK_a[a]	k_{RO^-}/dm^3 mol^{-1} s^{-1}
CH_3O^-	15.54	15.96
$ClCH_2CH_2O^-$	14.31	20.1
$CH{\equiv}C{-}CH_2O^-$	13.55	7.82 × 10^{-1}
$CF_3CH_2O^-$[b]	12.43	1.95 × 10^{-1}
$MeOC_6H_4O^-$	10.06	8.12 × 10^{-4}
$(CF_3)_2CHO^-$	9.30	1.05 × 10^{-4}
$(CF_3)_2C(OH)O^-$	6.76	8.98 × 10^{-6}
HPO_4^{2-}	6.51	1.08 × 10^{-5}
$CH_3CO_2^-$	4.60	1.26 × 10^{-6}
HCO_2^-	3.62	4.47 × 10^{-7}

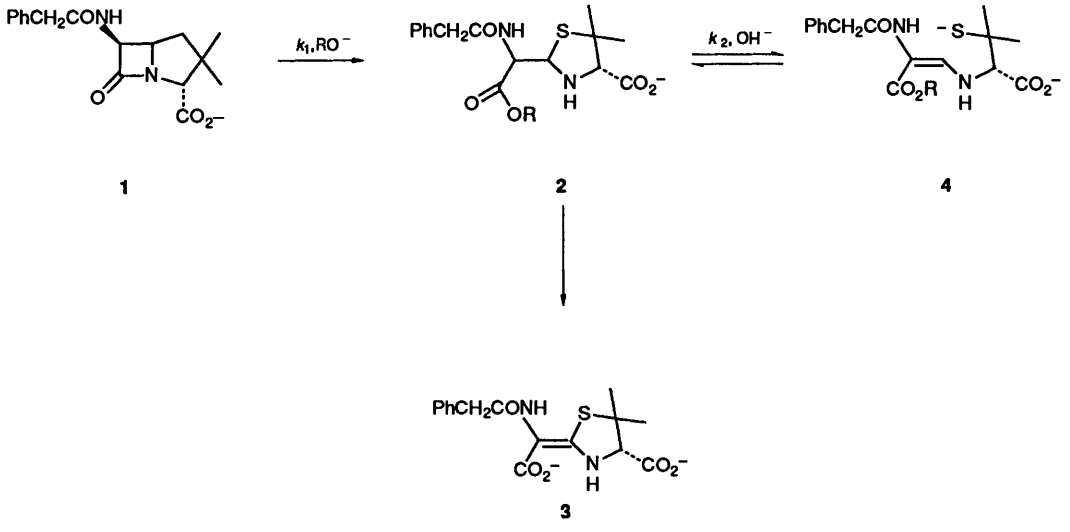
[a] pK_a of the conjugate acid of the alcohol; the values for the basic alkoxide ions are from P. Ballinger and F. A. Long, *J. Am. Chem. Soc.*, 1960, **82**, 795. [b] In D_2O k_{RO^-} = 6.18 × 10^{-2} dm^3 mol^{-1} s^{-1}.

Discussion

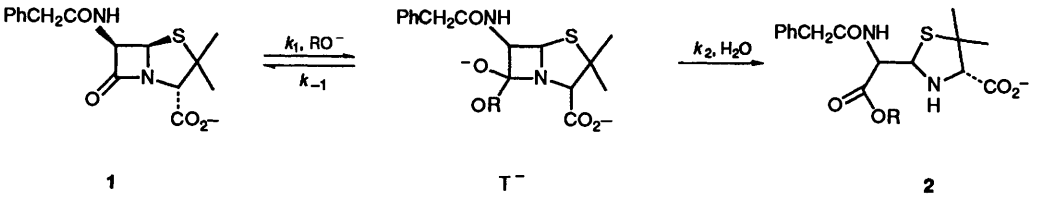
The pathway of the alkoxide-ion-catalysed hydrolysis of benzylpenicillin, **1** in aqueous solution is outlined in Scheme 2.

Alcohols facilitate hydrolysis by nucleophilic catalysis involving the intermediate formation of a penicilloyl ester, **2**, which undergoes base-catalysed hydrolysis to give penicilloic acid, **3**. For weakly acidic alcohols, such as methanol and ethanol, the intermediate ester **2** undergoes elimination across C5–C6, and opening of the thiazolidine ring to generate the penamaldate enamine derivative **4** at a rate that is competitive with ester hydrolysis.

The second-order rate constants for the alkoxide-ion-catalysed hydrolysis of benzylpenicillin are given in Table 1 together with data for other oxygen bases. The logarithms of these rate constants are plotted against the pK_a of the alcohol or oxygen acid in Fig. 3. There are two slopes; one, for weak oxygen bases, with a slope of Brønsted β value 0.38, is presumably indicative of a general-base-catalysed mechanism of hydrolysis, whereas the steeper slope, for alcohols of pK_a > 9, corresponds to a Brønsted β value of 0.97, which is indicative of nucleophilic rather than general-base catalysis. Basic alkoxide ions thus catalyse hydrolysis by alcoholysis and the intermediate formation of a penicilloyl ester. The reaction of benzylpenicillin with oxygen nucleophiles has been previously reported,[12] and that with phosphate is thought to involve the intermediate formation of a penicilloyl phosphate ester.[13] The Brønsted β values for the alcoholysis of acyl groups are generally 0.2–0.3 for rate-limiting formation of the tetrahedral intermediate.[14] This



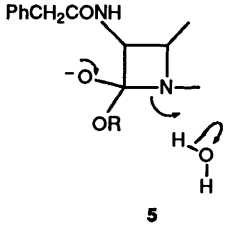
Scheme 2



Scheme 3

generally occurs when the attacking oxygen nucleophile is more basic than the leaving group, so that the tetrahedral intermediate would be expected to expel the leaving group to give products faster than it expels the nucleophile to regenerate the reactants. The much larger Brønsted β_{nuc} value shown for the alcoholysis of benzylpenicillin is indicative of rate-limiting breakdown of the tetrahedral intermediate T^- (Scheme 3). This suggests that breakdown of the intermediate T^- to regenerate the reactants (k_{-1}) is faster than breakdown by ring opening of the β-lactam (k_2) (Scheme 3). This is compatible with other studies which indicate that opening the four-membered ring is not a facile process.[1,15] The aminolysis of benzylpenicillin occurs by rate-limiting breakdown of the tetrahedral intermediate.[16,17] The second-order rate constants for the alcoholysis of benzylpenicillin are, in fact, similar to those observed for the uncatalysed aminolysis (Brønsted β_{nuc} = 1.0)[16] for the same basicity of nucleophile. Furthermore, it has been shown unambiguously[16] that the hydroxide-ion-catalysed aminolysis undergoes a change in rate-limiting step with increasing hydroxide ion concentration so that, at high base concentration, aminolysis proceeds with rate-limiting formation of the tetrahedral intermediate with a Brønsted β_{nuc} value of 0.3.[16] The rate constants for rate-limiting amine attack are 10^4 times greater than those observed for alcoholysis when the nucleophiles have a basicity of pK_a 8. It is clear that the observed second-order rate constants for the reaction of alkoxide ions with penicillin represent rate-limiting breakdown of the tetrahedral intermediate k_2 (Scheme 3).

The alcoholysis of benzylpenicillin with trifluoroethoxide shows a kinetic solvent isotope effect k_{H_2O}/k_{D_2O} of 3.16. This is also incompatible with rate-limiting attack of alkoxide, but suggests general-acid-catalysed breakdown of the intermediate by proton transfer from water to nitrogen, **5**.



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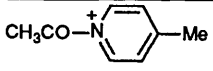
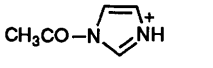
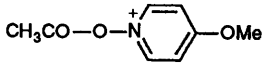
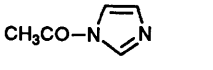
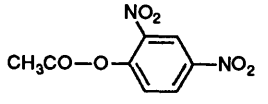
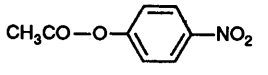
The traditional chemical view of penicillin as an effective antibiotic was that it was a good acylating agent because of enhanced reactivity, due to either strain in the four-membered ring, or reduced amide resonance. In Table 2 are shown the rate constants for the acylation of trifluoroethoxide ion by a variety of acylating agents. Penicillin is seen not to be particularly reactive towards this oxygen nucleophile. This is yet another simple demonstration that the high rate of reaction between penicillin and β-lactamase or transpeptidase enzymes is not due to the intrinsic reactivity of penicillin but is a result of the favourable non-bonded interactions between the substrate and enzyme which provide molecular recognition and binding energy to lower the activation energy.

Table 2 A comparison of the second-order rate constants for the reaction of 2,2,2-trifluoroethoxide ion with various acylating agents in water at 25 °C, unless stated otherwise

Acylating agent	k_1/dm^3 mol^{-1} s^{-1}	Ref.
	1.2 × 10^7	*a*
	6.5 × 10^5	*b*
CH_3CO–Cl	8.9 × 10^4	*c, d*
	4.6 × 10^4	*e*
	4.3 × 10^3	*b*
	4.0 × 10^2	*e*
	64	*f*
CH_3CO–OCH_2CF_3	20	*g*
CH_3CO–O–Ph	6.8	*e*
Benzylpenicillin	2.23 × 10^{-1}	*h*

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