# 104. Degradation of Cephaloridine on Alkaline Hydrolysis

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A kinetic study on the alkaline hydrolysis of cephaloridine (**1**) at pH 10.5 and 37° was carried out using ion-pair reversed-phase HPLC. The main resulting degradation products, the 7-epimer **2** of **1**, the $\Delta^2$-isomer **3** of **1**, and the 3-methylidene compound **4** were identified. The presence of a pyridinio group at C(3$^1$) results in a slightly increased formation constant for the 3-methylidene compound **4** and the 7-epimer **2**, and introduces a new reaction: the isomerization of the double bond at C(3) in the dihydrothiazine ring to C(2).

**Introduction.** – It was suggested by *Tipper* and *Strominger* [1] that the antibiotic activity of penicillins is based on their ability to acylate, by virtue of their reactive $\beta$-lactam grouping, the transamidases involved in the biosynthesis of the bacterial cell wall [2] [3]. We should note that, even though the biological activity of the $\beta$-lactam antibiotic is most markedly influenced by its ease of binding to the enzyme [4], its chemical reactivity should also be taken into account in order to compare its potential acylation ability. Some authors suggested that the basic hydrolysis constant of the $\beta$-lactam carbonyl group is related to the antibacterial activity of $\beta$-lactam antibiotics [5] [6]. This led some authors to perform kinetic studies on the basic hydrolysis of various cephalosporins.

Cephaloridine (**1**) is a first-generation cephalosporin with a pyridinio group at C(3$^1$) and a thienylacetamido moiety at C(7). It was the only first-generation agent to show significant nephrotoxicity. Subsequently, its clinical usefulness was seriously limited [7]. However, the antibiotic bears the same substituent at C(3$^1$) as do others with clinical uses (*e.g.* ceftazidime), so it would be interesting to investigate the effect of this substituent on the chemical reactivity of the $\beta$-lactam ring.

The earliest kinetic studies on this antibiotic [8] showed it to possess a high chemical reactivity relative to other antibiotics of the first cephalosporin generation (*e.g.* cephalexin, cephalotin, cefazolin). More recent research conducted in our laboratory by using HPLC and $^1$H-NMR on cefotaxime [9] showed the occurrence of another reaction in the basic hydrolysis of this cephalosporin, *viz.* the epimerization at C(7), which cannot be detected by the classical techniques (UV/VIS spectroscopy, iodometric method, *etc.*).

The present study investigates the mechanism of basic-hydrolysis degradation of cephaloridine (**1**) based on kinetic and degradation-product analyses.

**Results and Discussion.** – *Identification of the Degradation Products.* On alkaline hydrolysis of cephaloridine (**1**) at pH 10.5 and $I = 0.1$M, the degradation products **2**–**4**

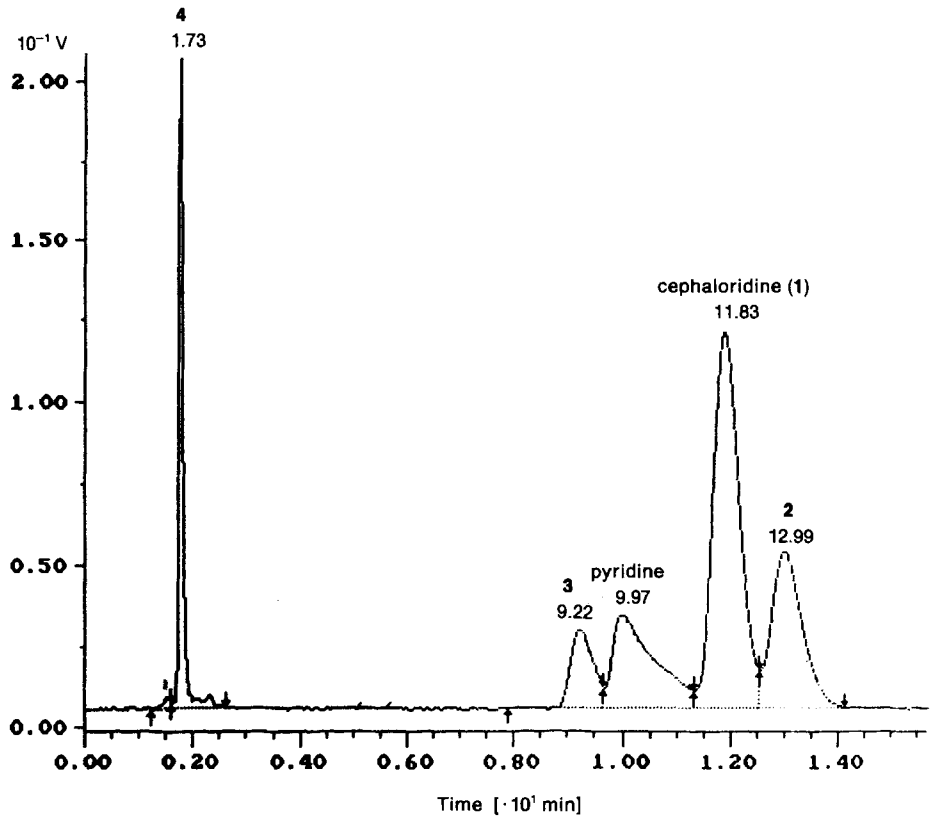



Fig. 1. *High-performance liquid chromatogram* ($\lambda_{abs}$ 260 nm) *of degradation products from cephaloridine* (**1**) *in aqueous solution at pH 10.5* (37°, reaction time 38.9 min)

and pyridine were obtained (see HPLC in *Fig. 1*). Their structures (see *Scheme 1*) were determined using spectroscopic data.

Product **2** ($t_R$ 12.99 min, *Fig. 1*) shows an UV spectrum very similar to that of cephaloridine (**1**; *Fig. 2*), which suggests that it preserves the $\beta$-lactam ring [10]. The $^1$H-NMR spectrum of **2** differs from that of **1** in an upfield shift of the signal of H–C(6) and H–C(7) (from 5.16 to 4.91 and from 5.68 to 4.95, resp.), as well as in a decreased coupling constant ($J$ = 4.7 Hz for **1** and 4.3 Hz for **2**). According to published data [10–12], this suggests that **2** is the 7-epimer of **1**.

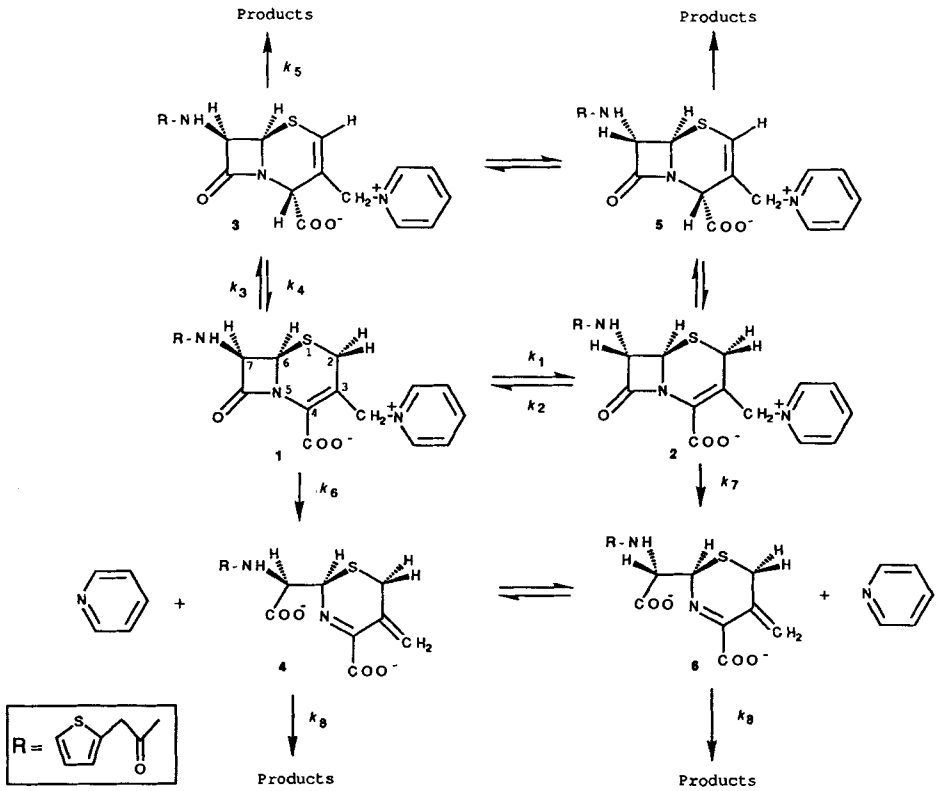
The UV spectrum (*Fig. 2*) of **3** ($t_R$ 9.22 min) reveals that the band in the region of 260 nm is slightly decreased. Comparison of the $^1$H-NMR spectra of **1** and **3** shows that the signals of H–C(6) and H–C(7) of **1** ($\delta$ 5.16 and 5.68, resp.) are shifted to $\delta$ 5.31 in the spectrum of **3**. Instead of the signals of $CH_2$(2) of **1** at $\delta$ 3.18 and $\delta$ 3.60, new signals are seen at $\delta$ 4.57 and 6.81 for **3**.

This is in accord with a C(2)=C(3) bond in **3** instead of the C(3)=C(4) bond in **1** [11].

The UV spectrum (*Fig. 2*) and the $t_R$ (9.97 min) of the pyridine from the degradation are identical with those of a pyridine soln.

The UV absorption at 260 nm of compound **4** ($t_R$ 1.73 min) is considerably decreased (*Fig. 2*) indicating an opened $\beta$-lactam ring [10]. The $^1$H-NMR spectrum shows 2*s*'s typical of olefin protons ($\delta$ 5.65 and 5.60 (2 H, $J$ = 0 Hz)) of an exocyclic double bond and 2*d*'s ($\delta$ 4.62 and 5.42 (2 H, $J$ = 2.5 Hz)) for H–C(6) and H–C(7) of the open ring [10]. The 3-methylidene compound **4** was previously reported to occur as a degradation product in the alkaline hydrolysis of cephalosporins with a good leaving group at position 3$^1$ [10] [13] [14].

*Scheme 1*




*HPLC Study. Kinetic Constants.* In the HPLC, the degradation products of **1** were all well resolved, which allowed for their ready quantification (*Fig. 1*). The areas of the chromatogram peaks were calculated with the aid of a programmable integrator.

The calibration constant of each compound was calculated from $[A]_0 = c_1A_1 + c_2A_2 + c_3A_3 + c_4A_4$, where $A_i$ denote peak areas (obtained at 260 nm and a preset time), $[A]_0$ is the initial antibiotic concentration, and $c_i$ is a constant of proportionality between each area and its matching concentration. Calibration constants were fitted by using a multi-variable nonlinear regression programme based on the *Marquardt* algorithm [15]. The best-fit values were as follows: $c_1 = 5.066E\text{-}8$ (**1**), $c_2 = 4.680E\text{-}8$ (**2**), $c_3 = 9.639E\text{-}8$ (**3**), and $c_4 = 6.540E\text{-}7$ (**4**). Once the different constants $c_i$ had been obtained, the concentration of each product was calculated. Rate constants were determined by using the kinetic data processing software GIT [16] [17], which performs numerical integration of the differential equations corresponding to a given kinetic scheme.

On the basis of the degradation products involved in the chromatogram depicted in *Fig. 1*, we developed the kinetic sequence shown in *Scheme 1*. It assumes that cephaloridine (**1**) can react *via* three pathways in an alkaline medium, namely *via* reversible epimerization at C(7) to yield the 7-epimer **2**, *via* nucleophilic attack of the $OH^-$

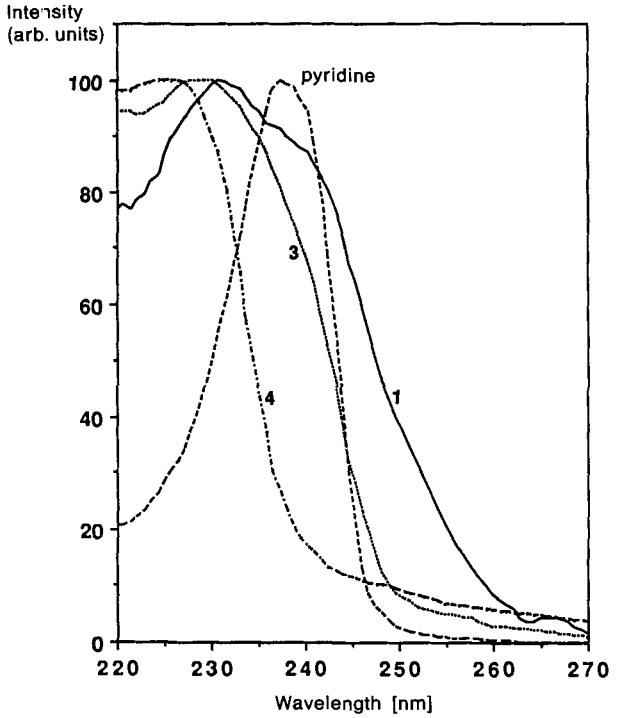



Fig. 2. *UV Spectra of cephaloridine* (**1**; —), its $\Delta^2$*-isomer* **3** (· · · ·), *pyridine* (– – –), *and 3-methylidene compound* **4** (–·–·). The spectra were normalized at the maximum of the spectrum.

ion on the carbonyl group of the β-lactam ring to give the methylidene compound **4** and pyridine, and *via* a reversible base-catalyzed isomerization of the $\Delta^3$-double bond to give $\Delta^2$-cephaloridine (**3**). The isomerization of a $\Delta^3$- to a $\Delta^2$-isomer introduces a new asymmetric center at C(4). However, it was reported that only a single stable isomer is produced [18], namely **3**, in which the $COO^-$ group lies below the dihydrothiazine ring.

The nucleophilic attack on the carbonyl group in the β-lactam ring of the 7-epimer **2** of cephaloridine should give a 3-methylidene compound **6** differing from **4** by its configuration at C(7). However, **6** was not detected due to its low concentration. Therefore, we considered $k_6 = k_7$ and did not calculate the kinetic rate constants of the reversible epimerization at C(7) of **4** and **6**. Chemically, one should also expect the $\Delta^2$-isomer **5** of the 7-epimer **2** by epimerization at C(7) of **3** or isomerization of the double bond in **2**. Inasmuch as the concentrations of compounds **2** and **3** are relatively small, compound **5** must be produced in a very low yield and hence precluded calculation of the constants involved in its formation.

Compound **3** may undergo a nucleophilic attack on the carbonyl group of the β-lactam ring to yield products containing an open ring. The concentration of **4** (**6**) decreased after a fairly long reaction time, thereby suggesting that it underwent subsequent degradation.

The different rate constants obtained were as follows ($h^{-1}$): $k_1 = 0.66 \pm 0.06$, $k_2 = 0.66 \pm 0.06$, $k_3 = 0.19 \pm 0.03$, $k_4 = 0.16 \pm 0.03$, $k_5 = 0.03 \pm 0.01$, $k_6 = 1.5 \pm 0.20$,

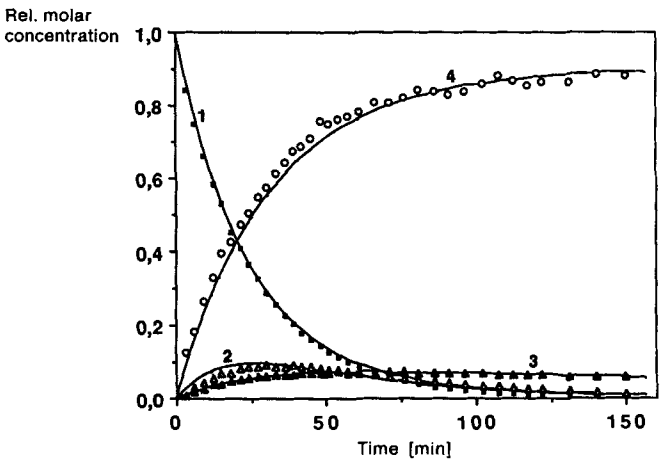



Fig. 3. *Time course of the alkaline degradation of cephaloridine* (**1**) *in carbonate buffer solution.* Points are the HPLC experimental values and continuous lines are the best theoretical fitting; cephaloridine (**1**; ■), 7-epimer **2** (△) $\Delta^2$-isomer **3** (▲), and 3-methylidene compound **4** (○).

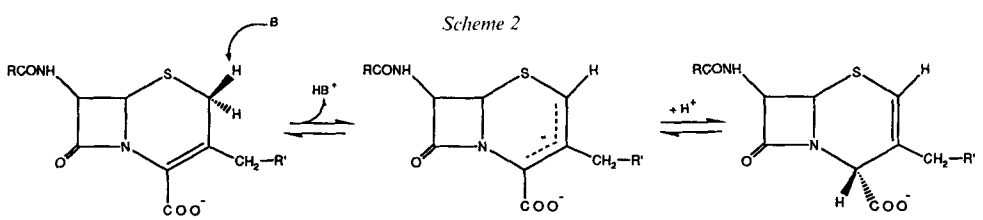
$k_7 = 1.5 \pm 0.20$, and $k_8 = 0.01 \pm 0.005$. *Fig. 3* shows the fitting of the experimental data to *Scheme 1* by using these rate-constant values.

Interestingly, the concentration of cephaloridine (**1**) and of its 7-epimer **2** coincided after a given time, which confirms that the epimerization is reversible with similar rate constants and equilibrium is definitely reached under the hydrolysis conditions used.

The kinetic constant of epimerization of cephaloridine (**1**) is slightly higher than that obtained for cefotaxime [9] (0.27 $h^{-1}$), a cephalosporin with an Ac group at C(3¹). This difference can be ascribed to the presence of the pyridinio group, which must stabilize the reaction intermediate involved in the epimerization at C(7). Even though some authors suggest the epimerization at C(6) in penicillins [19] [20] and at C(7) in cephalosporins [9] [12] [21], no such reaction had so far been reported in connection with **1**.

In studying the double-bond isomerization of cephalosporin esters, *Saab et al.* [22] obtained $k_3$ and $k_4$ values of 0.69 $h^{-1}$ and 0.32 $h^{-1}$, respectively, for the methyl ester of cefazolin at pH 7.4, 40°, and an ionic strength of 0.3M. These values are much greater than those obtained for **1**. Several autors [22] [23] suggested that the presence of a fully ionized carboxylate group prevents isomerization because when the carboxyl group is unesterified, the base-catalyzed attack at position 2 is inhibited (*Scheme 2*). However, the presence of a pyridinio group at C(3¹) in **1** may diminish the negative charge and hence favor the isomerization of the double bond.

*Scheme 2*



On the other hand, *Morin et al.* [18] suggested that the presence of a large substituent at C(3) most likely favors the $\Delta^2$-isomer in order to minimize the steric repulsion of two *cis*-arranged substituents on the double bond.

We detected the $\Delta^2$-isomer of ceftazidime [24], a cephalosporin with a pyridinio group at C($3^1$) and, recently, *Saikawa et al.* [25] and *Sugioka et al.* [12] reported the occurrence of this isomer in other cephalosporins containing an unesterified carboxyl group. Therefore, those cephalosporins which bear a pyridinio or related group at C($3^1$) need not to have their carboxyl group esterified to allow isomerization of the double bond in the dihydrothiazine ring.

*Yamana et al.* [8] obtained a hydrolysis constant value of $1 \cdot 10\ h^{-1}$ for cephaloridine (**1**) at pH 10.0. Such a value would correspond essentially to $k_6$ in the kinetic scheme since the study was carried out by using UV spectroscopy, the only reaction liable to decrease the band at 260 nm would be the formation of the 3-methylidene compound.

In summary, the basic hydrolysis of cephaloridine (**1**) proceeds primarily *via* the attack of the $OH^-$ ion on the $\beta$-lactam carbonyl group, which cleaves the ring, and the subsequent release of the pyridine group. We should note that this reaction possesses quite a large kinetic constant relative to other first-generation cephalosporins, in fact, the rate of cleavage of the $\beta$-lactam ring depends chiefly on the electron-withdrawing character of the $3^1$-substituent.

The epimerization at C(7) was observed in some cephalosporins but had not since been reported in connection with the basic hydrolysis of **1**. Another very important reaction is the isomerization of the double bond from position 3 to 2. This last reaction has been widely documented for cephalosporins containing an esterified carboxyl group at C(4), which endows them with oral bioavailability. However, there are few reports on cephalosporins with an unesterified carboxyl group.

Finally, we should emphasize that, even though the biological activity of the $\beta$-lactam antibiotic is primarily dictated by the goodness of fit with the enzyme [4], the chemical reactivity is another influential factor to be considered. We found that the presence of a pyridinio group at position $3^1$ increases the rate constants of basic hydrolysis and epimerization at C(7); in addition, it gives rise to the $\Delta^2$-isomer.

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## Experimental Part

*General.* Cephaloridine (**1**) was obtained from *Sigma* and used without further purification. $D_2O$ (99.8% D) was purchased from *Sigma*. High-performance liquid chromatography (HPLC): *Shimadzu-LC-9A* chromatograph with a *Rheodyne* model *7125* universal injector and *Waters-510* chromatograph a *Waters M-712* autoinjector; the former was equipped with an UV/VIS photodiode array detector (*Shimadzu SPD-M6A*) which allowed to record the UV spectra of the degradation products, the latter with a programmable multiwavelength detector (*Waters M-490*) set at 260 nm; *Spherisorb-ODS-2* column (10 µm 250 × 0.46 cm); eluent, MeCN/0.01M $NH_4OAc$ 12:88 (pH 6.50); flow rate, 1.40 ml/min; injection volume, 50 µl.

*Basic Degradation of Cephaloridine* (**1**) *for HPLC Analysis.* The concentration of **1** was $4.85 \cdot 10^{-4}$ M and the soln. maintained at 37° and stabilized at pH 10.5 by a carbonate-buffer soln. (ionic strength 0.1M). NMR Spectra: *Bruker-AMX-300* spectrometer; sample tube diameter, 5 mm; 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) as internal reference; chemical shift values ($\delta$) in ppm.

*Isolation of the Degradation Products from* **1**. A soln. of 35 mg of **1** in 2 ml of buffer of pH 10.5 and ionic strength 1.0M was kept at 37° for 1 h and then frozen. At a subsequent stage, the different products present in soln. were isolated by liquid chromatography. The eluates were lyophilized and the residues dissolved in 0.6 ml of $D_2O$ for analysis.

*Cephaloridine* (= *(6R,7R)-8-Oxo-3-[(pyridinio)methyl]-7-[(thien-2-yl)acetylamino]-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate*; **1**). $^1$H-NMR (300 MHz): 3.18, 3.60 (*AB*, $J$ = 17.0, $CH_2$(2)); 3.90 (*m*, thienyl-$CH_2$–CO); 5.16 (*d*, $J$ = 4.6, H–C(6)); 5.35, 5.54 (*AB*, $J$ = 14.0, $CH_2$–C(3)); 5.68 (*d*, $J$ = 4.8, H–C(7)); 7.02 (*d*, $J$ = 3.1, 2 H, thienyl); 7.35 (*m*, 1 H, thienyl); 8.10 (*m*, 2 H, pyridinio); 8.57 (*m*, 1 H, pyridinio); 8.94 (*d*, $J$ = 6.0, 2 H, pyridinio).

*7-Epicephaloridine* (= *(6R,7S)-8-Oxo-3-[(pyridinio)methyl]-7-[(thien-2-yl)acetylamino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate*; **2**). $^1$H-NMR (300 MHz): 3.14, 3.59 (*AB*, $J$ = 18.7, $CH_2$(2)); 3.91 (*m*, thienyl-$CH_2$–CO); 4.91 (*d*, $J$ = 4.3, H–C(6)); 4.95 (*d*, $J$ = 4.3, H–C(7)); 5.28, 5.41 (*AB*, $J$ = 14.0, $CH_2$–C(3)); 7.04 (*d*, $J$ = 5.1, 2 H, thienyl); 7.39 (*m*, 1 H, thienyl); 8.10 (*m*, 2 H, pyridinio); 8.57 (*m*, 1 H, pyridinio); 8.92 (*d*, $J$ = 5.3, 2 H, pyridinio).

*$\Delta^2$-Cephaloridine* (= *(6R,7R)-8-Oxo-3-[(pyridinio)methyl]-7-[(thien-2-yl)acetylamino]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate*; **3**). $^1$H-NMR (300 MHz): 3.92 (*m*, 2 H, thienyl-$CH_2$–CO); 4.57 (*s*, H–C(4)); 5.31 (*s*, H–C(6), H–C(7)); 5.34, 5.51 (*AB*, $J$ = 14.9, $CH_2$–C(3)); 6.81 (*s*, H–C(2)); 7.05 (*m*, 2 H, thienyl); 7.38 (*m*, 1 H, thienyl); 8.08 (*m*, 2 H, pyridinio); 8.58 (*m*, 1 H, pyridinio); 8.86 (*d*, $J$ = 6.2, 2 H, pyridinio).

*(2R)-2-{(R)-Carboxylato[(thien-2-yl)acetylamino]methyl}-5,6-dihydro-5-methylidene-1,3-thiazine-4-carboxylate* (**4**). $^1$H-NMR (300 MHz; numbering as for **1**): 3.38, 3.67 (*AB*, $J$ = 14.0 Hz, $CH_2$–C(2)); 3.88 (*m*, 2 H, thienyl-$CH_2$–CO); 4.62 (*d*, $J$ = 2.96, H–C(6)); 5.42 (*d*, $J$ = 2.40, H–C(7)); 5.60, 5.65 (2*s*, $J$ = 0, $CH_2$=C(3)); 7.01 (*m*, 2 H, thienyl); 7.33 (*m*, 1 H, thienyl).

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