Thiazolidine Ring Opening in Penicillin Derivatives. Part 2. Enamine Formation

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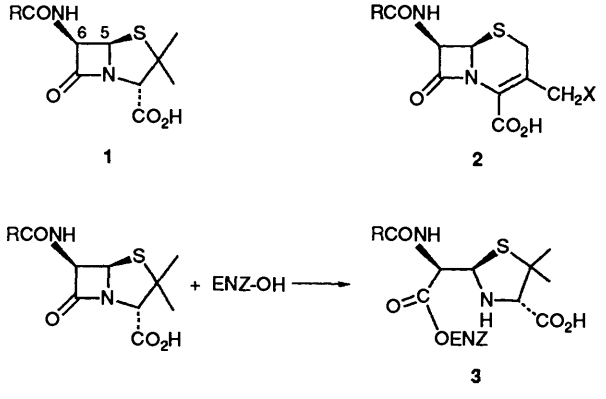
The alkaline hydrolysis of (3*S*,5*R*,6*R*)-methyl benzylpenicilloate, and the corresponding carboxamide and *N*-ethylamide, is accompanied by an absorbance increase at 285 nm. This is attributed to a competing elimination reaction across C6–C5 to open the thiazolidine ring and reversibly generate an enamine intermediate. Kinetic analysis and hydrolysis in D_2O do not indicate a significant build-up of this intermediate during hydrolysis of the methyl ester. However, over the pH range 4–11 the rate of thiazolidine ring opening is competitive with hydrolysis of the ester function. The deuterium solvent kinetic isotope effect on the ring closure reaction is 7.5.

The β-lactam antibiotics, such as penicillins (**1**) and cephalosporins (**2**), are effective antibacterial agents because they inhibit the enzymes required for the final stages of bacterial cell wall biosynthesis.[1] The cell wall is an alternating glycopolymer of *N*-acetylglucosamine and *N*-acetylmuramic acid with peptide bridges linking the latter residues on adjacent saccharide strands. The enzymes responsible for catalysing the formation of these peptide cross-links are membrane bound D-alanyl-D-alanine carboxypeptidases and transpeptidases.[2] It is these enzymes which are thought to be inhibited by the β-lactam antibiotics but a complete understanding of the mechanism of inhibition is unknown.[3] Model studies of inhibition have been based on a water soluble exocellular D-D-peptidase enzyme[2–4] and an X-ray crystallographic study of β-lactam antibiotics bound to this enzyme has recently been reported.[5] These enzymes are serine enzymes and the first chemical step in the reaction between the β-lactam and the D-D-peptidase is β-lactam ring opening and formation of an acyl enzyme (**3**) Scheme 1.[2] Interestingly there is no known residue which acts



Scheme 1

as a general-acid–base catalyst to facilitate the necessary proton transfers. The major difference between the reaction sequence outlined in Scheme 1 and that assumed to occur with the natural peptide substrate is that the leaving amino group, in the form of thiazolidine for penicillins, remains *covalently* bound to the enzyme.[6] It is not known why the acyl enzyme intermediate does not rapidly react with nucleophiles such as water. It is possible that the penicilloyl ester **3** undergoes another chemical step to reveal an electrophilic centre which could act as a 'second trap' for a nucleophilic group on the enzyme. The first paper in this series described the mechanism of the reaction of penicillins with alcohols *i.e.* a model reaction for the acylation outlined in Scheme 1.[7] The second paper described thiazolidine ring opening to generate an electrophilic iminium ion resulting in epimerisation at the C-5 position.[8] Herein we describe the elimination reaction across C6–C5 and the generation of an enamine intermediate. A preliminary report of this work has been published.[9]

Experimental

Materials.—The preparation of the penicilloyl ester **4** is described in the previous paper.[7] The penicilloyl amides **7** and **8** were prepared by treatment of benzylpenicillin with ammonia and ethylamine, respectively. NMR spectra were recorded in [$^2H_6$]dimethyl sulphoxide, and coupling constants are in Hz.

α-Amide of Benzylpenicilloic Acid (**7**). Benzylpenicillin sodium salt (3.56 g, 10 mmol) was dissolved in water (50 cm^3) and 35% ammonia (0.5 cm^3, 10 mmol) was added dropwise to this solution with stirring at room temperature over a period of 30 min. The solution was then stirred for a further 3 h. The mixture was then acidified with 50% phosphoric acid to pH 3.5 to give a solid which was filtered, washed with water and then recrystallised from 1:1 water:methanol. M.p. 108–110 °C; δ_H 1.16 (3 H, s, CH_3), 1.48 (3 H, s, CH_3), 3.53 (2 H, s, $COCH_2Ph$), 4.35 (1 H, t, *J* 8.1, 6-H), 4.87 (1 H, d, *J* 6.9, 5-H), 7.18 (1 H, s, NH_2), 7.25 (5 H, m, C_6H_5), 7.45 (1 H, s, NH_2) and 8.21 (1 H, d, *J* 9.3, CONH); ν_{max}(Nujol)/cm^{-1} 1525, 1645, 1701, 3314 and 3435; *m/z* (FAB, low resolution CI) 352 (M + H), 202 (Found: C, 51.65; H, 6.0; N, 11.4%. $C_{16}H_{21}N_3O_4$ requires C, 52.08; H, 6.23; N, 11.39%).

α-(N-Ethylamide) of Benzylpenicilloic Acid (**8**). The method used was that for the α-amide **7**, replacing ammonia with 70% ethylamine M.p. 102–104 °C; δ_H 0.98 (3 H, t, *J* 7.1, CH_2CH_3), 1.15 (3 H, s, CH_3), 1.50 (3 H, s, CH_3), 3.04 (2 H, m, CH_2CH_3), 3.25 (2 H, s, CH_2Ph), 4.35 (1 H, t, *J* 8.1, 6-H), 4.83 (1 H, d, *J* 6.8, 5-H), 7.22 (5 H, m, C_6H_5), 8.02 (1 H, t, *J* 5.5 NHEt) and 8.26 (1 H, d, *J* 9.2, CONH); ν_{max}(Nujol)/cm^{-1} 1536, 1649, 1719 and 3298; *m/z* (FAB, low resolution CI) 380 (M + H), 362 and 230.

Kinetics.—AnalaR grade chemicals were used exclusively in the preparation of buffers. Freshly boiled glass-distilled water was used throughout and the ionic strength was maintained at 1.0 mol dm^{-3} with potassium chloride except where otherwise indicated.

The pH of buffer solutions was measured with a Philips

PW 9409 digital pH meter equipped with a Russel type CEL glass combination electrode, calibrated against standard buffers of known pH at 30 °C. The electrode could be dipped into a reaction cuvette before and after a kinetic run to ensure the pH of the solution had not altered by more than 0.03 of a pH unit.

The spectrophotometer used for the majority of reactions was a Gilford model 2600 single-beam instrument which has a four cell compartment with an automatic cell change. The temperature in the cell compartment was maintained at 30 ± 0.1 °C by water circulated from a Haake water-bath to the cell block. Absorbance–time or UV spectra were plotted on a Hewlett-Packard 7225BX-Y plotter. Reactions were initiated by the addition of 25 mm^3 of substrate to 2.5 cm^3 of a temperature-equilibrated solution in a quartz cuvette, and the time-dependent change in absorbance monitored. Data was transferred to an Apple Europlus 2 or an IBM PC for analysis. Hard copies of results were printed on an Epson MX-80 printer and data stored on disk.

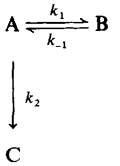
For very fast reactions where the half-life of the reaction was less than 2 s, the reactions were followed on a Nortech SF3A Mk. 4 stopped flow spectrometer. Reactants at twice the desired final concentration were placed in two piston-driven syringes. These feed into the reaction cell *via* coiled glass tubes immersed in a water-bath thermostatically maintained at 30 ± 0.1 °C. Absorbance changes after mixing were followed at 280–290 nm. The signal from the photomultiplier was transmitted to a Datalab DL 901 transient recorder which was automatically triggered by the outlet syringe, simultaneously causing a display on a Gould Advance OS 250B oscilloscope. Changes in absorbance *versus* time were output from the transient recorder to a Servogor 210 chart recorder.

Analysis of data. Data transferred from the Gilford 2600 to the Apple Europlus 2 were analysed using a program which calculates a first-order rate constant using an iterative non-linear least-squares procedure which treats the initial absorbance, final absorbance and rate constant as adjustable parameters. Besides accepting data from the Gilford, data can be entered from a disk file or manually on the keyboard. The experimental data may be compared graphically with the curve derived from the calculated parameters and edited where necessary.

Data transferred from the Gilford 2600 to an IBM PC was analysed using the ENZFITTER program for consecutive first-order reactions.

The data were also analysed manually by extrapolating the exponential decay of the intermediate back to zero time and correcting the absorbance contribution of the second reaction to that of the first and then calculating the pseudo-first-order rate constant from the corrected absorbance values. There was very good agreement between the various methods used.

The concentration of the intermediate B in the reaction sequence given in Scheme 2 has a time dependence given by



Scheme 2

eqn. (1) where $[A]_0$ is the initial concentration of A, t is the

$$[B] = \frac{k_1[A]_0}{\lambda_2 - \lambda_1}[\exp(-\lambda_1 t) - \exp(-\lambda_2 t)] \quad (1)$$

time and λ_1 and λ_2 are constants related to the kinetic rate constants given in Scheme 2 by eqns. (2) and (3).[10]

$$\lambda_1 = \frac{k_1 + k_2 + k_{-1} - [(k_1 + k_2 + k_{-1})^2 - 4k_{-1}k_2]^{\frac{1}{2}}}{2} \quad (2)$$

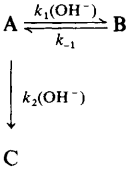
$$\lambda_2 = \frac{k_1 + k_2 + k_{-1} + [(k_1 + k_2 + k_{-1})^2 - 4k_{-1}k_2]^{\frac{1}{2}}}{2} \quad (3)$$

If the changes in concentration of B are determined by light absorption then the absorbance is given by eqn. (4) where ε is

$$A = \frac{\varepsilon k_1[A]_0}{\lambda_2 - \lambda_1}[\exp(-\lambda_1 t) - \exp(-\lambda_2 t)] \quad (4)$$

the extinction coefficient of B.

The reaction of the penicilloyl ester A to the enamine intermediate B and hydrolysis product C may be described by Scheme 3. Under the experimental conditions the conversion



Scheme 3

of the ester to product and intermediate would follow pseudo-first-order kinetics so that eqns. (5) and (6) hold.

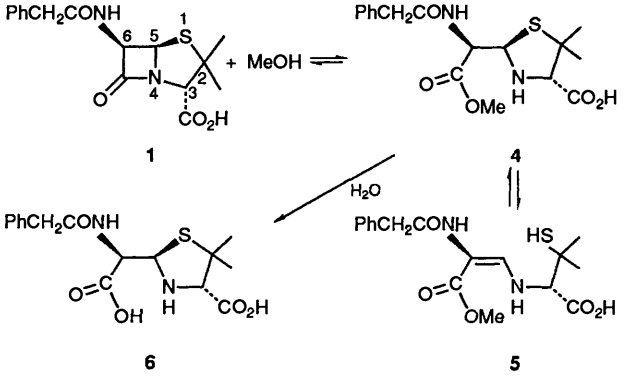
$$\lambda_1 + \lambda_2 = (k_1 + k_2)[OH^-] + k_{-1} \quad (5)$$

$$\lambda_1\lambda_2 = k_{-1}k_2[OH^-] \quad (6)$$

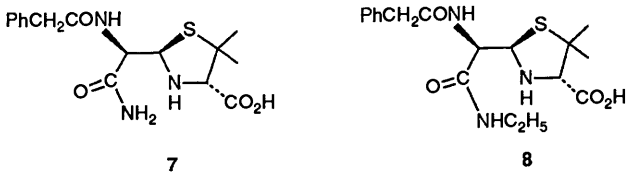
A plot of $(\lambda_1 + \lambda_2)$ against the concentration of hydroxide ion gives $(k_1 + k_2)$ as the slope and k_{-1} as the intercept, whereas a plot of $\lambda_1\lambda_2$ against hydroxide-ion concentration gives a slope of $k_{-1}k_2$. Hence all three rate constants can be determined, provided that k_1 is not too much smaller than k_2.

Results and Discussion

The alcoholysis of benzylpenicillin **1** with methanol in aqueous solutions of sodium hydroxide shows an absorbance increase, followed by a decrease, at 280 nm.[7] This was attributed to the penicilloyl ester intermediate **4** undergoing reversible elimination across C_6–C_5 to form the penamaldate **5**, in a process which is competitive with hydrolysis to give **6** (Scheme 4). To investigate this process in more detail the ester **4** and the



Scheme 4



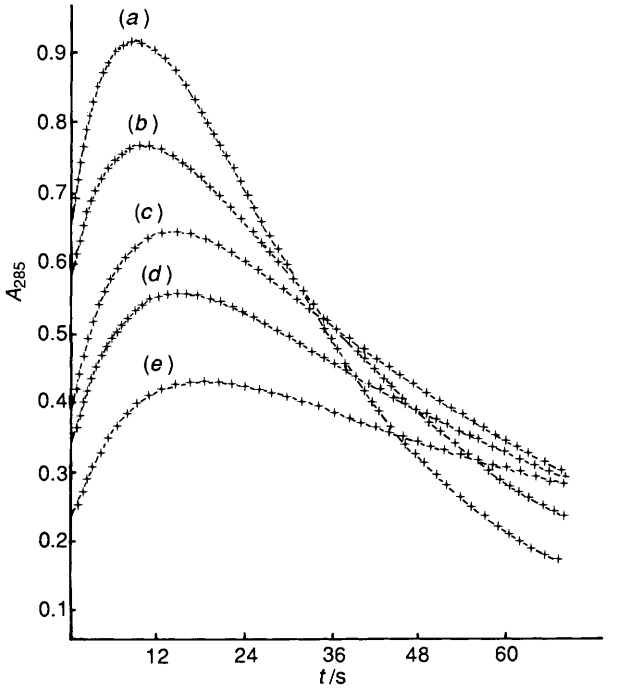


Fig. 1 The change in absorbance at 285 nm as a function of time for the hydrolysis of (5*R*,6*R*)-methyl benzylpenicilloate (1.0×10^{-3} mol dm^{-3}) at 30 °C in water. The solid lines are calculated from the equation and parameters given in the text. [NaOH] = (*a*) 0.10, (*b*) 0.06, (*c*) 0.04, (*d*) 0.03 and (*e*) 0.02 mol dm^{-3}.

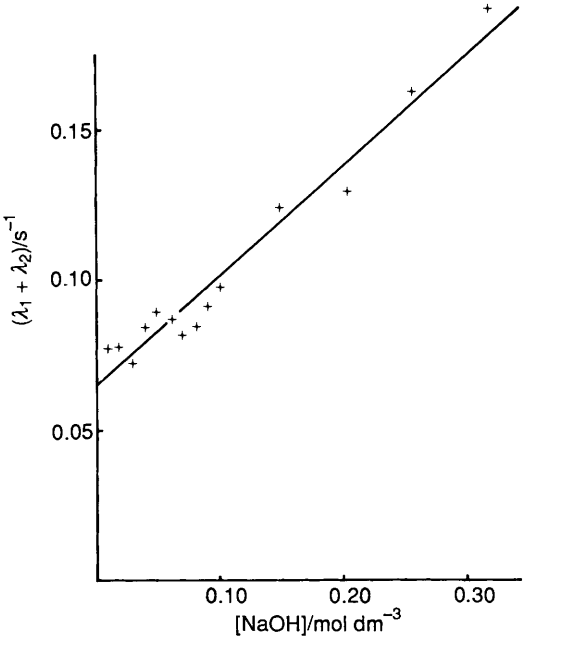


Fig. 2 A plot of a sum of the derived constants ($\lambda_1 + \lambda_2$) against the concentration of sodium hydroxide for the hydrolysis of (5*R*,6*R*)-methyl benzylpenicilloate

corresponding amides **7** and **8** were synthesised and their behaviour in aqueous solution examined.

The hydrolysis of (3*S*,5*R*,6*R*)-methyl benzylpenicilloate **4** is accompanied by an absorbance increase, followed by a decrease, at 285 nm with the absorbance maximum increasing with concentration of hydroxide ion (Fig. 1). The kinetic data were analysed according to Scheme 3 as described in the Experimental section.

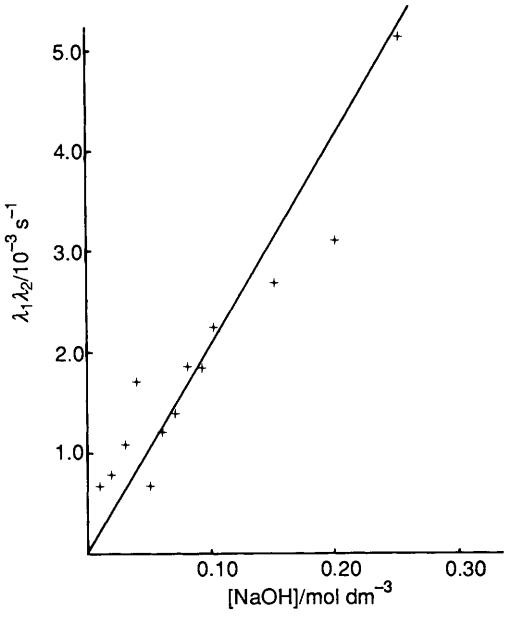


Fig. 3 A plot of the product of the derived constants ($\lambda_1\lambda_2$) against the concentration of sodium hydroxide for the hydrolysis of (5*R*,6*R*)-methyl benzylpenicilloate

The kinetic constants λ_1 and λ_2, obtained from the formation and decay of the chromophore at 285 nm, are related to the rate constants of Scheme 3 by eqns. (5) and (6). In Scheme 3 the penicilloyl ester 4 is represented by A, the penamaldate **5** by B and the hydrolysis product **6** by C.

A plot of ($\lambda_1 + \lambda_2$) against the concentration of hydroxide ion (Fig. 2) gives an intercept of 6.42×10^{-2} s^{-1} corresponding to k_{-1}, the pH-independent rate constant for ring closure of the enamine **5** to regenerate the starting material, eqn. (1). The products of the two constants $\lambda_1\lambda_2$ when plotted against the concentration of hydroxide ion (Fig. 3) gives a zero intercept and slope of $k_{-1}k_2$, eqn. (2). Hence the value of k_1, the second-order rate constant for the hydroxide-ion-catalysed formation of the enamine, is found to be 8.09×10^{-2} dm^3 mol^{-1} s^{-1} whilst that for hydrolysis of the methyl ester, k_2, is deduced to be 0.327 dm^3 mol^{-1} s^{-1}. The solid lines in Fig. 1 were calculated using these rate constants. The simplest interpretation of these observations is that (5*R*,6*R*)-methyl benzylpenicilloate **4** undergoes reversible elimination across C6–C5 to open the thiazolidine ring to give the enamine tautomer of methyl penamaldate **5**. The amount of enamine formed increases with increasing hydroxide-ion concentration which explains the increasing absorbance at 285 nm, the absorption maximum of penamaldates.[11] The calculated extinction coefficient of the penamaldate is 17 200 dm^3 mol^{-1} cm^{-1} which is in reasonable agreement with that observed for the mercury(II) salt of penamaldate.[11] The calculated equilibrium constant ($K = k_1/k_{-1}$) for formation of the enamine is 1.26 dm^3 mol^{-1}. In sodium hydroxide solution the equilibrium fraction of the penicilloyl ester **4** existing as the thiazolidine ring-opened enamine would be given by $K/(K + [OH^-]^{-1})$. However, the rate of ester hydrolysis is faster than enamine formation and the steady-state concentration of the enamine is given by eqn. (7), reaching a maximum value at the time, t_{max}, indicated by eqn. (8).

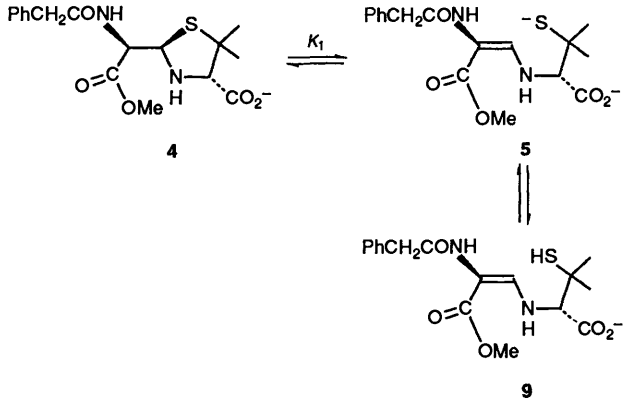
$$\frac{[5]}{[4]_0} = \frac{k_1}{\lambda_2 - \lambda_1}[\exp(-\lambda_1 t) - \exp(-\lambda_2 t)] \qquad (7)$$

$$t_{max} = \frac{1}{\lambda_1 - \lambda_2} \ln(\lambda_1/\lambda_2) \quad (8)$$

The fraction of the ester existing as the enamine never exceeds *ca.* 10%. This is substantiated by carrying out the hydrolysis reaction in $NaOD/D_2O$, neutralising at time t_{max} and observing, by NMR analysis, little deuterium incorporation into the C-6 hydrogen of the penicilloyl ester **4**. Similarly, the NMR spectrum of the neutralised but partially hydrolysed ester in water showed only a little epimerisation at C-6 and C-5. Both rate and equilibrium constants for formation of the enamine **5** are expected to depend upon the acidity of the C-6 hydrogen. Although there is no appearance of a chromophore at 280 nm from aqueous solutions of benzylpenicilloic acid **6** up to pH 13 there is a slow development in sodium hydroxide greater than 1 mol dm^{-3}. The absorbance maximum is dependent upon hydroxide ion concentration and the pseudo-first-order rate constants for the appearance of the chromophore are first order in hydroxide ion corresponding to a second-order rate constant of 4.50×10^{-4} dm^3 mol^{-1} s^{-1}, *ca.* 200 times slower than that for the corresponding ester. The rate retardation of the carboxylate anion compared with an ester substituent at C-6 is consistent with proton abstraction at C-6.

The penicilloyl amides **7** and **8** also show the appearance of the chromophore at 290 nm but, as expected, the absorbances are more stable. There is a decay of the chromophore, but at a rate which is much slower than that for the corresponding ester **4** and the rate constant for its formation is easily measured as a pseudo-first-order process. The observed pseudo-first-order rate constants show a non-linear dependence on hydroxide-ion concentration compatible with ionisation of the amide. Correction for this gives second-order rate constants of 0.115 and 0.15 dm^3 mol^{-1} s^{-1} for **7** and **8** respectively. The similarity of the rate constants compared with that for the ester **4** is also consistent with proton abstraction at C-6. The NMR spectrum of the amide **8** in $NaOD/D_2O$ shows the appearance of a singlet peak at δ 7.26 consistent with the vinylic hydrogen at C-5 of the enamine.

Although the intensity of the absorbance of the chromophore observed during the hydrolysis of the penicilloate ester **4** decreases with decreasing hydroxide-ion concentration, so that it is only *ca.* 0.01 for a 10^{-3} mol dm^{-3} solution of the ester **4** in 10^{-3} mol dm^{-3} sodium hydroxide, it reappears below pH 11. The pseudo-first-order rate constants for the appearance of the chromophore are first order in hydroxide ion and give a second-order rate constant similar to that obtained in solutions of sodium hydroxide. The absorbance change is independent of pH below pH 10.2 but decreases at higher pH. The simplest interpretation of these observations is that below pH 11 the penicilloyl ester **4** undergoes slow ring opening to form the enamine tautomer of methyl penamaldate **5** and that the thiol-



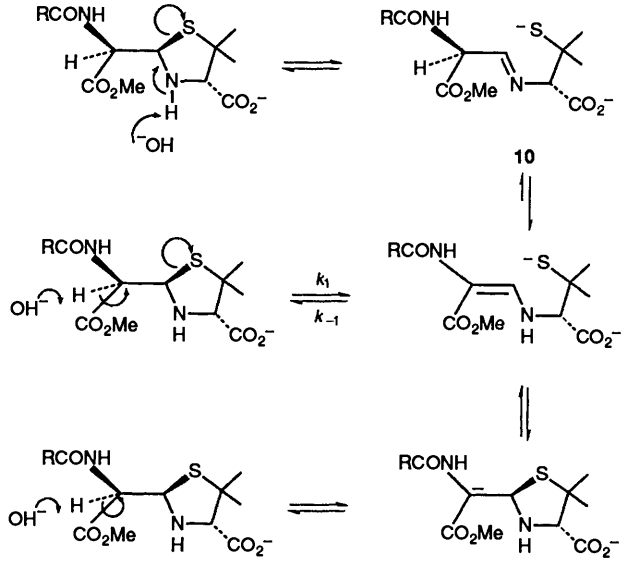
4 **5** **9**

Scheme 5

ate anion is then rapidly protonated to form the thermodynamically more stable thiol **9** at pH values below its pK_a (Scheme 5).

The equilibrium constant between the ester **4** and the enamine thiol **9** is given by K_1K_w/K_a where K_w and K_a are the ionisation constants of water and the thiol **9**, respectively. A pK_a of 11.0 for the thiol would indicate an equilibrium constant between **9** and **4** of 10^{-3}.

There are three possible mechanisms for the base-catalysed ring opening of the thiazolidine **4** to give the enamine **5** (Scheme 6).

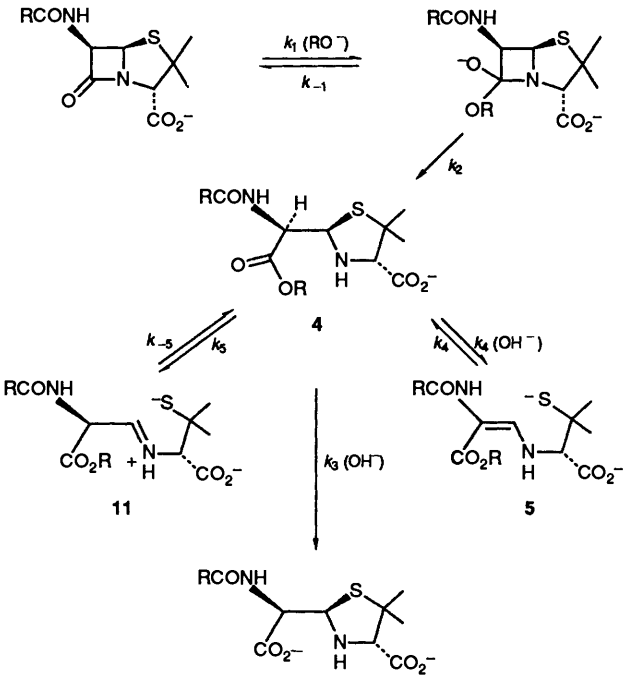


Scheme 6

A concerted E2-type process would generate the enamine directly and *anti*-elimination would give the *E* enamine. Ring opening could initially generate the imine **10** which then tautomerises to the more stable enamine. An equivalent process occurs during the epimerisation of benzylpenicilloic acid **6** with a second-order rate constant of 7.6×10^{-4} dm^3 mol^{-1} s^{-1}.[8] This is much slower than the rate constant of 8.1×10^{-2} dm^3 mol^{-1} s^{-1} for enamine formation from the ester **4** so it is unlikely to occur. Finally, elimination could occur by an E1cB-type process which could, in principle, generate both E and Z stereoisomers of the enamine.

Consistent with the concerted elimination process is the observation of a significant deuterium kinetic isotope effect on k_{-1}. The plot of $(\lambda_1 + \lambda_2)$ against base concentration in D_2O generates a line almost parallel to that found in H_2O whereas the product $\lambda_1\lambda_2$ plotted against deuteroxide ion concentration gives a slope 5.4 times less than that seen in H_2O (Fig. 3). The derived microscopic rate constants show that $k_{-1}^{H_2O}/k_{-1}^{D_2O} = 7.5$, compatible with a primary kinetic isotope effect on the protonation of C-6 by a water molecule, on which is superimposed a smaller secondary isotope effect on the generation of OD^- compared with OH^- (Scheme 6). The deuterium kinetic isotope effect on k_1 is the expected inverse one of 0.88 whereas the solvent isotope effect on the hydrolysis of the ester $k_2^{H_2O}/k_2^{D_2O}$ is 0.97.

The reactions of alkoxide ions with penicillins are summarised in Scheme 7. The initial alcoholysis proceeds by reversible formation of a tetrahedral intermediate which generates, in a rate-limiting step, the penicilloyl ester **4**. The ester **4** in turn undergoes thiazolidine ring opening by reversible base-catalysed elimination across C5–C6 to give an enamine **5**, in competition with hydrolysis of the ester function. At neutral pH the penicilloyl ester undergoes slow reversible unimolecular thiazolidine ring opening to generate an iminium ion **11** at a rate which is faster than that for hydrolysis of the ester function.



Scheme 7

Acknowledgements

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