NOTES

ISOLATION OF 7β-(5-HYDROXY-5-CARBOXYVARELAMIDO)-3-HYDROXYMETHYL-3-CEPHEM-4-CARBOXYLIC ACID FROM *STREPTOMYCES* SP.

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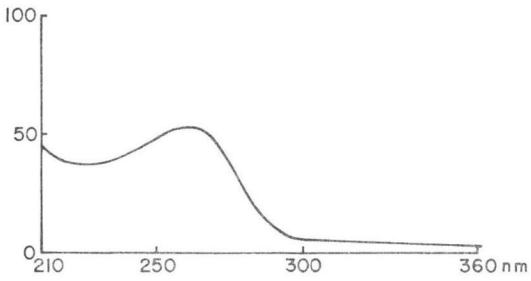
During the course of our screening work for new β-lactam antibiotics, we found that a *Streptomyces* strain numbered PA-41937 produces a substance active against *Escherichia coli* LS-1 (a mutant super sensitive to β-lactam antibiotics), sensitive to a cephalosporinase (from *Enterobacter cloacae*) and showing stronger acidic nature when compared with the known naturally occurring cephem compounds by paper electrophoresis. Since the acidic substance (tentatively called α-substance) was supposed to be a new cephem compound, it was isolated and the structure was elucidated to be 7β-(5-hydroxy-5-carboxyvarelamido)-3-hydroxymethyl-3-cephem-4-carboxylic acid (I). The strain probably belongs to *Streptomyces griseus* from preliminary taxonomical data and simultaneously produces four known cephem compounds, deacetylcephalosporin C[1], deacetyl-7-methoxycephalosporin C[2], deacetoxycephalosporin C[3] and deacetoxy-7-methoxycephalosporin C[4]. The isolation and structure elucidation of I are presented in this report.

The strain PA-41937 was fermented by submerged culture using a 30-liter jar fermentor containing 20 liters of medium consisting of tomato paste 2.0%, dextrin 2.0%, dried yeast 1.0% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 7.0 before sterilization) at 28°C for 72 hours under agitation of 300 r.p.m., aeration of 20 liters per minute and inner pressure of 0.5 kg/cm². The harvested broth was centrifuged by a Sharples centrifugal separator. The supernatant fluid (150 liters) obtained from 8 jar fermentors was adjusted to pH 7.0 and passed through a column (11 liters) of an Amberlite IRA-68 (Cl^-) (Rohm and Haas Co., Ltd.). The column was washed with water and eluted with 5% NaCl. The active eluate fraction when assayed by pulp disk agar diffusion method on an *E. coli* LS-1 assay plate were then passed through a column (4 liters) of a Diaion HP-20 (Mitsubishi Kasei Kogyo Co., Ltd.) at pH 3.5. The column was eluted with water and then with 50% methanol. From the active eluate with water, a crude mixture of deacetylcephalosporin C and deacetyl-7-methoxycephalosporin C was isolated, and these compounds were identified by direct comparison with the authentic specimens by HPLC. The active eluate with 50% methanol was adjusted to pH 7.0 by NaOH, concentrated and freeze-dried to give a crude powder (42 g). The crude powder was subjected to chromatography on a column (5×40 cm) of QAE-Sephadex A-25 (Pharmacia Fine Chemicals) with 0.2 M NaCl in 50 mM phosphate buffer, pH 7.0 (some 10 g portion could be charged for a run). Two active fractions appeared. From the former fraction, a crude mixture of deacetoxycephalosporin C and deacetoxy-7-methoxycephalosporin C which gave respective identical peaks on HPLC with those of the authentic specimens was isolated. The latter fraction was adsorbed on an HP-20 column at pH 3.5 and eluted with 50% methanol. Adjustment of pH to 7.0, evaporation and freeze-drying gave a residue (1.2 g). The residue was purified by chromatography on a cellulose column (Microcrystalline Cellulose, Avicel) with 70% *n*-propanol. Evaporation and freeze-drying of the active eluate gave a powder (250 mg). Final purification was achieved by preparative HPLC on a column (10×250 mm) of Nucleosil 10 C_{18} (Macherey-Nagel) with 10 mM phosphate buffer, pH 6.5. Peak fractions of the α-substance were collected. Adsorption and elution on an HP-20 AG column as above, adjustment of pH to 7.0 by NaOH, followed by freeze-drying gave the sodium salt of the α-substance (70 mg).

The sodium salt of the α-substance (I) is a colorless powder, easily soluble in water. It shows negative reaction to ninhydrin reagent. When

Fig. 1. UV spectrum of I sodium salt (H_2O).



tested by paper electrophoresis with 50 mM phosphate buffer, pH 7.0, at 10 volt/cm for 2 hours, it moved to the anode with Rm (relative mobility to cephamycin C) 2.0. It shows a maximum at 260.5 nm in the UV spectrum (Fig. 1). In the IR spectrum (Fig. 2), absorptions at 1745 cm^{-1} (β-lactam), 1645 cm^{-1} (amide) and 1590 cm^{-1} (carboxylate) are shown. The substance was hydrolyzed with constant boiling hydrochloric acid or 5 N NaOH at 110°C for 20 hours, and the hydrolysates were analyzed by an automatic amino acid analyzer. A small amount of glycine was detected, but α-aminoadipic acid was not detected in any amount in the both hydrolysates.

The ^1H and ^{13}C NMR spectra were recorded with a Varian XL-100 -12 A spectrometer in D_2O using tetramethylsilane as an external reference. When the ^1H and ^{13}C NMR data of I sodium salt were compared with those of deacetylcephalosporin C (II) sodium salt as a reference compound, close similarities were observed (Tables 1 and 2). Significant difference between NMR behaviors of I and II were shown as follows. (1) Compared with ^1H signal of 16-CH of II, a corresponding signal of I shifted to lower field ($\Delta\delta = +0.34$). (2) Compared with ^{13}C signals of 16-CH, 15-CH_2 and 17-COO^- of II, re-

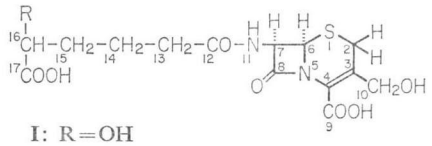
Table 1. ^1H NMR spectral data of I and II sodium salts (D_2O, external reference TMS).

δ (splitting pattern, J in Hz)		Assignment
I	II	
~2.16 (m)	~2.20 (m)	14-CH_2, 15-CH_2
~2.84 (m)	~2.85 (m)	13-CH_2
3.91 (d, 17.7)	3.89 (d, 17.6)	2-CH_2
4.11 (d, 17.7)	4.09 (d, 17.6)	
~4.47 (m)	~4.14 (m)	16-CH
4.73 (s)	4.70 (s)	10-CH_2
5.57 (d, 4.6)	5.55 (d, 4.6)	6-CH
6.07 (d, 4.6)	6.05 (d, 4.6)	7-CH

Table 2. ^{13}C NMR spectral data of I and II sodium salts (D_2O, external reference TMS).

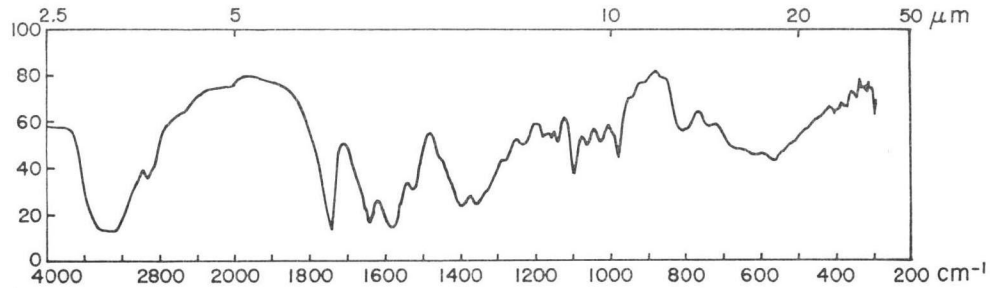
δ (multiplicity)		Assignment
I	II	
22.5 (t)	22.3 (t)	C-14
26.7 (t)	26.7 (t)	C-2
34.7 (t)	31.2 (t)	C-15
36.4 (t)	36.0 (t)	C-13
58.5 (d)	58.7 (d)	C-6
60.2 (d)	60.2 (d)	C-7
62.2 (t)	62.3 (t)	C-10
73.2 (d)	55.8 (d)	C-16
122.6 (s)	122.5 (s)	C-3
130.7 (s)	130.9 (s)	C-4
166.3 (s)	166.2 (s)	C-8
170.3 (s)	170.3 (s)	C-9
178.6 (s)	177.9 (s)	C-12
182.4 (s)	175.8 (s)	C-17

Scheme 1.



I: R=OH
II: R=NH_2 (Deacetylcephalosporin C)

Fig. 2. IR spectrum of I sodium salt (KBr).



markable downfield shifts were found for the corresponding signals of **I**; $\Delta\delta$ values were +17.4, +3.5 and +6.6, respectively. These signal shifts implied the replacement of an amino group on C-16 with a hydroxyl group. The CD spectrum of **I** sodium salt was substantially identical with that of **II** sodium salt; CD: $[\theta]_{355}$ 0, $[\theta]_{315}$ +230, $[\theta]_{305}$ +190, $[\theta]_{257.5}$ +26100, $[\theta]_{239}$ 0, $[\theta]_{225}$ −33800, $[\theta]_{196}$ 0 (*c* 0.0472, 10 mM phosphate buffer, pH 7.0).

I sodium salt was dissolved in methanol and methylated with diazomethane, and the product was subjected to measurement of FD/MS with a Hitachi RMU-8 GN spectrometer. A peak at *m/z* 403 (MH^+ of the dimethyl ester of **I**) was observed.

From the above data, we concluded the structure of **I** as shown in Scheme 1.

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