C-19393 S_2 AND H_2, NEW CARBAPENEM ANTIBIOTICS

II. ISOLATION AND STRUCTURES

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Two new β-lactam antibiotics, C-19393 S_2 (**1**) and H_2 (**2**), were isolated from the culture filtrate of *Streptomyces griseus* subsp. *cryophilus* nov. subsp. The structures were determined by spectral analysis as shown in Fig. 2. The antibiotics have broad antimicrobial activity and strongly inhibit β-lactamases. The minor product (**2**) is more stable than cephalosporin C in aqueous solution.

Our strategy of screening for new β-lactam antibiotics is directed toward isolating new compounds having (1) a broad antimicrobial spectrum, (2) tolerance to β-lactamases and (3) stability in aqueous solution. This paper deals with the isolation and structural elucidation of C-19393 S_2 (**1**) and H_2 (**2**)* which satisfy the above criteria.

The antibiotics, **1** and **2**, were isolated from the fermentation broth of *Streptomyces griseus* subsp. *cryophilus* nov. subsp. which was obtained from a soil sample collected in Sweden.[1]

Purification of the acidic, water-soluble antibiotics was carried out by chromatography using anion-exchange resins, high-porous resins, activated carbon and anion-exchange Sephadex. The last-named adsorbent was used to separate **1** and **2** by their difference in acidity. Reversed-phase HLC was used in the final stage of purification. Fig. 1 shows the isolation procedure for these antibiotics.

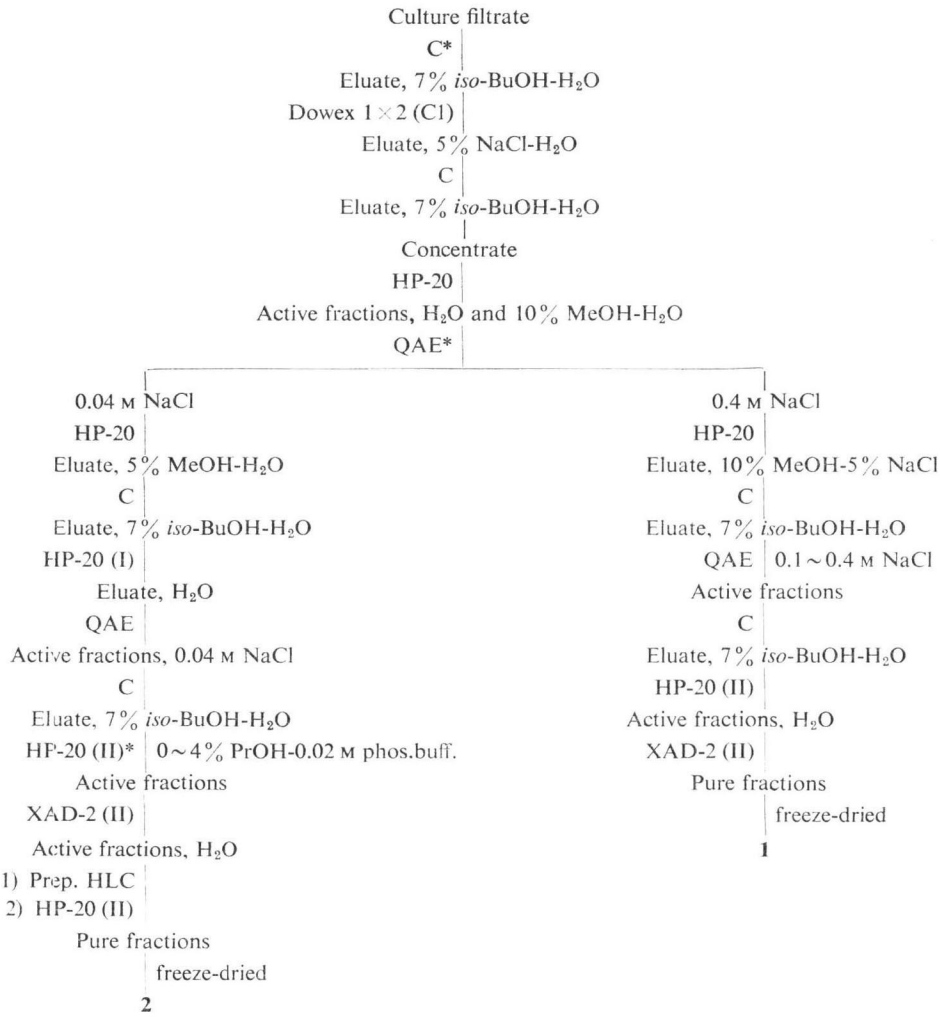
The active fractions were detected by bioassay using antimicrobial activity and inhibitory activity to β-lactamases and by chemical methods using TLC and HLC. The organism also produced a fair amount of penicillin N[2], epithienamycins[3] and olivanic acid derivatives[4] under various fermentation conditions.

The antibiotics, **1** ($[\alpha]_D^{24}-152°$) and **2** ($[\alpha]_D^{24}-141°$) were assumed to be β-lactam compounds from bioassay data, the isolation procedure and their IR spectra. They give a positive EHRLICH reaction and negative ninhydrin and GREIG-LEABACK reactions. Table 1 shows the chromatographic mobilities of **1** and **2** on TLC and HLC.

The molecular formula of **1** was assumed to be $C_{14}H_{16}N_2O_9S_2Na_2$ from elemental analysis and the number of carbons in its ^{13}C-NMR spectrum (Table 2). The IR spectrum of **1** indicated strong absorptions at ν_{max}^{KBr} 1770 (β-lactam carbonyl), 1700 (N-Ac), 1260 and 1050 cm^{-1} (sulfonate). The UV spectrum showed two maxima at $\lambda_{max}^{H_2O}$ 243.5 nm (ε 15,900) and 288 (13,200). The characteristic COTTON effects in the CD spectrum at $[\theta]_{nm}^{H_2O}$ 207 (ε −18,800), 234 (+47,000), 258 (−45,600) and 294 (−36,200) suggested the presence of a sulfoxide function. It was deduced from these spectral data that **1** has a

* A Dutch patent application on antibiotics C-19393 S_2 and H_2 (No. 8000628) has been open to public inspection since Aug. 5th, 1980.

Fig. 1. Isolation procedure of C-19393 S_2 (**1**) and H_2 (**2**).



* Abbreviations: C; activated carbon, QAE; QAE-Sephadex A-25 (Cl^- type), I; 50~100 mesh, II; 100~200 mesh in HP-20 and XAD-2.

similar structure to the antibiotic MM 4550[5] for which the chromophore is

$$^{-}OOC\text{–}\overset{|}{C}=\overset{|}{C}\text{–}\overset{\uparrow}{S}\text{–}CH=CH\text{–}N\text{–}.$$
O

^{1}H-NMR spectral studies (Table 3) confirmed the similarity of **1** to that of MM 4550. Two methyl signals were observed at $\delta^{D_2O}_{ppm}$ 1.66 (3H, s) and 1.73 (3H, s) in **1**. The H_8-proton signal at $\delta^{D_2O}_{ppm}$ 4.97 (1H, m) in MM 4550 was not observed in **1**. The other proton signals of **1** including those for *trans* vinyl protons were similar in chemical shifts and splitting patterns to those of MM 4550. The orientation of H_5 and H_6 in **1** was deduced to be *cis* from the coupling constants, $J_{5,6}$=6 Hz, $J_{4a,5}$=9 Hz and $J_{4b,5}$=10.5 Hz, assigned by proton-spin decoupling studies. Thus the signals in the ^{13}C-NMR spectrum were all unambiguously accommodated by structure **1** (Table 2).

The minor product (**2**) has the molecular formula $C_{14}H_{17}N_2O_6SNa$. Its spectral data were similar to those of **1**. Absorption maxima were observed at $\lambda^{H_2O}_{max}$ 244.5 nm (ε 14,300) and 290 (11,000) and the

Project (ISP)[6]. T agar, which was used additionally for characterization and for the maintenance of strain No. C-19393, was prepared as follows: Twenty grams each of oatmeal and tomato paste, and 2 g of Bovril (edible beef extract, Bovril Ltd., Burton-on-Trent) were boiled for 10 minutes in 1 liter of tap water and the mixture was filtered through gauze. The filtrate was adjusted to pH 7.0, made to 1 liter with tap water and supplemented with 20 g of Bacto agar (Difco Labs., Detroit). Agar slants were prepared after autoclaving at 120°C for 15 minutes.

Seed medium used for fermentation contained (g/liter): glucose 20, soluble starch 30, soybean flour 10, corn-steep liquor 10, Polypepton (Daigo Nutritive Chem., Osaka) 5, NaCl 3, and $CaCO_3$ (precipitated) 5. Fermentation medium contained (g/liter): glucose 30, soluble starch 30, soybean meal 15, cotton-seed meal 15, K_2HPO_4 0.6, KH_2PO_4 0.25, $CoCl_2$ 0.002, and Actcol (antifoam, Takeda Chem. Ind., Osaka) 0.5. The pH of the seed and fermentation media was adjusted to pH 7.0 with 2 N NaOH before sterilization.

Assay methods

Antibiotics C-19393 S_2 and H_2 were assayed by determining (1) antibacterial activity against mutants of *Escherichia coli* lacking chromosomal β-lactamase and penicillin-binding protein 1B[2] and (2) β-lactamase-inhibiting activity using *Klebsiella pneumoniae* as described by BROWN *et al.*[7] The minimum inhibitory concentrations were assayed by the conventional agar-dilution method using the medium described previously[8]. The synergistic action of C-19393 S_2 and H_2 with ampicillin and cefotiam was examined by the two-fold agar-dilution method using Trypticase soy agar (BBL, Baltimore).

Chemicals

Ampicillin is a product of our company. Cephaloridine is a product of Eli Lilly & Co. Cefotiam was prepared in our research division. Other chemicals are commercial products.

Results

Taxonomy of Strain No. C-19393

The taxonomic characterization was carried out according to the method recommended by the ISP[6]. Unless otherwise specified, the cultivation temperature was 28°C.

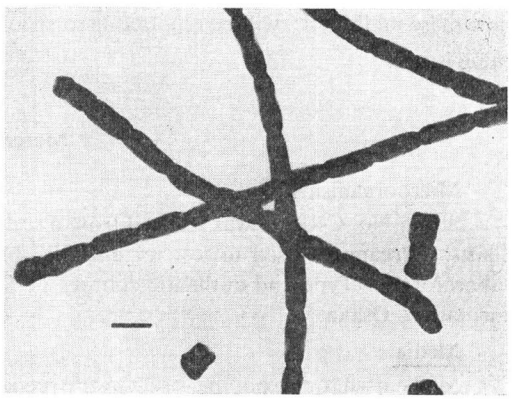
Morphological characterization

The strain produced aerial mycelium with tufts of straight to slightly wavy spore chains; it therefore belongs to the Section *Rectus-Flexibilis* (RF). The mature spore chains were generally long with more than 30 spores per chain. The spores were cylindrical ($0.35 \sim 0.55 \times 0.7 \sim 1.4$ μm) and their surfaces were smooth (Fig. 2).

Cultural characteristics

The cultural characteristics observed after a 2-weeks cultivation are shown in Table 1. The strain gave the most characteristic appearance on T agar; the color of the aerial mycelium was light grayish yellow and thus it belongs to the Yellow color-series. Neither melanin nor other soluble pigments were formed on any agar media tested.

Fig. 2. Electron microphotograph of spores of strain No. C-19393. Bar indicates 1 μm.



Physiological characteristics

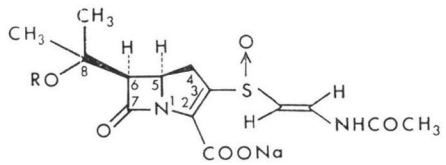
The physiological characteristics are shown in Table 2. The strain contained cell walls of type I (LL-diaminopimelic acid). It is unusual

tion due to a sulfate group in the IR spectrum of **2**.

The chemical relationship between **1** and **2** was confirmed by the conversion of **1** to **2** on mild hydrolysis. It was apparent that **2** has a hydroxy group instead of the sulfoxy group at the position 8.

The structures of **1** and **2** were thus determined to be [5R, 6R]-3-[(*E*)-2-acetamidoethenyl-sulfinyl]-6-[1-sulfooxy-1-methyl-ethyl-]-7-oxo-1-azabicyclo-(3,2,0)-hept-2-ene-2-carboxylic acid disodium salt and the corresponding 6-[1-hydroxy-1-methyl-ethyl-]compound, respectively, as shown in Fig. 2. The same structures have recently been proposed for carpetimycins B and A[6].

Fig. 2. Structures of C-19393 S_2 (**1**) and H_2 (**2**).



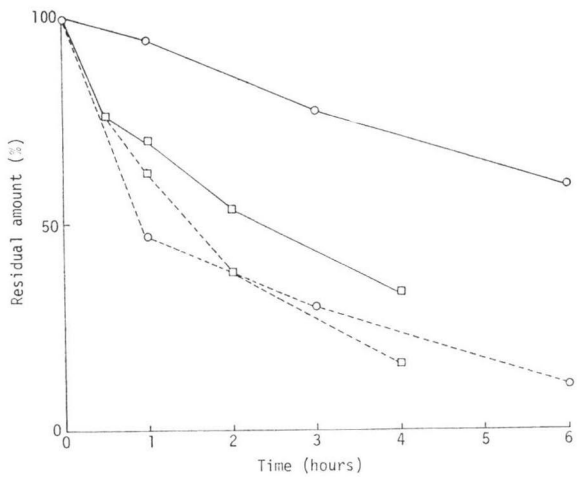
C-19393 S_2 (**1**): R=SO_3Na
C-19393 H_2 (**2**): R=H

Table 4. Stability ($T_{1/2}$)* of C-19393 S_2 (**1**) and H_2 (**2**), and other β-lactam antibiotics at 60°C in 0.05 M phosphate buffer.

Antibiotic	Concentration	pH			
		5	6	7	8
2	10 μg/ml	1.8	7.4	7.4	2.8
Cephalosporin C	〃	1.0	2.1	1.0	0.9
1	100 μg/ml	1.8	3.2	2.3	0.7
MM 17880	〃	0.6	1.9	3.8	3.9
Epithienamycin B	〃	0.43	1.1	2.2	4.8
Cephalosporin C	〃	1.6	1.5	1.5	1.5

* Half life time in hours.
Detection: HLC (System 1) was used.

Fig. 3. Stability of C-19393 H_2 (**2**) and S_2 (**1**), and cephalosporin C at 60°C in 0.05 M phosphate buffer (pH 7.0).
Detection: HLC, System 1) and 2). ○—○; **2**, 10 μg/ml, ○---○; Cephalosporin C, 10 μg/ml, □—□; **1**, 100 μg/ml, □---□; Cephalosporin C, 100 μg/ml.



It has been reported that thienamycin and the related carbapenem antibiotics are unstable substances in various aqueous solutions[7,8]. However, **2** appeared to be more stable than cephalosporin C, especially at neutral pH. Antibiotics **1** and **2** having a sulfoxide group, are more stable than epithienamycin B and MM 17880 in the acidic range but less stable in the basic range. The stability of these antibiotics is shown in Fig. 3 and Table 4. The dimethylhydroxy group may reduce the susceptibility to cleavage of the β-lactam ring.

Antibiotics **1** and **2** strongly inhibit β-lactamases and have synergistic effects with other β-lactam antibiotics.[1] Antibiotic **2** exhibits potent antimicrobial activity *in vitro*[1]. Our search for stable, effective carbapenem antibiotics is continuing.

Experimental

Isolation of **1** and **2**: The culture broth of *Streptomyces griseus* subsp. *cryophilus* nov. subsp. (1,000 liters, 5°C, pH 6.2~6.5) was filtered using Hyflo-Super Cel. The filtrate was applied to activated carbon (100 liters) and active components were eluted with 7% iso-BuOH-H_2O (700 liters). The eluate was chromatographed on Dowex 1×2 (Cl^- type, 12 liters) and eluted with 5% NaCl-H_2O (180 liters). The eluate was again desalted with activated carbon (25 liters). The concentrate (2 liters) was chromatographed on Diaion HP-20 (5 liters, Mitsubishi Kasei) with H_2O (5 liters) followed by 10% MeOH-H_2O (10 liters). Active fractions were loaded on QAE-Sephadex A-25 (Cl^- type, 3 liters). The components were separated at this stage by elution with 0.04 M NaCl and 0.4 M NaCl. The 0.04 M NaCl eluate, containing **2**, was chromatographed on HP-20 pretreated with 5% NaCl (2 liters) and active fractions were eluted with 5% MeOH-H_2O. After treatment with activated carbon (0.5 liters) the recovered antibiotic was chromatographed on HP-20 (50~100 mesh, 1 liter) with H_2O. Active fractions were again loaded on QAE-Sephadex and eluted slowly with 0.04 M NaCl. After desalination with activated carbon the antibiotic was chromatographed on HP-20 (50~100 mesh) with a 0~4% of gradient *n*-PrOH-0.02 M phosphate buffer (pH 6.3). Active fractions were desalted on a column of Amberlite XAD-2 (100~200 mesh). The antibiotic was loaded on a preparative HLC column of Lichrosorb RP-18 and eluted with 10% MeOH-0.02 M phosphate buffer (pH 6.3). The fractions giving a single peak by HLC were collected and desalted with HP-20. Pure fractions were concentrated and freeze-dried to give **2** as a white powder (2 mg).

The 0.4 M NaCl eluate, containing **1**, from QAE-Sephadex chromatography was applied to HP-20 pretreated with 5% NaCl (2 liters). Active fractions were eluted with MeOH: 5% NaCl (1: 9). After desalination with activated carbon the recovered antibiotic was chromatographed on QAE-Sephadex with a gradient of 0.1~0.4 M NaCl. After treatment with activated carbon the recovered antibiotic was loaded on HP-20 (50~100 mesh) and eluted with H_2O. Active fractions were chromatographed on XAD-2 (100~200 mesh) with H_2O. Pure fractions giving a single peak by HLC were combined and freeze-dried to give **1** as a white powder (9 mg).

1: $[\alpha]_D^{24}$ −152° (*c* 0.5, H_2O), UV $\lambda_{max}^{H_2O}$ 243.5 nm (ε 15,900) and 288 (13,200). IR ν_{max}^{KBr} 1770, 1700, 1260 and 1050 cm^{-1}. Anal. Calcd. for $C_{14}H_{16}N_2O_9S_2Na_2$ (466.41), C, 34.71; H, 3.75; N, 5.78; O, 33.03; S, 13.24; Na, 9.49. Found: C, 36.29; H, 3.72; N, 6.07; S, 13.13; Na, 9.70 (%).

2: $[\alpha]_D^{24}$ −141° (*c* 0.54, H_2O), UV $\lambda_{max}^{H_2O}$ 244.5 nm (ε 14,300) and 290 (11,000). IR ν_{max}^{KBr} 1770 and 1700 cm^{-1}. Anal. Calcd. for $C_{14}H_{17}N_2O_6SNa$ (364.36), C, 46.15; H, 4.70; N, 7.69; O, 26.35; S, 8.80; Na, 6.31. Found: C, 45.34; H, 4.98; N, 7.48; S, 8.51; Na, 6.15 (%).

Hydrolysis of **1**: A solution of **1** (67 mg) in 0.01 M phosphate buffer (pH 6.3, 67 ml) was allowed to stand at 60°C for 5 hours. The mixture was applied to HP-20 (100~200 mesh) and eluted with H_2O. The fractions containing **2** detected by HLC were collected and freeze-dried to give **2** (5 mg, 9.6%). The compound was identical with naturally occurring **2** by HLC, TLC, UV and IR spectra.

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