Hydrolysis of 7-Substituted Cephalosporins catalysed by β-Lactamases I and II from *Bacillus cereus* and by Hydroxide Ion

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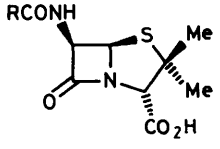
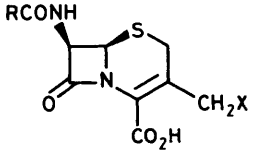
Kinetic parameters are reported for the *Bacillus cereus* β-lactamase I- and β-lactamase II-catalysed hydrolysis of a series of thirty-seven cephalosporins substituted in the 7-position. These are compared with the second-order rate constants for the hydroxide ion-catalysed hydrolysis of these derivatives. There is no significant dependence of the rate of the base-catalysed hydrolysis upon the nature of the side-chain substituent. For β-lactamase I, k_{cat}/K_m varies over 2×10^5 but for β-lactamase II the variation with substituents is only 10. For alkyl substituents, k_{cat}/K_m increases with chain length and passes through a maximum, for β-lactamase I this is with the undecyl derivative and for β-lactamase II the octylcephalosporin. For β-lactamase I, but not for β-lactamase II, the t-butylcephalosporin is a very poor substrate. There is no evidence for a significant cavity in either enzyme to host aromatic residues. An ionised carboxylate residue on the side-chain significantly reduces reactivity with β-lactamase I but not β-lactamase II. It is suggested that a carboxy group on β-lactamase I acts as a general catalyst facilitating β-lactam C–N bond fission.

The two major classes of β-lactam antibiotics are the penicillins (**1**) and the cephalosporins (**2**).† [1] Bacteria which are normally susceptible to these antibiotics may be rendered insusceptible if they can produce β-lactamases, enzymes that catalyse the hydrolysis of the β-lactam.

There are many different β-lactamases which have been classified on the basis of substrate profile (the ability to catalyse the hydrolysis of various β-lactam antibiotics *e.g.* penicillinase or cephalosporinase), on the basis of their sequence or on a mechanistic basis.[2] For example, the TEM2 β-lactamase from *E. coli* is very efficient and one molecule of the enzyme can catalyse the hydrolysis of 2 000 molecules of benzylpenicillin in 1 second.[3] However, this enzyme is more than 10 000 less reactive towards some cephalosporins.[4] Conversely, one molecule of the P99 β-lactamase can hydrolyse 2 000 molecules of cephaloridine in 1 second.[5] One mechanistic class comprises β-lactamases that are serine enzymes; this is subdivided into classes A and C based on the amino acid sequences.[2] The other mechanistic class of β-lactamases is distinguished by the requirement for zinc(II) ions and a lack of sequence homology to enzymes of the other classes. There are two well characterised but unrelated enzymes of this class B—β-lactamase II from *B. cereus*[6] and β-lactamase L-1 from *Pseudonomas maltophilia.*[7]



(1)



(2)

† An example of the systematic nomenclature system for the trivially named cephalosporins, includes 3-[(acetyloxy)methyl]-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid for benzylcephalosporin.

Besides the requirement of zinc(II) it appears there is a glutamic acid residue involved at the active site.[8]

Very little is known about the binding sites of any of the β-lactamases away from the active site. The previous paper[9] describes the effect of 6-substituted penicillins on the activity of *B. cereus* β-lactamase I. Herein, we compare the effects of 7-substituted cephalosporins on the activity of β-lactamase I and II from *B. cereus*.

Experimental

β-Lactamase II was purified from the culture supernatant of *B. cereus* 569/H/9.[10]

Synthesis of 7-Substituted Cephalosporins.—Preparation of the acetyl derivative (**2**; R = Me). A solution of 7-aminocephalosporanic acid (7-ACA) (2.72 g, 10 mmol) in water (40 ml) containing sodium hydrogencarbonate (2.1 g, 25 mmol) and acetone (30 ml) was cooled to 0—5 °C, stirred, and treated with a solution of acetic anhydride (2.0 g, 20 mmol) in acetone (20 ml). The mixture was maintained at 0—5 °C for 30 min with stirring. The acetone was removed on a rotary evaporator, and the pH of the solution was adjusted to pH 7 with sodium hydrogen carbonate. The solution was washed with ethyl acetate, acidified to pH 2 with dilute HCl, and twice extracted with ethyl acetate. The combined extracts were washed with a little cold water and dried ($MgSO_4$). The cephalosporanic acid in solution was converted into the sodium salt by the addition of a few drops of sodium 2-ethylhexanoate in butanol; the sodium salt precipitated from the solution. The precipitate was filtered, suspended in boiling acetone, and water added dropwise until solution was just obtained. Cooling this solution gave the white crystalline product, $\nu_{max.}$ 1 775 (β-lactam CO), 1 720 (ester CO), 1 665 (amide CO), 1 605 (carboxylate), and 3 250 cm^{-1} (amide NH); $\delta_H(D_2O)$ 5.10 (1 H, d, 6-H), 5.65 (1 H, d, 7-H), 3.50 (2 H, d, 2-H), 2.10 (3 H, s, Me), 4.80 (2 H, d, CH_2OCOMe), and 1.90 (3 H, s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 7 950) nm.

Preparation of Other Alkylamido Cephalosporins. Method A.[11] A solution of 7-ACA (2.72 g, 10 mmol) in water (40 ml) containing sodium hydrogencarbonate (2.1 g, 25 mmol) and acetone (30 ml) was cooled to 0—5 °C and treated with a solution of the acid chloride (10 mmol) in acetone (20 ml). After 15 min, a further portion of the acid chloride (2.5 mmol) was added and the mixture stirred a further 15 min. Work-up and recrystallisation was then as described above.

Method B. 7-ACA (5.44 g, 20 mmol) was suspended in boiling ethyl acetate (250 ml) and treated with the acid chloride (40 mmol). The mixture was stirred under reflux for 30 min, cooled, and filtered. The solution was treated with aniline (60 mmol), stirred for 1 h, and extracted several times with 3% aqueous sodium hydrogencarbonate. The combined extracts were washed with ethyl acetate, acidified to pH 2 with dilute HCl, and extracted with ethyl acetate. Work-up and recrystallisation was then as previously described.

Method C. A solution of dimethylformamide (DMF) (0.33 ml, 4.3 mmol) in dry dichloromethane was stirred under dry conditions, under nitrogen at −10 °C. Oxalyl chloride (0.34 ml, 3.9 mmol) was added dropwise and the solution stirred for a further 30 min resulting in a gelatinous precipitate. The carboxylic acid (4 mmol) was added followed by *N*-methylmorpholine (0.51 ml) and stirring continued for a further 30 min. In a separate dry flask, under nitrogen, to a suspension of 7-ACA (1.05 g, 0.4 mmol) in dry dichloromethane (8 ml) was added bis(trimethylsilyl)acetamide (1.91 ml, 7.5 mmol). The suspension was stirred for 1 h resulting in a clear solution. This was then transferred by syringe to the acid chloride solution at −10 °C. The mixture was stirred for 1 h whilst warming to room temperature, then poured into 50 ml of a 3% solution of sodium hydrogencarbonate. The mixture was shaken and the aqueous layer retained, washed with ethyl acetate, and acidified to pH 2 with dilute HCl. The product was extracted with ethyl acetate, converted into the sodium salt, and recrystallised as previously described.

Using these methods the following 7-substituted cephalosporins were prepared:

7-*Ethylcephalosporin* (**2**; R = Et), $\nu_{max.}$ 1 792 (β-lactam CO), 1 748 (ester CO), 1 652 (amide CO), 1 610 (carboxylate), and 3 300 cm^{-1} (amide NH); δ_H(DMSO) 5.05 (1 H, d, 6-H), 5.65 (1 H, q, 7-H), 3.50 (2 H, d, 2-H), 2.10 (3 H, m, Me), 1.05 (2 H, q, CH_2), 4.90 (2 H, d, C*H*$_2$OCOMe), 1.90 (3 H, s, CH_2OCOMe), and 8.75 (1 H, d, NH); $\lambda_{max.}$ 260 (ε 7 750) nm; (Found: C, 42.72; H, 4.21; N, 7.55. $C_{13}H_{15}N_2NaO_6S{\cdot}H_2O$ requires C, 42.3; H, 4.62; N, 7.61%).

7-*Propylcephalosporin* (**2**; R = Pr), $\nu_{max.}$ 1 765 (β-lactam CO), 1 740 (ester CO), 1 652 (amide CO), 1 628 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.70 (q to d in D_2O, 7-H), 3.50 (d, 2-H), 2.10 [m, $(CH_2)_2Me$], 0.9 [t, $(CH_2)_2$Me], 1.6 [m, $(CH_2)_2$Me], 4.90 (m, C*H*$_2$OCOMe), 1.90, (s, CH_2OCOMe), and NH (8.70, d, disappears in D_2O); $\lambda_{max.}$ 260 (ε 7 850) nm; (Found: C, 46.16; H, 4.73; N, 7.50. $C_{14}H_{17}N_2NaO_6S$ requires C, 46.15; H, 4.67; N, 7.69%).

7-*Butylcephalosporin* (**2**; R = Bu), $\nu_{max.}$ 1 758 (β-lactam CO), 1 738 (ester CO), 1 657 (amide CO), 1 628 (carboxylate), and 3 286 cm^{-1} (amide NH); δ(D_2O) 2.10 [m, $(CH_2)_3Me$], 1.2—1.8 [m, $(CH_2)_3$Me], 1.0 [m, $(CH_2)_3$Me], 4.90 (m, C*H*$_2$OCOMe), and 2.15 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 8 195) nm; (Found: C, 47.51; H, 5.03; N, 7.22. $C_{15}H_{19}N_2NaO_6S$ requires C, 47.62; H, 5.03; N, 7.41%).

7-*Pentylcephalosporin* (**2**; R = C_5H_{11}), $\nu_{max.}$ 1 765 (β-lactam CO), 1 735 (ester CO), 1 648 (amide CO), 1 611 (carboxylate), and 3 308 cm^{-1} (amide NH); δ(D_2O) 5.15 (d, 6-H), 5.70 (d, 7-H), 3.55 (q, 2-H), 2.20 [m, $(CH_2)_4Me$], 1.2—1.8 [m, $(CH_2)_4$Me], 0.9 [m, $(CH_2)_4$Me], 4.85 (m, C*H*$_2$OCOMe), and 2.10 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 7 670) nm; (Found: C, 46.59; H, 5.60; N, 6.83. $C_{16}H_{21}N_2NaO_6S{\cdot}H_2O$ requires C, 46.82; H, 5.61; N, 6.83%).

7-*Hexylcephalosporin* (**2**; R = C_6H_{13}), $\nu_{max.}$ 1 751 (β-lactam CO), 1 732 (ester CO), 1 653 (amide CO), 1 624 (carboxylate), and 3 305 cm^{-1} (amide NH); δ(D_2O) 5.05 (d, 6-H), 5.65 (d, 7-H), 3.40 (q, 2-H), 2.10 [m, $(CH_2)_5Me$], 1.25—1.8 [m, $(CH_2)_5$Me], 4.85 (d, C*H*$_2$OCOMe), and 2.00 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 8 170) nm; (Found: C, 49.98; H, 5.68; N, 6.80. $C_{17}H_{23}N_2NaO_6S$ requires C, 50.25; H, 5.67; N, 6.89%).

7-*Heptylcephalosporin* (**2**; R = C_7H_{15}), $\nu_{max.}$ 1 755 (β-lactam CO), 1 735 (ester CO), 1 668 (amide CO), 1 630 (carboxylate), and 3 305 cm^{-1} (amide NH); δ(DMSO; 400 MHz) 4.95 (d, 1 H, $J_{6,7}$ 5 Hz, 6-H), 5.55 (dd, 1 H, $J_{7,6}$ 5 and $J_{7\text{-NH}}$ 10 Hz, 7-H), 3.2 and 3.5 (qAB, *J* 18 Hz, 2-H), 0.90 (t, Me, 3 H), 1.2 [br s, $(CH_2)_4$, 8 H], 1.5 (t, CH_2, 2 H), 2.2 (m, CH_2CO, 2 H), 4.75 and 5.05 (qAB, 2 H *J* 12 Hz, C*H*$_2$OCOMe), 2.04 (s, 3 H, CH_2OCOMe), and 8.70 (d, 1 H, *J* 10 Hz, NH); $\lambda_{max.}$ 260 (ε 7 800) nm; (Found: C, 51.36; H, 5.98; N, 6.76. $C_{18}H_{25}N_2NaO_6S$ requires C, 51.43; H, 5.95; N, 6.67%).

7-*Octylcephalosporin* (**2**; R = C_8H_{17}), $\nu_{max.}$ 1 750 (β-lactam CO), 1 730 (ester CO), 1 649 (amide CO), 1 626 (carboxylate), and 3 292 cm^{-1} (amide NH); δ(DMSO) 5.03 (d, 6-H), 5.65 (q, 7-H), 3.50 (2-H), 2.10 [$(CH_2)_7Me$], 0.9—1.8 [br s, $(CH_2)_7$Me], 1.3 [m, $(CH_2)_7$Me], 4.90 (d, C*H*$_2$OCOMe), 2.05 (s, CH_2OCOMe), and 8.80 (d, NH); $\lambda_{max.}$ 260 (ε 7 420) nm; (Found: C, 52.43; H, 6.30; N, 6.38. $C_{19}H_{27}N_2NaO_6S$ requires C, 52.53; H, 6.22; N, 6.45%).

7-*Nonylcephalosporin* (**2**; R = C_9H_{19}), $\nu_{max.}$ 1 750 (β-lactam CO), 1 730 (ester CO), 1 650 (amide CO), 1 622 (carboxylate), and 3 298 cm^{-1} (amide NH); δ(D_2O) 5.06 (d, 6-H), 5.65 (d, 7-H), 3.55 (br s, 2-H), 1.1—1.8 [$(CH_2)_8$Me], 4.80 (d, C*H*$_2$OCOMe), 2.10 (s, CH_2OCOMe), and 8.80 (d, NH); $\lambda_{max.}$ 260 (ε 7 800) nm; (Found: C, 52.82; H, 6.48; N, 6.26. $C_{20}H_{29}N_2NaO_6S$ requires C, 53.57; H, 6.47; N, 6.25%).

7-*Decylcephalosporin* (**2**; R = $C_{10}H_{21}$), $\nu_{max.}$ 1 748 (β-lactam CO), 1 728 (ester CO), 1 650 (amide CO), 1 620 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(D_2O) 5.10 (d, 6-H), 5.65 (d, 7-H), 3.50 (br s, 2-H), 1.0—1.75 [$(CH_2)_9$Me], 4.82 (d, C*H*$_2$OCOMe), 2.15 (s, CH_2OCOMe), and 8.75 (d, NH); $\lambda_{max.}$ 260 (ε 7 845) nm.

7-*Undecylcephalosporin* (**2**; R = $C_{11}H_{23}$), $\nu_{max.}$ 1 750 (β-lactam CO), 1 730 (ester CO), 1 648 (amide CO), 1 622 (carboxylate), and 3 296 cm^{-1} (amide NH); δ(D_2O) 5.00 (d, 6-H), 5.65 (d, 7-H), 3.60 (m, 2-H), 1.2—1.8 [$(CH_2)_{10}$Me], 4.90 (d, C*H*$_2$OCOMe), and 1.90 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 7 830) nm; (Found: C, 55.39; H, 7.18; N, 5.84. $C_{22}H_{33}N_2NaO_6S$ requires C, 55.46; H, 6.93; N, 5.88%).

7-*Dodecylcephalosporin* (**2**; R = $C_{12}H_{25}$), $\nu_{max.}$ 1 745 (β-lactam CO), 1 725 (ester CO), 1 648 (amide CO), 1 620 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(D_2O) 5.05 (d, 6-H, mostly unresolved), 1.0—1.8 [$(CH_2)_{11}$Me], 2.10 (s, CH_2OCOMe), and 8.80 (d, NH); $\lambda_{max.}$ 260 (ε 7 855) nm.

7-*Tridecylcephalosporin* (**2**; R = $C_{13}H_{27}$), $\nu_{max.}$ 1 750 (β-lactam CO), 1 730 (ester CO), 1 649 (amide CO), 1 621 (carboxylate), and 3 298 cm^{-1} (amide NH); $\lambda_{max.}$ 260 (ε 7 870) nm; (Found: C, 57.24; H, 7.51; N, 5.41. $C_{24}H_{37}N_2NaO_6S$ requires C, 57.14; H, 7.34; N, 5.55%).

7-*Isobutylcephalosporin* (**2**; R = Bu^i), $\nu_{max.}$ 1 760 (β-lactam CO), 1 740 (ester CO), 1 650 (amide CO), 1 623 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.00 (d, 6-H), 5.60 (q, d in D_2O, 7-H), 3.40 (d, 2-H), 0.8 and 0.9 (d, CH_2CHMe_2), 1.95—2.55 (m, CH_2CHMe_2), 4.90 (d, C*H*$_2$OCOMe), 2.00 (s, CH_2OCOMe), and 8.95 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 9 500) nm.

7-*t-Butylcephalosporin* (**2**; R = Bu^t), $\nu_{max.}$ 1 765 (β-lactam CO), 1 740 (ester CO), 1 660 (amide CO), 1 610 (carboxylate), and 3 350 cm^{-1} (amide NH); δ(DMSO) 5.05 (d, 6-H), 5.55 (q to d in D_2O, 7-H), 3.50 (br s, 2-H), 1.30 (9 H, s, CMe_3), 4.95 (d, C*H*$_2$OCOMe), 2.00 (s, CH_2OCOMe), and 8.65 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 7 860) nm.

7-*Isopropylcephalosporin* (**2**; R = Pr^i), $\nu_{max.}$ 1 769 (β-lactam CO), 1 738 (ester CO), 1 650 (amide CO), 1 600 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.00 (d, 6-H), 5.65 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 0.9 and 1.1 (d, $CHMe_2$), 2.4 (m, $CHMe_2$), 4.95 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 8.70 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 760) nm.

7-*Phenylcephalosporin* (**2**; R = Ph), $\nu_{max.}$ 1 755 (β-lactam CO), 1 730 (ester CO), 1 640 (amide CO), 1 600 (carboxylate), and 3 305 cm^{-1} (amide NH); δ(DMSO) 5.10 (d, 6-H), 5.75 (q, d, in D_2O, 7-H), 3.40 (br s, 2-H), 7.50 and 7.95 (two peaks, m, Ph), 5.00 (m, CH_2OCOMe), 2.00 (s, CH_2OCOMe), and 9.25 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 12 650) nm.

7-*Benzylcephalosporin* (**2**; R = $PhCH_2$), $\nu_{max.}$ 1 770 (β-lactam CO), 1 743 (ester CO), 1 640 (amide CO), 1 604 (carboxylate), and 3 305 cm^{-1} (amide NH); δ(400 MHz; D_2O) 4.93 (d, 1 H, $J_{6,7}$ 5 Hz, 6-H), 5.49 (d, 1 H, $J_{6,7}$ 5 Hz, 7-H), 3.46 and 3.2 (m, qAB, 2 H, *J* 17.4 Hz, 2-H), 7.20 (m, 5 H, $PhCH_2CONH$), 3.52 and 3.58 (qAB, 2 H, *J* 14.5 Hz, $PhCH_2CONH$), 4.57 and 4.72 (qAB, 2 H, *J* 12.5 Hz, CH_2OCOMe), and 1.98 (s, 3 H, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 8 816) nm.

7-*Phenethylcephalosporin* [**2**; R = $Ph(CH_2)_2$], $\nu_{max.}$ 1 750 (β-lactam CO), 1 730 (ester CO), 1 640 (amide CO), 1 595 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(D_2O) 7.22 [s, $Ph(CH_2)_2$], 2.4—3.0 [m, $Ph(CH_2)_2$], 4.90 (d, CH_2OCOMe), and 2.15 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 9 193) nm.

7-*Phenylpropylcephalosporin* [**2**; R = $Ph(CH_2)_3$], $\nu_{max.}$ 1 760 (β-lactam CO), 1 750 (ester CO), 1 635 (amide CO), 1 595 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(D_2O) 5.05 (d, 6-H), 5.70 (d, 7-H), 3.45 (d, 2-H), 7.15 [s, $Ph(CH_2)_3$], 1.6—2.8 [m, $Ph(CH_2)_3$], 4.90 (d, CH_2OCOMe), and 2.15 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 7 460) nm.

7-*Cyclopropylcephalosporin* (**2**; R = C_3H_5), $\nu_{max.}$ 1 758 (β-lactam CO), 1 738 (ester CO), 1 640 (amide CO), 1 600 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.00 (masked, 6-H), 5.60 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 0.9 (m), 1.5 (m) [$(CH_2)_2CH$], 4.95 (AB, CH_2OCOMe), 2.00 (s, CH_2OCOMe), and 9.00 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 560) nm.

7-*Cyclobutylcephalosporin* (**2**; R = C_4H_7), $\nu_{max.}$ 1 756 (β-lactam CO), 1 742 (ester CO), 1 643 (amide CO), 1 610 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 4.96 (d, 6-H), 5.55 (q to d in D_2O, 7-H), 3.35 (d, 2-H), 2.1 (m), 3.4 (m) [$(CH_2)_3CH$], 4.90 (d, CH_2OCOMe), 2.10 (CH_2OCOMe), and 8.55 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 155) nm.

7-*Cyclohexylcephalosporin* (**2**; R = C_6H_{11}), $\nu_{max.}$ 1 750 (β-lactam CO), 1 735 (ester CO), 1 645 (amide CO), 1 622 (carboxylate), and 3 280 cm^{-1} (amide NH); δ(D_2O) 5.05 (d, 6-H), 5.65 (d, 7-H), 3.45 (br s, 2-H), 1.05—1.85 (broad complex m, C_6H_{11}), 4.90 (d, CH_2OCOMe), and 2.10 (s, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 9 440) nm; (Found: C, 49.88; H, 5.17; N, 6.68. $C_{17}H_{21}N_2NaO_6S$ requires C, 50.49; H, 5.19; N, 6.93%).

7-*Cyclohexylmethylcephalosporin* (**2**; R = $C_6H_{11}CH_2$), $\nu_{max.}$ 1 760 (β-lactam CO), 1 720 (ester CO), 1 630 (amide CO), 1 610 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.00 (d, 6-H), 5.50 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 1.90—2.10 (d, $C_6H_{11}CH_2$), 1.00 (br t, $C_6H_{11}CH_2$), 4.95 (d, CH_2OCOMe), and 8.75 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 560) nm.

7-(1-*Phenylpropyl*)*cephalosporin* [**2**; $MeCH_2(Ph)CH$], $\nu_{max.}$ 1 760 (β-lactam CO), 1 738 (ester CO), 1 658 (amide CO), 1 612 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.05 (d, 6-H), 5.55 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.35 [s, $MeCH_2(Ph)CH$], 3.55 [t, $CH(Ph)CH_2Me$], 0.9 [t, $CH(Ph)CH_2Me$], 1.5—2.4 [m, $CH(Ph)CH_2Me$], 4.95 (d, CH_2OCOMe), 2.05, (s, CH_2OCOMe), and 9.25 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 159) nm.

7-(2-*Phenylpropyl*)*cephalosporin* [**2**; R = $MeCH(Ph)CH_2$], $\nu_{max.}$ 1 758 (β-lactam CO), 1 738 (ester CO), 1 658 (amide CO), 1 622 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(DMSO) 5.05 (d, 6-H), 5.55 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 7.25 [s, $CH_2(Ph)CHMe$], 3.40 [m, $CH_2(Ph)CHMe$], 1.3 [d, $CH_2(Ph)CHMe$], 2.6 [d, $CH_2(Ph)CHMe$], 4.95 (d, CH_2OCOMe), 2.00 (s, CH_2OCOMe), and 9.25 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 10 150) nm.

7-*Biphenyl-4-ylmethylcephalosporin* (**2**; R = $PhC_6H_4CH_2$), $\nu_{max.}$ 1 762 (β-lactam CO), 1 730 (ester CO), 1 644 (amide CO), 1 590 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(DMSO) 5.00 (d, 6-H), 5.65 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.25—7.75 (m, PhC_6H_4), 5.05 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 9.80 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 28 400) nm.

7-*Styrylcephalosporin* (**2**; R = PhCHCH), $\nu_{max.}$ 1 760 (β-lactam CO), 1 740 (ester CO), 1 660 (amide CO), 1 615 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.70 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 7.3—7.7 (br s, *Ph*CHCH), 6.95 and 7.85 (AB, *J* 15 Hz, PhC*HCH*), 4.95 (CH_2OCOMe), 2.05 (s, CH_2OCOMe), and 9.65 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 25 650) nm.

7-(4-*t-Butylphenyl*)*cephalosporin* (**2**; R = *m*-$Me_3CC_6H_4$), $\nu_{max.}$ 1 760 (β-lactam CO), 1 738 (ester CO), 1 640 (amide CO), 1 605 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.0 (masked 6-H), 5.70 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 7.4—8.0 (AB, $Me_3CC_6H_4$), 1.25 (9 H, s, $Me_3CC_6H_4$), 5.00 (AB, CH_2OCOMe), 2.00 (s, CH_2OCOMe), and 9.30 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 640) nm.

7-(4-*Nitrophenyl*)*cephalosporin* (**2**; R = *p*-$O_2NC_6H_4$), $\nu_{max.}$ 1 760 (β-lactam CO), 1 740 (ester CO), 1 650 (amide CO), 1 610 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(DMSO) 4.95 (d, 6-H), 5.75 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 8.10 and 8.50 (AB, *p*-$O_2NC_6H_4$), 5.00 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 10.00 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 21 600) nm.

7-(3-*Nitrophenyl*)*cephalosporin* (**2**; R = *m*-$O_2NC_6H_4$), $\nu_{max.}$ 1 760 (β-lactam CO), 1 750 (ester CO), 1 646 (amide CO), 1 618 (carboxylate), and 3 290 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.70 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.50—8.15 (m, *m*-$O_2NC_6H_4$), 4.95 (d, CH_2OCOMe), 2.00 (s, CH_2OCOMe), and 9.60 (d, disappears in D_2O, NH); $\lambda_{max.}$ 270 (ε 9 300) nm.

7-(2-*Nitrophenyl*)*cephalosporin* (**2**; R = *o*-$O_2NC_6H_4$), $\nu_{max.}$ 1 760 (β-lactam CO), 1 755 (ester CO), 1 650 (amide CO), 1 622 (carboxylate), and 3 280 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.80 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 7.80, 8.50, and 8.85 (4 H at 1:2:1, *o*-$O_2NC_6H_4$); 5.05 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 9.80 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 7 200) nm.

7-(2-*Methylphenyl*)*cephalosporin* (**2**; R = *o*-MeC_6H_4), $\nu_{max.}$ 1 775 (β-lactam CO), 1 738 (ester CO), 1 640 (amide CO), 1 612 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.70 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.2—7.40 (m, MeC_6H_4), 2.45 (s, MeC_6H_4), 5.00 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 9.30 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 820) nm.

7-(3-*Methylphenyl*)*cephalosporin* (**2**; R = *m*-MeC_6H_4), $\nu_{max.}$ 1 780 (β-lactam CO), 1 740 (ester CO), 1 640 (amide CO), 1 620 (carboxylate), and 3 260 cm^{-1} (amide NH); δ(DMSO) 5.15 (d, 6-H), 5.80 (q to d in D_2O, 7-H), 3.50 (br s, 2-H), 7.40 (d, MeC_6H_4), 7.80 (d, MeC_6H_4), 2.35 (s, MeC_6H_4), 4.95 (AB, CH_2OCOMe), 2.05 (s, CH_2OCOMe), and 9.30 (d, disappears in D_2O, NH) $\lambda_{max.}$ 260 (ε 8 845) nm.

7-(4-*Methylphenyl*)*cephalosporin* (**2**; R = *p*-MeC_6H_4), $\nu_{max.}$ 1 760 (β-lactam CO), 1 738 (ester CO), 1 640 (amide CO), 1 612 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.10 (d, 6-H), 5.75 (q to d in D_2O, 7-H), 3.40 (d, 2-H), 7.30 and 7.80 (AB, MeC_6H_4), 2.40 (s, MeC_6H_4), 4.95 (AB, CH_2OCOMe),

2.10 (s, CH_2OCOMe), and 9.30 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 8 825) nm.

7-(4-*Carboxyphenyl*)*cephalosporin* (**2**; R = *p*-$HO_2CC_6H_4$), $\nu_{max.}$ 1 775 (β-lactam CO), 1 735 (ester CO), 1 640 (amide CO), 1 600, 1 595 (carboxylate), and 3 295 cm^{-1} (amide NH); δ(DMSO) 5.10 (d, 6-H), 5.75 (q to d in D_2O, 7-H), 3.40 (br s, 2-H), 8.30 ($HO_2CC_6H_4$), 4.95 (AB, CH_2OCOMe), 2.05 (s, CH_2OCOMe), and 9.10 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 11 070) nm.

7-(3-*Carboxyphenyl*)*cephalosporin* (**2**; R = *m*-$HO_2CC_6H_4$), $\nu_{max.}$ 1 780 (β-lactam CO), 1 735 (ester CO), 1 660 (amide CO), 3 280 cm^{-1} (amide NH); δ(D_2O) 5.05 (d, 6-H), 5.70 (d, 7-H), 3.50 (m, 2-H), 7.50, 7.95, and 8.30 ($HO_2CC_6H_4$), 4.80 and 4.95 (q, CH_2OCOMe), and 1.90 (s, CH_2OCOMe); $\lambda_{max.}$ 270 (ε 8 380) nm.

7-(2-*Carboxyphenyl*)*cephalosporin* (**2**; R = *o*-$HO_2CC_6H_4$), $\nu_{max.}$ 1 800 (β-lactam CO), 1 740 (ester CO), 1 670 (amide CO), and 3 300 cm^{-1} (amide NH); δ(D_2O) 5.25 (d, 6-H), 5.90 (d, 7-H), 3.60 (m, 2-H), 7.50—8.0 ($HO_2CC_6H_4$), 4.90 (d, CH_2OCOMe), and 2.10 (s, CH_2OCOMe); $\lambda_{max.}$ 270 (ε 10 100) nm.

7-(3,4,5-*Trimethoxyphenyl*)*cephalosporin* [**2**; R = $(MeO)_3C_6H_2$], $\nu_{max.}$ 1 760 (β-lactam CO), 1 728 (ester CO), 1 690 (amide CO), 1 600 (carboxylate), and 3 400 cm^{-1} (amide NH); δ(DMSO) 5.10 (d, 6-H), 5.70 (q to d in D_2O, 7-H), 3.45 (d, 2-H), 7.30 [s, $(MeO)_3C_6H_2$], 3.90 [9 H, s, $(MeO)_3C_6H_2$], 5.00 (d, CH_2OCOMe), 2.15 (s, CH_2OCOMe), and 9.60 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 9 650) nm.

7-(2-*Furyl*)*cephalosporin* (**2**; R = C_4H_3O), $\nu_{max.}$ 1 768 (β-lactam CO), 1 720 (ester CO), 1 643 (amide CO), 1 622 (carboxylate), and 3 300 cm^{-1} (amide NH); δ(DMSO) 5.20 (d, 6-H), 5.80 (q to d in D_2O, 7-H), 3.35 (br s, 2-H), 7.95 (s, C_4H_3O), 7.45, 6.75 (AB, C_4H_3O), 5.00 (d, CH_2OCOMe), 2.10 (s, CH_2OCOMe), and 9.30 (d, disappears in D_2O, NH); $\lambda_{max.}$ 260 (ε 15 600) nm.

7-(2-*Thenyl*)*cephalosporin* (**2**; R = $C_4H_3SCH_2$), $\nu_{max.}$ 1 750 (β-lactam CO), 1 723 (ester CO), 1 658 (amide CO), 1 620 (carboxylate), and 3 280 cm^{-1} (amide NH); δ(D_2O) 5.22 (d, 1 H, *J* 5 Hz, 6-H), 5.75 (d, 1 H, *J* 5 Hz, 7-H), 3.49 and 3.70 (d, 2 H, *J* 18 Hz, 2-H), 7.15 (m, 2 H, $C_4H_3SCH_2$), 7.45 (m, 1 H, $C_4H_3SCH_2$), 4.00 and 4.08 (qAB, 2 H, *J* 14 Hz, $C_6H_3SCH_2$), 4.84 and 5.02 (qAB, 2 H, *J* 12 Hz, CH_2OCOMe), and 2.24 (s, 3 H, CH_2OCOMe); $\lambda_{max.}$ 260 (ε 8 800) nm.

The kinetic analysis was carried out as described in the preceding paper.[9]

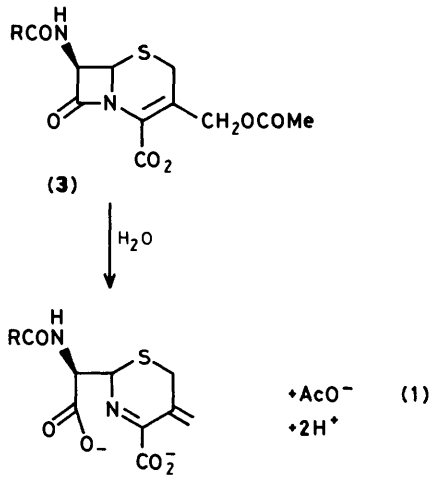
Results and Discussion

The hydrolysis of the cephalosporins (**3**) opens the β-lactam ring which is followed by expulsion of the acetate leaving group at C-3′ to give the conjugated imine as an identifiable product,[1,12] equation (1). This reaction is catalysed by hydroxide ion and the β-lactamase enzymes. In order to identify binding interactions between the substrate cephalosporin and the enzyme it is necessary to separate reactivity effects due to intrinsic ones, *e.g.* inductive and steric, of substituents on the susceptibility of the β-lactam carbonyl carbon towards nucleophilic attack.[13]

The second-order rate constants for the alkaline hydrolysis of the 7-substituted cephalosporins are given in Table 1. The variation in k_{OH} is less than five-fold for 37 substituents. It is apparent that inductive and steric effects of the 7β-acylamido substituents show little influence on the chemical reactivity of cephalosporins towards nucleophilic attack of hydroxide ion on the β-lactam carbonyl carbon.

The β-lactamase-catalysed hydrolysis of cephalosporins exhibits Michaelis–Menten kinetics equation (2), where E, S,

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} \text{Products} \qquad (2)$$



(1)

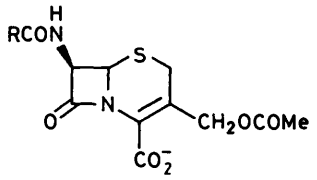
and ES are the enzyme, substrate, and the enzyme–substrate complex, respectively, and K_m and k_{cat} are the associated constants for this process. The second-order rate constant, k_{cat}/K_m, is not only the constant observed at low concentrations of substrate when the enzyme is not saturated with substrate, but it represents the free energy difference between the enzyme–substrate transition state and the free unbound substrate and enzyme.[13] If a series of substrates show the same chemical reactivity, *e.g.* as measured by their susceptibility towards hydroxide ion-catalysed hydrolysis, then variation in the parameter k_{cat}/K_m can be used to measure the binding energy between non-reacting parts of the substrate and the enzyme.[13] If the substituent introduces little change in the ground state energies, differences in k_{cat}/K_m reflect the differences in binding energy between the substituent and the enzyme in the transition state.[13–15]

The effect of the nature of the 7β-acylamido substituent on the *Bacillus cereus* β-lactamase I-catalysed hydrolysis of cephalosporins is significant. The kinetic parameters for this serine enzyme are given in Table 1. The second-order rate constant k_{cat}/K_m varies by greater than 2×10^5, whereas k_{cat} and K_m vary by greater than 700 and less than 1 000, respectively. Similar changes in substituents produce dramatically smaller effects in the *B. cereus* β-lactamase II-catalysed hydrolysis of cephalosporins. The kinetic parameters for this zinc-dependent enzyme are given in Table 2. The variations in k_{cat}/K_m, k_{cat}, and K_m are only 10, 3, and 9, respectively. In general, the cephalosporins are 10—100 fold better substrates for β-lactamase II than for β-lactamase I. The effects of substituents will now be compared in groups.

The Effect of Alkyl Substituents.—(i) *Length of alkyl chain.* A series of alkyl cephalosporins (**3**) were synthesised and hydrolysed using β-lactamase I and β-lactamase II as catalysts. As can be seen from Table 1, k_{cat}/K_m for β-lactamase I increases steadily with chain length reaching a maximum with the undecyl derivative which is nearly 30-fold more reactive than the methyl analogue. There is a sharp drop in reactivity with the dodecylcephalosporin. It is better to use k_{cat}/K_m to estimate binding energies rather than K_ms. The latter often underestimates the binding energy of a substituent since enzymes often use binding energy to lower the activation energy rather than using it to give lower K_ms and apparent tighter binding.[13,16,17]

The incremental Gibbs free energy of transfer of a substituent S from water to the enzyme, relative to hydrogen, is given by equation (3).[13] A plot of $\Delta\Delta G^{\ddagger}$ against the number of

Table 1. Second-order rate constants for the hydroxide ion-catalysed hydrolysis of 7β-substituted amidocephalosporins at 30 °C, I = 1.0M and the kinetic parameters for the *B. cereus* β-lactamase I-catalysed hydrolysis at 30 °C, pH 7.0



R	K_m/mol dm^{-3}	k_{cat}/s^{-1}	k_{cat}/K_m mol^{-1} dm^3 s^{-1}	k_{OH^-}/mol^{-1} dm^3 s^{-1}
Me	3.93×10^{-5}	2.90×10^{-2}	7.38×10^{2}	0.140
$MeCH_2$	3.25×10^{-5}	2.00×10^{-2}	6.15×10^{2}	0.129
$Me(CH_2)_2$	1.49×10^{-5}	1.50×10^{-2}	1.01×10^{3}	—
$Me(CH_2)_3$	4.91×10^{-5}	6.00×10^{-2}	1.22×10^{3}	0.131
$Me(CH_2)_4$	1.92×10^{-5}	7.40×10^{-2}	3.88×10^{3}	0.133
$Me(CH_2)_5$	1.68×10^{-5}	4.80×10^{-2}	2.86×10^{3}	0.128
$Me(CH_2)_6$	1.29×10^{-5}	5.20×10^{-2}	4.00×10^{3}	0.131
$Me(CH_2)_7$	0.85×10^{-5}	5.60×10^{-2}	6.59×10^{3}	0.134
$Me(CH_2)_8$	0.99×10^{-5}	7.20×10^{-2}	7.27×10^{3}	0.131
$Me(CH_2)_{10}$	0.35×10^{-5}	7.10×10^{-2}	2.03×10^{4}	0.065
$Me(CH_2)_{12}$	2.76×10^{-5}	7.50×10^{-2}	2.74×10^{3}	—
Ph	6.16×10^{-7}	7.77×10^{-3}	1.26×10^{4}	0.073
$PhCH_2$	4.70×10^{-5}	2.80×10^{-1}	5.96×10^{3}	0.089
$Ph(CH_2)_2$	1.47×10^{-5}	1.45×10^{-1}	9.88×10^{3}	0.084
$Ph(CH_2)_3$	2.61×10^{-5}	1.32×10^{-1}	5.07×10^{3}	0.108
Cyclo-C_6H_{11}	5.69×10^{-6}	1.60×10^{-2}	2.80×10^{3}	0.049
Cyclo-$C_6H_{11}CH_2$	9.93×10^{-5}	3.33×10^{-1}	3.36×10^{3}	0.059
Cyclopropyl	4.99×10^{-5}	5.80×10^{-2}	1.17×10^{3}	0.045
Cyclobutyl	7.22×10^{-5}	1.04×10^{-1}	1.46×10^{3}	0.049
Me_2CH	1.31×10^{-5}	1.27×10^{-2}	9.69×10^{2}	0.109
Me_3C	—	—	< 1.0	0.196
EtCH(Ph)	5.59×10^{-5}	7.00×10^{-2}	1.26×10^{3}	0.057
$MeCH(Ph)CH_2$	2.47×10^{-5}	1.46×10^{-1}	5.91×10^{3}	0.062
$PhC_6H_4CH_2$	2.78×10^{-5}	1.44×10^{-1}	5.18×10^{3}	0.176
$Me_3CC_6H_4$	1.08×10^{-4}	6.90×10^{-2}	6.38×10^{2}	0.041
p-$NO_2C_6H_4$	5.19×10^{-6}	8.40×10^{-2}	1.62×10^{4}	0.043
m-$NO_2C_6H_4$	1.02×10^{-5}	6.40×10^{-2}	6.27×10^{3}	0.038
o-$NO_2C_6H_4$	1.63×10^{-5}	1.48×10^{-3}	91	0.015
p-MeC_6H_4	2.22×10^{-5}	3.40×10^{-2}	1.53×10^{3}	0.060
m-MeC_6H_4	1.11×10^{-5}	4.14×10^{-3}	4.72×10^{2}	0.064
o-MeC_6H_4	1.92×10^{-5}	1.85×10^{-3}	96	0.050
p-$HO_2CC_6H_4$	8.26×10^{-5}	5.41×10^{-3}	~67	0.077
m-$HO_2CC_6H_4$			~40	
o-$HO_2CC_6H_4$			< 10	
3,4,5-Trimethoxyphenyl	1.27×10^{-5}	2.40×10^{-1}	1.90×10^{4}	0.074
Thenyl	3.86×10^{-5}	2.87×10^{-1}	7.46×10^{3}	0.085
Furyl	8.21×10^{-6}	3.50×10^{-3}	4.26×10^{2}	0.061

$$\Delta\Delta G^{\ddagger} = 5.71 \log_{10} \frac{(k_{cat}/K_m)_S}{(k_{cat}/K_m)_H} \quad (3)$$

methylenes in the alkyl side-chain is linear up to n = 10. The slope of this line is 0.88 kJ mol^{-1} per methylene, which may be compared with 1.46 kJ mol^{-1} observed for alkyl penicillins and the serine β-lactamase.[9]

There is a relatively small change in k_{cat} with alkyl chain lengths and k_{cat} is almost constant for the butyl and longer chain derivatives (Table 1).

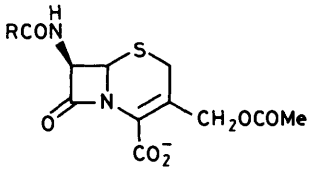
There is an even smaller dependence of reactivity on the length of the alkyl side-chain when the zinc dependent β-lactamase II is used as a catalyst (Table 2). There is an increase in k_{cat}/K_m with increasing chain length which reaches a maximum with the octylcephalosporin after which reactivity decreases. The increase in binding energy per methylene up to the octyl derivative is 0.46 kJ mol^{-1} for β-lactamase II, almost half of the value observed with the serine enzyme.

The contribution of the alkyl side-chain to the binding energy is small for both the serine and the zinc-dependent β-lactamase. The free energy of transferring a methylene group from water to a non-polar liquid is about 4 kJ mol^{-1}.[18] It has been estimated that the free energy change for transfer to the micellar phase is 2.7 kJ mol^{-1} [19] and the observed maximum value obtained from measurements with alkyl penicillins to micelles of cetyltrimethylammonium bromide is 3.0 kJ mol^{-1}.[20] By contrast the binding energy of a methylene unit to enzymes may be as high as 14 kJ mol^{-1}.[21,22] Although there appears to be a hydrophobic binding channel for alkyl side-chains the interaction between the alkyl group and the enzyme is not strong.

Maximum interaction varies with substrate and enzyme. For β-lactamase I and alkyl cephalosporins the maximum activity is shown by the undecyl derivative whereas with β-lactamase II this occurs with the octyl derivative. With β-lactamase I and alkyl penicillins the maximum activity is also shown by the octyl derivative.[9]

(ii) *Chain branching and cyclisation.* Making the 7β-alkylamido side-chain cyclic or branched has little effect upon reactivity except for the dramatic decrease observed with t-butylcephalosporin and β-lactamase I (Table 1). With the serine

Table 2. Kinetic parameters for the *B. cereus* β-lactamase II-catalysed hydrolysis of 7β-substituted cephalosporins at 30 °C, pH 7.0



R	K_m/mol dm^3	k_{cat}/s^{-1}	k_{cat}/K_m mol^{-1} dm^3 s^{-1}
Me	1.63×10^{-3}	152	9.33×10^4
$MeCH_2$	—	—	8.94×10^4
$Me(CH_2)_2$	—	—	9.00×10^4
$Me(CH_2)_4$	1.19×10^{-3}	210	1.76×10^5
$Me(CH_2)_5$	9.36×10^{-4}	174	1.86×10^5
$Me(CH_2)_6$	5.17×10^{-4}	126	2.44×10^5
$Me(CH_2)_7$	4.56×10^{-4}	154	3.38×10^5
$Me(CH_2)_9$	—	—	3.33×10^5
$Me(CH_2)_{10}$	6.45×10^{-4}	101	1.57×10^5
Me_3C	—	—	2.26×10^5
Me_2CH	—	—	8.30×10^4
Cyclo-C_6H_{11}	—	—	1.25×10^5
Cyclo-$C_6H_{11}CH_2$	1.67×10^{-3}	258	1.54×10^5
Cyclobutyl	—	—	1.02×10^5
Cyclopropyl	—	—	5.98×10^4
Ph	1.58×10^{-3}	237	1.50×10^5
$PhCH_2$	6.16×10^{-4}	188	3.05×10^5
$Ph(CH_2)_2$	8.12×10^{-4}	130	1.60×10^5
$Ph(CH_2)_3$	8.29×10^{-4}	148	1.79×10^5
EtCH(Ph)	—	—	3.21×10^5
$MeCH(Ph)CH_2$	—	—	9.61×10^4
p-MeC_6H_4	1.32×10^{-3}	328	2.52×10^5
m-MeC_6H_4	1.33×10^{-3}	370	2.78×10^5
o-MeC_6H_4	—	—	2.25×10^5
p-$HO_2CC_6H_4$	—	—	8.35×10^4
p-$NO_2C_6H_4$	—	—	2.44×10^5
m-$NO_2C_6H_4$	—	—	2.21×10^5
o-$NO_2C_6H_4$	—	—	2.36×10^5
p-$PhC_6H_4CH_2$	1.86×10^{-4}	121	6.54×10^5
p-$Me_3CC_6H_4$	—	—	4.16×10^4
3,4,5-Trimethoxyphenyl	—	—	2.19×10^5

enzyme, the cyclohexyl and hexyl; the cyclohexylmethyl and heptyl; the cyclopropyl, propyl, and isopropyl; and the cyclobutyl and butyl derivatives show similar reactivity for similar carbon numbers. Similar results are observed with the zinc-dependent enzyme, β-lactamase II (Table 2). However, the t-butylcephalosporin shows the expected reactivity with β-lactamase II but is a very poor substrate for β-lactamase I. It is over a 1 000-fold less reactive than the isopropyl derivative. The ratio of k_{cat}/K_m for β-lactamase II and β-lactamase I for this substrate (Tables 1 and 2) is greater than 2×10^5. Presumably, this is a reflection of an available binding site on β-lactamase I that is not accessible to the t-butyl group.

The Effect of Aromatic Residues.—Substituting a phenyl residue in the 7β side-chain of cephalosporins has little effect on their reactivity with β-lactamase II (Table 2). With β-lactamase I phenylcephalosporin is five-fold and 12-fold more reactive than cyclohexylcephalosporin and butylcephalosporin, respectively (Table 1). As the phenyl group is moved along an alkyl chain its effect decreases. Similarly, neither a thiophene nor furan residue enhances the reactivity of the cephalosporins. So, unlike α-chymotrypsin,[23] there does not seem to be a significant pocket in either β-lactamase to host an aromatic residue.

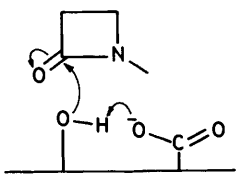
The Effect of Substituents in Phenyl Residue.—There is a large effect of substituents on the reactivity of 7-substituted phenylcephalosporins with β-lactamase I (Table 1). The 4-nitrophenyl derivative is over 3×10^3 times more reactive than the 2-carboxyphenylcephalosporin. Again, these observations are in marked contrast with the results with β-lactamase II where there is little dependence of k_{cat}/K_m upon the nature of the substituent (Table 2). With the serine enzyme, the 4-nitro substituent shows normal reactivity, k_{cat}/K_m being less than twice that for the parent phenylcephalosporin. The change in reactivity brought about by substituents is therefore a *decrease*, *i.e.* some of the substituted phenylcephalosporins are poor substrates. In general, k_{cat}/K_m decreases as the substituent moves from the *para* to *meta* to *ortho* positions. The *meta* and *ortho* substituted derivatives are three- to five-fold and 20- to 100-fold less reactive than the analogous *para* isomers, respectively. Superimposed on a steric effect there is a discernible polar or ionic effect of the ionised carboxylate group. The *ortho* carboxylate derivative is a very poor substrate for the serine enzyme.

The serine enzyme β-lactamase I is thought to catalyse the hydrolysis of β-lactam antibiotics by the intermediate formation of an acyl enzyme. This acylation process almost certainly requires a general base catalyst to remove a proton from the serine hydroxy proton and/or a general acid catalyst to protonate the β-lactam nitrogen to facilitate ring opening.[1,24] In contrast to serine proteases, there is no clear indication of the nature of these catalytic groups. However, recently it has been shown that β-lactamase I from *B. cereus* is inactivated by a water-soluble carbodi-imide and it is considered that this is mainly due to the conversion of the carboxy group of glutamic acid-168 into an amide.[25] It is not unambiguous that the carboxy group plays a mechanistic catalytic role because glutamic acid-168 is not conserved in all β-lactamases and its replacement by aspartic acid by mutagenesis has little effect on the enzyme's activity.[26] On the other hand, replacement of the conserved glutamic acid-166 by glutamine gives enzyme with little or no activity.[26] The pH dependence of k_{cat}/K_m for the *B. cereus* β-lactamase I-catalysed hydrolysis of cephaloridine is bell-shaped and corresponds to ionisable groups on the enzyme of 5.60 and 8.64. The lower pK_a could correspond to a carboxy group on a slightly non-polar environment.

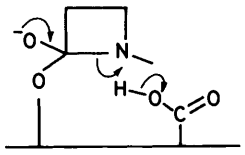
Crystallographic studies of the class A β-lactamases have been in progress for many years with little result until very recently.[27] It has been suggested, on the basis of the crystal structure of β-lactamase from *S. aureus*, that a lysine residue is involved in proton transfer.[28] This seems extremely unlikely because the lysine amino group will be protonated.

The present observation, that 2-carboxyphenylcephalosporin is a very poor substrate for β-lactamase I, is consistent with repulsion between the negatively charged carboxy group on the substrate and an anionic group near the catalytic centre of the enzyme. This is therefore also consistent with a carboxylate group being located at the active site of β-lactamase I. A possible role for this group is shown in (**4**) and (**5**). In other serine enzymes the more basic imidazole acts as a general base catalyst.[29] The alcoholysis of penicillins involves general acid-catalysed breakdown of the tetrahedral intermediate and breaking the C–N of the β-lactam is not a facile process.[1,30] It is therefore possible that the enzyme's carboxy group acts as a general acid catalyst facilitating C–N bond fission, (**5**). In cases where deacylation is rate limiting, the carboxylate can presumably act as a general base catalyst for ester hydrolysis. Another possibility, in view of the recent *X*-ray data,[28] is that the protonated amino group of lysine could act as a general acid catalyst in breakdown of the tetrahedral intermediate, (**6**).

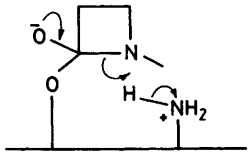
β-Lactamase II is readily inactivated by chelating agents and, in the absence of free zinc(II) ions, by agents modifying a



(4)



(5)



(6)

thiol group, a residue involved in metal ion co-ordination in β-lactamase II.[31] Until recently, no other inactivators of this enzyme have been reported. However, it has now been shown that a water-soluble carbodi-imide in the presence of a nucleophile inactivates *B. cereus* β-lactamase II by modifying the carboxy group of the enzyme glutamic acid-37 residue.[32] It was concluded that this residue represents a catalytically active group which acts as a general base catalyst.[32] The role of metal ions in proteolytic metalloenzymes is usually ascribed to that of an electrophile but this is not always unambiguous.[33]

In the present work, there is no indication that an ionised carboxy group on the substrate shows low reactivity because of repulsion by an anionic carboxy group on β-lactamase II. This could simply be due to a lack of proximity between these groups in the enzyme–substrate complex.

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