C-19393 E_5, A NEW CARBAPENEM ANTIBIOTIC

FERMENTATION, ISOLATION AND STRUCTURE

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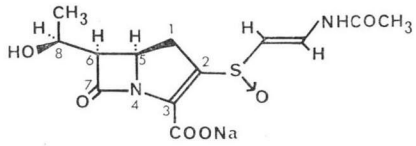
A new carbapenem antibiotic, C-19393 E_5, was isolated from the culture filtrate of *Streptomyces griseus* subsp. *cryophilus* C-19393 as a minor component. The chemical structure of the antibiotic was determined by comparing its spectral data with those of the known 5,6-*cis* carbapenem antibiotics and confirmed by partial synthesis from epithienamycin B as shown in Fig. 1. The antibiotic has a broad antimicrobial spectrum and shows strong inhibitory activity against β-lactamases.

In previous papers we have reported that *Streptomyces griseus* subsp. *cryophilus* C-19393 produces two novel carbapenem antibiotics, C-19393 H_2 and S_2,[1~3] together with penicillin N, epithienamycins (ETM) and olivanic acids. A subsequent investigation in search of new products of this organism led us to the discovery of another novel component, C-19393 E_5. The present paper deals with fermentation, isolation, structural elucidation and biological properties of C-19393 E_5 (Fig. 1).

Fig. 1. Structure of C-19393 E_5 sodium salt.



Materials and Methods

Microorganism

S. griseus subsp. *cryophilus* C-19393, maintained on T-agar,[1] was used in this study.

Fermentation

A pre-seed culture of 500 ml in a 2-liter Sakaguchi flask was inoculated into 100 liters of the seed medium in a 200-liter fermentor. After cultivation at 28°C for 2 days with aeration (70 liters/minute) and agitation (150 rpm), the culture was transferred into 1,200 liters of fermentation medium in a 2,000-liter fermentor and fermentation was carried out at 30°C for 3 days with aeration (840 liters/minute) and agitation (180 rpm).

Seed medium contained (g/liter): glucose 20, soluble starch 30, soy bean flour 10, corn steep liquor 10, Polypeptone (Daigo Nutritive Chem., Osaka) 5, NaCl 3, and $CaCO_3$ (precipitated) 5.

Fermentation medium contained (g/liter): glucose 30, soluble starch 30, soy bean meal 15, cotton seed meal 15, K_2HPO_4 0.6, KH_2PO_4 0.25, $CoCl_2 \cdot 6H_2O$ 0.002 and Actcol (Takeda Chem. Ind., Osaka) 0.5. Seed and fermentation media were adjusted to pH 7.0 with NaOH before sterilization.

Isolation

The fermentation broth was filtered after addition of Hyflo-Super Cel below 5°C. The filtrate (1,000 liters) was adjusted to pH 6.3 with phosphorus acid, loaded on Amberlite IRA-402 (Cl^- type, 25 liters), and the active components were eluted with 5% NaCl - H_2O (250 liters). The eluate was

chromatographed on Diaion HP-20 (20 liters) using the solvent system MeOH - 5% NaCl in H_2O (5: 95, 125 liters). The eluate was applied to activated carbon (5 liters), and the antibiotics were eluted with 7% *iso*-BuOH in H_2O (15 liters). The concentrate (2.5 liters) was washed with 1% tri-*n*-octylmethylammonium chloride - CH_2Cl_2 solution (1.25 liters) to remove impurities having a sulfonyloxy group. The aqueous layer was loaded on HP-20 (50~100 mesh, 1.5 liters) and eluted with MeOH - 5% NaCl in H_2O (5: 95, 9 liters). After desalination of the active fractions by chromatography on activated carbon (0.8 liters) the concentrate was chromatographed on QAE-Sephadex (0.25 liter) with a gradient system of 0.02~0.05 M NaCl in H_2O (2.5 liters). The fractions were analyzed by HPLC and divided into 3 groups. Each group was individually purified with HP-20 (100~200 mesh) to give C-19393 E_5 (5 mg) and H_2 (4 mg) and ETM B[4] (32 mg) as freeze-dried white powders.

Reverse-phase High-performance Liquid Chromatography (HPLC)

Model ALC/GPC 202/660 prepared by Waters Ass. Inc. was used. The analytical conditions were as follows; column: Radial Pak A, mobile phase: MeOH - 0.02 M phosphate buffer (pH 6.3), flow rate: 2 ml/minute, detection: UV absorbance detector at 254 nm. Applicable relationship was observed between peak heights and amounts of carbapenem antibiotics.

Microbial Assay Methods

Bioautographical detection of the antibiotic was carried out on nutrient agar plates seeded with *Escherichia coli* PG 8 lacking chromosomal β-lactamase and penicillin-binding protein 1B.[1] The minimum inhibitory concentration (MIC) was assayed by the conventional agar dilution method using the medium described previously.[1]

Oxidation of Epithienamycin B

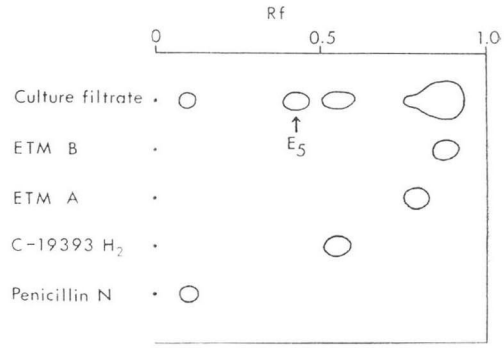
To a solution of ETM B (40 mg) in MeOH (20 ml) was added *m*-chloroperbenzoic acid (20 mg) and the mixture was stirred at 0~5°C for 30 minutes. After addition of 0.01 M phosphate buffer (pH 6.3, 100 ml), the organic solvent was removed. The aqueous solution was washed with AcOEt and concentrated. The concentrate (80 ml) was loaded on QAE-Sephadex A-25 (Cl^- type, 30 ml) for excluding impurities. The eluate obtained with 0.02 M NaCl (200 ml) was chromatographed on HP-20 (30 ml, pre-treated with 5% NaCl in H_2O) using the solvent system MeOH - 5% NaCl in H_2O (5: 95). Two fractions detected by HPLC were individually desalinated on HP-20, followed by activated carbon to give ETM B sulfoxide (6.9 mg) and its stereoisomeric sulfoxide (3.8 mg) as freeze-dried white powders.

Results and Discussion

Chromatographic Detection of the Products

The culture filtrate was treated with dichloromethane containing tri-*n*-octylmethylammonium chloride to remove compounds having a sulfonyloxy group.[5] The aqueous layer was then subjected to thin-layer chromatography (TLC) using the solvent system acetonitrile-water (4: 1). As shown in Fig. 2, an unknown spot, E_5, was detected just below that of C-19393 H_2.

Fig. 2. Bioautograms of the antibiotics produced by *S. griseus* subsp. *cryophilus* C-19393.



Culture filtrate (10 μl) was applied after treating with dichloromethane containing tri-*n*-octylmethyl ammonium chloride.
Solvent system; CH_3CN - H_2O (4: 1),
Test organism; *E. coli* PG 8.

Isolation and Characterization

C-19393 E_5 was isolated from the culture filtrate by similar methods as used for the purification of C-19393 H_2.[2] The chromatographies were carried out by using as adsorbents, anion-exchange resins, Sephadex, activated carbon

Table 1. Mobilities of C-19393 E_5 and related antibiotics.

Compound	HPLC*1 Retention time (minutes) Percentage of MeOH (%)			TLC*2 Rf value	
	2	4	8	PrOH - H_2O (4: 1)	CH_3CN - H_2O (4: 1)
C-19393 H_2		36.4	8.9	0.60	0.56
ETM B	18.0	11.4	5.2	0.75	0.87
ETM A	10.3	6.8	3.4	0.63	0.80
C-19393 E_5	7.6	5.3	2.8	0.57	0.45
C-19393 S_2	14.6	6.7	3.3	0.38	0.41
MM 13902	6.7	3.7		0.68	0.78
MM 4550	3.7	2.4		0.31	0.30
MM 17780	3.4	2.2		0.60	0.65

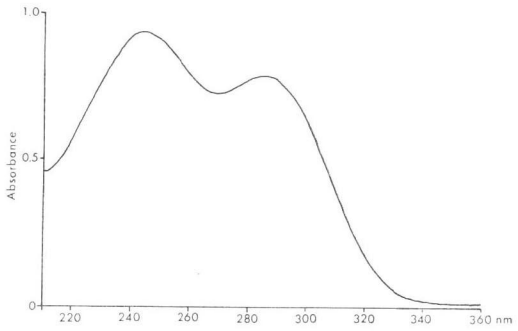
*1 Radial Pak A/MeOH - 0.02 M phosphate buffer (pH 6.3), 2 ml/minute.
*2 Spot film: Cellulose for TLC (Tokyo Kasei), Detection: Bioautography using *E. coli* PG 8.

Table 2. Stability of C-19393 E_5 and related antibiotics in 0.05 M phosphate buffer (100 μg/ml, 60°C).

Antibiotic	Half-life time. ($T_{1/2}$ (hour))*			
	pH 5	pH 6	pH 7	pH 8
C-19393 E_5	1.5	2.4	1.5	0.5
C-19393 H_2	1.0	4.8	4.7	1.4
ETM A	0.5	1.8	5.1	8.5
MM 4550	2.0	2.3	1.4	0.5
MM 13902	0.4	1.5	3.4	3.6

* The residual amount of the antibiotics was measured by HPLC.

Fig. 3. UV Spectrum of C-19393 E_5.



and high-porous resins. The active components in each eluate were detected by antimicrobial activity and HPLC.

The white powder of C-19393 E_5 showed positive Ehrlich reaction with blue color. Its specific rotation was $[\alpha]_D^{24}$ −126° (*c* 0.28, water). Table 1 shows the chromatographic mobilities of C-19393 E_5 allowing its distinction from the known 5,6-*cis* carbapenem antibiotics, ETM A,[4] ETM B, MM 17880,[6] MM 13902[7] and MM 4550,[7] which were isolated from this strain.

The stability of C-19393 E_5 and related antibiotics in aqueous solutions is shown in Table 2. C-19393 E_5 was more stable in the acidic than in the basic pH range.

Structural Elucidation

The UV spectrum of C-19393 E_5 in water showed maxima at 244 nm (ε 13,800) and 287 (11,500) as shown in Fig. 3. The CD spectrum in water indicated characteristic COTTON effects at 234 nm (+ 31,800), 260 (−28,300), 290 (−22,500) and 305 (−17,500). These spectral data suggested the presence of the same chromophore; $^-OOC-\overset{|}{C}=\overset{|}{C}-\overset{\uparrow O}{S}-CH=CH-\overset{|}{N}-$ as revealed on structural elucidation of C-19393 H_2 and S_2.[2] The IR spectrum (Fig. 4) showed absorptions at 1765 cm^{-1} (β-lactam carbonyl), 1700 cm^{-1} (*N*-acetyl) and in the finger print region similar to those of C-19393 H_2. The chemical

Fig. 4. IR Spectrum of C-19393 E_5 (KBr).

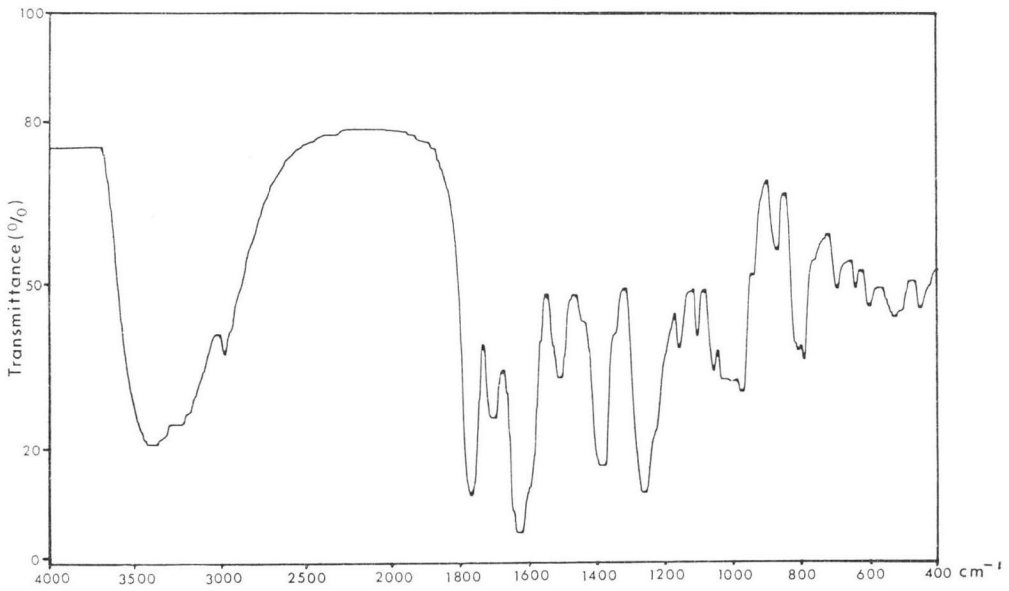
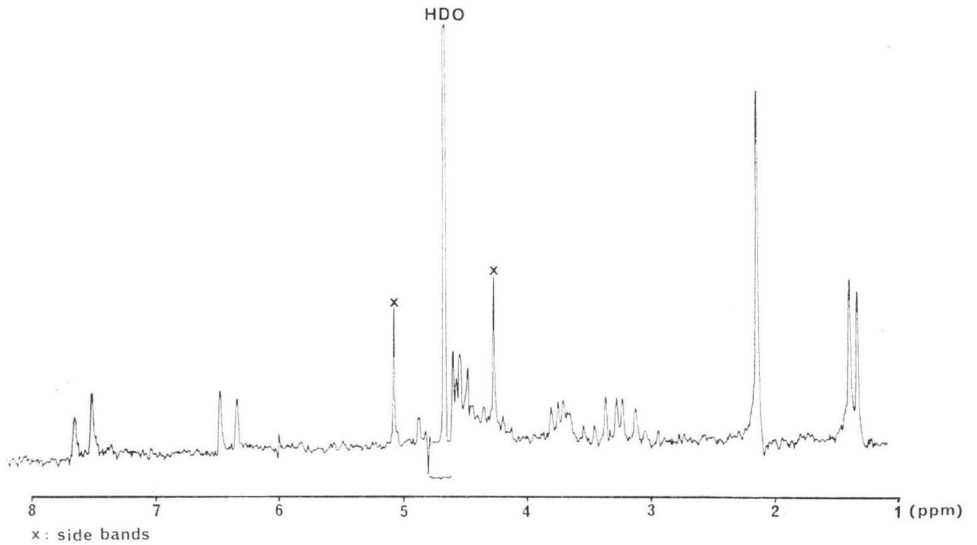


Fig. 5. PMR Spectrum of C-19393 E_5 (100 MHz).



shifts and splitting patterns (δ ppm, J in Hz) in the FT-PMR spectrum in deuterium oxide (Fig. 5) were assigned to the functional groups as follows; 1.39 (d, J=6, 8-CH_3), 2.18 (s, NHCOCH_3), 3.11 and 3.43 (dd×2, $J_{1a,5}$=10.5, $J_{1a,b}$=18, $J_{1b,5}$=9, 1-CH_2), 3.75 (dd, $J_{6,5}$=5 (*cis* coupling), $J_{6,8}$=9.5, H_6), 4.32 (m, H_8), 4.50 (m, H_5), 6.44 (d, J=14 (*trans* coupling), S–CH=) and 7.61 (d, J=14, N–CH=). In dimethyl sulfoxide-d_6, signals were observed at 6.31 (d, J=14, S–CH=), 7.24 (dd, J=14, 11, N–CH=) and 10.54 (d, J=11, N*H*CO). The chemical shifts of the methyl groups have been assigned to $\delta^{D_2O}_{ppm}$ 1.36, 1.47 (s×2, 8-(CH_3)$_2$) and 2.16 (s, NHCOCH_3) in C-19393 H_2[2] and to $\delta^{D_2O}_{ppm}$ 1.37 (d, 8-CH_3) and 2.09 (s, NHCOCH_3)* in ETM B.[4] Compared to C-19393 E_5, the PMR data of ETM B revealed an up-

* The data were measured in our laboratories using XL-100 (Varian) for direct comparison.

field shift of the *trans* vinyl protons and of H_5 (similar to $\Delta\delta$ observed between MM 13902 and its sulfoxide, MM 4550),[7] whereas the other proton signals showed almost the same chemical shifts for the two compounds. From these findings the chemical structure of C-19393 E_5 was assumed to be ETM B sulfoxide.

This assignment was confirmed by conversion of ETM B to C-19393 E_5. ETM B was oxidized by *m*-chloroperbenzoic acid to give ETM B sulfoxide and its stereoisomeric sulfoxide, UV; $\lambda_{max}^{H_2O}$ 251 nm (ε 15,200 and 288 (sh, 7,360), CD; $[\theta]_{nm}^{H_2O}$ 237 (−38,800), 266 (+7,280), 281 (+9,710) and 294 (+4,246). The former compound was identical with naturally occurring C-19393 E_5 as revealed by TLC, HPLC, UV, CD, IR and PMR spectra and antimicrobial activities.

The structure of C-19393 E_5 was thus determined as shown in Fig. 1. The stereochemistry of the sulfoxide will be described as having the *R*-configuration.[8]

Antimicrobial Activity

The antimicrobial spectrum of C-19393 E_5 was compared with that of C-19393 H_2 as shown in Table 3. C-19393 E_5 exhibits strong antibacterial activities against a wide variety of Gram-positive and Gram-negative bacteria. It showed almost equal or higher activity against Gram-positive bacteria compared to C-19393 H_2, but was generally less active against Gram-negative bacteria. The β-lactamase inhibitory activities of C-19393 E_5 were also observed at strikingly low concentrations as described in the following paper.[8]

Table 3. Antimicrobial spectra of C-19393 E_5 and H_2.

Organism	MIC (μg/ml)	
	C-19393 E_5	C-19393 H_2
E. coli NIHJ JC2	1.56	0.2
P. vulgaris IFO 3988	3.13	3.13
P. morganii IFO 3168	12.5	0.78
P. mirabilis ATCC 21100	1.56	3.13
K. pneumoniae IFO 3318	12.5	0.78
P. aeruginosa IFO 3080	12.5	6.25
A. faecalis IFO 13111	3.13	3.13
A. calcoaceticus IFO 12552	6.25	1.56
S. typhymurium IFO 12529	1.56	0.2
S. marcescens IFO 12648	12.5	0.78
C. freundii IFO 12681	3.13	0.2
E. cloacae IFO 12937	50	1.56
B. cereus IFO 3466	1.56	12.5
B. sphericus IFO 12622	1.56	3.13
S. aureus FDA 209P	0.78	0.39
M. luteus IFO 3232	0.2	0.39

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