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Intramolecular general acid catalysis in the aminolysis of β-lactam antibiotics

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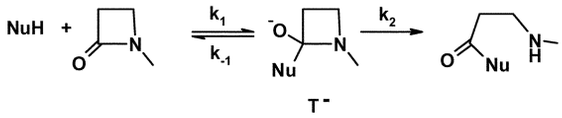
Received 3rd November 2003, Accepted 21st January 2004
First published as an Advance Article on the web 9th February 2004

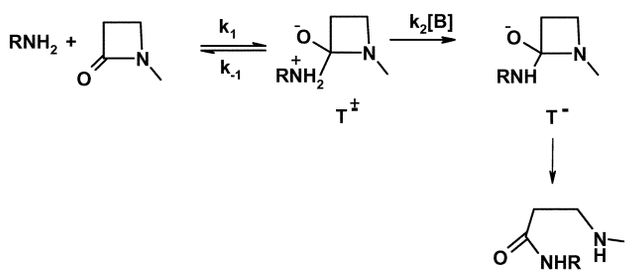
The rate of aminolysis of benzylpenicillin and cephaloridine by hydroxylamine, unlike other amines, shows only a first order dependence on amine concentration. The rate enhancement compared with that predicted from a Bronsted plot for other primary amines with benzylpenicillin is greater than 10^6. This is much more than an α-effect and is compatible with rate-limiting formation of the tetrahedral intermediate due to a rapid intramolecular general acid catalysed breakdown of the intermediate. For cephaloridine, the rate enhancement is greater than 10^4 which demonstrates that β-lactam C–N bond fission and expulsion of the leaving group at C3′ are not concerted.

Introduction

The reactions of β-lactam antibiotics and their derivatives have been extensively studied.[1] For example, nucleophilic substitution at the β-lactam carbonyl centre of penicillins (**1**) occurs, in water, with amines,[2–4] alcohols[5] and thiols[6] in competition with that by hydroxide ion. These are acyl transfer processes involving covalent bond formation between the carbonyl carbon and the nucleophile and C–N bond fission of the β-lactam. In general, 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] The relative sequence of bond making and breaking between heavy atoms in the aminolysis of β-lactam antibiotics is a result of subtle effects often involving proton transfer. A step-wise process for aminolysis occurs through the formation of a tetrahedral intemediate, resulting from the attack on the carbonyl centre by an amine, which gives rise to a large change in the pK_a of the amine NH as a result of covalent bond formation. Proton transfer from the amine nucleophile to a base catalyst thus occurs *after* full covalent bond formation, as it changes from a thermodynamically unfavourable to a favourable process. Hence aminolysis usually requires general base catalysis to remove a proton from the attacking amine and this is the dominant term in the rate law – in fact it is experimentally difficult to determine the rate constant for any uncatalysed reaction.[2,4] With penicillins (**1**) and cephalosporins (**2**) this proton transfer occurs after initial C–N bond formation in a rate-limiting step which is diffusion controlled[2–4] (Scheme 2). The aminolysis of β-lactam antibiotics also requires β-lactam C–N bond 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 ß-lactam is lost and proton transfer to nitrogen changes from an unfavourable to a thermodynamically favourable process. Thus many of these reactions require general acid catalysis and protonation of the amine nitrogen leaving group to facilitate C–N bond fission and avoid amine anion expulsion. Although the release of strain energy, which accompanies ring opening, could possibly decrease the need for protonation, C–N fission in

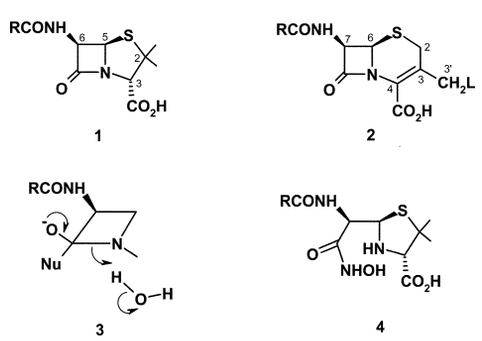


Scheme 1



Scheme 2

penicillins appears to require some form of catalysis.[1–3] For example, the alcoholysis[5] and thiolysis[6] of penicillins occur with rate limiting breakdown of the tetrahedral intermediate facilitated by proton transfer from solvent (**3**) water to the departing amine. Exceptionally, the thiolysis of some cephalosporins (**2**) appears to involve the breakdown of the tetrahedral intermediate by the expulsion of an enamine anion.[7]



We have been interested in developing inhibitors of the metallo β-lactamase enzymes[8] and one of these studies involved generating hydroxamic acid derivatives (**4**)[9] and part of that work required a kinetic analysis of the hydroxyaminolysis of β-lactam antibiotics which is reported here.

Experimental

Materials

Benzylpenicillin (sodium salt), cephaloridine (sodium salt), ferric chloride, methoxylamine hydrochloride and hydroxylamine hydrochloride were of general reagent grade and purchased from Sigma. Other materials were of AnalaR grade. Freshly boiled deionised water was used throughout and the ionic strength maintained at 0.5 mol dm^{-3} with potassium

DOI: 10.1039/b313900j

chloride unless otherwise stated. The buffers were prepared by partial neutralisation of the amine hydrochloride just prior to the kinetic run.

Kinetics

The hydroxylaminolysis of benzylpenicillin and cephaloridine were carried out with 0.025 to 0.125 mol dm^{-3} hydroxylamine used as a reagent and buffer. The reactions were initiated by the addition of 25 μl of aqueous 1.50×10^{-1} mol cm^{-3} of benzylpenicillin or 1.35×10^{-2} mol cm^{-3} of cephaloridine to 3.0 cm^3 of the aqueous hydroxylamine solution preincubated at 30 ± 0.05 C. The disappearance of the β-lactam was followed spectrophotometrically on a CARY 300 spectrophotometer at 240 nm for benzylpenicillin and at 260 nm for cephaloridine. The pH of all solutions was checked before and after each kinetic experiment and if it had changed by more than 0.03 the experiment was rejected.

Product analysis

Hydroxylaminolysis of benzylpenicillin and cephaloridine gives the corresponding hydroxamic acid. Hydroxamic acid concentration was estimated by the ferric hydroxamate complex formation.[10] The molar absorptivity of an iron(III) hydroxamate complex at 482 nm is about 1000 nM^{-1} cm^{-1}, *n* being the number of hydroxamate groups bonded per iron(III) ion. The molar absorptivity of the hydroxamate complex from benzylpenicillin was taken as 3000 M^{-1} cm^{-1}. Aliquots of the reaction mixture were withdrawn at different time intervals and the generation of the benzylpenicillin hydroxamate derivative determined at 482 nm. ^{1}H NMR chemical shifts of the benzylpenicillin hydroxamate derivative were determined from the spectrum of the reaction solution as follows: δ_H(400 MHz, D_2O) 1.09 (3H, *s*, 2-α-CH_3), 1.45 (3H, *s*, 2-β-CH_3), 3.33 (1H, *s*, 3-H), 3.54 (2H, *s*, Ph–CH_2), 4.11 (1H, *d*, 5-H, $J_{5,6}$ 8.99), 4.80 (1H, *d*, 6-H, $J_{6,5}$ 8.99), 7.23 (5H, *m*, Ph)

Results and discussion

The reaction of benzylpenicillin and cephaloridine in aqueous solutions of hydroxylamine gives the hydroxamic acid (**4**) as indicated from the ^{1}H NMR spectrum and the negative ion ES-IMS observed mass for example of 366 for benzylpenicillin. The corresponding pseudo-first-order rate constants, k_{obs}, for this process were determined from the exponential decrease in the UV absorbance at 240 nm (benzylpenicillin) and at 260 nm (cephaloridine) of solutions using excess hydroxylamine as both buffer and nucleophile. These rate constants increase linearly with total amine concentration, $[RNH_2]_{tot}$ and shows no evidence of a second order term in amine (Fig. 1 and 2). The intercepts of the plot of k_{obs} against total hydroxylamine

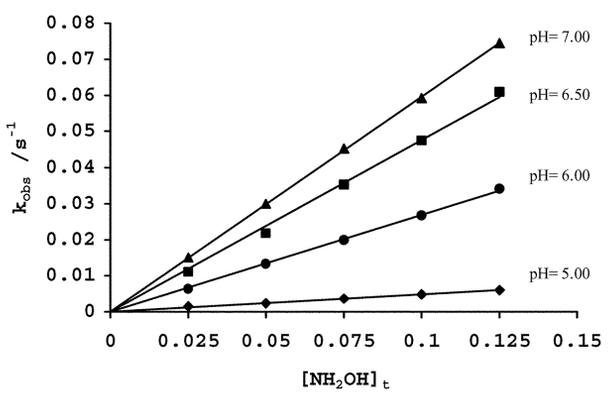


Fig. 1 Plot of the observed pseudo first order rate constant, k_{obs}, for the hydroxyaminolysis of benzylpenicillin against the total concentration of hydroxylamine at pH indicated at 30.0 °C and ionic strength 0.5 mol dm^{-3}.

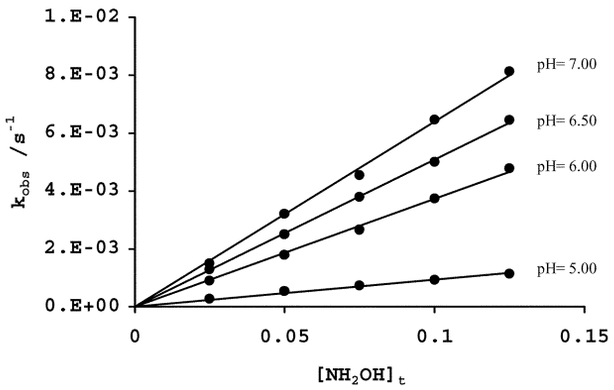


Fig. 2 Plot of the observed pseudo first order rate constant, k_{obs}, for the hydroxyaminolysis of cephaloridine against the total concentration of hydroxylamine at pH indicated at 30.0 °C and ionic strength 0.5 mol dm^{-3}.

concentration correspond well to the calculated first-order rate constant for the background hydrolysis. The rate law for aminolysis was further elucidated by determining the rates at different pHs and hence different ratios of the free hydroxylamine to its protonated form. The slopes of plots of the pseudo-first-order rate constants, k_{obs}, against the total hydroxylamine concentration are designated k_{cat}, eqn. (1) and increase with increasing pH. The dependence of k_{cat} upon the fraction of hydroxylamine present as the free base in the buffer solution, α, is given by eqn (2).

$$k_{obs} = k_{OH^-}[OH^-] + k_{cat}[NH_2OH]_{tot} \quad (1)$$

$$k_{cat} = k_{NH_2OH}\,\alpha + k_{NH_3OH^+}(1 - \alpha) \quad \text{where } \alpha = [NH_2OH]/[NH_2OH]_{tot} \quad (2)$$

A plot of k_{cat} against α gives a positive intercept equal to k_{NH_2OH} at α =1 and an intercept at α = 0 which is indistinguishable from zero (Fig. 3 and Fig. 4). This indicates that the reactive form of hydroxylamine is the free base form, and that there is no reaction by the protonated form. The rate law for the aminolysis of penicillin and cephaloridine by hydroxylamine in aqueous solution is effectively reduced to eqn. (3) with k_{NH_2OH} = 0.696 dm^3 mol^{-1} s^{-1} for benzylpenicillin and k_{NH_2OH} = 0.0864 dm^3 mol^{-1} s^{-1} for cephaloridine.

$$k_{obs} = k_{OH^-}[OH^-] + k_{NH_2OH}[NH_2OH] \quad (3)$$

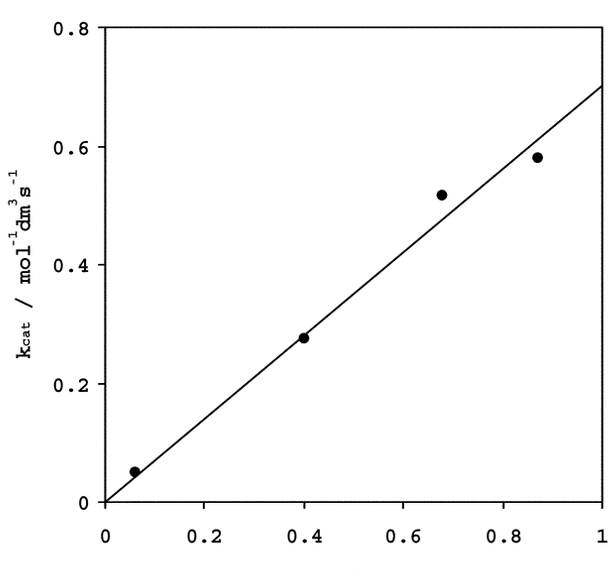


Fig. 3 Plot of the second-order rate constant, k_{cat}, for the hydroxyaminolysis of benzylpenicillin against the fraction of free base, *α*, of hydroxylamine.

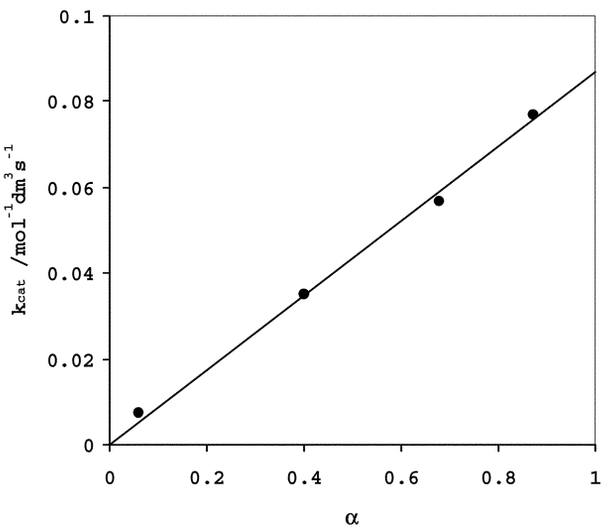


Fig. 4 Plot of the second-order rate constant, k_{cat}, for the hydroxyaminolysis of cephaloridine against the fraction of free base, α, of hydroxylamine.

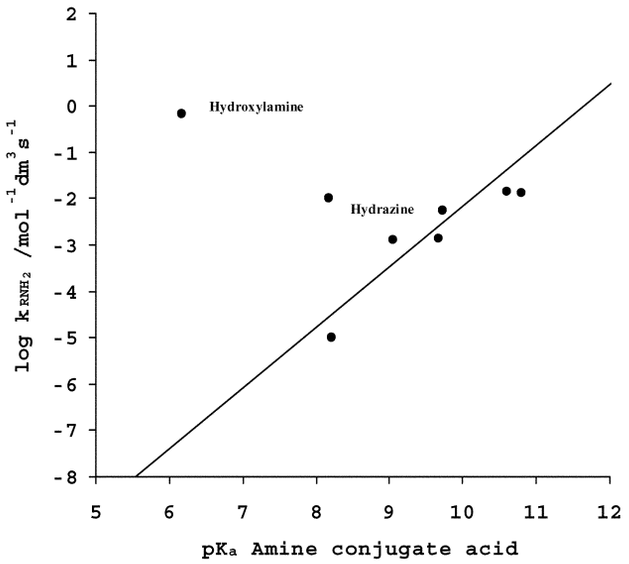


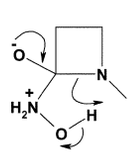
Fig. 5 Bronsted plot for the second order rate constants for uncatalysed aminolysis of benzylpenicillin as a function of the pK_a of the amine conjugate acid, the points for hydroxylamine and hydrazine are identified.

Hydroxylamine reacts with these antibiotics by nucleophilic attack on the β-lactam carbonyl carbon to displace the thiazolidine/dihydrothiazolidine amine to generate the hydroxamic acid by the generalised mechanism outlined in Scheme 1.

Normally, the rates of aminolysis of penicillins and cephalosporins are dominated by base catalysed reactions, either reflected in second order terms in amine concentration or a rate dependence on hydroxide ion concentration[2–4] reflecting the mechanism shown in Scheme 2. Neither of these terms are seen with aminolysis of benzylpenicillin or cephaloridine with hydroxylamine for which only the second order rate constants, corresponding to a first order dependence on amine concentration could be determined. The analogous rate constants for normal amines are, in fact, difficult to measure because they make such a small contribution to the overall rate law. However they can be determined and the second order rate constants for the aminolysis of benzylpenicillin increase with increasing basicity of the amine and a plot of log k_{RNH_2} against the pK_a of the amine conjugate acid (Fig. 5) shows a Bronsted $\beta_{nuc} = 1.0$.[3] The rate constant for hydroxylamine shows an enormous positive deviation from this line based on an amine of the same pK_a. A rate enhancement of more than 10^6 is apparent for hydroxylamine which is far greater than that expected for the α-effect.[11] For example, hydrazine also shows a positive deviation but with a rate enhancement of 300.[4] Furthermore, unlike hydroxylamine, the reaction of hydrazine with benzylpenicillin is very strongly base catalysed.[4] Taken together, these facts suggest a different rate limiting step for hydroxylamine compared with other amines.

The large dependence of the rate of the uncatalysed aminolysis of penicillins and cephalosporins upon the basicity of the amine with a Bronsted $\beta_{nuc} = 1.0$ indicates that there is an effective unit positive charge on the amine nucleophile in the transition state, compatible with rate limiting breakdown of the tetrahedral intermediate (Scheme 1).[3] This means that the rate of regeneration of reactants by expulsion of the amine is faster than opening of the strained β-lactam ring ($k_{-1} >> k_2$), (Scheme 1). This mechanism is strongly supported by the observation[4] of a change in rate limiting step with increasing base concentration as k_2[B] becomes >> k_{-1} (Scheme 2). From this, the microscopic rate constants for formation and breakdown of the tetrahedral intermediate can be elucidated and a separate Bronsted plot derived for the k_1 step—the rate of formation of the tetrahedral intermediate. The second order rate constant for the hydroxyaminolysis of benzylpenicillin and cephaloridine shows a rate enhancement of just 100 fold, a deviation from this plot compatible with a simple α-effect.

It therefore appears that hydroxylamine reacts with rate limiting formation of the tetrahedral intermediate which requires $k_2 >> k_{-1}$. The most likely explanation of this is that the breakdown step k_2 is facilitated by intramolecular general acid catalysis (**5**). The pK_a of the OH group in the tetrahedral intermediate $T^{\pm}$ is estimated to be about 4 so proton transfer to the departing amine N is thermodynamically favourable. Previous examples of intramolecular general acid catalysis from the attacking nucleophile have been observed with ethylenediamine[2] and 2-mercaptoethylammonium ion,[12] which show rate enhancements of 100 and 1400 respectively, from that predicted for amines of similar basicity. To further demonstrate that intramolecular general acid catalysis is taking place and that the enhanced rate with hydroxylamine is not simply an α-effect, the aminolysis of benzylpenicillin was studied using the *O*-methyl derivative of hydroxylamine, methoxylamine. This amine lacks the necessary hydrogen, present in hydroxylamine, to donate a proton to the departing β-lactam amine as in (**5**). The rate of reaction of benzylpenicillin in aqueous solutions of methoxylamine, up to 0.05 M, corresponds to simple hydrolysis with no aminolysis/buffer catalysis observable. The maximum second order rate constant must be less than 5×10^{-6} $dm^3mol^{-1}s^{-1}$. Although methoxylamine (pK_a of conjugate acid 4.6) is less basic than hydroxylamine, the lack of reaction shows that there is not a significant α-effect. Therefore the suggestion of intramolecular general acid catalysis with hydroxylamine is substantiated.



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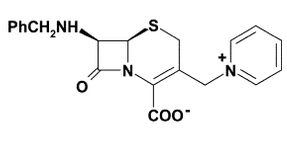

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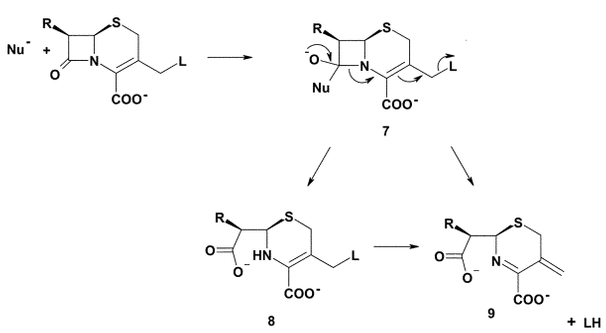
Interestingly similar observations were made with the aminolysis of cephaloridine (**6**) with hydroxylamine. The rate law shows only a first order dependence on hydroxylamine concentration and the second order rate constant, $k_{NH_2OH} = 0.0864$ $dm^3mol^{-1}s^{-1}$, is 6×10^4 fold greater than that estimated for the uncatalysed aminolysis of cephaloridine by an amine of pK_a 6.17. Again this is much greater than the expected α-effect and is attributed to intramolecular general acid catalysis by proton transfer from the terminal hydroxyl group of the attacking hydroxylamine to the departing β-lactam amine (**5**).

Cephaloridine, as other cephalosporins, possesses a potential leaving group at the C3′ position (L = pyridine), which is expelled during the hydrolysis of the β-lactam ring (Scheme 3) giving the exo-methylene compound (**9**). It has been suggested, based on quantum mechanical calculations, that β-lactam C–N bond fission and breakdown of the tetrahedral intermediate is concerted with the departure of the leaving group, L, at the C3′ position (**7**). An interesting feature of our observation of intramolecular general acid catalysis is that the two processes can not be coupled (Scheme 3) and that the tetrahedral intermediate breaks down by proton transfer to the departing nitrogen (**5**) to generate an intermediate enamine (**8**) which subsequently, in a separate step, expels pyridine to give the α,β-unsaturated imine (**9**). Other experimental observations have led to similar conclusions.[13]



Scheme 3

Acknowledgements

We thank the University of Huddersfield for financial support.

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