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Thiol-catalysed hydrolysis of cephalosporins and possible rate-limiting amine anion expulsion†

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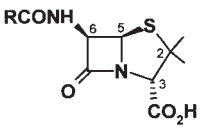
ABSTRACT: The rates of thiolysis of cephalosporins were investigated by high-performance liquid chromatography and ^{1}H NMR spectroscopy. Thiols catalyse the hydrolysis through the formation of a thioester intermediate and the catalytically reactive form of the thiol is the thiolate anion. Variation of nucleophilic reactivity by changing the basicity of the thiolate anion generates a Brønsted β_{nuc} value of 1.22 with cephaloridine, indicating that the breakdown of the tetrahedral intermediate is the rate-limiting step. The effect of C3′ substituents on the rate of thiolysis of cephalosporins generates a large Hammett ρ of ca 12, which is compatible with C—N bond fission occurring without protonation of the β-lactam nitrogen. Solvent kinetic isotope effects k_{H_2O}/k_{D_2O} of ca 1.1 also indicate that solvent water probably does not act as a general acid catalyst facilitating breakdown of the tetrahedral intermediate by protonating the departing amine. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: cephalosporins; thiol-catalysed hydrolysis; rate-limiting amine anion expulsion

INTRODUCTION

The reactions of β-lactam antibiotics and their derivatives have been extensively studied.[1–4] The β-lactam ring of penicillins (**1**) shows susceptibility towards attack by nucleophilic reagents in water, such as amines, alcohols and thiols, in competition with that by hydroxide ion. Nucleophilic substitution at the carbonyl centre of β-lactams is an acyl transfer process involving covalent bond formation between the carbonyl carbon and the nucleophile and C—N bond fission of the β-lactam. Previous studies have indicated that covalent bond formation to the incoming nucleophile occurs before β-lactam C—N bond fission, resulting in the reversible formation of a tetrahedral intermediate (Scheme 1).[1–4] The rate-limiting step in these reactions is thus commonly ring opening and breakdown of the tetrahedral intermediate.[2–6]



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The timing of bond making and breaking in acyl transfer reactions is a result of subtle effects often involving proton transfer. For stepwise processes involving the formation of a tetrahedral intemediate resulting from the attack on a carbonyl centre by a nucleophile with an ionizable hydrogen, there is a large change in the latter's pK_a as a result of covalent bond formation. Proton transfer from the nucleophile to a base catalyst thus occurs *after* full covalent bond formation, as it changes from a thermodynamically unfavourable to a favourable process. For example, aminolysis usually, but not always, requires general base catalysis to remove a proton from the attacking amine which occurs after formation of the tetrahedral intermediate in a rate-limiting step which is diffusion controlled.[2–4] More acidic nucleophiles such as alcohols and thiols may have a significant concentration of the ionized basic form present in solution which acts as the nucleophile and therefore proton removal is not necessary.

Acyl transfer involving β-lactam antibiotics requires C—N fission and expulsion of an amine. Formation of the tetrahedral intermediate also changes the basicity of the leaving group amine, as amide resonance in the β-lactam is lost and proton transfer to nitrogen changes from an unfavourable to a thermodynamically favourable process. Hence many of these reactions require general acid catalysis and protonation of the amine nitrogen leaving group. In addition, the tetrahedral intermediate may be stabilized by metal ion coordination to the leaving group.[4] Although the release of strain energy, which accompanies ring opening, could possibly decrease the need for protonation, C—N fission in penicillins appears to require some form of catalysis.[1–3] For

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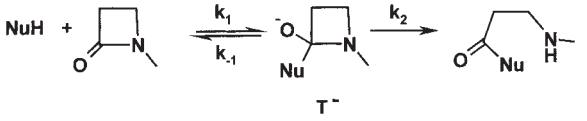
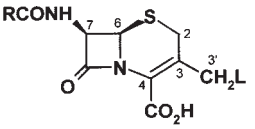


Scheme 1

example, the alcoholysis[5] and thiolysis[6] of penicillins occur with rate-limiting breakdown of the tetrahedral intermediate facilitated by proton transfer from solvent water to the departing amine.



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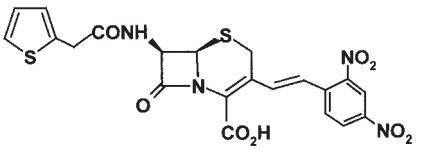
Cephalosporins (**2**) are different from penicillins in that the β-lactam is fused to a six-membered dihydrothiazine and breakdown of the tetrahedral intermediate involves the formal expulsion of an enamine.[3] However, thermodynamically protonation of enamines generally occurs on carbon, not nitrogen, to generate an iminium ion. Furthermore, by analogy with enols and alcohols, the nitrogen anion of deprotonated enamines is expected to be more stable than the analogous amine anion.

It has been suggested[7] that the mechanism of hydrolysis of the cephalosporin nitrocefin (**3**) catalysed by the binuclear zinc enzyme β-lactamase from *B. fragilis* occurs with amine anion expulsion which, in this case, is facilitated by extensive resonance stabilization. We wished to explore this possibility in non-enzyme catalysed reactions of cephalosporins which contain the standard substituents at C3 of the dihydrothiazine leaving group. We have studied the thiolysis of cephalosporins because we have previously demonstrated[6] that the analogous reaction with penicillins occurs with rate-limiting breakdown of the tetrahedral intermediate with C—N fission and expulsion of the thiazolidine amine leaving group.



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EXPERIMENTAL

Materials

Thiols (2-mercaptoethanol, methyl 3-mercaptopropionate, 3-mercaptopropane-1,2-diol, mercaptoethylamine [aminoethanethiol (AET)], methyl 2-mercaptoacetate (MT), 2,2,2-trifluoroethanethiol, sodium hydrogensulfide, thiophenol), buffers and the cephalosporins were purchased from Sigma and other materials were of AnalaR grade. Freshly boiled deionized water was used throughout and the ionic strength maintained at 0.5 mol dm^{-3} with potassium chloride. Deuterium oxide (99.9% D) was obtained from Sigma. Owing to the high volatility and the oxidation of some of the thiols used in this study (particularly when alkaline solutions of thiols are used), the concentration of thiol in solution was measured just before use by Ellmann's method.[8] In order to minimize disulfide formation the experiments were carried out under argon and if the loss of thiol was still significat sealed ampoules were used.

High-performance liquid chromatography (HPLC)

A Shimadzu LC-9A chromatograph with a Rheodyne model 7125 universal injector and a Shimadzu SPD-M6A UV–visible photodiode-array detector were used for the HPLC experiments. The column was Spherisorb ODS (25 × 0.46 cm i.d., 5 μm) and the eluent was 0.1 mol dm^{-3} aqueous NH_4OAc–MeCN (88:12) at a flow-rate of 1.5 ml min^{-1}.

The kinetics of the reaction were studied by following the decrease in HPLC peak areas of the cephalosporin (ca 2.0×10^{-4} mol dm^{-3}) at 260 nm at the desired temperature. The reactions were initiated by addition of 200–2000 μl of a stock solution of the thiol (ca 0.5 mol dm^{-3}), prepared just before the kinetic run, to 25 ml of buffer solution (0.2 mol dm^{-3}) and at a constant ionic strength of 0.5 mol dm^{-3} (with KCl) containing the desired amount of cephalosporin (20–40 mg) preincubated at the desired temperature ± 0.1 °C. The final concentration of thiol was between 20 and 100 times greater than that of the cephalosporin. Reactions where the concentration of thiol was fairly high were initially frozen by withdrawing 1 ml of the reaction mixture at appropriate time intervals adding 200 μl of 1 M HCl to lower the pH to 7.0, and immediately freezing the sample in liquid nitrogen. The samples were kept in the freezer until they were injected (during the same day) into the HPLC system. Assays were repeated if the pH change over the course of a reaction was >0.03. All reactions were repeated two or three times in order to obtain a reliable value, with an estimated error of ± 5% in the second-order rate constant. The kinetics of the hydrolysis reactions were also studied using the same buffer solutions and technique. The decrease in HPLC peak areas for cephalosporin was followed at 260 nm and at the desired temperature using carbonate or MOPS [3-(*N*-morpholino)propanesulfonic acid] as buffer (0.2 mol dm^{-3}) at the same pH (or pD) as the thiolysis reaction. The buffer solutions were preincubated at constant temperature prior to the kinetic run.

Deuterium solvent kinetic isotope effects

The second-order rate constants of thiolysis of cephaloridine and cephalothin with 2-mercaptoethanol and

2-mercaptoethylamine were also determined in D_2O solutions. In order to have an accurate concentration of the thiol in H_2O and D_2O, the pK_a values of the thiols were determined by potentiometric titration in H_2O and D_2O. The pD was measured with a standard microelectrode using pD = pH meter reading + 0.40.

NMR spectroscopy

The NMR spectra were obtained on a Bruker AMX-300 spectrometer. Sample tubes of 5 mm diameter were used containing 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) as internal reference. Chemical shift values (δ) are given in ppm and coupling constants are in hertz.

Determination of free thiol content

Aliquots of the reaction mixture ([thiol] = 4.0×10^{-3}–2.0×10^{-2} mol dm^{-3}, [cephalosporin] = 2.0×10^{-4} mol dm^{-3}) were diluted between 2- and 10- fold and then assayed for free thiol content by using the method of Ellmann.[8] A stock solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 41.3 mg in 10 ml of 0.1 mol dm^{-3} phosphate buffer (pH 7.0), was prepared and stored in the dark. Samples of the diluted reaction (100–150 µl) were withdrawn at different times, mixed with 2.5 ml of phosphate buffer (pH 8.0) and 40 µl of stock DTNB solution were added. The final thiol concentration was therefore ca 1×10^{-4} mol dm^{-3}, in order to have an initial absorbance value between 1.2 and 1.6. After 2 min, the absorbance at 412 nm was measured against a blank solution lacking thiol. A new blank was prepared for every measurement.

RESULTS AND DISCUSSION

The hydrolysis of cephaloridine (**4**) is catalysed by thiols and the observed pseudo-first-order rate constants, k_{obs}, increase linearly with total thiol concentration, $[RSH]_{tot}$. The intercept of the plot of k_{obs} against total thiol concentration corresponds well with the calculated first-order rate constant for the background hydrolysis. The rate law for the thiol catalysed hydrolysis is given by Eqn (1) and the slopes of the plots are designated k_{cat} [Eqn (2)] and increase with increase in pH. The dependence of k_{cat} on the fraction of thiol present as the free base in the buffer solution, α, is given by Eqn (2).

$$k_{obs} = k_{OH^-}[OH^-] + k_{buffer}[buffer] + k_{RSH}[RSH] + k_{RS^-}[RS^-] \quad (1)$$

$$k_{obs} = k_{OH^-}[OH^-] + k_{buffer}[buffer] + k_{cat}[RSH]_{tot}$$

where

$$k_{cat} = k_{RS^-}\alpha + k_{RSH}(1 - \alpha) \quad (2)$$

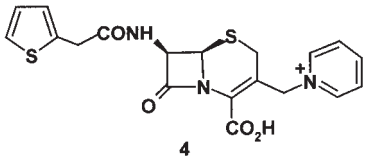
and

$$\alpha = [RS^-]/[RSH]_{tot}$$

A plot of k_{cat} against α gives a positive intercept equal to k_{RS^-} at $\alpha = 1$ and an intercept at $\alpha = 0$ which is indistinguishable from zero. This indicates that the reactive form of the thiol is the thiolate anion, and that there is no reaction by the neutral, undissociated thiol. A plot of the observed pseudo-first-order rate constant, k_{obs}, against the concentration of the thiolate anion of 2-mercaptoethanol is shown in Fig. 1. The second-order rate constant for thiolysis, k_{RS^-}, is given by the slope of this graph. The rate law for the hydrolysis of cephaloridine in aqueous buffers of thiol is effectively reduced to Eqn (3):

$$k_{obs} = k_{OH^-}[OH^-] + k_{buffer}[buffer] + k_{RS^-}[RS^-] \quad (3)$$

It seems likely that the thiolate anions react with cephalosporins by nucleophilic attack on the β-lactam carbonyl carbon to displace the dihydrothiazine enamine to initially generate a thioester. The reaction of thiols with cephaloridine (**4**) in water is outlined in Scheme 2 and although the presence of the thioester intermediate (**5**) could not be identified by ^{1}H NMR, it is compatible with analogous studies with penicillin[6] and observations



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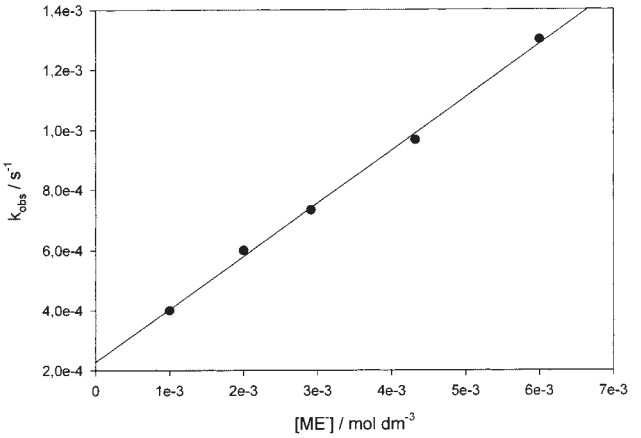
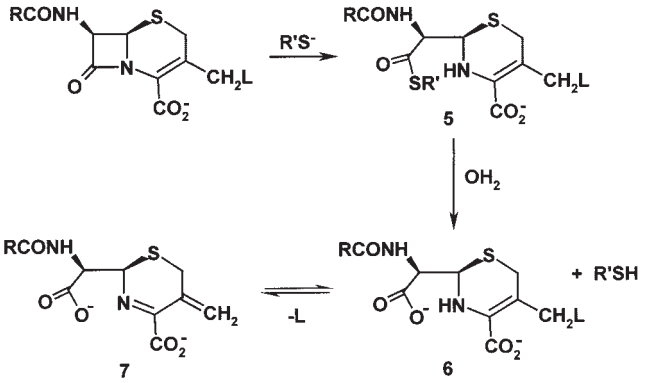


Figure 1. Plot of the observed pseudo-first-order rate constant, k_{obs}, against the concentration of thiolate anion of 2-mercaptoethanol at pH 10.10 at 30.0 °C and ionic strength 0.5 mol dm^{-3}



Scheme 2

described later. In the case of cephaloridine (**4**), the leaving group L (pyridine) is rapidly expelled from the initial enamine (**6**) to generate the unsaturated imine (**7**). Evidence for a thioester intermediate has been directly observed in some other reactions, e.g. in the hydrolysis of methyl thiohippurate[9] and *N-trans*-cinnamoylimidazole,[10] in addition to being deduced from kinetic observations.[11,12]

The second-order rate constants for the reaction of cephaloridine with a variety of thiols were determined and found to increase with increasing basicity of the thiol (Table 1). A plot of $\log k_{RS^-}$ against the pK_a of the thiol (Fig. 2) has a least-squares slope of $\beta_{nuc} = 1.22 \pm 0.02$. This large dependence of the rate upon basicity of the thiolate anion is indicative of nucleophilic rather than general-base catalysis. The second-order rate constant for AET and MT show a slight positive deviation from the line, whereas a negative deviation is observed in the case of hydrogen sulfide.

Table 1. Second-order rate constants for the reaction of thiolate anions with cephaloridine at 30.0 °C and total ionic strength 0.5 mol dm^{-3} (KCl)

Thiol	pK_a	k_{RS^-} (mol^{-1} dm^3 s^{-1})
2-Mercaptoethanol (ME)	9.61[a]	1.64×10^{-1}
Methyl 3-mercaptopropionate (MMP)	9.33[b]	1.25×10^{-1}
3-Mercaptopropane-1,2-diol (MTG)	9.28[c]	1.85×10^{-1}
2-Aminoethanethiol (AET)	8.50[d]	6.40×10^{-2}
Methyl 2-mercaptoacetate (MT)	7.83[e]	1.47×10^{-2}
2,2,2-Trifluoroethanethiol (TFET)	7.30[a]	2.31×10^{-4}
Sodium hydrogensulfide (NaHS)	6.88[f]	7.57×10^{-5}
Thiophenol (TP)	6.43[a]	5.08×10^{-5}

[a] Ref. 13.
[b] Ref. 14.
[c] Ref. 15.
[d] Determined by photometric titration.
[e] Ref. 16.
[f] Ref. 17.

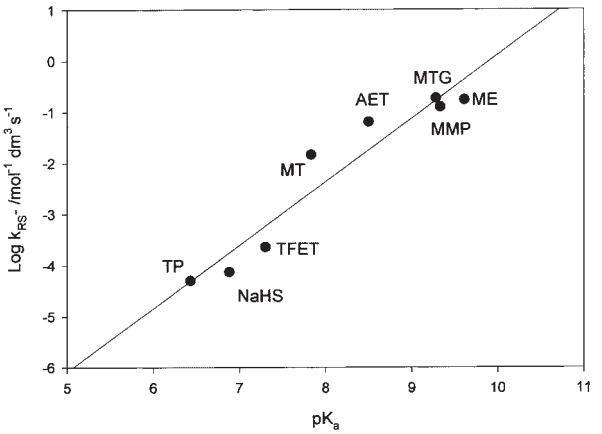
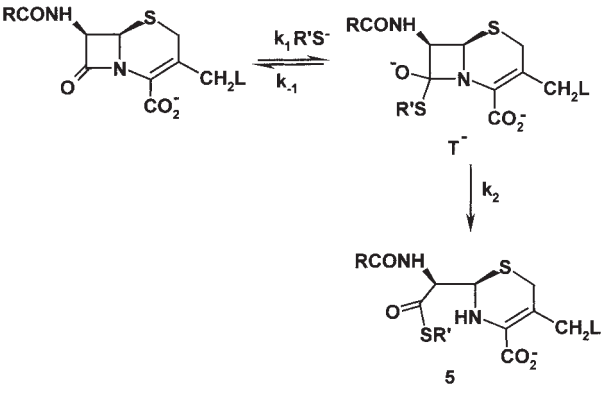
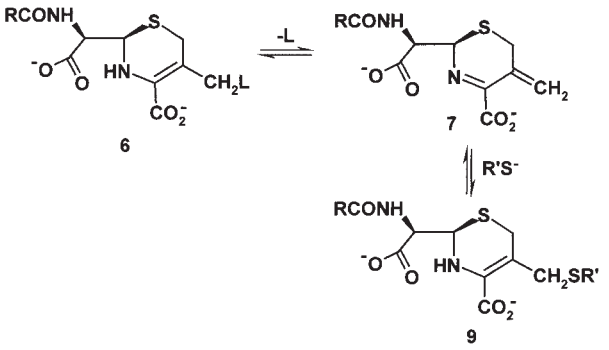


Figure 2. Plot of the second-order rate constant, k_{RS^-}, for the thiolysis of cephaloridine against the pK_a of the corresponding thiol. Temperature 30.0 °C and ionic strength 0.5 mol dm^{-3}. Thiols: ME, 2-mercaptoethanol; MMP, methyl 3-mercaptopropionate; MTG, 3-mercaptopropane-1,2-diol; AET, aminoethanethiol; MT, methyl 2-mercaptoacetate; TFET, 2,2,2-trifluoroethanethiol; NaHS, sodium hydrogensulfide; TP, thiophenol

The Brønsted β_{nuc} of 1.22 indicates that the negative charge on the thiolate anion in the reactant is effectively removed in the transition state and, perhaps, has gained some effective positive charge. The Brønsted β_{nuc} values for the thiolysis of other acyl groups are generally 0.2–0.3 when the rate-determining step is attack on the carbonyl group and is often observed with basic thiols and good leaving groups expelled from the acyl centre.[14,18] Much larger values of about 1.0 are observed when breakdown of tetrahedral intermediate is rate limiting, as seen with weakly basic thiols and poor leaving groups.[14,18] The Brønsted β_{nuc} value of 1.22 obtained for the thiolysis of cephaloridine therefore suggests rate-limiting breakdown of the tetrahedral intermediate $\mathbf{T}^-$ (Scheme 3) to form the thioester. This means that the rate of regeneration of reactants by expulsion of the thiolate anion is faster than opening of the strained β-lactam ring ($k_{-1} \gg k_2$) (Scheme 3). Full conversion of the thiolate anion to a thioester (**5**) is expected[14] to change the effective charge on sulfur from -1 to $+0.4$, corresponding to a β_{nuc} of 1.4.



Scheme 3



Scheme 4

Table 2. Second-order rate constants for thiolysis with the thiolate anion of 2-mercaptoethanol, k_{RS^-}, and for the hydroxide ion-catalysed hydrolysis, k_{OH^-}, of cephalosporins as a function of substituents at C3′, at 30 °C, $I = 0.5$ mol dm^{-3} (KCl)

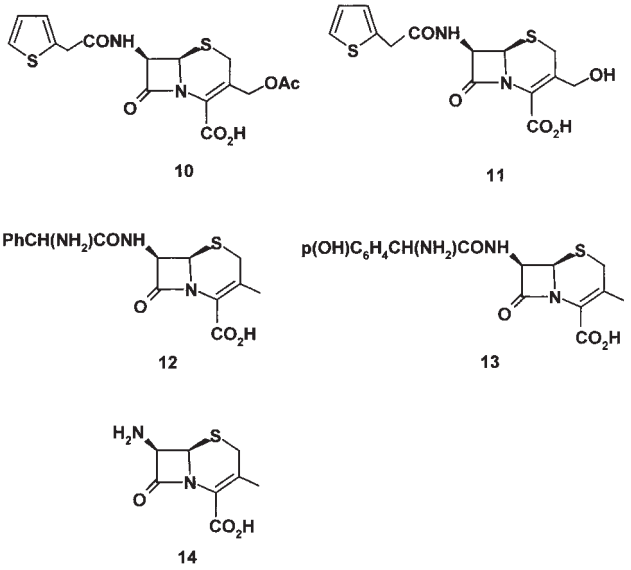
C3′ substituent	k_{RS^-} (mol^{-1} dm^3 s^{-1})	k_{OH^-} (mol^{-1} dm^3 s^{-1})	σ_I[a]
Pyridinium	1.64×10^{-1}	6.49×10^{-1}	0.60
OAc	7.88×10^{-3}	9.36×10^{-2}	0.38
OH	2.95×10^{-6}	6.51×10^{-2}	0.24
H	$< 1 \times 10^{-7}$	2.90×10^{-2}	0.0

[a] Ref. 22, pp. 251–254.

The observed β_{nuc} value of 1.2 is thus indicative of significant C—N fission and thioester formation in the transition state. The Brønsted β_{nuc} for the thiolysis of benzylpenicillin is smaller at 0.9 but still compatible with rate-limiting breakdown of the tetrahedral intermediate.[6]

In addition to hydrolysis and thiolysis of cephaloridine, a small amount of Δ^2–Δ^3 isomerization and C7 epimerization occur. The rates of these processes could be followed by both ^{1}H NMR and HPLC. For example, at pH 10.5, 30.0 °C and ionic strength 0.5 M with 0.01 M mercaptoethanol, Δ^2–Δ^3 isomerization occurs about 100 times more slowly than thiolysis, which is also about 20 times faster than C7 epimerization. Also, the initial hydrolysis product (**6**) may expel a leaving group L at C3′ to generate an α,β-unsaturated imine (**7**) (Scheme 4), which is then converted to the C3′ thioether adduct (**9**) as a result of Michael addition of the mercaptoethanol.[19] The second-order rate constant for this process, (**7**) to (**9**), which was shown to be reversible, is 24.1 mol^{-1} dm^3 s^{-1}.

The effect of C7 and C3 substituents on the rate of the thiol-catalysed hydrolysis of cephalosporins was also investigated using cephalothin (**10**), desacetylcephalothin (**11**), cephalexin (**12**), cephadroxil (**13**), 7-amino desacetylcephalosporanic acid (**14**) and nitrocefin (**3**). Thiolysis



was observed when the C3 substituent was hydroxymethyl or acetoxymethyl but not when it was methyl or substituted ethylidene. The second-order rate constants for thiolysis of various cephalosporins with mercaptoethanol are given in Table 2. The absence of thiolysis with some cephalosporins indicates that the rates of alkaline hydrolysis and thiolysis have a very different dependence on the nature of the C3 substituent. For thiolysis to be seen, its rate must be greater than that for hydrolysis promoted by hydroxide ion and buffer.

We shall first consider the effect of C3 substituents on the rate of alkaline hydrolysis of cephalosporins. In general, the second-order rate constants for the hydroxide ion-catalysed hydrolysis of cephalosporins are similar to those of penicillins. This similarity indicates that the non-planarity of the β-lactam nitrogen does not significantly affect amide resonance since the nitrogen is 0.4 Å out of the plane defined by its substituents in penicillins, whereas in the cephalosporins it deviates by 0.2–0.3 Å.[1] The kinetic similarity also indicates that having a leaving group at C3′ does not significantly affect the reactivity of cephalosporins. There are several experimental observations which indicate that the reaction is not concerted and the expulsion of the leaving group at C3′ occurs after β-lactam ring opening.[1,3,19] Electron-withdrawing substituents attached to the β-lactam nitrogen increase the rate of hydrolysis and give a Brønsted β_{lg} of -0.6.[20] The rate-limiting step in the alkaline hydrolysis of cephalosporins appears to be the *formation* of the tetrahedral intermediate. In the stepwise mechanism, breakdown of the tetrahedral intermediate generates the enamine followed by expulsion of the leaving group at C3′ to give the conjugated imine.[3,19]

The second-order rate constants for the hydroxide ion-catalysed hydrolysis of cephalosporins are correlated with σ_I for C3′ substituents and give a Hammett ρ_1 of 2.17 for (Fig. 3). Several substituents at C3, e.g. CH_3, H, CH_2CO_2Et and Cl, are not expelled but a single correlation occurs for substituents (not shown in Fig. 3, but previously published[1,20]) irrespective of whether or not they are expelled during the reaction, which is compatible with separate steps for C—N bond fission and β-lactam ring opening and expulsion of a leaving group.[1,19,20] The

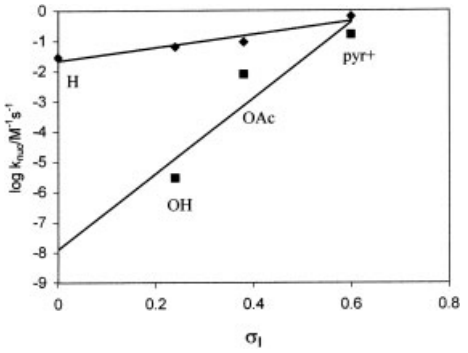
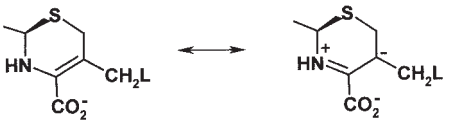


Figure 3. Hammett plot for the second-order rate constants for hydroxide ion-catalysed hydrolysis and for the thiolysis of cephalosporins with the anion of 2-mercaptoethanol as a function of σ_I for the C3′ substituent; pyr^+ = pyridinium ion; OAc = acetate

Hammett ρ_I for the reaction rates usually bears no exact and direct relationship to the transition-state structure because the model to define σ_I and the reaction being studied are dissimilar. However, a positive ρ_I value indicates the removal of effective positive charge at the reaction centre on going from the reactant to the transition state and is compatible with the conversion of a nitrogen with an effective charge of +0.7 in the amide of cephaloridine to a neutral one in the tetrahedral intermediate.[21,22]

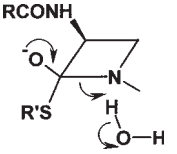
Second-order rate constants, k_{RS^-}, for the thiolysis of cephalosporins by 2-mercaptoethanol as a function of substituents at C3′ are shown in Table 2. Although the data are limited, there is an enormous dependence of the rate of thiolysis upon the nature of the C3′ substituent. The thiolysis rate constant increases dramatically as the electron-withdrawing character of the substituent increases. A Hammett plot of the second-order rate constants for the thiol-catalysed degradation by 2-mercaptoethanol of the same cephalosporins is shown in Fig. 3. The apparent Hammett ρ_I value, based on just three data points, for the thiolysis of cephalosporins is 12, indicative of a large increase in negative charge which is developed at the reaction centre on passing from the reactant to the transition state. Despite the imprecision involved, it is apparent from Fig. 3 that the transition-state structures for hydroxide ion-catalysed hydrolysis and thiolysis are significantly different. As described earlier, the dependence of the rate constants for thiolysis of cephaloridine on the basicity of the thiol anion generates a $\beta_{nuc} = 1.22$, indicating that the rate-limiting step for thiolysis is breakdown of the tetrahedral intermediate to expel the leaving group by C—N bond fission. The effect of substituents at C3′ on the rate gives some indication of the charge development on the nitrogen of the enamine leaving group. Amide resonance in the β-lactam causes a significant development of positive charge on the β-lactam nitrogen which is removed upon formation of the tetrahedral intermediate. However, subsequent cleavage



Scheme 5

of the β-lactam C—N bond, to give the ring-opened product enamine (**5**), generates some negative charge density at C3 due to enamine resonance (Scheme 5). It is therefore not surprising that a reaction proceeding with rate-limiting breakdown of the tetrahedral intermediate shows a significant dependence in the nature of the C3 substituent. The presence of electron-withdrawing substituents at the C3 position stabilizes the negative charge increase on the β-lactam nitrogen, lowering the energy of the transition state and thus increasing the rate. However, the observed ρ value is so high that it indicates the generation of a significant negative charge on the β-lactam nitrogen leaving group in the transition state and so could indicate that carbon–nitrogen bond fission occurs without protonation of the β-lactam nitrogen.

If the breakdown of the tetrahedral intermediate of cephalosporins does not involve the protonation of the departing β-lactam nitrogen, a small solvent kinetic isotopic effect (SKIE) would be expected in the thiolysis of cephalosporins. Four solvent kinetic isotope effects were determined (Table 3) for the thiolysis of cephalosporins. A SKIE $k_{RS^-}(H_2O)/k_{RS^-}(D_2O)$ of 1.17 is obtained for the thiolysis of cephaloridine by 2-mercaptoethanol determined in water at 30.0 °C and a value of 1.09 was obtained with cephalothin. The zwitterionic thiolate anion of 2-mercaptoethylamine also shows a SKIE of 1.1 with both cephalosporins, despite the possibility of intramolecular general acid catalysis from the ammonium group. For comparison, the SKIE for the thiolysis of benzylpenicillin is 2.3, which is much more clearly compatible with proton transfer to nitrogen, with water acting as a general acid catalyst donating a proton to the departing nitrogen facilitating C—N fission (**15**).[6] A similar situation was observed with the alcoholysis of penicillin.[5]



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The major effect determining the solvent isotope effect on the equilibrium formation of the tetrahedral intermediate comes from removing the negative charge and solvation of the attacking thiolate anion giving rise to an inverse isotope effect which is compensated by the normal isotope effect for the development of the negative charge on the oxyanion (Scheme 3).[13] Both the hydrogen-bonding

Table 3. Solvent kinetic isotope effects, $k_{RS^-}(H_2O)/k_{RS^-}(D_2O)$, for the thiol-catalysed hydrolysis of cephalosporins at 30 °C and $I = 0.5$ mol dm^{-3} (KCl)

Cephalosporin	k_{RS^-} (mol^{-1} dm^3 s^{-1}): 2-mercaptoethanol	SKIE (ME)	k_{RS^-} (mol^{-1} dm^3 s^{-1}): 2-mercaptoethylamine	SKIE (AET)
Cephaloridine (H_2O)	1.64×10^{-1}	1.17	6.40×10^{-2}	1.12
Cephaloridine (D_2O)	1.40×10^{-1}		5.73×10^{-2}	
Cephalothin (H_2O)	7.88×10^{-3}	1.09	3.03×10^{-3}	1.09
Cephalothin (D_2O)	7.25×10^{-3}		2.78×10^{-3}	

ability and the basicity of sulfur are less than those for analogous oxygen derivatives because of the large size and low electron density of sulfur. The thiolysis of *p*-nitrophenyl acetate with a weakly basic thiolate anion proceeding with rate-limiting breakdown of the tetrahedral intermediate and expulsion of the leaving group shows a kinetic solvent isotope effect $k_{H_2O}/k_{D_2O} = 0.88$.[14]

A rate enhancement of ca 1400 is observed for the thiolysis of benzylpenicillin by thiols with a substituent capable of acting as an intramolecular general acid catalyst, such as 2-mercaptoethylammonium ion, which is able to transfer a proton to the departing β-lactam nitrogen in the transition state.[23] However, with cephalosporins, 2-mercaptoethylamine (AET) fits the Brønsted plot (Fig. 2), consistent with no intramolecular general acid catalysis by proton transfer to the β-lactam nitrogen of cephalosporins. Together with the relatively small SKIE value, this is compatible with rate-limiting breakdown of the tetrahedral intermediate expelling the β-lactam nitrogen as the anion.

The reason why there is no observed thiolysis of cephalexin (**12**), cephadroxil (**13**), 7-aminodesacetylcephalosporanic acid (**14**) and nitrocefin (**3**) is because the value of the second-order thiolysis rate constant is so small compared with that for alkaline hydrolysis. The thiolysis-catalysed degradation of cephalosporins with non-polar substituents at the C3′ position is negligible compared with the hydrolysis reaction. Cephalosporins with polar substituents at C3 position have second-order thiolysis rate constants similar to those for hydrolysis. The predicted rate constant for thiolysis by 2-mercaptoethanol for a CH_3 substituent at C3 is at least 10^4-fold less than that for hydroxide ion-catalysed hydrolysis. Nucleophilic attack on the β-lactam ring involves covalent bond formation between the carbonyl carbon and the nucleophile and subsequent bond fission of the β-lactam ring (Scheme 1). The rate-limiting step for thiolysis, and also for alcoholysis[23] and aminolysis,[3] of cephalosporins is the breakdown of the tetrahedral intermediate ($k_{-1} > k_2$), whereas for alkaline hydrolysis[1] it is the formation ($k_2 > k_{-1}$). As basic thiolate anions are better nucleophiles than hydroxide ion, the rate of formation of the tetrahedral intermediate, k_1, with thiolate anions is generally greater than that for hydroxide ion from the same acyl centre.[14] However, with cephalosporins the overall observed second-order rate constant, k_{RS^-}, is smaller than k_{OH^-}. Hence the ratio k_2/k_{-1} must be <1 for thiolate anions, compatible with rate-limiting breakdown of the tetrahedral intermediate. Either the k_2 steps are different for hydroxide ion and thiolate anion, in both magnitude and perhaps mechanism, or the relative magnitude of the k_{-1} steps, expulsion of the attacking nucleophile, is significantly different. Breakdown of the tetrahedral intermediate in the forward direction, the k_2 step, involves the formal expulsion of the same leaving group although the acyl centres become either a carboxylic acid or thioester. The rate of expulsion of the leaving group, k_2, from the tetrahedral intermediate anion, T^-, when the attacking nucleophile is a thiolate anion (Nu = SR, Scheme 1) is probably slower than that for oxygen nucleophiles (Nu = OH, Scheme 1). The ability of sulfur to provide some electron 'push' to expel the leaving group is less than that for oxygen, reflecting the reluctance of sulfur to form double bonds to carbon. For example, chloride ion expulsion from $ROCH_2Cl$ is 1500-fold faster than that from $RSCH_2Cl$.[25] In addition, the rates of breakdown of the intermediate in the reverse direction, the k_{-1} step, to expel either the attacking thiolate anion or hydroxide anion are significantly different. Thiolate anions are generally better leaving groups than analogous oxygen anions. The difference in rate-limiting step for thiolysis, alcoholysis and aminolysis compared with alkaline hydrolysis could be due to the higher degree of solvation of hydroxide ion compared with the other nucleophiles. The thiolate and alkoxide anions and also amines are better leaving groups than the hydroxide anion, which makes k_{-1} greater for RO^-, RS^- and RNH_2 than for OH^-.

It has been suggested that the binuclear metallo-β-lactamase from *B. fragilis* catalyses the hydrolysis of nitrocefin (**3**) by the first active site zinc ion acting as a Lewis acid which stabilizes the oxyanion of the tetrahedral intermediate whilst the second zinc ion facilitates C—N cleavage by stabilizing the β-lactam nitrogen which is thought to be expelled as an anion.[7] This mechanism was anticipated earlier from non-enzymatic metal ion-catalysed hydrolysis of β-lactams.[3] The work reported here appears to establish the principle that rate-limiting enamine anion expulsion is possible in the β-lactam ring-opening reactions of cephalosporins. The pK_a of enamines in water is not known, but enols are about

5 pK_a units stronger acids than alcohols. A reasonable estimate for enamines is therefore about 20, and with the strongly electron-withdrawing pyridinium ion attached to the allylic carbon may be significantly lower.

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REFERENCES

1. Page MI. *Adv. Phys. Org. Chem.* 1987; **23**: 165; Page MI. In *The Chemistry of β-Lactams*, Page MI (ed). Blackie: Glasgow, 1992; 129–147; Page MI. *Acc. Chem. Res.* 1984; **17**: 144–151.
2. Martin AF, Morris JJ, Page MI. *Chem. Commun.* 1979; 298; Gensmantel NP, Page MI. *Chem. Commun.* 1978; 374–375; Morris JJ, Page MI. *J. Chem. Soc., Perkin Trans. 2* 1980; 220–224; Gensmantel NP, Page MI. *J. Chem. Soc., Perkin Trans. 2* 1982; 147–153.
3. Page MI, Proctor P. *J. Am. Chem. Soc.* 1984; **106**: 3820.
4. Gensmantel NP, Proctor P, Page MI. *J. Chem. Soc., Perkin 2* 1980; 1725–1732.
5. Davis AM, Proctor P, Page MI. *J. Chem. Soc., Perkin Trans. 2* 1991; 1213–1217.
6. Llinas A, Donoso J, Vilanova B, Frau J, Munoz F, Page MI. *J. Chem. Soc., Perkin Trans. 2* 2000; 1521–1525.
7. Wang Z, Fast W, Valentine AM, Benkovic SJ. *Curr. Opin. Chem. Biol.* 1999; **3**: 614–622; Wang Z, Fast W, Benkovic SJ. *Biochemistry* 1999; **38**: 10013–10023; Wang Z, Fast W, Benkovic SJ. *J. Am. Chem. Soc.* 1998; **120**: 10788–10789; McManus-Munoz S, Crowder MW. *Biochemistry* 1999; **38**: 1547–1553.
8. Ellmann GL. *Arch. Biochem. Biophys.* 1959; **82**: 70–74.
9. Lowe G, Williams A. *Biochem. J.* 1965; **96**: 189–193; Lowe G, Williams A. *Biochem. J.* 1965; **96**: 194–198; Lowe G, Williams A. *Biochem. J.* 1965; **96**: 199–203.
10. Brubacher LJ, Bender ML. *J. Am. Chem. Soc.* 1966; **88**: 5871–5889.
11. Weiss J. *Chem. Ind. (London)* 1937; **15**: 685–687.
12. Smith L, Kimmel JR, Brown DM, Thompson EOP. *J. Biol. Chem.* 1955; **215**: 67–75.
13. Jencks WP, Salvesen K. *J. Am. Chem. Soc.* 1971; **93**: 4433–4436.
14. Hupe DJ, Jencks WP. *J. Am. Chem. Soc.* 1977; **99**: 451–464.
15. DeBrabander HF, VanPoucke LC, Eeckhaut Z. *Inorg. Chim. Acta* 1971; **5**: 473–480.
16. Lienhard GE, Jencks WP. *J. Am. Chem. Soc.* 1966; **88**: 3982–3995.
17. Widmer M, Schuarenback G. *Helv. Chim. Acta* 1964; **47**: 266–272.
18. Fersht AR. *J. Am. Chem. Soc.* 1971; **93**: 3504–3515.
19. Buckwell SC, Page MI, Longridge J. *Chem. Commun.* 1986; 1039; Buckwell SC, Page MI, Waley SG, Longridge J. *J. Chem. Soc., Perkin Trans. 2* 1988; 1823–1827.
20. Proctor P, Gensmantel NP, Page MI. *J. Chem. Soc., Perkin Trans. 2* 1982; 1185–1191.
21. Williams A. *Adv. Phys. Org. Chem.* 1992; **27**: 1–55.
22. Page MI, Williams A. *Organic and Bio-organic Mechanisms*. Longman: Harlow, 1997; 52–79.
23. Llinas A, Vilanova B, Muñoz F, Donoso J. *J. Mol. Catal.* 2001; **175**: 3–16.
24. Davies KJ, Page MI. *Chem. Commun.* 1990; 1448–1449.
25. Böhme H. *Ber. Dtsch. Chem. Ges B.* 1941; **74**: 248–258.

