150. The Degradation Mechanism of an Oral Cephalosporin: Cefaclor

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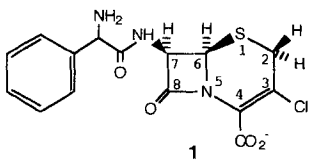
The degradation of cefaclor (**1**), an oral cephalosporin antibiotic, was studied at 37° in a neutral aqueous medium by HPLC and ^{1}H-NMR. Under these conditions, **1** underwent intramolecular aminolysis by the 7-side-chain NH_2 group on the β-lactam moiety to give a piperazine-2,5-dione. The most prominent peak in the HPLC profile of a degradation solution from **1** was isolated by prep. HPLC. Mechanistically, the formation of this degradation product *cis*-**11** from **1** involves the contraction from a six-membered cephem ring to a five-membered ring, which presumably takes place *via* a common episulfonium ion intermediate **9** (see *Scheme*). Loss of the Cl-atom from 3-chloro-3-cephem is a general reaction subsequent to β-lactam ring opening.

Introduction. – Cephalosporins are a major group of β-lactam antibiotics used in clinical medicine. They inhibit the transpeptidases and carboxypeptidases, two types of enzymes involved in the synthesis of bacterial cell walls [1–3]. *Tipper* and *Strominger* believe that the inhibition results from acylation of a serine group in the enzyme [1]. Since acylation of the enzyme is required for antibacterial action to succeed, the reactivity of the β-lactam ring of cephalosporins can be used as a measure of their antibiotic activity. The chemical reactivity of the β-lactam ring has been thoroughly investigated by several authors [4–8] in terms of the cephalosporin substituent in position 3 in the dihydrothiazine ring and of the function present in position 7.

Cefaclor (**1**) is an orally administered semi-synthetic cephalosporin. Its chemical structure is similar to that of cefalexin (**2**). Substitution of the Me group in position 3 of the dihydrothiazine ring in **2** by a Cl-atom gives a compound, **1**, with a potent antibacterial effect, particularly against *Haemophilus influenzae* [9]. Cefaclor (**1**) possesses two ionizing groups with $pK_{a1} = 1.5$ and $pK_{a2} = 7.17$, corresponding to the dissociation of the carboxy and α-amino group, respectively [10].

Nakashima et al. [11] studied the degradation of **1** in an aqueous medium and found that, at neutral pH, the antibiotic acts essentially *via* an intramolecular attack of the NH_2 group in the side chain at C(7) on the β-lactam carbonyl function. They observed the formation of dimers at high cefaclor (**1**) concentrations (above 10 mM) as the result of intermolecular aminolysis. *Dimitrovska et al.* [12] studied the degradation in kinetic terms and found the pH-degradation rate profile to be similar to those for other cephalosporins with related chemical structures. However, neither of these authors characterized the degradation product of **1** in aqueous medium.

Indelicato et al. [13] isolated and identified a diketopiperazine compound **3** in the degradation of the 4-nitrobenzyl (pNB) ester of cefaclor (**1**) in nonaqueous medium



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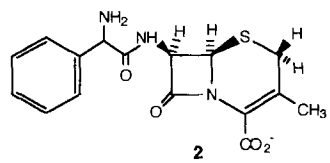

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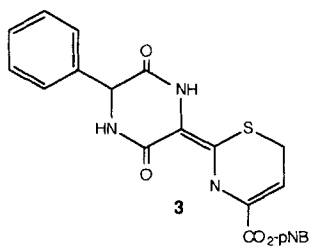

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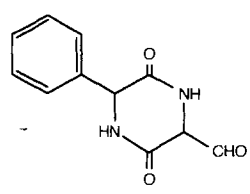

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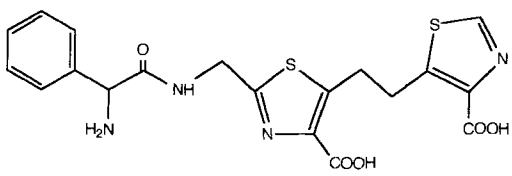

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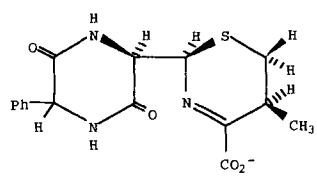

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(refluxing benzene). The elemental analysis of the degradation product revealed the absence of Cl from its formula, thereby suggesting that the loss of the Cl-atom was subsequent to the opening of the β-lactam ring. *Dinner* [14] isolated 3-formyl-3,6-dihydro-6-phenylpyrazine-2,5(1*H*,4*H*)-dione (**4**), one of the degradation products of **1** in acidic medium. Recently, *Baertschi et al.* [15] isolated and identified another degradation product of **1** in acidic medium (0.1N HCl over two months): compound **5**. This compound is formed by condensation of two degradation products of **1** that undergo contraction of their six-membered rings. None of the degradation products of **1** in a neutral medium has been isolated to date; therefore, it does not allow one to put forward a plausible hydrolysis mechanism for this cephalosporin.

In this work, the hydrolysis of cefaclor (**1**) was thoroughly studied. Most of the degradation products of the antibiotic were detected, isolated, and characterized. From the results, a mechanism for the hydrolysis reaction is proposed.

Experimental. – 1. *General.* Cefaclor (**1**) was purchased from *Sigma*, while cefalexin was complimentarily supplied by *Glaxo España*. Both were used without further purification. Deuterium oxide (99.8% D) was obtained from *Sigma*. β-Lactamase I from *Bacillus cereus* was supplied by *Speywood Ltd.*, England, and used as received. The buffering material was reagent-grade, and distilled H_2O was used throughout. HPLC: *Shimadzu-LC-9A* chromatograph equipped with a *Rheodyne-7125* universal injector and a *Shimadzu-SPD-M6A* UV/VIS photodiode array detector; isolation of degradation product *cis*-**11** (see *Fig. 1*) using a *Spherisorb ODS-2* (5 µm) column (25 × 1.0 cm) and 0.1M NH_4OAc/MeCN 96:4 at a flow-rate of 1.3 ml/min, injected volume 200 µl; monitoring of the rate of cefaclor (**1**) degradation by measuring the remaining drug concentration using a *Spherisorb ODS-2* (5 µm) column (25 × 0.46 cm) and 0.1M NH_4OAc/MeCN 97:3 at a flow-rate of 1.1 ml/min, injected volume 50 µl. ^{1}NMR Spectra: *Bruker-AMX-300* spectrometer, sample tube of 5 (^{1}H, ^{13}Cl) or 10 (^{35}Cl) mm i.d.; chemical shifts δ in ppm rel. to 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) as internal reference, coupling constants J in Hz; for

^{1}H, initial concentration of **1** $8 \cdot 10^{-3}$ M in D_2O, at 37°, soln. stabilized at pD 6.4 (pD = $-\log[D^+]$) with phosphate buffer at an ionic strength of 0.5M; ^{1}H assignments based on [7] [16–19]; for ^{13}C, initial concentration of **1** $2 \cdot 10^{-2}$ M in H_2O/D_2O 90:10, at 37°, soln. stabilized at pH 6.4 with phosphate buffer; determination of ^{13}C multiplicities by the distortionless enhancement bipolarization transfer (DEPT) method [20]; ^{13}C,^{1}H correlations from two-dimensional heteronuclear experiments (H,C-COSY) designed to detect correlations due to coupling through one chemical bond [21]; ^{13}C assignments based on [22] [23]; for ^{35}Cl, initial concentration of **1** 10^{-2} M in H_2O/D_2O 90:10, at 37° soln. stabilized at pH 6.4 with phosphate buffer of ionic strength 1.0M; cephalosporin numbering is used for all compounds. Mass spectra: *Fisons (VG) Quattro II SQ* spectrometer, using positive-ion electrospray ionization; scanning of the mass range 50–1200 Da/e within 3 s; mobile phase MeOH/H_2O 1:1 containing 0.1% of HCOOH; source temp. 50°; sample loop volume 10 µl.

2. *Determination of Primary Amino Groups in the Side Chain.* The NH_2 group was quantified by using the trinitrobenzenesulfonic acid assay as implemented by *Satake et al.* [24], with slight modifications. A 100-µl aliquot of the degrading soln. containing **1** at an initial concentration of $3.6 \cdot 10^{-3}$ M was added to 1 ml of 0.2M phosphate buffer at pH 7.9. Then, 1 ml of a 0.2% (*v*/*v*) aq. 2,4,6-trinitrobenzenesulfonic acid soln. was added and the mixture allowed to stand at r.t. in the dark for 35 min, after which the absorbance of the resulting orange soln. was measured at 420 nm against a blank consisting of 100 µl of reaction soln. without **1**, 1 ml of phosphate buffer, and 1 ml of a similarly treated trinitrobenzenesulfonic acid soln. VIS spectral measurements: *Uvikon-940* spectrophotometer, 1.0-cm cell.

3. *Determination of Thiol.* Aliquots (100 µl) of the reaction mixture containing an initial cefaclor (**1**) concentration of $1.7 \cdot 10^{-4}$ M were assayed for thiol by using the method of *Ellman* [25].

4. *Structure Elucidation of the Degradation Products of Cefaclor* (**1**).

4.1. *2-{[(Amino)phenylacetylamino]carboxylatomethyl}-5-chloro-3,6-dihydro-2*H*-1,3-thiazine-4-carboxylate* (**6**). The ^{1}H-NMR (D_2O) recorded during the hydrolysis of cefaclor (**1**) catalyzed by the enzyme β-lactamase I from *Bacillus cereus* at pD 6.4, 37°, and $I = 0.5$M, started to change within 1 min. The reaction yielded intermediate **6** (see below, *Scheme*). ^{1}H-NMR (D_2O, 25°): see *Table 1*; the differences to the ^{1}H-NMR of **1**, *i.e.*, upfield shift of H–C(6) and H–C(7) ($\Delta\delta = \delta(\mathbf{6}) - \delta(\mathbf{1}) = -0.58$ and -1.05, resp., $\Delta J = +1.1$ Hz) and $\Delta J(CH_2(2)) = -0.8$ Hz, suggest that the four-membered ring of **1** was opened and that C(3)=C(4) was preserved. Similar compounds have been detected in the enzyme hydrolysis of other cephalosporins [26] [27].

4.2. *5-Chloro-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylate* (**8**). The hydrolysis of cefaclor (**1**) in neutral D_2O medium (pD 6.4) produced an intermediate D-**8** with the following

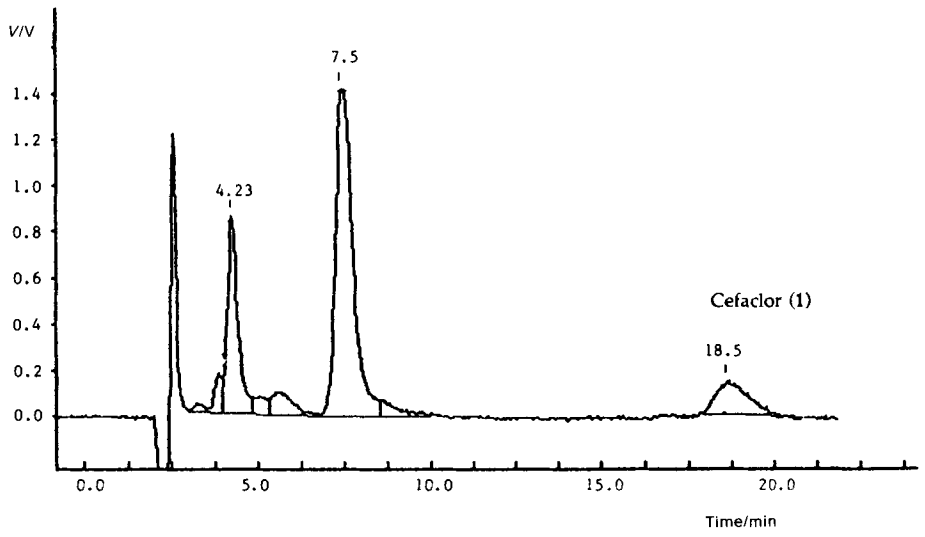


Fig. 1. *High-performance liquid chromatogram of degraded cefaclor* (**1**) *in aqueous solution* (pD 6.4, 37°, λ_{abs} 212 nm). Reaction time 7 h.

Table 1. *^{1}H-NMR Data* (D_2O, 25°) *of the Antibiotics and Their Degradation Products*[a])

	CH_2(2)	H–C(5)	H–C(6)	H–C(7)	PhC*H*	Ph	Other signals
1[b])	3.79 (*d*, *J* = 17.8), 3.35 (*d*, *J* = 17.8)		5.14 (*d*, *J* = 4.3)	5.69 (*d*, *J* = 4.3)	5.18 (*s*)	7.52 (*s*)	
2[b])	3.46 (*d*, *J* = 18.0), 3.03 (*d*, *J* = 18.0)		5.03 (*d*, *J* = 4.2)	5.64 (*d*, *J* = 4.2)	5.20 (*s*)	7.53 (*s*)	1.84 (*s*, Me(3))
6	3.44 (*d*, *J* = 17.0), 3.22 (*d*, *J* = 17.0)		4.56 (*d*, *J* = 5.4)	4.64 (*d*, *J* = 5.4)	5.16 (*s*)	7.52 (*s*)	
8	3.48[c]) (*dd*, *J* = 3.6, 14.5), 3.20[c]) (*dd*, *J* = 4.8, 14.5)		5.54 (*d*, *J* = 2.4)	4.56 (*d*, *J* = 2.4)	5.60 (*s*)	7.50 (*s*)	H–C(3) not determined
trans-**11**		6.28 (*dd*, *J* = 3.0, 5.0)	4.53 (*d*, *J* = 3.0)		5.30 (*s*)	7.50 (*s*)	5.0[d]) (H–C(2)); 3.94 (*dd*, *J* = 4.7, 11.8, 1 H), 3.89 (*dd*, *J* = 3.7, 11.8, 1 H CH_2OH)
cis-**11**[b])		6.31 (*dd*, *J* = 3.0, 2.4)	4.53 (*d*, *J* = 3.0)		5.30 (*s*)	7.50 (*s*)	5.0[d]) (H–C(2)); 3.99 (*dd*, *J* = 4.2, 11.7, 1 H), 3.83 (*dd*, *J* = 6.0, 11.7, 1 H, CH_2OH)
13[b])	3.09[c]) (*dd*, *J* = 4.0, 13.6, H_β), 2.68[c]) (*dd*, *J* = 4.6, 13.6, H_α)		5.36 (*d*, *J* = 2.6)	4.48 (*d*, *J* = 2.6)	5.59 (*s*)	7.50 (*s*)	1.22 (*d*, *J* = 7.0, Me–C(3)); 2.80 (*m*, H–C(3))

[a]) For convenience, the cephalosporin numbering is used (see **1** and *cis*-**11** in the *Scheme*); for systematic names, see *Experimental*.

[b]) ^{1}H-NMR chemical shifts [ppm] of these compounds were obtained from the isolated compounds. All other signals were estimated from the spectra of mixtures.

[c]) In H_2O.

[d]) The signals at 5.0 ppm were obtained from the two-dimensional NMR experiment.

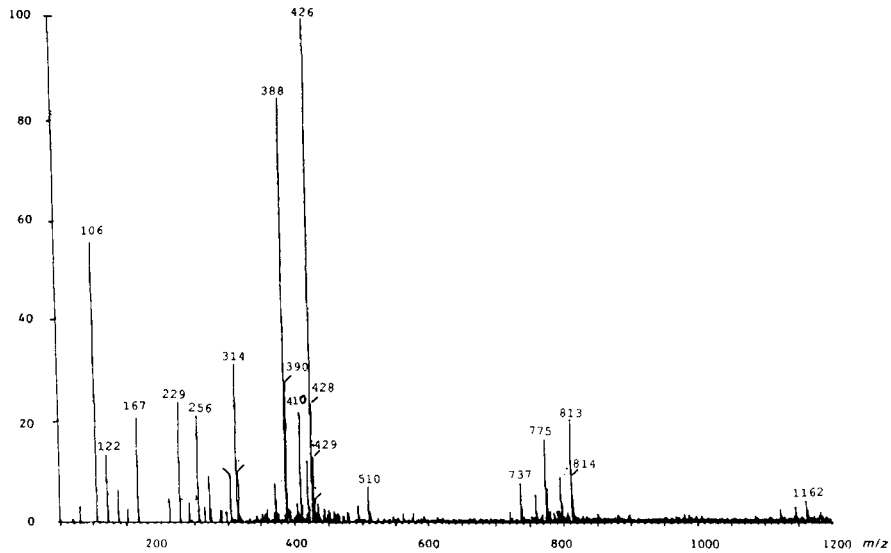


Fig. 2. *ES-MS of* m/z *388, the protonated molecule of* **1**

^{1}H-NMR: 3.20, 3.48 (*AB*, J = 14.5, CH_2(2)); 4.56 (*d*, J = 2.4, H–C(7)); 5.54 (*d*, J = 2.4, H–C(6)); 5.60 (*s*, PhC*H*NH_2); 7.50 (*s*, Ph). In H_2O, **8** was obtained, with a slightly different ^{1}H-NMR: see *Table 1* (*AB* → *ABX*); the *ABX* system reveals uptake of a proton at C(3); $\Delta\delta = \delta(\mathbf{8}) - \delta(\mathbf{1}) = +0.40$ for H–C(6) suggests C(4)=C(5) (**8**) rather than C(3)=C(4) (**1**). Structure **8** is also supported by reported data [14].

In a complementary experiment, cefalexin (**2**) was hydrolyzed in D_2O at pD 6.4; the ^{1}H-NMR data of the isolated degradation product D-**13** were consistent with structure **8**. In H_2O **13** was obtained. ^{1}H-NMR: see *Table 1*; confirmation of the uptake of a proton at C(3) since the *s* of Me–C(3) of D-**13** became a *d* (J = 7.0 Hz) in **13**.

According to the coupling constants (< 5 Hz) observed in the hydrolysis product of cefalexin (**2**) and cefaclor (**1**), the proton uptake at C(3) occurred on the α face leading to an equatorial(eq)/axial(ax) and an eq/eq relationship with the 2 H–C(2) (*cf.* corresponding values for $J_{eq,ax}$ and $J_{eq,eq}$ (= 2–5 Hz) and $J_{ax,ax}$ (= 7–9 Hz) in cyclohexane [28]). Uptake from the β side would lead to an ax/eq and ax/ax relationship.

4.3. cis-*2-(3,6-Dioxo-5-phenylpiperazin-2-yl)-2,5-dihydro-5-(hydroxymethyl)-1,3-thiazole-3-carboxylate* (cis-**11**). In the HPLC (*Fig. 1*) of the degradation mixture obtained from **1** at pD 6.4, a major product appeared at t_R 7.5 min. This HPLC fraction was isolated and lyophilized: *cis*-**11**. UV/VIS: no band at 260 nm, suggesting a structure with an open β-lactam ring. ^{1}H-NMR: see *Table 1*; the signal of H–C(2) was superimposed by the H_2O peak, but determined by 2D-NMR; homonuclear decoupling revealed the ^{1}H,^{1}H-couplings H–C(5)/H–C(6), H–C(5)/H–C(2), CH_2–C(2)/CH_2–C(2), and CH_2–C(2)/H–C(2), H,C-COSY correlated the C- and H-atoms directly bonded to each other (see *Table 2*). ^{13}C-NMR: see *Table 2*; confirmation of 14 C-atoms, quaternary C(3) of the azomethine missing (broad resonance due to a longer relaxation time (nuclear quadrupole coupling and

Table 2. *^{13}C-NMR Data of* cis-**11** *in H_2O/D_2O and Correlation to ^{1}H-NMR Signals* (δ, ppm)

	COO^-	C(7)[a])	C(10)[a])	C_{ipso}	H–C_p	H–C_o	H–C_m	H–C(5)	H–C(2)	CH_2–C(2)	H–C(9)	H–C(6)
^{13}C-NMR	175.9	172.2	169.7	138.2	131.2	131.1[b])	130.1[b])	85.0	65.1	64.9	61.0	60.8
^{1}H-NMR	–	–	–	7.5	7.5	7.5	7.5	6.32	5.0	3.93–3.83	5.28	4.53

[a]) Assignments may be interchanged. [b]) Double intensity.

π-electron polarization)), the pulse interval used in the *Fourier* experiment did not allow enough time for complete relaxation; a DEPT experiment confirmed the attribution of CH_2OH to the signal at 64.9 ppm. ES-MS: see *Fig. 2*; 388 ($C_{15}H_{15}N_3O_5KS^+$, MH^+), 426 (MK^+), 775 (M_2H^+), 813 (M_2K^+); indication of trimeric species at Da/e *ca.* 1150 (these adduct ions are the result of an ES phenomenon and do not indicate the presence of these species in the solid).

Results and Discussion. – Several authors have shown that hydrolysis in neutral or alkaline medium of cephalosporins with a phenylglycine residue in the side chain at C(7) give piperazine-2,5-dione as a result of the intramolecular attack of the NH_2 group bonded to that side chain on the β-lactam carbonyl group [14] [29–31]. This reaction can be selectively studied by monitoring the disappearance of free NH_2 groups by reaction with trinitrobenzenesulfonic acid (TNBS) [23]. *Fig. 3* shows that the disappearance of the NH_2 group from the side chain at C(7) during the degradation of cefaclor (pD 6.4, 37°, $I = 0.5$M) follows a pseudo-first-order kinetics with $k_1 = 3.9 \cdot 10^{-3}$ min^{-1}. We studied the degradation of cefaclor (**1**) by HPLC at the same pD and temperature and obtained a pseudo-first-order kinetic constant $k_2 = 4.0 \cdot 10^{-3}$ min^{-1} (*Fig. 3*). The apparent first-order constants obtained with the two methods are full coincident, which suggests that the degradation of **1** at pD 6.4 is dominated by spontaneous aminolysis. *Bundgaard* [30] found that, at pH 5–8.5, cefalexin (**2**) degrades by 90–100% *via* intramolecular aminolysis; this is consistent with our results for **1**. Our ^{1}H-NMR data led to kinetic constants similar to those obtained by HPLC – by monitoring the signals of H–C(7) (5.7 ppm) and CH_2(2) (3.5 ppm) – which allowed the concentration of unhydrolyzed **1** to be determined (see *Fig. 4*).

Fig. 4 shows the changes in the ^{1}H-NMR spectrum of **1** on degradation: an intermediate with spectroscopic properties suggesting opening of the β-lactam ring is formed. The intermediate evolves to two major products that differ from **1** in a downfield shift in one

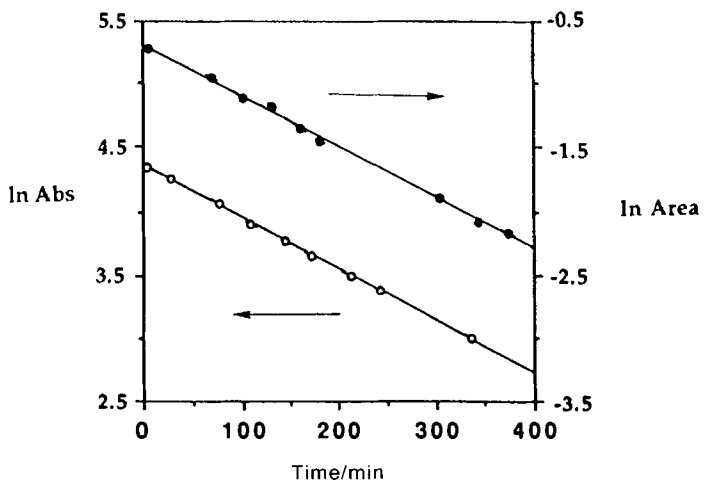


Fig. 3. *First-order plots for* a) *amino-group disappearance* (○) *during the degradation of cefaclor* (**1**) *in 0.4*M *phosphate buffer, pD 6.4, at 37°* (Abs refers to the absorbances produced by subjecting equal aliquots of the reaction solution to the TNBS acid assay) *and* b) *degradation of* **1** (●) *at pD 6.4, 37°, and an ionic strength of 0.5*M (determined by HPLC assay)

of the protons (H–C(6) or H–C(7), 6.4–6.2 ppm) and in decreased coupling constants for the protons at C(2). The hydrolysis in H_2O revealed that the signals for $CH_2(2)$ split and the *d* obtained at 6.3 ppm in D_2O was a twofold *d* here. These products were identified as the isomers **11**.

Indelicato et al. [13] found that, during the formation of the diketopiperazine from cefaclor (**1**), a Cl-atom was released. They suggested that this took place in a reaction subsequent to the cleavage of the β-lactam ring. We recorded the ^{35}Cl-NMR spectrum for our reaction mixture at pD 6.4 at 37°, which exhibited a *s* at 0 ppm corresponding to the Cl-atom in its Cl^- form; this confirms that the Cl-atom is also released in the degradation of **1**. The spectrum also revealed the release of the Cl-atom in the hydrolysis of **1** catalyzed by the enzyme β-lactamase I.

Based on the changes in the ^{1}H-NMR spectrum for cefaclor (**1**) and the compounds identified, the following transformations are proposed to account for the degradation of the antibiotic in neutral medium (see *Scheme*). The NH_2 group in the side chain at C(7) of **1** attacks the β-lactam carbonyl group to give compound **7** (not detected). The carbonyl group might also be attacked by H_2O to form the corresponding cephalosporoate **6**; however, the k_1 and k_2 values obtained reveal that the principal reaction in the nonenzymatic hydrolysis of **1** is intramolecular aminolysis.

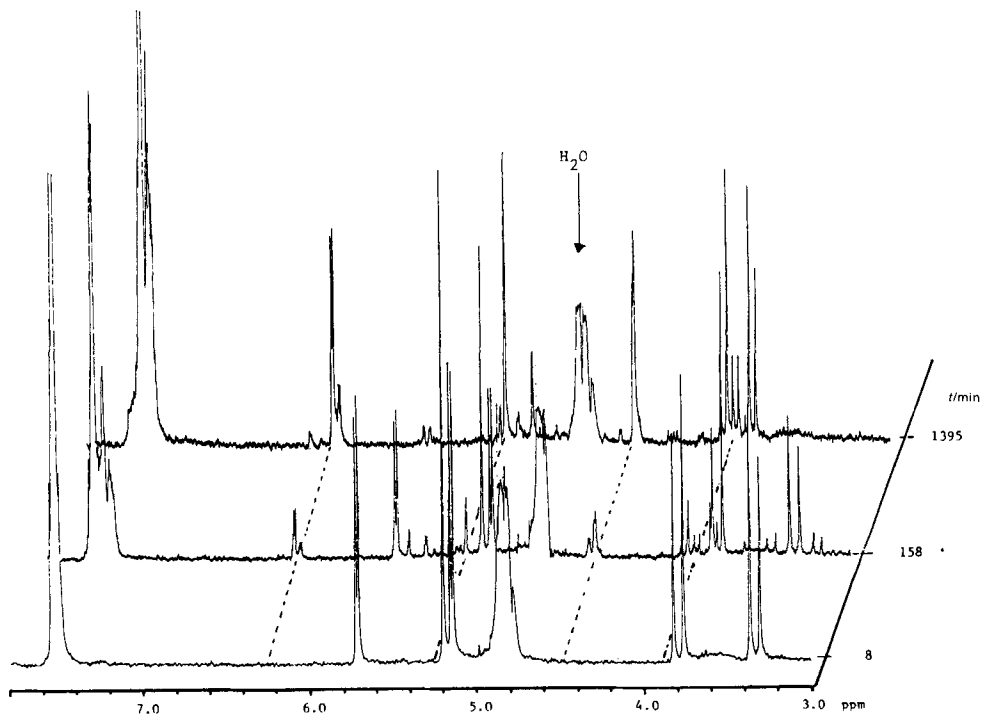
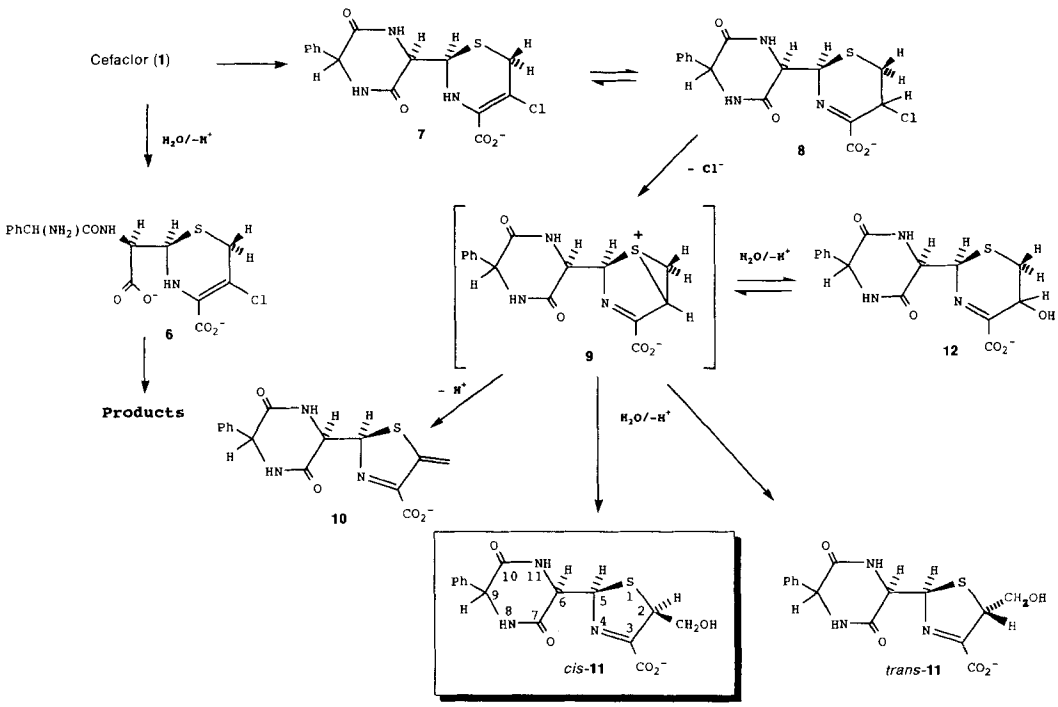


Fig. 4. *Change in intensities of ^{1}H-NMR signals during degradation of cefaclor* (**1**) *in 0.4* M *phosphate buffer solution* (pD 6.4, 37°, I = 0.5M)

Scheme



Compound **7** is in a formal equilibrium with the tautomer **8** (detected by ^{1}H-NMR). The uptake of the proton at C(3) can take place *via* the α or β face of the six-membered ring. Only one isomer was detected, *i.e.*, **8**, suggesting that the uptake of the proton at C(3) depends upon thermodynamic and/or kinetic factors, favoring the formation of one of the isomers.

Subsequently, a 1,3-*trans*-ring attack of the S-atom at C(3) takes place, the Cl-atom being released and an episulfonium ion **9** formed. Contraction from a six- to five-membered ring *via* an episulfonium intermediate was previously observed in the synthetic interconversion of cepham and penam systems [32]. *Baertschi et al.* [15] previously considered the formation of this type of intermediate in the degradation of cefaclor (**1**) in an acidic medium.

Compound **9** can evolve to **10** by releasing a proton. The formation of this compound has been suggested in the hydrolysis of cefaclor in an acid medium [15]; however, we failed to detect it in this work. Alternatively, a H_2O molecule might attack position 3 of the episulfonium ion to form compound **12**, which would be in equilibrium with **9**. This compound could not be detected either, probably because it was produced in amounts below the detection limits of the ^{1}H-NMR technique. Finally, the attack of a H_2O molecule in position 2 of compound **9** would lead to contraction of the six-membered ring and yield compound **11**. In this work, we detected two isomers of **11**. As previously pointed out by *Cooper* and *Spry* [33], the formation of isomeric penams can indicate that the episulfonium ion **9** occurs in two configurations. Based on the spectroscopic data for

the two compounds formed in the hydrolysis of **1**, the peak at t_R 7.5 min (*Fig. 1*) was assigned to *cis*-**11**, whereas that at t_R 4.23 min was ascribed to *trans*-**11**.

Application of the method of *Ellman* [25] to the degradation of **1** in a neutral medium revealed that no degradation product with an open six-membered ring was produced since no free thiol group was detected.

For the elimination of the Cl-atom in the hydrolysis of **1** in benzene, *Indelicato et al.* [13] proposed the opening of the four-membered ring – by attack of a nucleophile on the β-lactam carbonyl group –, followed by elimination of H–C(6) and release of the Cl-atom; subsequently, H–C(7) would be removed. However, in neutral medium, we found H–C(6) and H–C(7) not to be eliminated; therefore, the mechanism given in [13] can just take place in an alkaline medium or in the presence of a base abstracting such protons. To check the mechanism proposed in [13], we carried out the hydrolysis of **1** in an alkaline medium (pD 10.5, 37°, $I = 0.5$M) and found that the Cl-atom was released (^{35}Cl-NMR: *s* at 0 ppm that appeared gradually as the reaction developed) and that the signals of H–C(6) and H–C(7) disappeared as the reaction progressed.

It is interesting to note that the synthesis of cefaclor (**1**) was promoted by the need to increase the chemical reactivity of the four-membered ring in a structure (cefalexin (**2**)) that could be administered orally, also increasing its antibacterial activity. In a neutral medium, **1** reacts *via* intramolecular aminolysis, which makes it scarcely stable in relation to other cephalosporins without an NH_2 group in the side chain in position 7. It should be noted that, after the four-membered ring has been cleaved, the Cl-atom is released from its structure. In neutral medium, the release takes place *via* an episulfonium ion; in alkaline medium, it proceeds *via* elimination reactions. *Blaszczak et al.* [34] compared the chemical reactivity and microbiological activity of 1-carba-1-dethiacephalosporins and cephalosporins and found loracarbef (the carba analogue of **1**) to have similar *MIC* values. From our results it follows that the hydrolysis of loracarbef in neutral medium or under enzymatic catalysis cannot yield compound **11** owing to the absence of the S(1)-atom of the dihydrothiazine ring, responsible for the formation of the episulfonium ion.

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