# The Effect of the Carboxy Group on the Chemical and β-Lactamase Reactivity of β-Lactam Antibiotics

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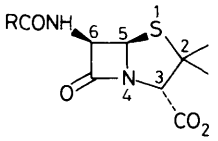
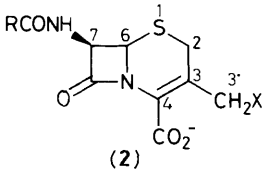
Kinetic parameters are reported for the *Bacillus cereus* β-lactamase I and β-lactamase II catalysed hydrolysis of esters and lactones of penicillins and cephalosporins. These are compared with the second-order rate constants for the hydroxide-ion catalysed hydrolysis of the same derivatives. The second-order rate constant, $k_{cat}/K_m$, for the hydrolysis of the cephalosporin lactone catalysed by β-lactamase I is 50 times greater than that for an analogous cephalosporin and is $3 \times 10^4$ times greater than that for hydroxide-ion catalysed hydrolysis, a ratio similar to that for cephalosporins with a carboxylate group at C-4. The methyl ester of benzyl penicillin, but not the corresponding cephalosporanate, is a substrate for β-lactamase I. All ester derivatives are much poorer substrates for β-lactamase II. The cephalosporin lactone and, to a lesser extent, the methyl ester of benzyl penicillin can obviously bind to β-lactamase I even though they do not possess a formal anionic site at C-4 and C-3 respectively. The esterification of the carboxy group at C-3 in penicillins induces neighbouring-group participation by the C-6 acylamido side chain to give an oxazolinone intermediate. This is attributed to different Brönsted $\beta_{lg}$ dependency for alkaline hydrolysis and intramolecular acylamido participation, which exhibits rate limiting C–N bond fission of the β-lactam.

Until recently, a basic tenet of the chemistry of β-lactam antibiotics, such as penicillins (**1**) and cephalosporins (**2**), had been that their presumed enhanced reactivity was due to either strain in the four-membered ring or reduced amide resonance. It is now generally accepted that the chemical reactivity of β-lactam antibiotics is not unusual and that there is little evidence to support the importance of strain or reduced amide resonance as major factors in determining either their biological or chemical reactivity.[1] In fact, the four-membered ring does not open readily and the strain energy of the ring does not significantly facilitate C–N bond cleavage.[2]




(**1**)

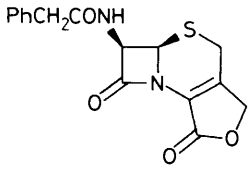



(**2**)

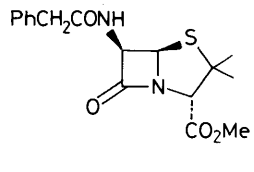
Another commonly accepted assumption is the necessity of an anionic group, such as carboxylate, at C-3 in penicillins (**1**) and at C-4 in cephalosporins (**2**) for effective biological activity. For example, esterification of the C-3-carboxylate of penicillins greatly decreases antibacterial activity.[3]

The major cause of bacterial resistance to the normally lethal action of penicillins and cephalosporins is the bacteria's ability to produce β-lactamase enzymes which catalyse the hydrolysis of the β-lactam to biologically inactive products. It is usually assumed that good substrates or inhibitors of β-lactamases also require the presence of a carboxylate, or other anionic residue. This prime recognition site is presumed to have a complementary positively charged residue on the β-lactamase enzyme.

We report herein the effect of esterification of the carboxylate group at C-3 in penicillins (**4**) and at C-4 in cephalosporins (**5**) and compare their reactivity towards the β-lactamase enzymes from *Bacillus cereus* with that of the lactone (**3**). The intrinsic electronic effects of these structural changes on nucleophilic attack on the β-lactam carbonyl carbon and ring opening are determined by the relative rates of alkaline hydrolysis. It is also reported that esterification of the C-3 carboxy group in penicillin (**1**) induces neighbouring-group participation by the C-6 amido group.




(**3**)

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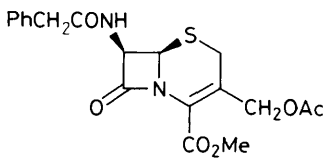



(**5**)

## Experimental

$^1$H N.m.r. spectra were measured on a Bruker 270 MHz FT spectrometer with tetramethylsilane as an internal standard. I.r. spectra were taken as Nujol mulls or potassium bromide discs.

*Synthesis of the lactone* (**3**). The lactone was prepared according to the method of Cocker *et al.*[4] and recrystallised from ethanol, m.p. 208–209 °C (lit.,[4] 210 °C); δ([$^2H_6$]DMSO) 3.49, 3.54, 3.58, and 3.63 (2 H, AB system, 2-H), 5.08 (1 H, d, 6-H, $J_{6,7}$ 7 Hz), 5.85 (1 H, dd, 7-H, $J_{7,6}$ 7 Hz), 4.96 (2 H, s, 3'-$CH_2$), 3.77 (2 H, s, Ph$CH_2$), 7.27–7.31 (5 H, m, Ph), 9.20 (1 H, d, NH); $\lambda_{max}$ 256 (ε 7 800) nm; $\nu_{max}$ 1 795, 1 780 (γ-lactone), 1 758 (β-lactam) 1 665, and 1 540 (CONH) cm$^{-1}$.

*Synthesis of 7-Phenylactamidocephalosporanic Acid Methyl Ester* (**5**). The ester was prepared according to the method of Cocker *et al.*,[4] m.p. 177–178 °C, (lit.,[4] 178–180 °C).

*Synthesis of Benzylpenicillin Methyl Ester* (**4**). The ester was

**Table.** Second order rate constants for the hydroxide ion catalysed hydrolysis of β-lactam derivatives at 30 °C, I = 1.0 mol $dm^{-3}$ and the kinetic parameters *B. cereus* β-lactamase I and II catalysed hydrolysis at 30 °C, pH 7.0.

| Substrate | $k_{OH^-}$/$mol^{-1}$ $dm^3$ $s^{-1}$ | $k_{cat}/K_m$/$dm^3$ $mol^{-1}$ $s^{-1}$ β-lactamase I | β-lactamase II |
|---|---|---|---|
| Lactone of 7-phenylactamido-cephalosporanic acid (**3**) | 11.6 | $3.23 \times 10^5$ | $1.50 \times 10^3$ |
| Benzylpenicillin methyl ester (**4**) | 2.49 | $7.03 \times 10^3$ | <10 |
| 7-Phenylacetamido-cephalosporanic acid methyl ester (**5**) | 4.17 | $<1 \times 10^2$ | $<1 \times 10^2$ |
| Benzylpenicillin (**1**)[a] R = $PhCH_2$ | $1.54 \times 10^{-1}$ | $4.17 \times 10^7$ | $3.19 \times 10^5$ |
| 7-Phenylacetamido-cephalosporanic acid (**2**)[b] R = $PhCH_2$ | $8.90 \times 10^{-2}$ | $5.96 \times 10^3$ | $3.05 \times 10^5$ |

[a] Data from reference 6. [b] Data from reference 8.

prepared according to the method of Gensmantel *et al.*,[5] m.p. 88–89 °C (lit.,[5] 90–92 °C).

*Materials.*—All reagents used were AnalaR grade. β-Lactamase types I and II from *Bacillus cereus* 569H were obtained from Porton Products.

*Kinetics.*—The kinetics of the enzyme-catalysed reactions were analysed as previously described.[6] The kinetics of the alkaline hydrolysis were studied in aqueous solution by monitoring the change in u.v. absorption.

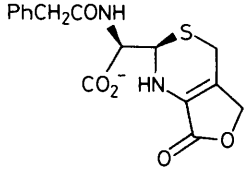
The reactions were initiated by the addition of 25 $mm^3$ of a stock solution ($10^{-2}$ mol $dm^{-3}$) of the substrate, dissolved in acetonitrile, to 2.5 $cm^3$ of the aqueous buffer or sodium hydroxide solution, pre-incubated at 30 ± 0.05 °C, with thorough mixing. The kinetics were thus measured using $10^{-4}$ mol $dm^{-3}$ substrate in 1% v/v acetonitrile–water. The disappearance of the substrate was followed spectrophotometrically using a Gilford 2600 spectrophotometer. For the lactone (**3**) the wavelength of absorption was at 256 nm, for the methyl ester (**4**) at 235 and 310 nm whilst for the methyl ester (**5**) it was at 260 nm. The data from the spectrophotometer were fed directly into a BBC microcomputer from which the rate constants were calculated using an iterative non-linear least-squares method which treated first-order rate constants and the absorbances at time zero and infinity as disposable parameters.

*N.M.R. Study.*—The lactone (**3**) (8.0 mg, $2.4 \times 10^{-5}$ mol) was dissolved in [$^2H_3$]acetonitrile to give a $8 \times 10^{-2}$ mol $dm^{-3}$ solution, to which was added an aqueous ($D_2O$) solution of β-lactamase I (0.3 mg) in 200 $mm^3$ of phosphate buffer pD 7.4.

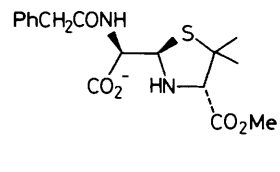
## Results

*Enzyme Catalysed Hydrolysis.*—The lactone (**3**) is a good substrate for β-lactamase I but a poor one for the metalloenzyme β-lactamase II. Saturation kinetics are not observed with either β-lactamase I or II and the Michaelis constant $K_m$ for both enzymes is estimated to be greater than $5 \times 10^{-3}$ mol $dm^{-3}$. The second-order rate constants $k_{cat}/K_m$ are given in the Table from which it can be seen that the lactone (**3**) is a substrate for β-lactamase I 50 times better than the corresponding cephalosporin (**2**) with a free carboxylate residue.

The β-lactamase I catalysed hydrolysis of the lactone (**3**) was also studied by high field n.m.r. spectrometry which showed that the β-lactam ring opened with the lactone intact. The n.m.r. spectrum of the hydrolysis product is consistent with structure




(**6**)




(**7**)

(**6**). Most characteristic is the 3′-$CH_2$ which changes δ from a singlet at 4.97 for (**3**) for a doublet centred at 4.80. The product spectrum is not consistent with either of the possible ring-opened lactone analogues of (**6**) which would show either an exocyclic methylene at C-3 as a doublet centred at δ 5.69 or the corresponding hydroxy acid with the 3′-$CH_2OH$ at δ 4.26.[7]

The cephalosporin methyl ester (**5**) is not a substrate for β-lactamase I. The rate of hydrolysis at pH 7 is the same in the absence or presence of enzyme. The maximum value for $k_{cat}/K_m$ for the β-lactamase I catalysed hydrolysis of (**5**) is $<1 \times 10^2$ $dm^3$ $mol^{-1}$ $s^{-1}$.

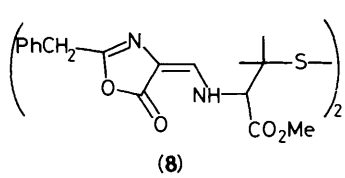
Benzylpenicillin methyl ester (**4**), however, is a substrate and the kinetics of the decrease in absorbance at 235 nm give a second-order rate constant $k_{cat}/K_m$, of $7.03 \times 10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$ for β-lactamase I (Table).

*Hydroxide-ion Catalysed Hydrolysis.*—The initial product of hydrolysis of the lactone (**3**) is the β-lactam ring-opened derivative (**6**) with the lactone still intact and the second order rate constant for hydroxide ion catalysed hydrolysis is 11.6 $dm^3$ $mol^{-1}$ $s^{-1}$ (Table).

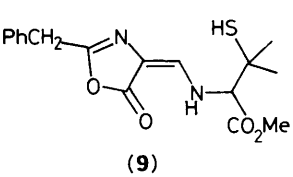
The alkaline hydrolysis of benzylpenicillin methyl ester (**4**) is accompanied by an increase in absorbance at 235 and 310 nm. The rate of production of the chromophore at 310 nm is first order in sodium hydroxide and the second-order rate constant is 2.49 $dm^3$ $mol^{-1}$ $s^{-1}$ (Table). That this chromophore is generated directly from the intact penicillin and not from the hydrolysis product (**7**) was shown by adding base to (**7**) formed from the β-lactamase catalysed hydrolysis of (**4**) at pH 7. The absorbance at 310 nm was proportional to the amount of β-lactam remaining during the enzyme-catalysed reaction.

The products were purified by removing the methyl penicilloic acid (**7**) by elution through Amberlite XAD-2 with water following by 25:75 v/v acetone–water. The latter fraction was freeze dried and then purified by reversed-phase preparative h.p.l.c. eluting with 15% acetonitrile pH 7 buffer.

The n.m.r. spectrum of the isolated product was consistent with the oxazolinone disulphide dimer (**8**). The vinylic hydrogen




(**8**)




(**9**)

at C-5 occurs at δ 8.25 and $\lambda_{max}$ at 316 nm. It is known that penicillenic acid disulphide is stable and its formation is favoured at high pH.[9] The disulphide (**8**) presumably arises from oxidation of the penicillenic acid derivative (**9**) which has a characteristic u.v. absorption at 310 nm.[10] Using an extinction coefficient of $2 \times 10^4$, the second-order rate constant for the hydroxide-ion catalysed hydrolysis of the β-lactam is 2.32 $dm^3$ $mol^{-1}$ $s^{-1}$ and that for the ring-closure

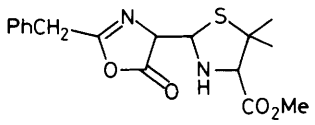
reaction 0.17 $dm^3$ $mol^{-1}$ $s^{-1}$. The amount of oxazolinone formed is independent of pH.

The alkaline hydrolysis of the cephalosporin methyl ester (**5**) followed first-order kinetics and although it is accompanied by Δ3–Δ2 isomerisation and hydrolysis of the methyl ester, there was no significant oxazoline formation. The second-order rate constant for the hydroxide-ion catalysed reaction is 4.17 $dm^3$ $mol^{-1}$ $s^{-1}$ (Table).

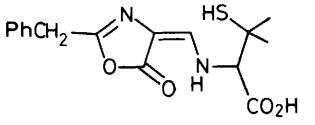
## Discussion

*Alkaline Hydrolysis.*—Esterification of the carboxy residue at C-3 in penicillins (**1**) and at C-4 in cephalosporins (**2**), to give the derivatives (**3**), (**4**), and (**5**), increases the rate of the hydroxide-ion catalysed hydrolysis of the β-lactam. The increase in the second-order rate constant is 16–130 fold, in line with the Brönsted $B_{1g}$ value of −0.5 reported for penicillins.[11] The esters and lactones are more electron withdrawing than the carboxylate group making the β-lactam carbonyl carbon more susceptible to nucleophilic attack and they are also expected to increase the ease of C–N bond cleavage.[1]

A surprising observation is that the alkaline hydrolysis of benzylpenicillin methyl ester (**4**) is accompanied by neighbouring-group participation by the amido group at C-6. The isolated product is the dimer (**8**) which presumably arises from the oxidation of the penicillenic acid derivative (**9**) which in turn is probably formed from the oxazolinone thiazolidine intermediate (**10**).




(**10**)




(**11**)

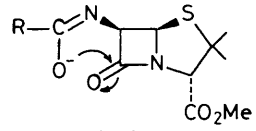
Penicillins with amido side chains at C-6 rearrange to penicillenic acid (**11**) in weakly acidic solution.[1,11–13] However, nucleophilic attack of the amido side chain on the β-lactam carbonyl carbon is not observed in the neutral or alkaline hydrolysis of penicillins in aqueous solution.[1,11] H.p.l.c. and u.v. analysis indicates that there is less than 1% penicillenic acid formed during the alkaline hydrolysis of benzylpenicillin.

Benzylpenicillenic acid (**11**) is not very stable in aqueous solution at high and low pH[9] and hydrolyses in acidic solution to give a mixture of penamaldic and penillic acids.[10] Between pH 6 and 13 the main products are penicilloic acid,[14] the normal hydrolysis product of penicillin, but at higher pH carbon–nitrogen bond cleavage rapidly occurs to give D-penicillamine and 2-benzyl-4-hydroxymethyleneoxazol-5(4*H*)-one.[15] Additionally, benzylpenicillenic acid is readily oxidised to the disulphide, which has a similar u.v. spectrum to that of the monomer.[12,14] Benzylpenicillenic acid methyl ester, (**9**), however, is much more stable in alkaline solution, remaining unchanged for over 16 h in 0.1 mol $dm^{-3}$ sodium hydroxide at 30 °C.

Neighbouring amide groups are very effective intramolecular nucleophiles in the reactions of carboxylic acid esters.[16] Oxazolinones are intermediates in the hydrolysis of activated esters of *N*-benzoylglycine.[17,18] There is a change in mechanism in the alkaline hydrolysis of these derivatives with changing $pK_a$ of the leaving group. Esters with poor leaving groups, high $pK_a$ alcohols, are hydrolysed by rate-limiting attack of hydroxide on the ester carbonyl and exhibit a Brönsted $\beta_{1g}$ of −0.35. Substituted phenyl esters with good leaving groups are hydrolysed *via* an oxazolinone intermediate and exhibit a Brönsted $\beta_{1g}$ of −0.8 consistent with rate limiting breakdown of the tetrahedral intermediate.[17]

It is therefore conceivable that intramolecular nucleophilic attack by the ionised C-6 amide in the methyl ester of benzylpenicillin is competitive with intermolecular attack by hydroxide on the β-lactam carbonyl, whereas it is not in the hydrolysis of benzylpenicillin, (**1**), itself. Esterification of the C-3 carboxy group lowers the $pK_a$ of the thiazolidine amine in the hydrolysis product by 2 units.[9] It is probable therefore that oxazolinone formation has a greater dependence on the $pK_a$ of the β-lactam leaving group amine than does hydrolysis initiated by nucleophilic attack of hydroxide ion on the β-lactam. Neighbouring-group participation is therefore observed for benzylpenicillin methyl ester (**4**) but not for benzyl penicillin (**1**) itself.

A simple interpretation of this change in mechanism would be that direct nucleophilic attack by hydroxide ion involves rate-limiting formation of the tetrahedral intermediate[1] whereas oxazolinone formation proceeds with rate-limiting breakdown and consequently has a greater dependence on the $pK_a$ of the leaving group. Nucleophilic attack by the ionised amide on the β-lactam carbonyl (**12**) generates the tetrahedral intermediate (**13**). Rate-limiting ring opening of the β-lactam requires the rate of C–N bond fission in the four-membered ring of (**13**) to be slower than that of C–O bond fission and expulsion of the amide anion.

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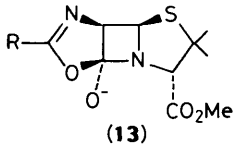


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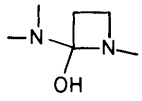



(**14**)

There are many pieces of evidence which suggest that opening the four-membered ring is not the expected facile process.[1,2] The alcoholysis of penicillins shows a Brönsted $\beta_{nuc}$ value of 0.95 for the reaction with alkoxide ions and a solvent isotope effect $k_{H_2O}(RO^-)/k_{D_2O}(RO^-)$ of 3.9, both of which are consistent with rate-limiting breakdown of the tetrahedral intermediate.[1,19] Hydrolysis of azetidin-2-ylideneammonium salts generates a tetrahedral intermediate (**14**) in which exocyclic C–N bond fission may occur faster than endocyclic C–N bond fission.[2]

The kinetic solvent isotope effect for the hydroxide-ion catalysed reaction is $k_{H_2O}(OH^-)/k_{D_2O}(OD^-) = 0.78$ which is consistent with previously reported values for nucleophilic attack on the β-lactam carbonyl[1] and oxazolinone formation.[17]

*β-Lactamase Catalysed Hydrolysis.*—The cephalosporin lactone (**3**) is a substrate for β-lactamase I 50 times better than the corresponding cephalosporin (**2**) with a free carboxylate residue (Table). Is this enhanced activity due to better binding interactions with the enzyme or to an intrinsic reactivity factor of the lactone substrate? The effect of changes in substrate structure on enzyme catalytic activity are often used to identify specific binding sites between parts of the enzyme and substrate. Changes in substrate structure can induce different intrinsic 'chemical' effects such as inductive, resonance and steric ones as well as different extrinsic molecular interactions between the substrate and enzyme. An important step towards understanding enzyme catalysis is to separate these effects.[20]

The hydrolysis of the β-lactam antibiotics catalysed by β-lactamases almost certainly involves nucleophilic attack upon the β-lactam carbonyl carbon. For β-lactamase I this nucleophile is a serine hydroxy group[21] but is unknown for β-lactamase II.[22] One method of determining the 'chemical' effect of structural

changes in β-lactam antibiotics on intrinsic reactivity is therefore to determine the susceptibility of the β-lactam towards nucleophilic attack. The magnitude of these effects will, of course, depend upon the nature of the nucleophile and comparisons of non-enzyme and enzyme catalysed processes may be complicated by different rate-limiting steps or even mechanisms in the two processes. For example, electron withdrawing substituents attached to the nitrogen of β-lactams will increase the rate of nucleophilic attack on the β-lactam carbonyl carbon. However, their expected effect of also increasing the ease of carbon–nitrogen bond cleavage is complicated by their opposing effect on basicity and protonation of nitrogen.[2] Despite these reservations, we have chosen to use the hydroxide-ion catalysed hydrolysis of the β-lactams as a measure of the intrinsic effects of substituents.

The second-order rate constant, $k_{cat}/K_m$, for the β-lactamase I catalysed hydrolysis of benzylpenicillin is $10^8$ times greater than that for the hydroxide-ion catalysed hydrolysis (Table). This is the typical ratio shown by nearly all penicillins (**1**).[6] The ratio is less for cephalosporins[8] and for the phenylacetamido derivative (**2**) (R = $PhCH_2$ and X = OAc) it is $7 \times 10^4$, despite the similar chemical reactivity of cephalosporins and penicillins (Table).

For the lactone (**3**) the ratio of $k_{cat}/K_m$ to $k_{OH}$ for β-lactamase I is $3 \times 10^4$ whereas it is $3 \times 10^3$ for benzylpenicillin methyl ester, (**4**), and less than 25 for the cephalosporin methyl ester, (**5**), (Table). The rate enhancement for hydrolysis brought about by the enzyme is therefore as great for the lactone (**3**) as it is for cephalosporins with a carboxylate group at C-4. This indicates that either the C-4-carboxylate in cephalosporins is not a primary and necessary recognition site for substrates to show high reactivity with β-lactamase or that the lactone residue contributes a similar binding energy to that of the carboxylate. The former explanation could be the reason for the lower reactivity of cephalosporins compared with penicillins in their β-lactamase catalysed hydrolysis.[6,8] The geometrical relationship between the carboxylate residues and the β-lactam carbonyl group is different in penicillins and cephalosporins.[1,23] It could therefore be that the necessary close proximity of the β-lactam carbonyl carbon of the cephalosporin to the enzyme's serine hydroxy group prevents interaction of its C-4 carboxylate with a cationic group on the enzyme as presumably occurs with penicillins. Esterification of the carboxylate group in penicillin decreases the apparent enzyme rate enhancement by $10^5$ (Table).

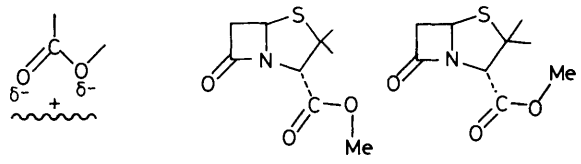
Neither the cephalosporin lactone (**3**), the cephalosporin methyl ester (**5**), or benzylpenicillin methyl ester (**4**) are good substrates for β-lactamase II. This is particularly interesting in view of the generally poor recognition of other structural changes in the substrate by the metalloenzyme.[8]

The carbonyl group of esters, lactones and carboxylic acids is highly polarised but it is difficult to quantify this in terms of 'atomic charge'. For example, despite the traditional view of resonance in carboxylic acids and carboxylate anions, it has recently been calculated[24] that the change in relative negative charge on the oxygens upon ionisation of carboxylic acids is only 0.1–0.2.

Furthermore, the charges on both oxygens of the neutral carboxylic acid are large, more negative than −1, and much greater than in conventional chemical thought which derives mostly from the magnitudes of total dipole moments.

Small ring lactones must adopt the s-*trans* conformation at the ester group which is estimated to be between 16 and 35 kJ $mol^{-1}$ less stable than the s-*cis* isomer.[25] The dipole moment of s-*cis*-methyl formate is only 1.98 D and considerably smaller than the 4.60 D for the s-*trans* rotamer.[26] This high value for the dipole moment is similar to the 3.90 and 4.2 D reported for γ-butyrolactone.[27]

It therefore appears that the two oxygen atoms of the lactone in the cephalosporin lactone (**3**) carry considerable negative charge and could interact with a suitably placed positive charge such as lysine 234 or the dipole of an α-helix in the enzyme (**15**). Compatible with the former suggestion is the observation that $k_{cat}/K_m$ for the hydrolysis of the lactone (3) decreases by a factor of 100 between pH 8 and 10. The negative charge is reduced for the s-*cis*-conformations of the esters (**4**) and (**5**) and any favourable binding energy between the enzyme and the s-*trans*-conformation of the ester is insufficient to compensate the unfavourable conformational change such as in (**16**) to (**17**). It would be of interest to know whether the lactone is a general mimic of the carboxylate group. The cephalosporin lactone, however, is a poorer substrate than cephalosporin C for *Streptomyces* R61 DD-peptidase.[28].




(**15**) (**16**) (**17**)

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