# 98. High Performance Liquid Chromatography (HPLC) of Natural Products Part VI[1])

## Sulfoxides of Penicillin N and Cephalosporin C and their Role in Biosynthesis of β-Lactam Antibiotics

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*Summary*

Pure penicillin N α-sulfoxide (**1**) and penicillin N β-sulfoxide (**2**) were obtained by HPLC and tested as substrates for deacetoxycephalosporin C synthetase (DXCS). Neither one of the sulfoxides was utilized under conditions of conversion of penicillin N (**8**) to deacetoxycephalosporin C (**9**). The cephalosporin C α- and β-sulfoxides (**3** and **4**, resp.) were also prepared. Relative stabilities of the sulfoxides **3** and **4** are discussed by interpretation of the $^{13}$C-NMR spectra.

In a series of publications from our laboratories we have shown a convenient way to follow the isolation and identification of different metabolites from the fermentation broth using HPLC [2]. We have applied a similar technique to the study of cell-free biosynthesis of β-lactam antibiotics [1].

A penicillin N sulfoxide had been first suggested by *Abraham & Newton* (1965) [3] and later by *Troonen et al.* (1976) [4] as a possible intermediate in the conversion of penicillin N (**8**) to deacetoxycephalosporin C (**9**). These assumptions were made on the basis of analogy with the nonbiological conversion of penicillin to cephalosporins first described by *Morin et al.* (1963) [5]. *Baldwin et al.* (1980) [6] mentioned, however, that there was no evidence of cephalosporin formation when β-sulfoxide **2** or **5** of penicillin N and isopenicillin N, respectively, were added to protoplast lysates prepared from *Cephalosporium* unlike in the case where synthetic samples of (L-2-amino-6-adipyl)-L-cysteinyl-D-valine (**6**), isopenicillin N (**7**), or penicillin N (**8**) were added as precursors of the formation of deacetoxycephalosporin C (**9**).

One of us [7] has suggested that biosynthesis of penicillin N (**8**), deacetoxycephalosporin C(**9**), and closely related substances could possibly be explained as proceeding

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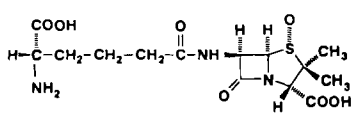

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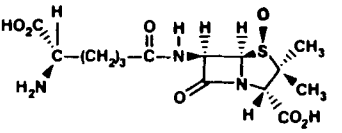

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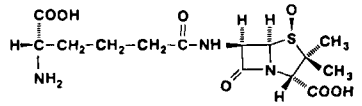

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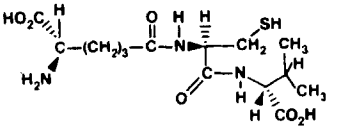

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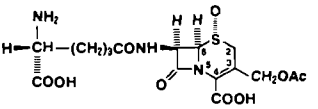

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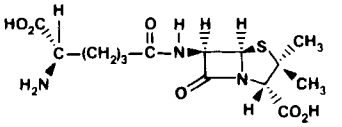

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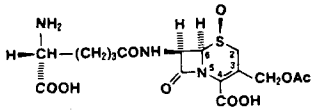

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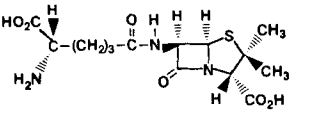

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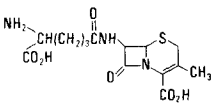

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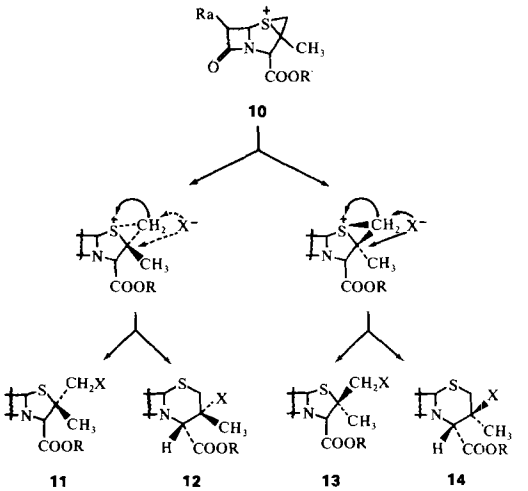
*via* a common thiiranium ion (**10**) (Ra = (2-amino-6-adipyl)amino, R = H; see *Scheme*). The formation of the final products most likely depends upon conditions in fermentation processes. In the course of our studies of the biosynthesis of $\beta$-lactam antibiotics, we decided to search for compounds of this general type in fermentation broths of both *Penicillium* and *Cephalosporium*.

So far we have found only the intermediate **12** (R = H, X = OH, see *Scheme*). However, this compound is a shunt metabolite and not a precursor. Hydroxypenicillins and cephalosporins are inherently unstable. Their sulfoxides, however, show an improved stability [8] as shown first by *Cooper* [9] in the case of penicillins. Therefore, we decided to oxidize the fermentation broths of penicillin and cephalosporin, hoping to transform these unstable metabolites into the corresponding, more stable sulfoxides. The hope was to examine the oxidized reaction mixture by HPLC and to look for sulfoxides of hydroxypenams **11** and **13** (Ra = (2-amino-6-adipyl)amino, R = H, X = OH) and of hydroxycephams **12** and **14** (see *Scheme*).

In order to ascertain the identity of such compounds, model $\alpha$- and $\beta$-sulfoxides of penicillin N and cephalosporin C had to be prepared for determination of their reten-

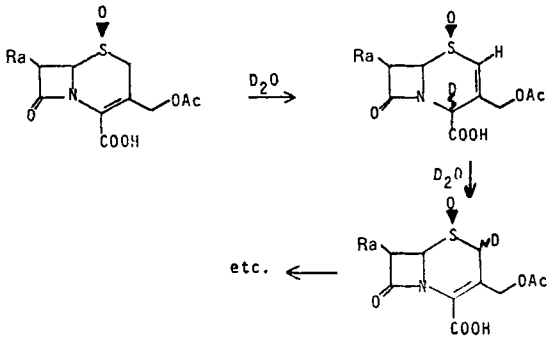
Scheme. *Possible Formation of Hydroxypenams* **11** *and* **13** *and Hydroxycephams* **12** *and* **14** *from a Hypothetical Thiiranium Ion* **10** *(X = OH)*




tion times (see *Exper. Part*). Repeated examination of the oxidized broth by HPLC in a variety of solvent systems did not reveal the presence of compounds others than penicillin N $\alpha$- and $\beta$-sulfoxides (**1** and **2**, resp.) and cephalosporin C $\alpha$- and $\beta$-sulfoxides (**3** and **4**, resp.).

The availability of penicillin N $\alpha$- and $\beta$-sulfoxides prompted us to recheck the suitability of the former as a substrate for deacetoxycephalosporin C synthetase (DXCS). As already shown by *Baldwin* [6], the $\beta$-sulfoxide **2** was not a substrate for the enzyme. In addition, we tested the suitability of the $\alpha$-sulfoxide **1** as a precursor of cephalosporin in the identical cell-free system. Here again the outcome was negative. We, therefore, rechecked the quality of the enzyme extract in the ring enlargement experiment using penicillin N (**8**); the formation of deacetoxycephalosporin C (**9**) proceeded with usual efficiency (see *Fig. 1* in the *Exper. Section*).

The $^{13}$C-NMR spectra of cephalosporin C $\alpha$- and $\beta$-sulfoxides (**3** and **4**, resp.) at 20 MHz (see *Fig. 7* and *9*, resp., in the *Exper. Part*) failed to show resonances for all of the C-atoms. When the compounds were subjected to examination by a 62.9-MHz instrument, a fully assigned spectrum could be obtained. The $D_2O$ solution of the $\alpha$-sulfoxide **3** exhibited an almost identical $^{13}$C-NMR spectrum at 20 and 62.9 MHz. However, when the stale $D_2O$ solution of the $\beta$-sulfoxide **4** was examined at 62.9 MHz it was apparent that the sample had undergone chemical change: The C(2) resonance was absent and a new peak occurred at 73.8 ppm, a position virtually identical with the chemical shift of C(6) of the $\alpha$-sulfoxide **3**. This suggests epimerization of **4** to **3**. Some other peaks indicated that other unidentified species had been formed. When a fresh solution of the $\beta$-sulfoxide **4** was prepared, it was found that the C(2) resonance disappeared within 5–8 hours. These changes suggest that in the case of the $\beta$-sulfoxide **4** there is a rapid tautomeric exchange of the C(2) protons for deuterium. $^2$H-NMR




Ra = (2-amino-6-adipyl)amino.

spectra of both the stale and fresh $D_2O$ solutions of **4** indicated the presence of vinyl deuterium in the state solution. This rapid tautomeric exchange of the C(2) protons is illustrated above. There is no indication for such a change taking place in the $\alpha$-sulfoxide **3**.

We thank *J. DeHoniesto* and *J. W. Paschal,* for their help with the $^{13}$C-NMR spectra and *T. K. Elzey* with the $^{1}$H-spectra.

## Experimental Part

1. *General Remarks.* $^{2}$H-NMR spectra were recorded using a *Bruker WH* 360 MHz NMR spectrometer. $^{13}$C-NMR spectra were recorded using a 20 MHz *Varian FT 80A* spectrometer.

2. *HPLC Conditions.* All chromatograms were obtained using a *M6000A* pump, *U6K* septumless injector, differential refractometer *R401,* all from *Waters Associates,* Milford, Massachusetts. In addition, a *Fisher Omniscribe* strip chart recorder (*Fisher Scientific,* Cincinnati, Ohio) was used. Spectrally pure solvents were obtained from *Burdick E. Jackson,* Muskegon, Michigan, or *J. T. Baker.* Only deionized and glass-distilled $H_2O$ was used. Conditions for HPLC chromatography of different compounds are described in *Fig. 1* and *2.*

3. *Preparation of Partially Purified DXCS.* Partially purified DXCS was obtained in this series from *C. acremonium* strain *92G-AD-11* derived from strain *M8650.* Strain *92G-AD-11* produces cephalosporin C at a yield several fold greater than strains previously employed. Cells were grown and extracted as described before [2].

4. *Cell-free Biosynthesis of Deacetoxycephalosporin C* (**9**) *from Penicillin N* (**8**). Each reaction mixture contained the final concentrations of these ingredients: 0.04 mM $FeSO_4 \cdot 7H_2O$ (120 µl), 0.67 mM ascorbic acid (120 µl), 0.83 mM ATP (120 µl), 0.20 mM hydrogen Z 2-oxoglutarate (α-K6; 120 µl), 1 mg/ml of substrate (120 µl), 2.5 ml of partially purified enzyme. Ingredients were added in the order described above. Immediately after addition of enzyme, the pH of the mixture was checked and a few µl of 0.5M tris-HCl ( = tris (hydroxymethyl)methylammonium chloride; pH 8.5) were added to bring the pH exactly to pH 7.2. Total volume of the mixture was 3.1 ml. The reactions were then incubated at 25° and 250 r/min in a 10-ml flask. Samples were withdrawn at times indicated in *Fig. 1;* each sample was analyzed for **9** by HPLC as previously described [1].

5. *Preparation, Isolation, and Characterization of α- and β-Sulfoxides.* 5.1. *Penicillin N Sulfoxides* (**1** *and* **2**). 5.1.1. α-*Sulfoxide* **1**. A solution of 100 mg of freshly prepared penicillin N (**8**) in 5 ml of $H_2O$/acetone 3:2 was treated with 100 mg of oxone (potassium peroxymonosulfate, *Aldrich 898*) and stirred at r.t. for 45 min. After lyophilization, the crude product was shown by $^{1}$H-NMR to be a mixture **1**/**2** of 3:1. The isomers were first separated by HPLC as shown in *Fig. 2* and *3;* after lyophilization, the final purification could be achieved by additional prep. HPLC. In both cases, we used *µ-Bondapak C-18 (Waters)* on a 4 × 300-mm column with pyridine AcOH/$H_2O$ 0.4:0.4:99.2 as a mobile phase; index of refraction was used for detection. The purity of **1** was checked by $^{1}$H-NMR (360 MHz): see *Fig. 4.*

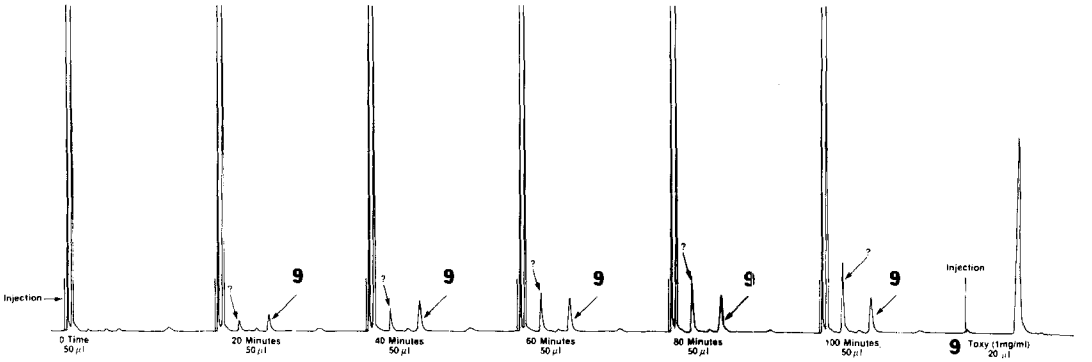



Fig. 1. *Biosynthetic Formation of Deacetoxycephalosporin C* (**9**) *from Penicillin N* (**8**) *as Shown by HPLC.* UV range 254 nm (0.2); chart speed 5 mm/min; flow rate 4 ml/min; column size 4 × 150 mm; packing: *DuPont Zorbax Microamine;* p.s.i. 2700; solvent: AcOH/MeOH/MeCN/$H_2O$ 2:4:7.5:86.5. For the reaction mixture, see text.

5.1.2. *β-Sulfoxide* **2**. To a solution of fresh **8** (100 mg) in 2.0 ml of $H_2O$ were added 50 mg of sodium periodate. The pH was adjusted to 5.1–5.5 with phosphate buffer (pH 7) from the initial pH value of 3.5. The solution was kept at r.t. for 45 min and lyophilized. The $^1$H-NMR of the crude product in $D_2O$ indicated it to be mostly **2**. The compound was further purified by HPLC (*Fig. 2* and *3*). Its purity was checked by $^1$H-NMR (360 MHz): see *Fig. 5*.

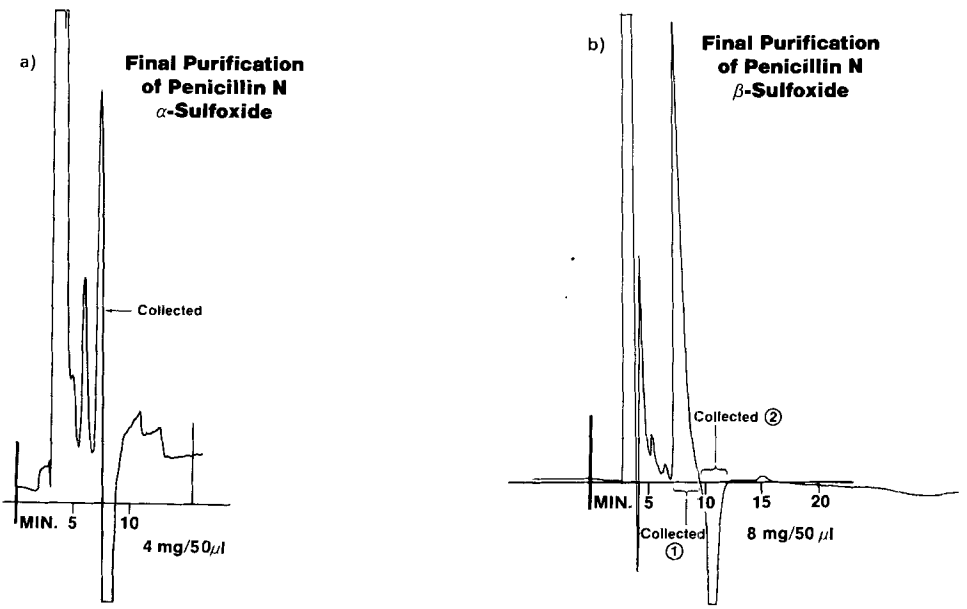



Fig. 2. *Final Purification of Penicillin N α-Sulfoxide* (**1**) *and Penicillin N β-Sulfoxide* (**2**) *by HPLC.* a) Attenuation $\Delta$RI (16×); chart speed 5 mm/min; flow rate 1 ml/min; column size 4 × 300 mm; packing: *μ-Bondapak C-18;* solvent: Py/AcOH/$H_2O$ 0.4:0.4:99.2; p.s.i. 800. b) Except for the attenuation $\Delta$RI (32×), the same conditions as in *Fig. 2a* were used.

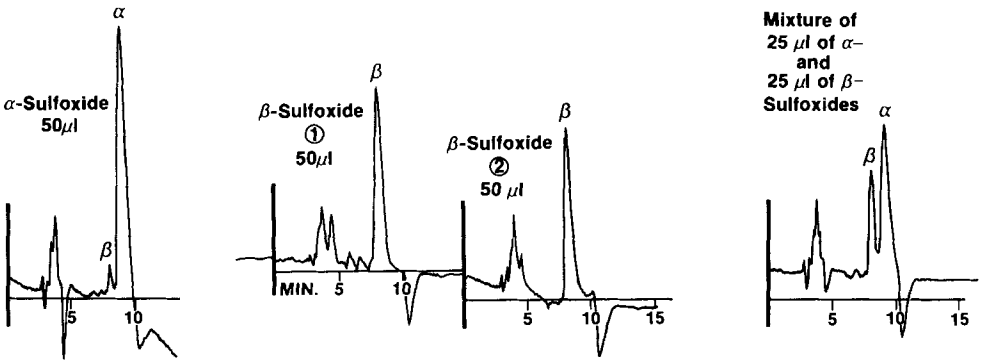



Fig. 3. *Comparison of Penicillin N α- and β-Sulfoxides* (**1** and **2**, resp.) *on HPLC.* Except for the attenuation $\Delta$RI (8×), the same conditions as in *Fig. 2a* were used.

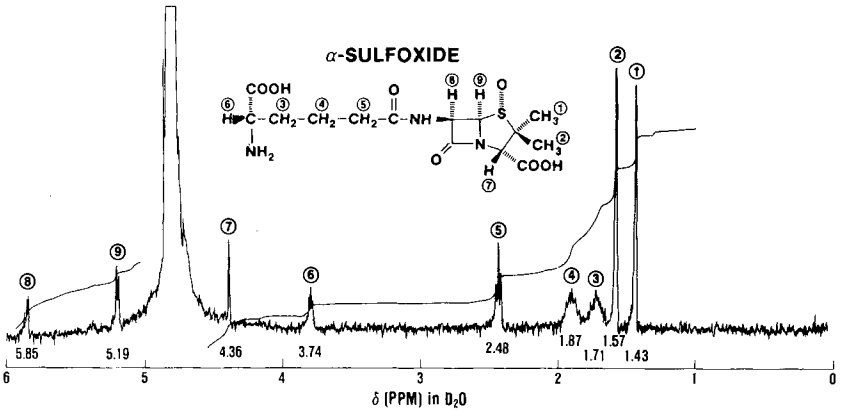



Fig. 4. *$^1$H-NMR (360 MHz) of Penicillin N α-Sulfoxide* (**1**) *in $D_2O$*

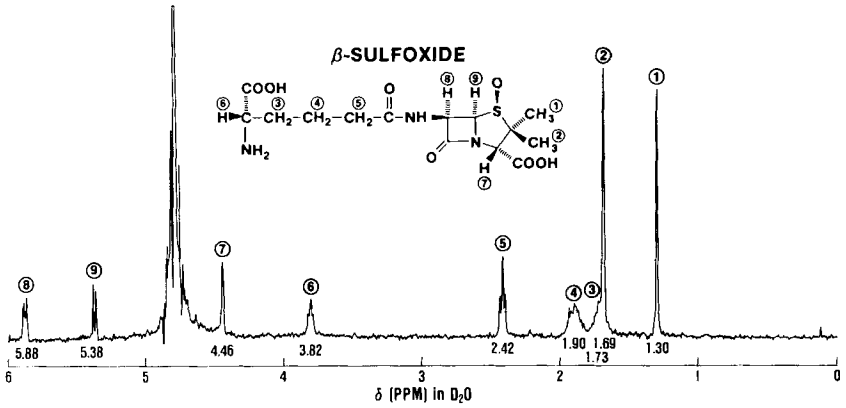



Fig. 5. *$^1$H-NMR (360 MHz) of Penicillin N β-Sulfoxide* (**2**) in $D_2O$

5.2. *Sulfoxides* **3** *and* **4** *of Cephalosporin C.* To a solution of 940 mg (4.4 mmol) of sodium periodate in 6.0 ml of $H_2O$ was added a solution of 1890 mg (4 mmol) of cephalosporin C in 12.0 ml of $H_2O$. The pH was 5.1. After the temp. rose to 29°, the mixture was lyophilized and subjected to prep. HPLC (*Waters Associates Prep LC/System 500* with *PrepPak 500/C$_{18}$;* isocratic solvent system containing $H_2O$/MeOH HCOOH 99:0.5:0.5). A total of 40 100-ml fractions were collected at 100 ml/min. Fractions were lyophilized individually and combined after recording of the $^1$H-NMR. Fractions 12–17 weighed 228 mg and represented *cephalosporin C α-sulfoxide* (**3**). Fractions 20–34 weighed 832 mg and represented *cephalosporin C β-sulfoxide* (**4**), **3**: For $^1$H-NMR and $^{13}$C-NMR; see *Fig. 6* and *7*, resp. **4**: For $^1$H-NMR and $^{13}$C-NMR: see *Fig.* 8 and *9*, resp.

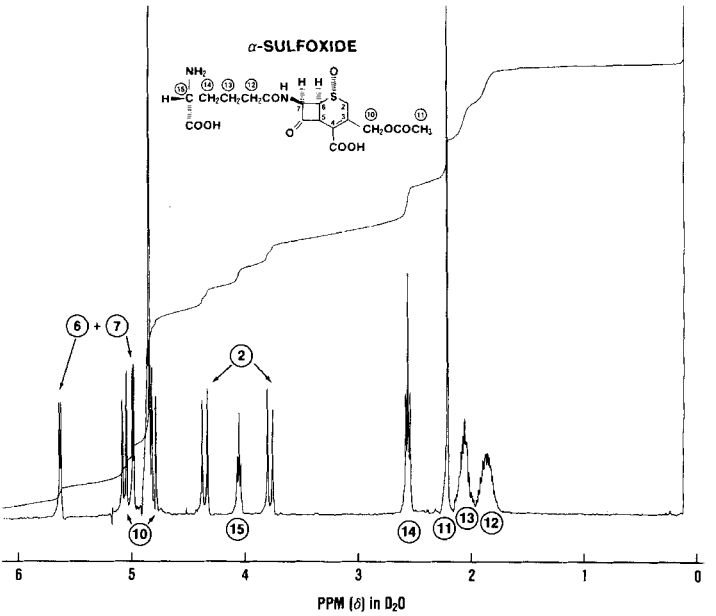



Fig. 6. *$^1$H-NMR of Cephalosporin C α-Sulfoxide* (**3**) *in $D_2O$*

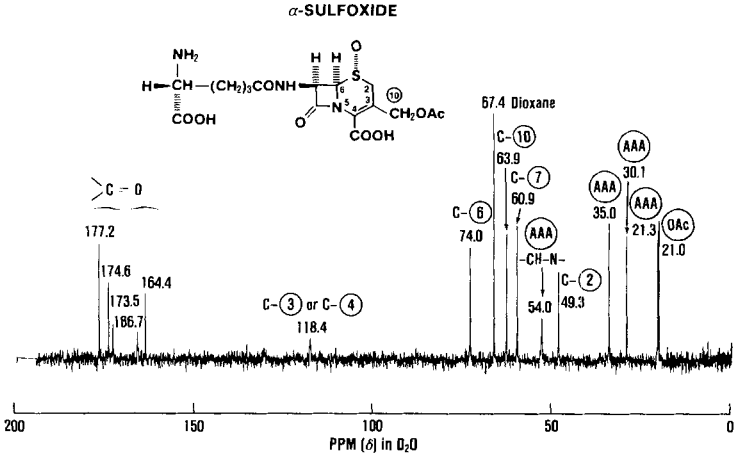



Fig. 7. *$^{13}$C-NMR (20 MHz) of Cephalosporin C α-Sulfoxide* (**3**) *in $D_2O$.* AAA = Signals of the (2-amino-6-adipyl)amino moiety.

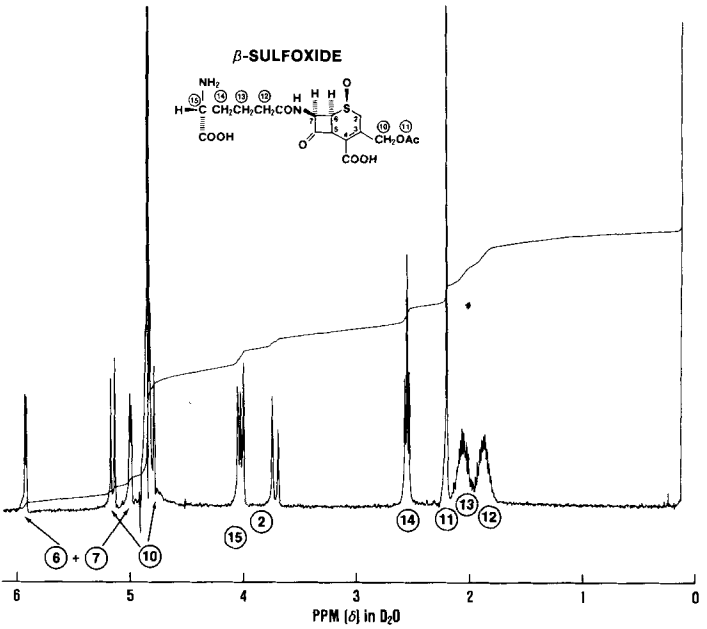



Fig. 8. *$^1$H-NMR (360 MHz) of Cephalosporin C β-Sulfoxide* (**4**) *in $D_2O$*

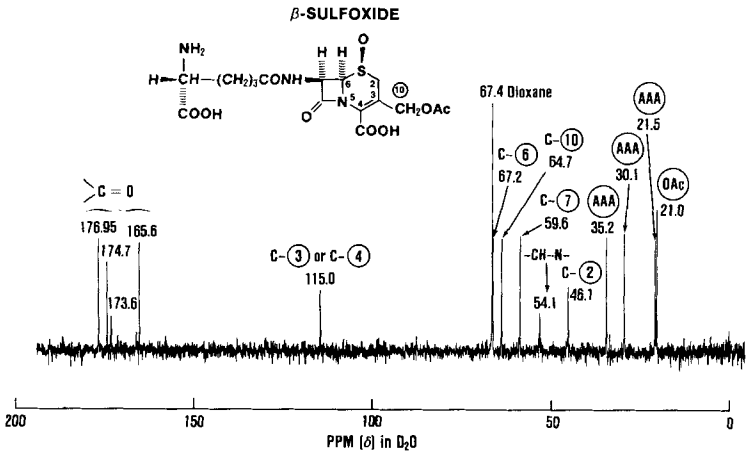



Fig. 9. *$^{13}$C-NMR (20 MHz) of Cephalosporin C β-Sulfoxide* (**4**) *in $D_2O$.* AAA = Signals of the (2-amino-6-adipyl)amino moiety.

## REFERENCES

[1] *N. Neuss, D.M. Berry, J. Kupka, A.L. Demain, S.W. Queener, D.C. Duckworth & L.L. Huckstep*, J. Antibiot. *35*, 580 (1982).
[2] *R.D. Miller, L.L. Huckstep, J.P. McDermott, S.W. Queener, S. Kukolja, D.O. Spry, T.K. Elzey, S.M. Lawrence & N. Neuss*, J. Antibiot. *34*, 984 (1981).
[3] *E.P. Abraham & G.G.F. Newton*, 'The Cephalosporins', in Advances in Chemotherapy *2*, 23–90 (1965).
[4] *H. Troonen, P. Roelants & B. Boon*, J. Antibiot. *29*, 1258 (1976).
[5] *R.B. Morin, B.G. Jackson, R.A. Mueller, E.R. Lavagnino, W.B. Scanlon & S.L. Andrews*, J. Am. Chem. Soc. *85*, 1896 (1963).
[6] *J.E. Baldwin, M. Jung, P. Singh, T. Wan, S. Haber, S. Herchen, J. Kitchin, A.L. Demain, N.A. Hunt, M. Kokshake, T. Konowi & M. Yoshida*, Phil. Trans. R. Soc. Lond. B *289*, 169 (1980).
[7] *S. Kukolja, S.R. Lammert, M.R. Gleissner & A.I. Ellis*, J. Am. Chem. Soc. *97*, 3192 (1975).
[8] *D.O. Spry*, J. Org. Chem. *1979* 3084.
[9] *R.D.G. Cooper*, J. Am. Chem. Soc. *94*, 1018 (1972).