β-Lactamase-catalysed hydrolysis of cephalexin: evolution of the cephalosporoate intermediate

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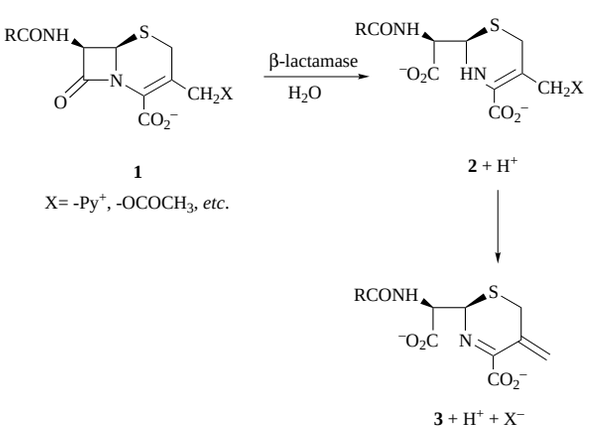
Hydrolysis of cephalexin catalysed by P99 β-lactamase from *Enterobacter cloacae* has been studied at pD 6.4 and 8.0. The enzymatic hydrolysis product is the cephalosporoate intermediate, which in aqueous media undergoes tautomerization of the double bond in the dihydrothiazine ring from position 3–4 to 4–5, with the consequent uptake of a proton at C(3). This latter process is governed by steric constraints. NMR results show that the cephalosporoate compound bears the aromatic group, present in the side chain at C(7), folded over the dihydrothiazine ring. Under these conditions the proton is chiefly taken up by the α face of the dihydrothiazine ring. Subsequently, the six-membered ring can be subjected to several reactions according to the pH.

Introduction

β-Lactamases are enzymes that bacteria produce to defend themselves against the action of antibiotics containing the four-membered β-lactam ring. These enzymes are important in medicine because they represent the main cause of antibiotic resistance. The main division of β-lactamases is into serine enzymes and zinc enzymes; the former have an active-site serine and function by a covalent mechanism, whereas the latter are metalloenzymes containing at least one Zn^{2+} ion per active subunit and appear to involve only non-covalent intermediates.[1,2] Comparison of amino acid sequences has led to the separation of the serine enzymes into two classes, called class A and class C. All zinc β-lactamases are often lumped together as class B enzymes, although only a few of them are known to have closely related amino acid sequences. The OXA β-lactamases are allocated to class D, their sequences differing greatly from those of other β-lactamases, but they are serine enzymes.[3]

β-Lactamases catalyse the hydrolytic cleavage of the β-lactam ring of both penicillins and cephalosporins, the products of fragmentation lack antibiotic activity. It has been found that the hydrolysis of cephalosporins **1** containing good leaving groups at the 3′-position catalysed by β-lactamases yields in solution cephalosporoate intermediates **2** which retain the 3′ substituent (Scheme 1). The 3′ substituent is then eliminated in a non-enzyme-catalysed reaction to yield the well-known 5-*exo*-methylene-1,3-thiazine **3** as a final product.[4–8] On the other hand, the β-lactamase-catalysed hydrolysis of cephalosporins with poor or no 3′-leaving groups yields the cephalosporoate compound.[4–8] To date, there is no evidence of studies regarding the cephalosporoate intermediate in aqueous media.



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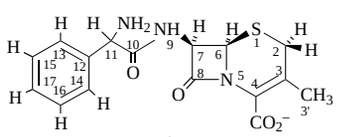
X= -Py+, -OCOCH3, *etc.*

Scheme 1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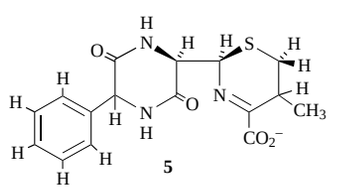
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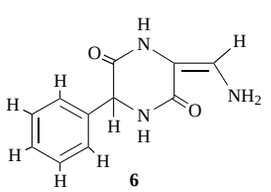
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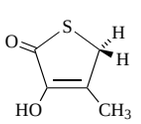
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Cephalexin **4** is an orally administered cephalosporin widely used in the treatment of mild to moderate respiratory tract, skin and soft-tissue infections.[9,10] Structurally, it is a 7-(α-amino-α-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid. The presence of a methyl group at C(3) hinders the occurrence of the *exo*-methylene compound in the enzyme-catalysed hydrolysis of this cephalosporin and thereby allows the evolution of the cephalosporoate compound to be studied.

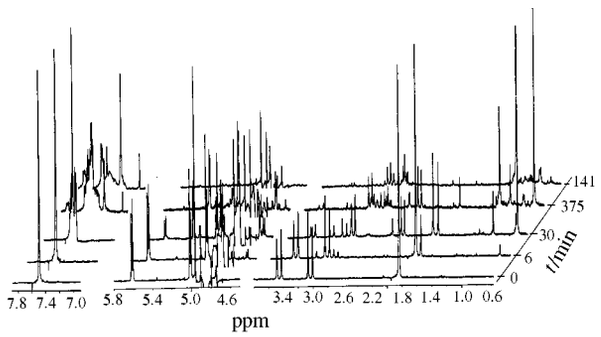
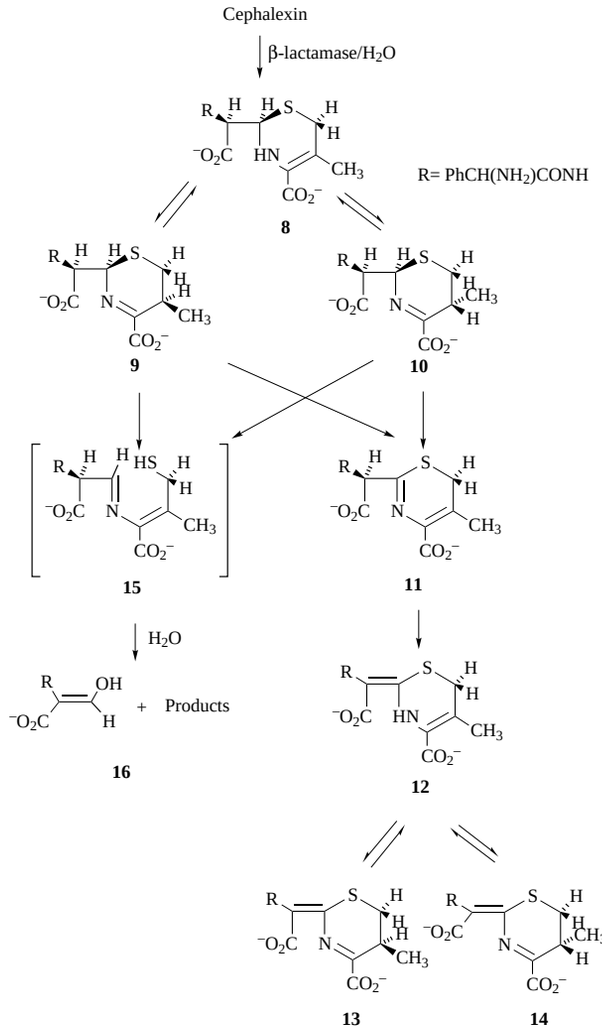


Fig. 1 Change in intensities of ^{1}H NMR signals during degradation of cephalexin in the presence of P99 β-lactamase in 0.4 M phosphate buffer solution (pD 8.0, 37 °C)

Studies by Bundgaard[11,12] on the non-enzymatic hydrolysis of the former cephalosporin in aqueous media revealed that the predominant degradation reaction at neutral pH was an intramolecular aminolysis of the β-lactam carbonyl moiety by the C(7) side chain amino group in which the piperazine-2,5-dione **5** was formed. This compound tends at neutral pH towards the occurrence of 3-aminomethylene-6-phenylpiperazine-2,5-dione **6** and 3-hydroxy-4-methylthiophen-2(5*H*)-one **7**.

In the present work a study of the enzymatic hydrolysis of cephalexin in addition to the evolution undergone by the compounds obtained has been carried out. ^{1}H NMR is a useful technique for determining both the groups involved in the degradation of antibiotics and the structural elucidation of the products obtained,[6,13,14] whereas the use of 2D NMR (NOESY) experiments[15–20] allows the determination of the interproton distances in the isolated products. Semi-empirical calculations have been performed in order to determine the conformation of detected products according to the interproton distances data obtained from the NOESY experiment. Finally, a degradation mechanism regarding this antibiotic in the presence of the P99 enzyme from *Enterobacter cloacae* has been established.



Scheme 2

Results and discussion

The enzymatic hydrolysis of cephalexin at pD 6.4 and 8.0 (37 °C) has been studied by means of ^{1}H NMR. The enzyme employed was *Enterobacter cloacae* P99 (class C). The pH dependence of k_{cat}/K_M gives two pK_a values of about 6.0 and 10.0.[21] The former values have been assigned to groups in the free enzyme which govern the enzymatic activity.

Fig. 1 shows the NMR spectrum corresponding to cephalexin at pD 8.0 at different reaction times. The addition of P99 enzyme modifies the spectrum giving rise to the cephalosporoate intermediate (compound **8**, Scheme 2). The spectroscopic data obtained for this compound (compound **8**, Table 1) are similar to those given by Pratt and Faraci[4,5] involving cephalosporins with a methyl group at C(3).

At pD 8.0 it is observed that the cephalosporoate intermediate leads to a second compound. This compound is differentiated from the cephalosporoate intermediate by a downfield shift in the proton bonded to C(6) (5.33 ppm) in addition to an upfield shift in the signals corresponding to the methyl (0.71 ppm) and the CH_2(2) (2.26 and 2.93 ppm) groups. These changes in the spectrum are indicative of a shift in the double bond of the dihydrothiazine ring from position 3–4 to 4–5, with consequent uptake of a deuterium at C(3). When hydrolysis was carried out in water (see Table 1, compound **9**) the uptake of a proton at C(3) was confirmed—the presence of a H at C(3) modified the signal corresponding to the methyl group, which then appeared as a doublet (J = 7.0 Hz), whereas signals corresponding to the CH_2(2) group became an ABX system. These facts suggest the formation of either compound **9** or **10**. A conformational study, described below, was carried out, and has demonstrated finally that this compound corresponds to structure **9** in Scheme 2.

Table 2 lists the interproton distances for cephalexin and compound **9** calculated from the NOESY experiment and theoretical calculations. The structures analysed by semi-empirical methods were selected after a conformational analysis, as described in the Experimental section. It can be seen from Table 2 that the experimental values are coincident with those calculated using PM3 and AM1 semi-empirical methods.

Values of the interproton distances calculated for the first compound formed from the cephalosporoate intermediate (compound **9**, Table 2) indicate the methyl and phenyl groups are very close. Taking into account that the NOE effect is detected between the methyl group and the C(2)-Hβ (see Fig. 2), whereas the former effect is not detected between the methyl group and the C(2)-Hα it can be deduced that the C(3)-H proton lies below (α face) the six-membered ring, and thus is assigned to compound **9** in Scheme 2. Values in the literature for analogous compounds[22] place the methyl group at 1.1 ppm; however, in compound **9** this signal is shifted to 0.71 ppm due to the ring effect of the phenyl group upon the methyl group.

In Fig. 1 it can be seen that a compound other than **9** forms during the reaction with a slight downfield shift of the methyl group, whereas the signals corresponding to the CH_2 group are now closer (system AB in which $\nu/J \leqslant 1.0$). Protons bonded to C(6) and C(7) appear at 5.27 and 4.62 ppm, respectively, and in the spectrum obtained in water (compound **10**, Table 1) a proton at C(3) is seen. These facts seem to suggest the formation of another isomer of compound **9**, *viz.*, one in which the C(3)-H proton lies above (β face) the six-membered ring

Table 1 ^{1}H NMR data (D_2O) for cephalexin and degradation products

	CH_2(2)	CH_3(3′)	H-C(6)	H-C(7)	PhC*H*-	Ph	Other signals
4[a]	3.03 (d, *J* 17.7) 3.46 (d, *J* 17.7)	1.84 (s)	5.03 (d, *J* 4.2)	5.62 (d, *J* 4.2)	5.20 (s)	7.51 (s)	
8	2.92 (d, *J* 17.0) 3.20 (d, *J* 17.0)	1.77 (s)	4.47 (d, *J* 5,1)	4.60 (d, *J* 5.1)	5.20 (s)	7.51 (s)	
9[a]	2.93 Hα (dd) (*J* 13.5, 4.5) 2.26 Hβ (dd, *J* 13.5, 2.5)	0.71 (d, *J* 7.0)	5.33 (d, *J* 2.4)	4.65 (d, *J* 2.4)	5.15 (s)	7.2, 7.3 (br)	2.70 (m) C(3)-H
10	2.30 Hβ (dd) (*J* 13.5, 5.7) 2.24 Hα (dd, *J* 13.5, 7.2)	1.04 (d, *J* 7.0)	5.27 (d, *J* 3.0)	4.62 (d, *J* 3.0)	5.15 (s)	7.2, 7.3 (br)	2.60 (m) C(3)-H
17[a]	3.09 Hβ (dd) (*J* 13.6, 4.0) 2.68 Hα (dd, *J* 13.6, 4.6)	1.22 (d, *J* 7.0)	5.36 (d, *J* 2.6)	4.48 (d, *J* 2.6)	5.59 (s)	7.50 (br)	2.80 (m) C(3)-H
18[a]	2.98 Hβ (dd) (*J* 13.7, 4.6) 2.71 Hα (dd, *J* 13.7, 6.8)	1.19 (d, *J* 7.0)	5.34 (d, *J* 2.3)	4.48 (d, *J* 2.3)	5.22 (s)	7.50 (br)	2.80 (m) C(3)-H

[a] ^{1}H NMR chemical shifts of these compounds were obtained from the isolated compounds at pD 6.4. All other signals were estimated from the spectra of mixtures.

Table 2 Internuclear distances from NOESY spectra[a]

			Model	
Diagonal	Cross peak	INTRA method	PM3	AM1
(*a*) Cephalexin				
C(3)-CH_3	C(2)-Hβ	2.41	2.51	2.58
C(3)-CH_3	C(2)-Hα	2.67	2.78	2.71
C(3)-CH_3	Ph	>5.00[b]	7.10	7.05
C(2)-Hβ	C(2)-Hα	1.77	1.77	1.81
C(2)-Hα	C(6)-H	2.88	3.12	3.07
C(6)-H	C(7)-H	2.32	2.42	2.44
(*b*) Compound **9**				
C(3)-CH_3	C(2)-Hβ	2.43	2.56	2.55
C(3)-CH_3	C(3)-H	2.36	2.43	2.49
C(3)-CH_3	Ph	3.00	3.00	4.00
C(2)-Hβ	C(3)-H	2.61	2.55	2.59
C(2)-Hβ	C(2)-Hα	1.77	1.76	1.81
C(2)-Hα	C(3)-H	2.71	2.35	2.36
C(2)-Hα	C(6)-H	2.65	3.71	3.23
C(6)-H	C(7)-H	2.50	2.79	2.65

[a] The distances are in Å and the estimated error in the INTRA method is ±0.3 Å, which is the value when the error in the ratio of the integrated intensities of the cross peak to the diagonal peak is 50%. [b] Cross peaks disappear in the noise for distances greater than 5 Å, thus establishing the upper limit on distance determination.

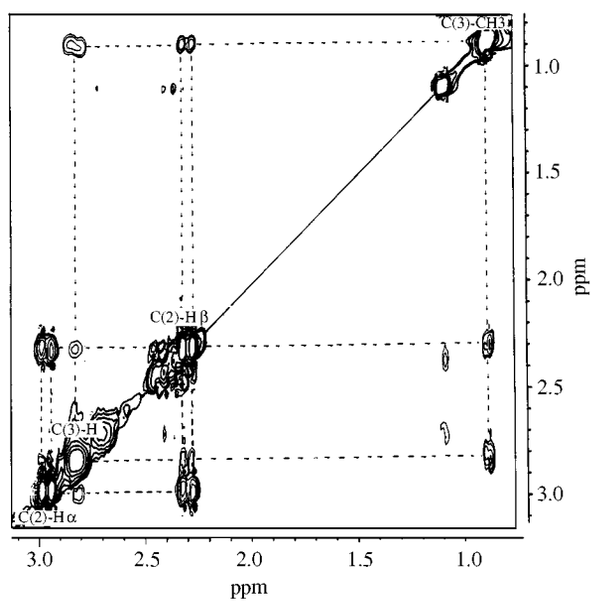


Fig. 2 Expanded fingerprint region of the phase-sensitive NOESY spectrum of compound **9** and **10** in D_2O. The experimental conditions are given in the Experimental section. Only the NOESY connectivities of the major compound **9** are plotted.

(compound **10**, Scheme 2). The 2D NOESY spectrum confirms this assumption since the NOE effect between the methyl and phenyl groups is not detected, which would explain the downfield shift in the methyl group in relation to that observed in compound **9**. On the other hand, the fact that the methyl group shows an NOE effect with the two protons bonded to C(2) indicates that the conformation of this compound corresponds to that described in Scheme 2. The concentration of compound **10** obtained during the enzymatic hydrolysis was insufficient to perform a quantitative analysis of the NOESY experiment.

After a long reaction time (Fig. 1, t = 375 min) it should be emphasized that an intermediate with more relevant spectroscopic data [3.32, 3.70 (2H, AB, *J* 17.1), 1.83 ppm (3H, s)] forms. The former values suggest that under these pD conditions the abstraction of the protons bonded to C(6) and C(7) has taken place, whereas the double bond of the six-membered ring is located once more at position 3–4. Finally, it is observed that this intermediate evolves towards other compounds in which the methyl group resonances are within the region 1.0–1.2 ppm, and the signals corresponding to the geminal protons between 2.6 and 3.0 ppm. A further discussion of the former signals will be considered.

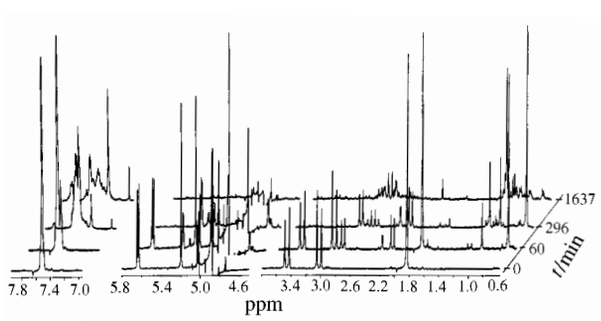


Fig. 3 Change in intensities of ^{1}H NMR signals during degradation of cephalexin in the presence of P99 β-lactamase in 0.4 M phosphate buffer solution (pD 6.4, 37 °C)

In Fig. 3 is depicted the spectrum of cephalexin at pD 6.4. The addition of the P99 enzyme initially leads to the occurrence of compound **9**, and later **10**. At this pD the formation of cephalosporoate is not detected. Compounds **9** and **10** evolve towards other compounds with a methyl resonance between 1.0 and 1.2 ppm and CH_2 protons between 2.68 and 2.85 together

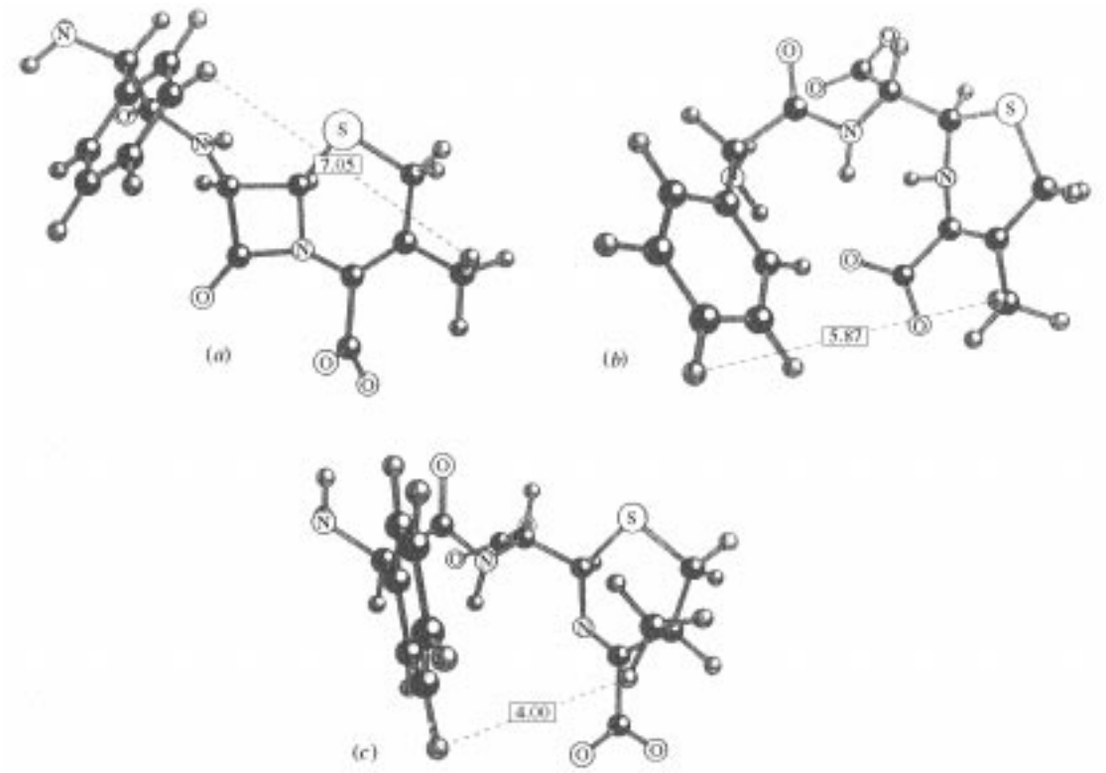


Fig. 4 Conformation of cephalexin (*a*), cephalosporoate compound (*b*) and compound **9** (*c*) in aqueous solution as determined by NMR spectroscopy and conformational calculations

with signals at 4.5–6.0 ppm. These changes detected in the reaction mixture will be discussed later.

In addition, the enzymatic hydrolysis of cephalexin at pD 5.5 was carried out, and the results are coincident with those obtained at pD 6.4.

On the basis of the evolution of the spectrum and according to the identified products, Scheme 2 is proposed for the enzymatic hydrolysis of cephalexin and the further degradation of the products obtained.

According to Scheme 2 cephalexin **4** is catalytically hydrolysed by the P99 enzyme giving rise to the cephalosporoate compound **8**. At pD 5.5 and 6.4 the formation of the cephalosporoate compound is not detected, since the concentration of the protons in the medium prevents the accumulation of the former intermediate. The cephalosporoate compound **8** in aqueous media at low enough pH undergoes a shift in the double bond of the dihydrothiazine ring, from position 3–4 to 4–5, with the consequent uptake of a proton at C(3). Experimentally, it has been observed that the uptake of the proton by the α face (compound **9**) is more favourable than by the β face (compound **10**), indicating that this reaction is controlled by steric constraints. The calculated distance between the methyl and phenyl groups is smaller than 4 Å in compound **9**, whereas in cephalexin this distance is larger than 7 Å. Therefore, at some stage of the enzymatic hydrolysis an approach of the phenyl group towards the methyl group has taken place. The semiempirical calculations indicate that in cephalexin the phenylglycine group lies above the four-membered ring. The existence of two cyclic groups bonded together means approach of the phenyl group towards the six-membered ring within 7 Å is not feasible [see Fig. 4(*a*)]. Nevertheless, in the cephalosporoate compound, once the β-lactam ring is cleaved, free rotation about the C(6)–C(7) bond is easier, allowing in turn a greater proximity of the phenyl group with regard to the methyl group [Fig. 4(*b*)]. In this situation, a possible uptake of a proton at C(3) would preferably occur at the α face of the six-membered ring yielding compound **9** [Fig. 4(*c*)], since the bulky phenyl group is found on the other side of the ring.

Hydrolysis of cephalexin with another serine enzyme, β-lactamase I from *Bacillus cereus* (class A enzyme), was carried out and the same results as those obtained with the P99 enzyme (mainly compound **9** was formed) were observed. It can be deduced that the folding of the aromatic group of the side chain at C(7) over the six-membered ring must occur in the cephalosporoate compound. However, it is not certain from these results whether the cephalosporoate compound is formed folded at the active site of the enzyme or outside the enzyme. On the other hand, the formation of compound **10** is observed as the reaction develops, indicating the existence of a kinetic process which opens the folded cephalosporoate compound, allowing the uptake of the hydrogen by the β face of the dihydrothiazine ring.

In a study on the enzymatic hydrolysis of cephalosporin analogues of cephalexin by Pratt and Faraci[4] the chemical degradation of the cephalosporoate compound was not detected. This fact can be attributed to the experimental conditions since the former study was undertaken at pD 9.4 and at 5 °C, when the uptake of the hydrogen in the cephalosporoate cannot be detected.

Experimentally, the reversibility between compounds **9** and **8** (or between **10** and **8**) has been observed, as dissolving compound **9** (previously isolated from water) in D_2O–NaOD causes both the disappearance of the C(3) methine proton signal and the conversion of the doublet corresponding to the methyl group into a singlet. The above mentioned facts indicate that elimination of the hydrogen at C(3) has taken place, with the consequent uptake of a deuterium at the former position.

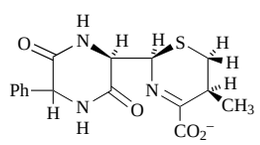
Both compounds **9** and **10** are subject to several reactions depending on the pD value.

At slightly basic pD the disappearance of both the C(6)-H and C(7)-H protons is observed, and therefore the evolution of compound **9** (**10**) should take place *via* abstraction of the proton bonded to C(6)-H yielding intermediate **11** (not detected). This compound could be subjected to a second abstraction of the C(7)-H proton yielding compound **12**. The spectroscopic values of the intermediate detected at long reaction times

(Fig. 1, $t = 375$ min) would be in agreement with this structure, hence corroborating this mechanism. Finally, compound **12** would be in tautomeric equilibrium with compounds **13** and **14**. The latter products detected at pD 8.0 suggest the existence of compounds with the methyl resonances in the range 1.0–1.2 ppm. On the other hand, signals detected in the region 2.6–3.0 indicate that the opening of the six-membered ring has not taken place (AB system with J 13 Hz). Also, the spectrum obtained in water confirmed the presence of a proton at C(3). All these spectroscopic signals are in agreement with the proposed structures (**13** and **14**).

At neutral pD and long reaction time (Fig. 3, $t = 296$ min) the formation of a major compound which is not coincident with that observed at pD 8.0 is detected, which coincides, however, with the one detected at pD 5.15. Under these conditions a cleavage of the six-membered ring may take place, a reaction which has been described by Mobashery and Johnston[8] and by other authors at neutral[12] and acidic[23] pH, yielding structures **15**, which would evolve to compound **16** and other degradation products.[24]

Bundgaard[11] found that at pH 5–8.5, cephalexin degrades 90–100% *via* intramolecular aminolysis, yielding the piperazine-2,5-dione compound **5**. However, this author did not indicate whether one or both isomers at C(3) were present. For this reason the non-enzymatic hydrolysis of cephalexin was performed at pD 6.4. The formation of two compounds **17** and **18**



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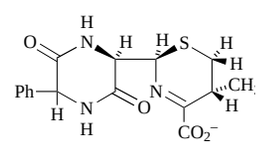

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was observed by ^{1}H NMR (Table 1). Compounds **17** and **18** are regarded as the isomers of the diketopiperazine compound. From a mechanistic point of view, after the opening of the four-membered ring, which in this case is due to an intramolecular attack, a shift in the double bond of the dihydrothiazine ring and the uptake of a proton at C(3) (reaction analogous to that detected in the enzymatic hydrolysis) occurs. However, the uptake of a proton is carried out on both faces with approximately the same probability. This is due to the fact that the diketopiperazine ring formed is in a conformation which does not hinder the uptake of a proton at C(3) by the β face of the six-membered ring, and therefore neither face prevents the approach of protons at C(3).

In summary, cephalosporoates formed in the hydrolysis of cephalosporins not bearing a leaving group at position 3′, undergo tautomerization of the double bond in the dihydrothiazine ring, from position 3–4 to 4–5 in aqueous media, with the consequent uptake of a proton at C(3). The uptake of this proton is governed by steric constraints, as folding of the aromatic ring on the side chain at C(7) over the dihydrothiazine ring has been observed, thus enhancing the uptake of H by the α face of the six-membered ring. In the course of the reaction the cephalosporoate compound is unfolded allowing the uptake of a proton by the β face. Uptake of a proton in cephalosporoates or their analogues is of great importance in other reactions undergone by cephalosporin(penicillin) in clinical use. For instance, it has been observed that the hydrolysis of cefaclor in acidic[24] or neutral[25] media takes place *via* intermediates analogous to structures **9** and **10**.

Experimental

Materials

Cephalexin was a gift from Glaxo. β-Lactamase P99 from *Enterobacter cloacae* was purchased from Speywood Ltd., England, and used as received. The specific activity of the enzyme was 425 mol of cephalosporin C hydrolysed per second per mol of enzyme at pH 8.5 (50 mM TAPS †–NaOH buffer) at 30 °C. Deuterium oxide (99.8% D) was obtained from Sigma. The buffering material was reagent-grade and distilled water was used throughout.

NMR Experiments

^{1}H NMR (300 MHz) and ^{13}C NMR (300 MHz) spectra were recorded on a Bruker AMX-300 spectrometer. A sample tube of 5 mm i.d. was used. Chemical shift values (δ) are quoted in parts per million (ppm) downfield from 3-(trimethylsilyl)-propane-1-sulfonic acid (DSS). Coupling constants, J, are in Hz. pD values were obtained by adding 0.4 to pH meter readings.[26] The multiplicities of carbon resonances were determined by the distortionless enhancement bipolarization transfer (DEPT) method.[27] Pulse sequences and parameters used to collect and process the two-dimensional (2D) NMR data are described below.

Nuclear Overhauser enhancement spectroscopy (NOESY). Phase-sensitive NOESY spectra were acquired using the method of States *et al.*[19] The mixing time was 500 ms. The data consisted of 512 points in t_1 and 2048 points in t_2. The FIDs were multiplied in both dimensions with a sine–bell function and zero-filled once in t_1 before Fourier transform. The phase corrections of the 1D spectrum, acquired using the same experimental conditions, were used for phasing after the first Fourier transform. Interproton distances were evaluated *via* the INTRA method.[18] The method, which was first suggested by Bodenhausen and Ernst,[20] uses the ratio between the cross peak and the diagonal peak and is independent of the initial rate approximation. The correlation time used in the calculation was computed using a known distance (1.77 Å) between the geminal protons of the six-membered ring.

Computational methods

Semi-empirical calculations were carried out using the PM3[28–30] and AM1[31] methods as implemented in the AMPAC 5.0 software package.[32] These methods have previously been used in conformational studies on β-lactam antibiotics.[33] The software was run on a Silicon-Graphics Iris Indigo XZ4000 computer. The input for the cephalexin was chosen in such a way that the phenyl ring was lying above the β-lactam system (on the β face); this structure is the global minimum in the potential energy surface obtained in the conformational analysis of the former ring. Structure **9** was obtained from the previous structure by increasing the N(5)–C(8) distance and adding oxygen at the C(8) position and allowing a further total optimization. From the conformational study carried out in structure **9**, the structure which best agreed with that obtained by the NOESY experiment was chosen, since the structure corresponding to the global minimum presented a slightly stretched side chain at C(7). Nevertheless, the energy of the chosen structure in relation to that of the global minimum was lower by 2.5 kcal mol^{-1}.

Cephalexin degradation

Cephalexin (80 mg) was dissolved in 10 ml of H_2O at 37 °C containing 0.4 M sodium phosphate at pH 7.0 and <1.0 mg of solid P99 β-lactamase was added. ^{1}H NMR spectra were immediately taken after the former addition and subsequently

† TAPS = 3-[tris(hydroxymethyl)methylamino]propane sulfonic acid.

recorded at appropriate intervals. When the desired product was formed the solution was frozen (1 h for compound **9**). Next the solution was lyophilized and the residue dissolved in 1.0 ml of D_2O for analysis. Spectroscopic data for cephalexin and the isolated compound are given below. Assignments were carried out on the basis of reported data.[13,34]

Cephalexin. ^{1}H NMR data are listed in Table 1. δ_C 14.5 (C-3′), 24.3 (C-2), 53.0 (C-6), 53.9. (C-11), 54.6 (C-7), 118.0 (C-4), 123.3 (C-13 and C-14), 125.3 (C-15 and C-16), 125.0 (C-17), 125.1 (C-12), 133.8 (C-3), 170.9 (C-10), 160, 166.1 (4-COO^- and C-8).

Compound 9. ^{1}H NMR data are listed in Table 1. δ_C 19.1 (C-3′), 29.9 (C-3), 31.45 (C-2), 58.8 (C-11), 60.0 (C-6), 63.0 (C-7), 130.5 (C-13 and C-14), 132.0 (C-15 and C-16), 132.6 (C-17), 134.9 (C-12), 172 (C-10), 177.0, 177.1 (4-COO^- and C-8) and 175 (C-4).

Enzymatic reaction

Enzymatic hydrolysis of cephalexin was carried out in the presence of 0.4 M phosphate (pD 6.4 and 8.0) at 37 °C, with a substrate concentration of 0.006 g ml^{-1} and a final enzyme concentration of 6.26×10^{-6} M (pD 6.4) and 1.23×10^{-5} M (pD 8.0). After acquisition of an initial spectrum, a 40 μl solution of P99 was added to 1 ml of the substrate solution. Spectra were then acquired at timed intervals.

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