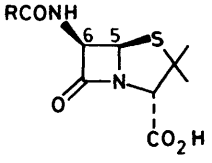
Hydrolysis of 6-Alkyl Penicillins catalysed by β-Lactamase I from *Bacillus cereus* and by Hydroxide Ion

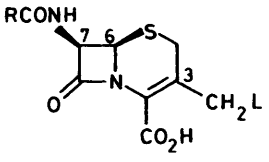
Stephen C. Buckwell and Michael I. Page*
Department of Chemical and Physical Sciences, The Polytechnic, Queensgate, Huddersfield, West Yorkshire, HD1 3DH
Jethro L. Longridge
I.C.I. Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG

Second-order rate constants for the hydroxide ion-catalysed hydrolysis of 6-alkyl penicillins are independent of the length of the alkyl side-chain and replacement of the amido by an amino group decreases the susceptibility to nucleophilic attack on 6-aminopenicillanic acid only three-fold. *B. cereus* β-lactamase I catalyses the hydrolysis of 6-alkyl penicillins with values of k_{cat}/K_m which are at least 50-fold greater than that shown by 6-aminopenicillanic acid. For the enzyme-catalysed reaction k_{cat}/K_m increases with increasing chain length, reaching a maximum with hexylpenicillin, and then decreases. The binding energy of the alkyl group is weak, only 1.45 kJ mol^{-1} per methylene residue. Although there appears to be a recognition site for the amido group there is no specific pocket in β-lactamase I for the recognition of hydrophobic residues in the 6-acylamido side-chain of penicillins.

One of the major factors responsible for the resistance of pathogenic bacteria to the normal lethal action of penicillins, (**1**),† cephalosporins, (**2**) where L is a potential leaving group, and other β-lactam antibiotics is the bacteria's ability to produce hydrolytic enzymes, β-lactamases, which catalyse the hydrolysis of the β-lactam to biologically inactive products.



(**1**)



(**2**)

The majority of β-lactamases produced by Gram-positive organisms are inducible and extracellular, but membrane-bound enzymes have been described.[1] Gram-negative organisms may produce an inducible or a constitutive β-lactamase found in the periplasm. The β-lactamases may be either chromosomally or plasmid coded and the genes are easily transferred which accounts for their facile spreading among bacteria.[2]

There appear to be at least three evolutionarily distinct classes of β-lactamases.[3] Classes A and C have an active site serine and molecular weights of about 30 000 and 39 000, respectively. Class B β-lactamase is a zinc metalloenzyme, has a molecular weight of about 25 000, and is produced by *Bacillus cereus*.

Although several types of β-lactamases are known, none appears to catalyse the hydrolysis of acyclic peptides,[4] although some acyclic depsipeptides may be substrates.[5] The specificity towards the β-lactam is therefore thought to be high but there is a variety of activities with individual β-lactamases towards substituted penicillins and cephalosporins.[6]

There is rather limited knowledge of active site residues and the identification of catalytic groups has, until recently, come mainly from chemical modification and inhibitor studies.[7] Some of the conclusions have been shown to be based on experimental artifacts.[8] In fact, the only residue for which there is convincing evidence is that for serine. The catalytic involvement of a serine has been deduced from covalent modification of the enzyme by inhibitors[9] and from site-specific mutagenesis.[10] Until this year, there were no complete high-resolution *X*-ray crystallographic structures of β-lactamase available.[11]

As a contribution towards elucidating the important binding sites in β-lactamases we report kinetic parameters for the hydrolysis of 6-substituted penicillins (**1**) in which substituents are varied systematically. In the following papers similar observations are described for 7- and 3-substituted cephalosporins, respectively.[12,13]

† Examples of the systematic nomenclature systems for the trivially named penicillins include 3,3-dimethyl-6-octanamido-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid for heptylpenicillin and 3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]-heptane-2'-carboxylic acid for benzylpenicillin.

Experimental

^{1}H N.m.r. spectra were measured either at 60 MHz on a Perkin-Elmer R12B n.m.r. spectrometer or at 400 MHz on a Bruker FT n.m.r. spectrometer (courtesy of Leeds University and ICI Pharmaceuticals, Macclesfield), with $SiMe_4$ as internal standard. I.r. spectra were run on Nujol mulls with a Perkin-Elmer 197 i.r. spectrophotometer calculated against the 1 602 cm^{-1} polystyrene band. U.v. spectra were run on a Gilford model 2000 u.v.–visible spectrophotometer calculated against standard holmium oxide.

Synthesis of 6-Alkyl Penicillins.—The alkyl penicillins were synthesised by the acylation of 6-aminopenicillanic acid (6-APA).

Method A. An anhydrous solution of the required acid chloride (15 mmol) and triethylamine (2.0 ml, 15 mmol) in dioxane (30 ml) and acetone (10 ml) was cooled to 0 °C with stirring. To this solution was added dropwise methyl chloroformate (1.5 ml, 15 mmol) maintaining the temperature at 0 °C for 45 min. A cooled solution prepared from 6-APA (2.16 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in water (30 ml) was added to the mixed anhydride solution. The mixture was stirred for 1 h at 0—5 °C, then 70 ml of a 3% sodium hydrogen carbonate solution was added. The resulting clear solution was extracted twice with ether (2 × 50 ml). The aqueous solution

was covered with a layer of ether, acidified to pH 2 with dilute HCl, then extracted with ether (2 × 50 ml). The combined ethereal extracts were washed with ice–water (25 ml), dried, and filtered. The sodium or potassium salt was prepared by the addition of a few drops of the appropriate salt of 2-ethylhexanoic acid, and the precipitate filtered.

Method B. The required acid chloride (15 mmol) was dissolved in dioxane (30 ml) and triethylamine (3.0 ml) was added. The mixture was stirred for 10 min at 0 °C. Methyl chloroformate (1.5 ml) dissolved in dioxane (20 ml) was then added, and the mixture stirred for a further 1 h at 0 °C. A solution of 6-APA (3.36 g, 15 mmol) and triethylamine (4.0 ml) in water (30 ml) was then added and this mixture stirred for 1.5 h whilst warming to room temperature. The solution was adjusted to pH 8 by the addition of a 3% solution of sodium hydrogen carbonate, and washed twice with ether. Work-up then proceeded as on the previous method.

Variation on the Method of Purification.—Using either of the above preparations to the point at which the required alkyl penicillin was in ethereal solution, the solvent was removed on a rotary evaporator resulting in a yellow oil. The oil was dissolved in water and purified by chromatography using macroporous polystyrene–divinylbenzene copolymer beads in a preparative column (20 × 2.5 cm), connected to a u.v. detector at 230 nm. The product was eluted in 10% acetonitrile solution and the organic solvent removed on a rotary evaporator. The aqueous solution was freeze-dried resulting in the acid form of the required penicillin.

Using the above methods the following compounds were prepared:

Potassium salt of 6-*methylpenicillin* (**1**; R = **Me**) $\nu_{max.}$ 1 780 (β-lactam CO), 1 660 (amide CO), and 1 600 cm^{-1} (carboxylate); δ(D_2O) 1.51 (Me, s), 1.63 (Me, s), and 2.40 (Ac, s); (Found: C, 40.38; H, 4.49; N, 9.28; S, 10.62. $C_{10}H_{13}KN_2O_4S$ requires C, 40.54; H, 4.39; N, 9.46; S, 10.84%).

Potassium salt of 6-*ethylpenicillin* (**1**; R = Et) $\nu_{max.}$ 1 780, 1 680, and 1 610 cm^{-1}; δ(D_2O) 1.51 (Me, s), 1.63 (Me, s), 1.09 (Me, t), and 2.34 (CH_2CO, q); (Found: C, 40.31; H, 4.97; N, 8.19. $C_{11}H_{15}KN_2SO_4{\cdot}H_2O$ requires C, 40.31; H, 5.19; N, 8.53%).

Potassium salt of 6-*propylpenicillin* (**1**; **R** = C_3H_7) $\nu_{max.}$ 1 784, 1 650, and 1 590 cm^{-1}; δ(D_2O) 1.52 (Me, s), 1.60 (Me, s), 1.04 ($MeCH_2$, m), 2.35 (CH_2CO, t), and 4.23 (1 H, s); (Found: C, 42.06; H, 5.58; N, 8.15. $C_{12}H_{17}KN_2O_4S{\cdot}H_2O$ requires C, 42.06; H, 5.53; N, 8.12%).

Potassium salt of 6-*butylpenicillin* (**1**; R = C_4H_9) $\nu_{max.}$ 1 784, 1 660, and 1 600 cm^{-1}; δ(D_2O) 1.50 (Me, s), 1.52 (Me, s), 0.8—2.0 [$Me(CH_2)_2$, m], 2.35 (CH_2CO, t), 4.23 (1 H, s), 5.48 (2 H, 5- and 6-H); (Found: C, 45.84; H, 5.38; N, 8.11. $C_{13}H_{19}KN_2S$ requires C, 46.10; H, 5.61; N, 8.28%).

Potassium salt of 6-*pentylpenicillin* (**1**; R = C_5H_{11}) $\nu_{max.}$ 1 790, 1 675, and 1 608 cm^{-1}; δ(D_2O) 1.50 (Me, s), 1.61 (Me, s), 0.7—2.4 [$Me(CH_2)_3$, m], 4.24 (1 H, s), and 5.50 (2 H, m, 5- and 6-H); (Found: C, 45.25; H, 6.28; N, 7.54. $C_{13}H_{21}KN_2O_4S{\cdot}H_2O$ requires C, 45.25; H, 6.22; N, 7.54%).

Potassium salt of 6-*hexylpenicillin* (**1**; R = C_6H_{13}) $\nu_{max.}$ 1 790, 1 720, and 1 630 cm^{-1}; δ(D_2O) 1.48 (Me, s), 1.59 (Me, s), 0.8—2.0 [$Me(CH_2)_5$, m], 4.20 (1 H, s), and 5.50 (2 H, 5- and 6-H).

Potassium salt of 6-*heptylpenicillin* (**1**; R = C_7H_{15}) $\nu_{max.}$ 1 790, 1 675, and 1 605 cm^{-1}; δ(D_2O) 1.50 (Me, s), 1.61 (Me, s), 0.7—2.0 [$Me(CH_2)_5$, m], 2.28 (CH_2CO, s), 4.20 (1 H, s), and 5.50 (2 H, 5- and 6-H); (Found: C, 49.20; H, 6.59; N, 6.88. $C_{16}H_{25}KN_2O_4S{\cdot}\frac{1}{2}H_2O$ requires C, 49.20; H, 6.52; N, 7.17%).

Potassium salt of 6-*nonylpenicillin* (**1**; R = C_9H_{19}) $\nu_{max.}$ 1 780, 1 670, and 1 600 cm^{-1}; δ(D_2O) 1.52 (Me, s), 1.61 (Me, s), 0.88 ($MeCH_2$, 3 H), 1.30 [$(CH_2)_6$, m], 2.30 (CH_2CO), 4.23 (1 H, s), and 5.60 (2 H, 5- and 6-H); (Found: C, 50.30; H, 7.35; N, 6.40. $C_{18}H_{29}KN_2O_4S{\cdot}H_2O$ requires C, 50.70; H, 7.30; N, 6.60%).

Potassium salt of 6-*undecylpenicillin* (**1**; R = $C_{11}H_{23}$) $\nu_{max.}$ 1 795, 1 675, and 1 605 cm^{-1}; δ(D_2O) 1.54 (Me, s), 1.62 (Me, s), 1.30 [$(CH_2)_8$, m], 0.88 (Me, t), 2.30 (CH_2CO, t), 4.26 (1 H, s), and 5.61 (2 H, 5- and 6-H); (Found: C, 52.80; H, 7.62; N, 6.10. $C_{20}H_{33}KN_2O_4S{\cdot}H_2O$ requires C, 52.80; H, 7.71; N, 6.16%).

Kinetics.—Materials used. All reagents used were AnalaR grade. β-Lactamase I from *B. cereus* 569/H, a constitutive overproducer of the enzyme, was obtained from the Sigma Chemical Company as a preparation containing some of the metalloenzyme β-lactamase II. The latter was excluded from reaction by the addition of EDTA to solutions used. The specific activity of the enzyme was 2.0 × 10^3 mol of benzylpenicillin (**1**; R = $PhCH_2$) hydrolysed per second per mol of enzyme at pH 7.0, 30 °C using 2.6 × 10^4 as the molar absorbance of *B. cereus* β-lactamase I at 280 nm.[14]

The spectrophotometer used for the majority of reactions was a Gilford model 2600 single-beam instrument which has a four-cell compartment with an automatic cell change. The temperature in the cell compartment was maintained at 30 ± 0.1 °C by water circulated from a Haake water-bath to the cell block. Absorbance–time or u.v. spectra were plotted on a Hewlett-Packard 7225B X-Y plotter. Reactions were initiated by the addition of 25 μl of substrate to 2.5 cm^3 of a temperature equilibrated solution in a quartz cuvette, and the time-dependent change in absorbance monitored. Data was transferred to an Apple Europlus2 for analysis. Hard copies of results were printed on an Epson MX-80 printer and data stored on floppy disk.

For very fast reactions such as at high enzyme or base concentration where the half-life of the reaction was less than 5 seconds, the reactions were followed on a Nortech SF3A Mk. 4 stopped flow spectrometer. Reactants at twice the desired final concentration were placed in two piston-driven syringes. These feed into the reaction cell *via* coiled glass tubes immersed in a water-bath thermostatically maintained at 30 ± 0.1 °C. Optical density changes after mixing were followed at 230 nm. The signal from the photomultiplier was transmitted to a Datalab DL 901 transient recorder which was automatically triggered by the outlet syringe, simultaneously causing a display on a Gould Advance OS 250B oscilloscope. Changes in absorbance *versus* time were output from the transient recorder to a Servogor 210 chart recorder.

Analysis of data. Data transferred from the Gilford 2600 to the Apple Europlus2 was analysed in the following ways.

Pseudo-first-order rate constants. The programme calculates a first-order rate constant using an iterative non-linear least-squares procedure which treats the initial absorbance, final absorbance, and rate constant as adjustable parameters according to the method of Deming[15] and Wentworth.[16] Besides accepting data from the Gilford, data can be entered from a disk file or manually on the keyboard. The experimental data may be compared graphically with the curve derived from the calculated parameters and edited where necessary.

Michaelis–Menten constants K_m, k_{cat}, *and* k_{cat}/K_m. The common linear transformations of the Michaelis–Menten equation (Lineweaver–Burke, Eadie–Hofstee, Hanes, *etc.*) have for many years been used to calculate enzyme kinetic parameters. All these plots tend to distort the data to some extent[17] but the advent of laboratory microcomputers has led to a rectangular hyperbolae curve fitting to enzyme kinetic data which has resulted in some comments[18] that the argument concerning the relative merits of the different linear transformations is now obsolete.

Initial rates. Using a single enzyme stock solution and different substrate concentrations values of 5—0.5 K_m were obtained. The initial slope was determined from the plot of (S) *versus* time and from this Michaelis constants were derived. The single advantage of this method is that side-effects such as

product inhibition, if it occurs, are eliminated. It does however suffer from the following disadvantages. (i) The process is very time consuming, (ii) the initial slope determination is open to severe error, as is the precise calculation of substrate concentration, (iii) it is very costly in terms of consumption of enzyme, (iv) only small numbers of data points are generated. To improve the estimation of initial rates the fitting of a polynomial curve was attempted since the derivative when $t = 0$ gives an estimate of the slope at the start of the reaction.

Plot expansion. If product inhibition is not significant then essentially all the data required can be generated by a single enzyme/substrate reaction. The reaction catalysed by β-lactamase is essentially irreversible and the products are not effective inhibitors of the enzyme.[19] The substrate concentration can be determined from the optical density at any given point, and the corresponding rate can be determined as a tangent to that point in the same way as initial rate. The Gilford model used is interfaced to the Hewlett-Packard X-Y plotter. A facility on the Gilford is that any given portion of the plot obtained can be expanded to A4 size. This allows for more accurate determination of the tangent to any point so long as the initial and final optical densities from the reaction are known. The procedure is therefore to plot accurately the substrate u.v. spectra before reaction, run the enzyme reaction and then expand the x, y co-ordinates as necessary to determine accurately tangents for a series of optical densities. A wavelength scan of the products from the reaction then allows determination of the change in extinction coefficient.

The Gilford is also interfaced to the Apple Europlus2 microcomputer and data can be transferred to the Apple under software control. The following programs were used to analyse the data:

'*Diffenz*'. This program is an extension of the plot expansion method described above. The substrate concentration S at time t is given by $(Abs_t - Abs^{\infty})/\Delta\varepsilon$ where Abs^{∞} is the absorbance of the products and $\Delta\varepsilon$ is the difference in extinction coefficients between substrate and product. If the reaction curve is divided into many points (S_n, t_n), then S_n can be determined and the rate calculated with reasonable accuracy at any point from $\Delta S_n/\Delta t = \Delta Abs/(\Delta\varepsilon\Delta t)$ in moles per unit time. A 'blocking' facility may be used where Δt has a pre-determined value since it was found that this value could affect the size of K_m determined. From these rate data, Michaelis constants were determined in the software using Lineweaver–Burke, Eadie–Hofstee, and Hanes Plots. The method allows hundreds of data points to be generated from a single enzyme–substrate reaction and, in general, very good reproducibility is found. Constants derived from standard reagents such as cephaloridine or benzylpenicillin agreed with literature values and with those obtained by the initial-rate method.

'*Gildydx*'. This is an adaptation of Waleys' 'chord' technique[20] and in some circumstances generates more accurate rate data than the previous program, but at a cost of the number of data points that can be analysed. The facility on the Gilford for calculating tangents at any point in the reaction is utilised. Essentially, the Gilford does this by using linear regression on a specified number of data points either side of S_n. It can be shown that the slope of such a line is a good estimate of the slope of the tangent to S_n.

Because the program uses so much of the Apple memory it is not possible to analyse as many data points as with 'Diffenz'. The advantage of the program is that it obtains accurate rate data when the optical density change occurring is very small or when the reaction trace is particularly 'noisy'.

'*Intenz*'. This program uses an integrated form of the rate equation, given by:

$$V_{max.}\,\Delta T = -K_m \ln\frac{[S]}{[S_0]} - \Delta[S]$$

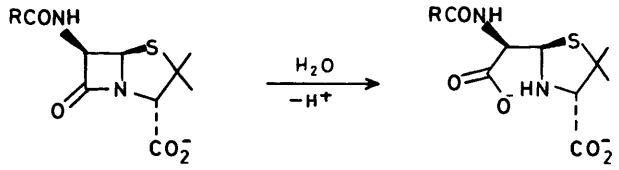
which for this program is arranged into the linear form:

$$-\frac{\Delta T}{\Delta[S]} = \frac{1}{V_{max.}} + \frac{K_m}{V_{max.}}\cdot\frac{\Delta \ln [S]}{\Delta[S]}$$

Although it is claimed that the use of the integrated rate equation has the advantage that if product inhibition occurs then the plot will not be linear,[21] competitive product inhibition will also give a linear plot.

Results and Discussion

β-Lactamase catalyses the hydrolysis of penicillins to give the biologically inactive penicilloic acid derivative:



The results of the β-lactamase I-catalysed hydrolysis of the alkyl penicillins were analysed assuming Michaelis–Menten kinetics:

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} \text{Product}$$

where E, S, and ES respectively, are the enzyme, substrate, and enzyme–substrate complex. Values of k_{cat}, K_m, and k_{cat}/K_m determined at 30 °C in 0.1 M phosphate buffer at pH 7.0 are given in the Table.

Within experimental error, the second-order rate constants for the hydroxide ion-catalysed hydrolysis of the 6-alkyl penicillins are independent of the alkyl substituent. Acylation of the 6-amino substituent increases the rate of alkaline hydrolysis three-fold (Table). By contrast, the β-lactamase-catalysed hydrolysis of alkyl penicillins is up to 300-fold more efficient, as measured by k_{cat}/K_m than that of 6-aminopenicillanic acid.

The second-order rate constant k_{cat}/K_m is directly related to the free energy of activation—the difference in free energy between the free unbound substrate and enzyme and the transition state.[22] In a series of substrates in which substituents which do not affect chemical reactivity are varied, the difference in the free energies of activation of the enzyme-catalysed reaction, corrected for differences in ground state energies of the substrates, gives the free energy of binding between the substituent and the enzyme in the transition state.[22,23] The chemical reactivity of the 6-alkyl penicillins is clearly the same, as indicated by their susceptibility to nucleophilic attack by hydroxide ion (Table). The favourable binding energy interaction between the 'non-reacting' part of the penicillin molecule—the 6-alkyl side-chain—and the enzyme is used to lower the activation energy for the enzyme-catalysed reaction. The rate enhancement brought about by β-lactamase is indicated by comparing k_{cat}/K_m and k_{OH} (Table) which gives ratios of 4×10^7 to 3×10^8. These ratios are similar to those observed for the serine enzymes such as α-chymotrypsin, although, of course, β-lactamase is not strictly a peptidase.[24] It is sometimes stated that the logic of serine enzymes converting their amide substrates into ester, acyl enzyme, intermediates is that the esters are chemically more reactive and more susceptible to hydrolysis. In the case of β-lactamase and penicillins this argument is not valid because penicillins are as reactive as esters towards nucleophilic attack.[25,26]

Although 6-aminopenicillanic acid (6-APA) is a poor

Table. Second-order rate constants for the hydroxide ion-catalysed hydrolysis of 6-alkyl penicillins and the kinetic parameters for the *B. cereus* β-Lactamase I catalysed hydrolysis at 30 °C, I = 0.1, pH 7.0

	$K_m/10^{-4}$M	k_{cat}/s^{-1}	$k_{cat}/K_m/10^6$ dm^3 mol^{-1} s^{-1}	k_{OH^-}/ dm^3 mol^{-1} s^{-1}
6-APA	5.35	58.6	0.11	0.039
Me	1.21	771	6.37	0.138
$MeCH_2$	0.66	1 110	16.81	0.116
$Me(CH_2)_2$	1.46	4 070	27.90	0.109
$Me(CH_2)_3$	1.03	3 530	34.27	0.116
$Me(CH_2)_5$	1.30	4 690	36.08	0.119
$Me(CH_2)_6$	0.56	1 490	26.61	0.126
$Me(CH_2)_7$	2.30	1 580	6.87	0.125
$Me(CH_2)_8$	1.98	2 010	10.15	0.132
$PhCH_2$	0.614	2 560	41.70	0.154

[a] I = 0.05M KCl.

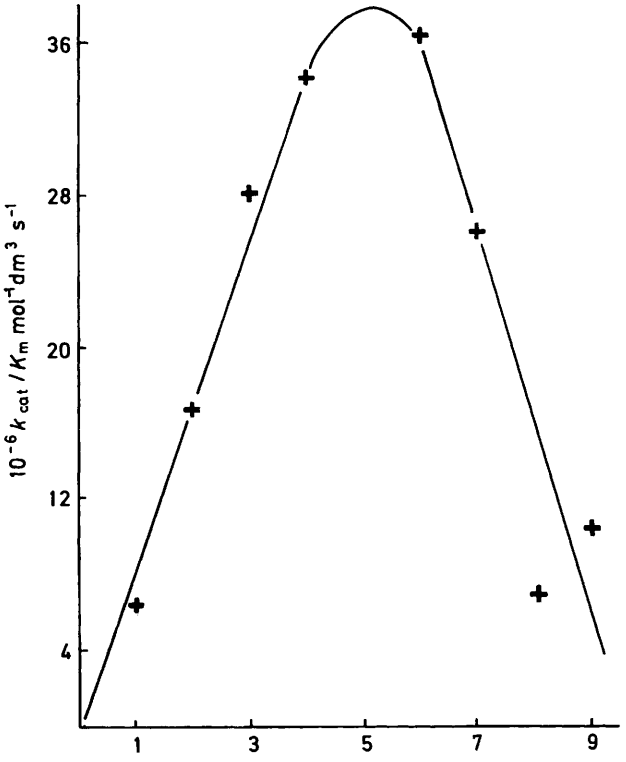


Figure. Second-order rate constants k_{cat}/K_m for the β-lactamase I-catalysed hydrolysis of 6-alkyl penicillins at 30 °C and pH 7.0 as a function of the length of the alkyl side-chain

substrate for β-lactamase, the rate enhancement brought about by the enzyme is still significant, a factor of 3 × 10^6 (Table). 6-APA is thus a better substrate than the cephalosporins for β-lactamase I[12] and the k_{cat}/K_m for 6-APA shows that, although the binding energy contribution from the 6-acylamido side-chain is significant, the major recognition site comes from elsewhere in the penicillin molecule. Similarly, although benzylpenicillin is the best substrate (Table) it is not significantly better than the alkyl penicillins. There does not appear to be a specific pocket in β-lactamase I for the recognition of hydrophobic residues in the 6-acylamido side-chain. The k_{cat}/K_m for benzylpenicillin is high and approaching the diffusion-controlled limit but the variation in this rate constant shown by the other penicillins indicates that this limit has not been reached.

The variation of the second-order rate constants, k_{cat}/K_m, with the length of the 6-alkyl side-chain is illustrated in the Figure. The rate constant increases steadily with increasing chain length, reaching a maximum with the hexyl derivative, and then decreases. Similar behaviour is shown by 7-substituted cephalosporins, except that the maximum occurs with the octyl derivative.[12] From these observations it is possible to estimate the free energy of transfer of the methylene group from water to the enzyme in the transition state, using equation (1), where n refers to the number of methylene units in the side-chain and H

$$\log_{10}(k_{cat}/K_m)_n = \frac{n \times \Delta\Delta G^{\ddagger}}{2.303\,RT} + \log(k_{cat}/K_m)\mathrm{H} \quad (1)$$

refers, formally, to the 6-formyl derivative. A plot of $\log_{10}(k_{cat}/K_m)$ against the number of methylenes is linear up to n = 4 and corresponds to a free energy of transfer of 1.46 kJ mol^{-1} per methylene. This is a relatively small value[23,27] and is a quantitative indication of the weak interaction between the alkyl side-chain and the enzyme. The free energy of transferring a methylene group from water to various non-polar environments is 4 kJ mol^{-1} to non-polar liquids,[28] 3 kJ mol^{-1} to micelles,[29] and 14 kJ mol^{-1} to some enzymes.[30]

There is no particular trend in the individual parameters k_{cat} and K_m and it has been noted, in any case, that it is more meaningful to use the second-order rate constant k_{cat}/K_m to interpret specificity.[31]

Acknowledgements

We thank the S.E.R.C. for support and the award of a CASE Studentship to S. C. B.

References

1 B. K. N. Nielsen and J. O. Lampen, *Biochemistry*, 1983, **22**, 4652.
2 K. G. H. Dyke, in 'β-Lactamases,' eds. J. M. T. Hamilton-Miller and J. T. Smith, Academic Press, London, 1979, p. 291.
3 R. P. Ambler, *Philos. Trans. Roy. Soc. (Lond.)*, 1980, **289**, 321; S. Bergstrom, S. Olsson, and S. Normark, *J. Bacteriol.*, 1982, **150**, 528.
4 A. K. Saz, D. L. Lewery, and L. L. Jackson, *J. Bacteriol*, 1961, **82**, 298.
5 R. F. Pratt and C. P. Govardhan, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 1302.
6 K. Bush and R. B. Syke, *Antimicrob. Agents Chemother.*, 1986, **30**, 6.
7 C. Little, E. L. Emanuel, J. Gagnan, and S. G. Waley, *Biochem. J.*, 1986, **240**, 215.
8 B. L. Wolozin, R. Myerowitz, and R. F. Pratt, *Biochim. Biophys. Acta*, 1982, **701**, 153.
9 R. F. Pratt and M. J. Loosemore, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 4145; V. Knott-Hunziker, S. G. Waley, B. Orlek, and P. G. Sammes, *FEBS Lett.*, 1979, **99**, 59.
10 G. Dalbadie-McFarland, L. W. Cohen, A. D. Riggs, C. Morin, K. Itakura, and J. H. Richards, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 6409; I. S. Sigal, W. F. DeGrado, B. J. Thomas, and S. R. Petleway, *J. Biol. Chem.*, 1984, **259**, 5327.
11 O. Herzberg and J. Moult, *Science*, 1987, **236**, 694.

12 S. C. Buckwell, M. I. Page, S. G. Waley, and J. L. Longridge, following paper.
13 S. C. Buckwell, M. I. Page, J. Longridge, and S. G. Waley, *J. Chem. Soc., Perkin Trans. 2*, 1988, 1823.
14 P. A. Kiener, V. Knott-Hunziker, S. Petursson, and S. G. Waley, *Eur. J. Biochem.*, 1980, **109**, 575.
15 W. E. Deming, 'Statistical Adjustment of Data,' J. Wiley and Sons, New York, 1964.
16 W. E. Wentworth, *J. Chem. Educ.*, 1965, **42**, 96, 162.
17 A. Cornish-Bowden, *Biochem. J.*, 1974, 721; J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, 1965, **240**, 863; M. Markus, *FEBS Lett.*, 1976, **63**, 225; A. Cornish-Bowden, 'Principles of Enzyme Kinetics,' Butterworths, 1970, p. 168.
18 P. C. Engel, in 'The Chemistry of Enzyme Action,' ed. M. I. Page, Elsevier, Amsterdam, 1984, p. 73.
19 P. A. Kiener and S. G. Waley, *Biochem. J.*, 1978, **169**, 197.
20 S. G. Waley, *Biochem. J.*, 1981, **193**, 1009.
21 H. Gutfreund, 'Enzymes: Physical Principles,' Wiley, Interscience, 1972.
22 R. P. Bell, J. E. Critchlow, and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1974, 245.
23 M. I. Page, in 'The Chemistry of Enzyme Action,' ed. M. I. Page, Elsevier, Amsterdam, 1984, pp. 1—54.
24 M. I. Page and S. Buckwell, in 'Peptides and Proteases, Recent Advances,' eds. R. L. Schowen and A. Barth, Pergamon, Oxford, 1987, p. 241.
25 M. I. Page, *Adv. Phys. Org. Chem.*, 1987, 165.
26 M. I. Page, *Acc. Chem. Res.*, 1984, **17**, 144.
27 M. I. Page, *Biochem. Biophys. Res. Commun.*, 1976, **72**, 456.
28 P. Molyneux, C. T. Rhodes, and J. Swarbick, *Trans. Faraday Soc.*, 1965, **61**, 1043.
29 N. P. Gensmantel and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1982, 155.
30 W.-T. Tsui and A. R. Fersht, *Nucl. Acids Res.*, 1981, **9**, 4627.
31 M. I. Page, in 'The Chemistry of Enzyme Action,' ed. M. I. Page, Elsevier, Amsterdam, 1984, p. 1; W. P. Jencks, *Adv. Enzymol.*, 1975, **43**, 219; R. J. Leatherbarrow and A. R. Fersht, in 'Enzyme Mechanisms,' eds. M. I. Page and A. Williams, Royal Society of Chemistry, London, 1987, p. 78; M. I. Page, in 'Accuracy in Molecular Processes,' eds. T. B. L. Kirkwood, R. F. Rosenberger, and D. J. Galas, Chapman and Hall, London, 1986, p. 37.

Received 30*th November* 1987; *Paper* 7/2114