Hydrolysis of 3-Substituted Cephalosporins catalysed by β-Lactamases I and II from *Bacillus cereus* and by Hydroxide Ion

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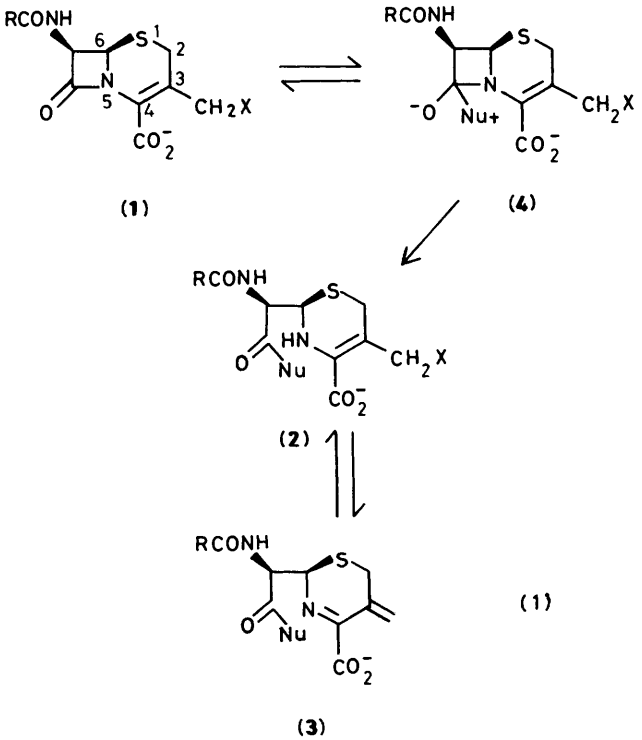
Second-order rate constants for the alkaline hydrolysis of 3-thiol substituted cephalosporins are independent of the pK_a of the thiol over a pK_a range of 9. If there is a leaving group at C-3′ it is expelled after the β-lactam ring is opened and the expulsion of the leaving group does not enhance the rate of β-lactam C–N bond fission. The zinc enzyme β-lactamase II is about a 100-fold better catalyst than the serine enzyme β-lactamase I for the hydrolysis of the same cephalosporin. The second-order rate constant k_{cat}/K_m for both β-lactamase enzymes shows no dependence on the nature of the substituent at C-3′ which is not explicable by the different chemical reactivity of the cephalosporins. There is no evidence for a significant recognition site in either enzyme for the C-3′ substituent. The kinetic parameters k_{cat} and K_m for the β-lactamase I-catalysed hydrolysis may be complicated by the formation of intermediates.

Many bacteria overcome the normal fatal encounter with β-lactam antibiotics by the production of membrane-bound and excreted enzymes which catalyse the hydrolysis of the β-lactam. These β-lactamase enzymes (E.C. 3.5.2.6) are of at least three types,[1] classes A and C are both serine enzymes whilst class B β-lactamases are zinc ion-dependent. Little is known about the detailed mechanism of action of these enzymes and until this year[2] there were no crystallographic data which were sufficiently resolved to allow a description of the enzymes in atomic detail.

The serine β-lactamases have no amino acid sequence relationship with the known serine proteases. In particular, there is no conserved histidine residue which is used by all serine proteases as a general acid–base catalyst.[3] There is no unambiguous evidence of the nature of the proton donor or acceptor group which is presumed to be necessary for acylation and deacylation of the serine β-lactamases.[4] The previous two papers attempted to map out some of the important binding sites for substituents at C-6 in penicillins[5] and at C-7 in cephalosporins.[6] This paper reports observations on the effect of the C-3 substituent in cephalosporins on the activity of the serine and zinc β-lactamases from *B. cereus*.



Many cephalosporins (**1**) differ from penicillins by having a substituent at C-3′ which is expelled when nucleophiles attack the carbonyl carbon of the β-lactam [equation (1)].[7] It has often been suggested[8] that the antibacterial activity of cephalosporins is very dependent on the nature of the group at C-3′. Although it has been claimed[9,10] that departure of the leaving group at C-3′ is concerted with nucleophilic attack at the β-lactam carbonyl carbon, experimental evidence is compatible with a stepwise process involving formation of a tetrahedral intermediate (**4**) and an enamine intermediate (**2**).[11–16] Furthermore, the enamine (**2**) and imine (**3**) have been shown to be in equilibrium.[17]

It is conceivable, although chemically unlikely, that conversion of the enamine (**2**) into the conjugated imine (**3**) by expulsion of the leaving group at C-3′ is facilitated enzymatically by general acid–base catalysis.[18] Even if there is no chemical catalysis there may be a binding site on β-lactamases for cephalosporin C-3′ substituents which facilitates catalyses.[19] The purpose of this work was to elucidate and separate chemical catalysis and recognition of the C-3′ substituent by the serine and zinc β-lactamases from *B. cereus*.

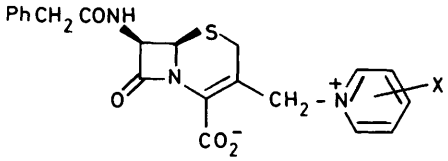
Experimental

Kinetics.—The kinetic analysis was carried out as described previously.[5]

Materials.—β-Lactamase II was purified from the culture supernatant of *B. cereus* 569/H/9.[20]

3′-*Substituted Cephalosporins.*—Most literature methods[21] produced inadequate yields and the most successful method used was the variation of a patent method[22] which uses trimethylsilyl iodide to cleave the acetate at C-3′.

Preparation of 7-Phenylacetamido 3′-Pyridinium Substituted Cephalosporins.—The sodium salt of 7-phenylacetamidocephalosporanic acid (0.42 g) was suspended in dry dichloromethane (9 cm^3). Trimethylsilyl iodide (1.4 g) was added followed by pyridine (0.67 g) and the mixture heated under reflux under argon for 2 h. The mixture was cooled to −15 °C then stirred with a solution of potassium iodide (0.6 g in 2.5 cm^3 water) and dilute HCl (2.5 cm^3) added. This mixture was left in an ice-bath for 2 h, with occasional shaking. The solvents were removed on a rotary evaporator, the residue was redissolved in distilled water, and the solution filtered. The solution was then loaded onto a styrene divinylbenzene preparative-scale column connected to a u.v. detector set to 260 nm. The inorganic salts were eluted in water followed by elution of the organic materials with 25% methanol–water. The methanol was removed on a rotary evaporator and the remainder freeze-dried. The resulting solid material was dissolved in the minimum amount of water and loaded onto a Dowex 1X-400 mesh column connected to the u.v. detector. The products were eluted in water, freeze-dried, and characterised. Using this method the following derivatives were prepared.



(5)

3′-*Ethylpyridinium derivative* (**5**; X = Et), δ(D_2O) 5.15 (d, 6-H), 5.70 (d, 7-H), 3.40 (d, 2-H), 7.35 (s, *Ph*CH_2CONH), 3.65 (s, PhCH_2CONH), 5.40 (m, C$H_2$$NC_5H_4$Me), 8.00 (m, CH_2N-$C_5H_4CH_2$Me), 8.45 (m), 8.85 (d, $CH_2NC_5H_4CH_2$Me), 2.95 (q, $CH_2NC_5H_4CH_2$Me), and 1.30 (t, $CH_2NC_5H_4CH_2$*Me*); $\lambda_{max.}$ 275 (ε 8 600) nm.

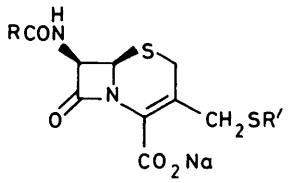
Pyridinium derivative (**5**; X = H), δ(D_2O) 5.10 (d, 6-H), 5.55 (d, 7-H), 3.40 (d, 2-H), 7.30 (s, *Ph*CH_2CONH), 3.60 (s, PhCH_2CONH), 5.45 (m, C$H_2NC_5H_5$), 8.10 (m, $CH_2NC_5H_5$), 8.50 (m, $CH_2NC_5H_5$), and 8.95 (d, $CH_2NC_5H_5$); $\lambda_{max.}$ 260 (ε 8 200) nm.

3′-*Bromopyridinium derivative* (**5**; X = Br), δ(D_2O) 5.10 (d, 6-H), 5.65 (d, 7-H), 3.45 (br s, 2-H), 7.25 (s, *Ph*CH_2CONH), 3.55 (s, PhCH_2CONH), 5.45 (m, C$H_2NC_5H_4$Br), 8.15 (m, $CH_2NC_5H_4$Br), 8.60 (m, $CH_2NC_5H_4$Br), and 8.90 (m, $CH_2NC_5H_4$Br), (2:1:1); $\lambda_{max.}$ 275 (ε 7 300) nm.

Conversion of 7-Aminocephalosporanic Acid (ACA) into the 3′-Pyridine Analogue.—To a mixture of 7-ACA (2.25 g) and potassium thiocyanate (0.73 g) in dry acetonitrile (12 ml) was added dropwise trifluoromethanesulphonic acid (3.4 g) below 15 °C with cooling in an ice-bath. The mixture was stirred for 30 min at 15—18 °C. The solution was added to a mixture of pyridine (6 ml) and water (8 ml) at 30 °C with stirring, then immediately poured into a cold stirred mixture of isopropyl alcohol (IPA) (45 ml) and ether (60 ml), and cooled in ice. The resulting yellow precipitate was filtered, washed with IPA–ether, and stirred for 1 h in water, (50 ml). The precipitate was filtered, dissolved in methanol–water, and passed down the Dowex 1X-400 column. The methanol component was removed on a rotary evaporator and the product freeze-dried resulting in a pale yellow powder: 1 800 (β-lactam CO) and 1 620 cm^{-1} (carboxylate); δ(X) 4.95 (d, 6-H), 5.55 (d, 7-H), 3.55 (d, 2-H), 5.25 (d, C$H_2NC_5H_5$), 8.15 (m, $CH_2NC_5H_5$), 8.60 (m, $CH_2NC_5H_5$), and 9.06 (m, $CH_2NC_5H_5$); $\lambda_{max.}$ 265 (ε 12 450) nm.

Preparation of 3′-Thiol Substituted Cephalosporins.—The most satisfactory method involved initial displacement of the C-3′ acetate group in 7-ACA by thiols followed by acylation of the 7-amino group.[23]

Example. To anhydrous acetonitrile (14 ml) was suspended 7-ACA (2.72 g) and benzenethiol (1.2 ml). (Glacial acetic was occasionally used in place of the acetonitrile to improve solubility.) To this suspension was added boron trifluoride–diethyl ether complex (4.3 g) resulting in a clear brown solution. The mixture was stirred under reflux for 2 h, cooled over ice, and water (14 mls) added. The pH of the solution was adjusted to 4 with dilute ammonia solution. The solid which precipitated was filtered and washed with a little water followed by a little acetone. The conversion with benzenethiol and many other thiols was found to proceed in quantitative yield. Acylation of these derivatives gave the following compounds.



(6)

3′-*Carboxyethylthio 7-heptyl derivative* [**6**; R = C_7H_{15}, R′ = $(CH_2)_2CO_2H$], $\nu_{max.}$ 1 755 (β-lactam CO), 1 700 (acid CO), 1 640 (amide CO), 1 600 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(D_2O) 0.9 (t, *Me*C_6H_{13}CONH), 1.1—2.0 [s, Me(CH_2)$_5$$CH_2$CONH], 2.40 [t, Me$(CH_2)_5CH_2$CONH], 4.40 [d, C$H_2S(CH_2)_2CO_2H$], 1.60 (t, $CH_2SCH_2CH_2CO_2H$), 2.75 (t, $CH_2CH_6CH_2CO_2H$); $\lambda_{max.}$ 270 (ε 9 660) nm.

3′-*Ethoxycarbonylmethylthio 7-heptyl derivative* (**6**; R = C_7H_{15}, R′ = CH_2CO_2Et), $\nu_{max.}$ 1 770 (β-lactam CO), 1 730 (ester CO), 1 640 (amide CO), 1 600 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 0.9 [t, *Me*(CH_2)CO], 1.1—1.95 [s, Me(CH_2)$_5$$CH_2$CO], 2.40 [t, Me$(CH_2)_5CH_2$CO], 4.75 (m, C$H_2SCH_2CO_2$Et), 2.0 (m, CH_2SCH_2CO_2Et), 2.0 (m, CH_2SCH_2-CO_2CH_2Me), 1.1—1.3 (t, $CH_2SCH_2CO_2CH_2$*Me*), and 9.15 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 9 240) nm.

3′-*Isopropylthio 7-benzyl derivative* (**6**; R = $PhCH_2$, R′ = $CHMe_2$), $\nu_{max.}$ 1 755 (β-lactam CO), 1 650 (amide CO), 1 600 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(D_2O) 5.10 (d, 6-H), 5.65 (d, 7-H), 3.45 (2-H), 7.30 (s, *Ph*CH_2CONH), 2.4—3.6 (PhCH_2CONH), 3.9—4.2 (m, CH_2SC*H*Me_2), and 1.3 (d, CH_2SCHMe_2); $\lambda_{max.}$ 270 (ε 11 400) nm.

7-*Benzyl 3′-S-thioacetate derivative* (**6**; R = $PhCH_2$, R′ = COMe), $\nu_{max.}$ 1 750 (β-lactam CO), 1 740 (thiolester CO), 1 660 (amide CO), 1 600 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(CD_3OD) 4.95 (d, 6-H), 5.60 (d, 7-H), 3.40 (m, 2-H), 7.30 (s, *Ph*CH_2CONH), 3.50 (s, PhCH_2CONH), 4.80 (d, CH_2SCOMe), and 1.90 (s, CH_2SCOMe); $\lambda_{max.}$ 264 (ε 9 070) nm.

7-*Benzyl 3′-thiophenol derivative* (**6**; R = $PhCH_2$, R′ = Ph), $\nu_{max.}$ 1 770 (β-lactam CO), 1 650 (amide CO), 1 600 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 4.90 (d, 6-H), 5.55 (q to d in D_2O, 7-H), 3.45 (br s, 2-H), 7.25 (*Ph*CH_2CONH), 3.50 (s, PhCH_2CONH), 4.30 (m, C$H_2SC_6H_5$), 7.30 ($CH_2C_6H_5$), 9.00 (d, disappears in D_2O, NH); $\lambda_{max.}$ 264 (ε 9 500) nm.

3′-*Benzylthio 7-benzyl derivative* (**6**; R = $PhCH_2$, R′ =

CH_2Ph), $\nu_{max.}$ 1 745 (β-lactam CO), 1 660 (amide CO), 1 600 (carboxylate), and 3 285 cm^{-1} (amide NH); δ(DMSO) 4.95 (d, 6-H), 5.50 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.35 (*Ph*CH_2CONH), 3.65 (s, PhC*H*$_2$CONH), 3.80 (m, CH_2SCH_2Ph), 7.20—7.35 (m, CH_2SCH_2*Ph*), and 9.00 (d, disappears in D_2O, NH); $\lambda_{max.}$ 265 (ε 8 400) nm.

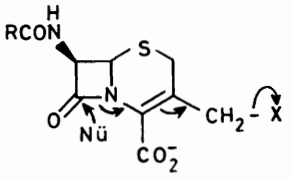
3′-Butylthio 7-benzyl derivative [**6**; R = $PhCH_2$, R′ = $(CH_2)_3Me$], $\nu_{max.}$ 1 760 (β-lactam CO), 1 660 (amide CO), 1 600 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(CD_3OD) 5.00 (d, 6-H), 5.60 (d, 7-H), 3.45 (d, 2-H), 7.30 (m, *Ph*CH_2CONH), 3.60 (s, PhC*H*$_2$CONH), 4.70 [in CD_3OD, C*H*$_2$S$(CH_2)_3$Me], 0.9 [d, $CH_2S(CH_2)_3$*Me*], 1.20—1.60 [m, $CH_2S(CH_2)_3Me$], and 3.30 (m, 2 H); $\lambda_{max.}$ 270 (ε 10 400) nm.

3′-Ethoxycarbonylmethylthio 7-benzyl derivative (**6**; R = $PhCH_2$, R′ = CH_2CO_2Et), $\nu_{max.}$ 1 770 (β-lactam CO), 1 728 (ester CO), 1 650 (amide CO), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.05 (d, 6-H), 5.60 (q to d in D_2O, 7-H), 3.45 (s, 2-H), 7.35 (m, *Ph*CH_2CONH), 3.80 (s, PhC*H*$_2$CONH), 4.80 (m, C*H*$_2$SCH$_2$CO$_2$Et), 2.0 (m, $CH_2SCH_2CO_2Et$), 2.0 (m, $CH_2SCH_2CO_2CH_2Me$), 1.20 (t, $CH_2SCH_2CO_2CH_2$*Me*), and 9.10 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 10 500) nm.

7-Benzyl 3′-t-butylthio derivative (**6**; R = $PhCH_2$, R′ = CMe_3), $\nu_{max.}$ 1 755 (β-lactam CO), 1 650 (amide CO), 1 600 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.00 (d, 6-H), 5.50 (q to d in D_2O, 7-H), 3.45 (q, 2-H), 7.30 (s, *Ph*CH_2CONH), 3.50 (PhC*H*$_2$CONH), 4.30 (m, C*H*$_2$SCMe$_3$), 3.50 (br s, CH_2SCMe_3), and 9.00 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 11 400) nm.

Results and Discussion

Alkaline Hydrolysis.—The major structural difference between cephalosporins (**1**) and penicillins is that the five-membered thiazolidine ring of penicillins is replaced by a six-membered dihydrothiazine in cephalosporins and the degree of pyramidalisation of the β-lactam nitrogen is generally smaller in cephalosporins. In addition, many of the cephalosporins have a leaving group, *e.g.* acetate, pyridine, and thiol, at C-3′ and expulsion of these groups occurs during the hydrolysis of the β-lactam, equation (1).[24] There have been many suggestions, apparently supported by theoretical calculations,[9,10] that nucleophilic attack upon the β-lactam carbonyl carbon is concerted with the leaving group at C-3′, (**7**), and that the presence of the leaving group at C-3′ enhances chemical reactivity.[25]



(**7**)

In general, the second-order constants for the hydroxide ion-catalysed hydrolysis of cephalosporins are similar to those of penicillins. Not only does this observation indicate that the non-planarity of the β-lactam nitrogen does not significantly affect reactivity, as the nitrogen is 0.4 Å out of the plane in penicillins whereas in cephalosporins it deviates by 0.2—0.3 Å,[7] but it also suggests that having a leaving group at C-3′ does not significantly affect the reactivity of cephalosporins. This is confirmed by the observations presented here. In the Table are shown the second-order rate constants for the hydroxide ion-catalysed hydrolysis of a series of cephalosporins with different substituents and leaving groups at C-3′. The acidities of the thiols potentially to be liberated at C-3′ cover a range of 9 pK_a units and yet the second-order rate constants are almost invariant. Although there are some reservations about using pK_a as an indication of nucleofugality,[26] the equilibrium constants between the enamine (**2**) and imine (**3**) are correlated with the pK_a of the thiol by a Brönsted βl_g of 0.7.[17] The Brönsted plot for the data shown in the Table is obviously scattered but the slope is less than 0.1. There is little or no change in the effective charge on the leaving group on going from the ground state to the transition state. This is compatible with a stepwise process and not with the concerted mechanism, (**7**). Futhermore, the rates of hydrolysis of cephalosporin with substituents at C-3′ which are expelled and those which are not (X = H) (see Table) are similar. It appears that the effect of substituents at C-3′ on the rate of alkaline hydrolysis of cephalosporins is purely inductive and that the expulsion of the leaving group at C-3′ occurs *after* β-lactam ring opening.[7] There is no significant coupling of the processes so that there is a significant lowering of the activation energy for carbon–nitrogen bond fission in the β-lactam *because* a group at C-3′ is expelled. It is conceivable that in some cases intermediate enamine (**2**) formed by β-lactam carbonyl carbon–nitrogen bond fission could be so unstable and that its lifetime is so short as to preclude its existence and therefore the breakdown of tetrahedral intermediate is enforced to be concerted. However, the general conclusion is that expulsion of a leaving group at C-3′ does not significantly enhance the rate of β-lactam carbon–nitrogen fission.

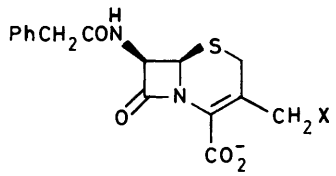
β-*Lactamase-catalysed Hydrolysis.*—The kinetic parameters for the β-lactamase I and II-catalysed hydrolysis of 3′-substituted cephalosporins are given in the Table. The second-order rate constants k_{cat}/K_m for the serine enzyme are generally 10^3—5×10^5-times greater than those for alkaline hydrolysis, whereas those for the zinc enzyme are 2×10^6—10^7-fold greater. The zinc enzyme is generally 10—100-fold more efficient at hydrolysing cephalosporins than the serine enzyme.

The values of k_{cat}/K_m for the β-lactamase I-catalysed hydrolysis of cephalosporins with thiol groups at C-3′ show little or no dependence on the pK_a of the thiol (see Table). For a change in pK_a of 9 units k_{cat}/K_m varies only three-fold but even this variation is not systematic. There is no evidence that cephalosporins which have better leaving thiol groups at C-3′ are better substrates. Cephalosporins with pyridines at C-3′ are better substrates than those with thiols at C-3′ for β-lactamase I (see Table). However, this approximate 10-fold increase in reactivity is similar to the faster rate observed with hydroxide ion. We therefore conclude that this is a 'chemical' effect due to the positively charged pyridinium substituent increasing susceptibility to nucleophilic attack and not due to any favourable interaction with the enzyme. Again there is no dependence of k_{cat}/K_m upon the pK_a of the pyridine at C-3′, and this general lack of a correlation is confirmed by k_{cat}/K_m for the 3-methyl derivative where there is no leaving group. The 3-methylcephalosporin is as good a substrate as cephalosporins with acetate or thiol 'leaving' groups at C-3′.

There is a similar lack of dependence of k_{cat}/K_m for the zinc enzyme-catalysed hydrolysis upon the pK_a or nature of the group at C-3′. There is no obvious recognition site in this enzyme for C-3′ substituents.

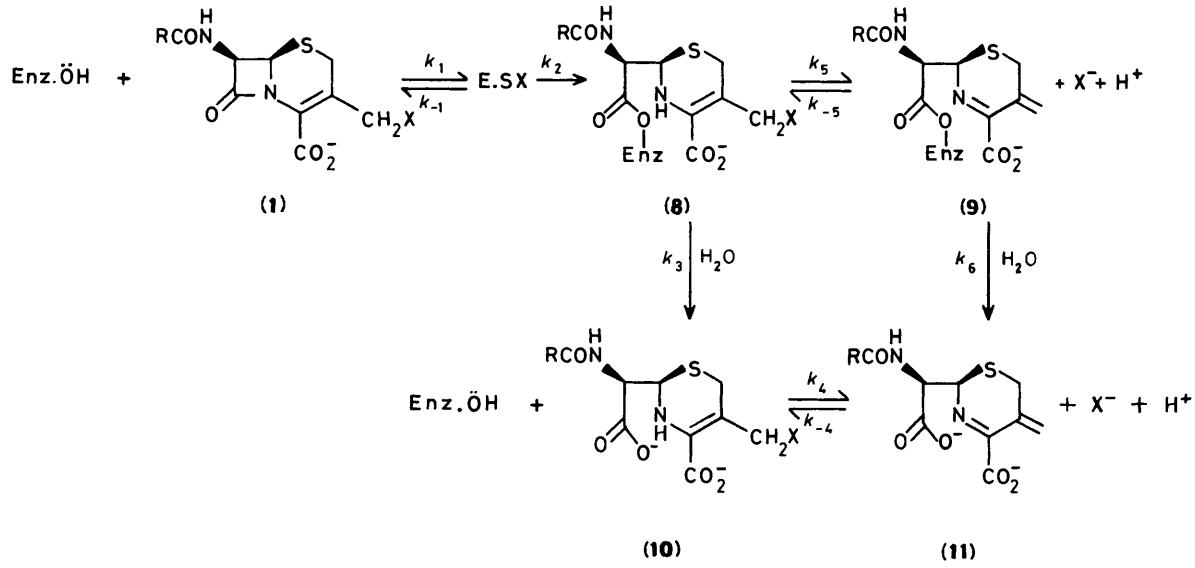
There is a complication with the interpretation of the steady-state kinetic parameters for the β-lactamase I-catalysed hydrolysis because turnover of the enzyme may be limited by a variety of steps. There are several pieces of evidence which suggest that the β-lactamase I-catalysed hydrolysis of penicillins and cephalosporins proceeds by the formation of an intermediate acyl-enzyme.[16,27–31] In addition to this intermediate, other covalently linked enzyme intermediates may be

Table. Second-order rate constants k_{OH} for the hydroxide ion-catalysed hydrolysis of 3′-substituted benzylcephalosporins at 30 °C, I = 1.0M, and the kinetic parameters for the *B. cereus* β-lactamase I- and II-catalysed hydrolysis at 30 °C, pH 7.0



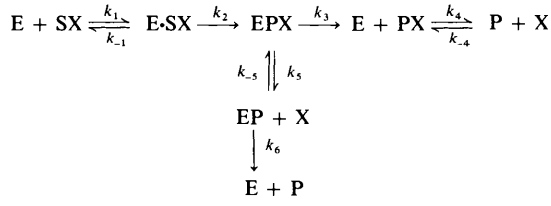
			β-Lactamase I			β-Lactamase II
X	pK_a of XH	k_{OH}/mol dm^{-3} s^{-1}	k_{cat}/s^{-1}	K_m/mol dm^{-3}	k_{cat}/K_m/mol^{-1} dm^3 s^{-1}	k_{cat}/K_m/mol^{-1} dm^3 s^{-1}
SCOMe	3.62	0.033	8.20×10^{-2}	4.90×10^{-5}	1.67×10^{3}	1.19×10^{5}
SPh	6.55	0.027	0.27	8.13×10^{-5}	3.27×10^{3}	—
$SCH_2CO_2C_2H_5$	7.95	0.033	0.304	5.64×10^{-5}	5.39×10^{3}	3.04×10^{5}
SCH_2Ph	9.43	0.034	0.174	5.35×10^{-5}	3.25×10^{3}	1.09×10^{5}
$SCHMe_2$	10.86	0.027	0.142	4.23×10^{-5}	3.36×10^{3}	1.36×10^{5}
$SCMe_3$	11.22	0.051	3.00×10^{-2}	6.17×10^{-6}	4.86×10^{3}	2.50×10^{5} [a]
$S(CH_2)_3Me$	12.40	0.027	0.142	6.81×10^{-5}	3.05×10^{3}	2.84×10^{4}
3-Bromopyridine	2.84	0.540	4.37	5.85×10^{-5}	7.45×10^{4}	—
Pyridine	5.17	0.426	2.81	4.39×10^{-5}	6.44×10^{4}	—
3-Ethylpyridine	5.70	0.444	5.60	1.71×10^{-4}	3.27×10^{4}	—
OCOMe	4.76	0.094	0.280	4.70×10^{-5}	5.96×10^{3}	3.03×10^{5} [b]
H	—	0.029	3.20×10^{-2}	3.17×10^{-5}	1.03×10^{3}	—

[a] k_{cat} = 5.09 s^{-1} and $K_m = 2.03 \times 10^{-5}$ mol dm^{-3}. [b] k_{cat} = 188 s^{-1} and $K_m = 6.20 \times 10^{-4}$ mol dm^{-3}.



Scheme 1.

formed. The relative rates of alkaline hydrolysis of 3-substituted cephalosporins reported here and other observations[7,16] indicate that the leaving group at C-3′ is expelled after the β-lactam ring is opened. Furthermore, Pratt[32] and ourselves[17] have shown that expulsion of the leaving group in the enamine (**2**) to give the imine (**3**) is a reversible process. Hydrolysis of the acyl-enzyme intermediate (**8**) (Scheme 1) may occur before or after expulsion of the leaving group at C-3′.[14,33] Turnover of the enzyme may therefore occur by k_3 (with the 'leaving' group at C-3′ still attached) and the subsequent non-enzyme-catalysed conversion of (**10**) into (**11**) or by k_6 when the leaving group has departed. The steady-state kinetic parameters reported in the Table could therefore arise from k_{cat} corresponding to rate-limiting acylation k_2, deacylation k_3 or k_6, or a mixture of these. Scheme 1 is simplified to Scheme 2 where SX is a cephalosporin (**1**), EPX is the acyl-enzyme (**8**), PX is the enamine (**10**), EP is conjugated imine of the acylenzyme (**9**), and P is the imine (**11**).



Scheme 2.

If $k_3 \gg k_5$, the steady-state kinetics will be that for a normal enzyme-catalysed reaction involving the formation of a covalent intermediate, with k_{cat}, K_m, and k_{cat}/K_m given by equations (2), (3), and (4)

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (2)$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \cdot \frac{k_3}{k_2 + k_3} \quad (3)$$

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \quad (4)$$

This is certainly the situation for the 3-methylcephalosporin because $k_5 = 0$. With good leaving groups at C-3′ such as acetate k_5 could be greater than k_3 and k_{-5} is probably negligible.[17,34] In this case, regeneration of the enzyme occurs by k_6 which would be reflected in the value of k_{cat} and independent of the nature of X. It has been shown that the acyl-enzymes (**8**) and (**9**) from *S. aureus* hydrolyse at different rates.[33] There are some indications that the equilibrium between (**8**) and (**9**) could cause a change in the rate-limiting step with time because adding thiols to the enzyme-catalysed reaction can remove the observed pre-steady state slower reaction.[4]

A possible explanation for the low sensitivity to substituents at C-3′ is that the C-4 carboxylate displaces the leaving group at C-3′ to give a lactone as substrate. The lactone *is* a very good substrate but lactonisation of the substrate does not occur before hydrolysis because the n.m.r.-observable products are (**10**) or (**11**) whereas the product from the β-lactamase I-catalysed hydrolysis of the lactone gives a β-lactam ring-opened product but with the lactone ring intact. Furthermore the values of k_{cat}/K_m are similar even for those substituents at C-3′ which are not or cannot be displaced.

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