EPONEMYCIN[†], A NEW ANTIBIOTIC ACTIVE AGAINST B16 MELANOMA

I. PRODUCTION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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Streptomyces hygroscopicus No. P247-71 (ATCC 53709) produced a novel antibiotic eponemycin which exhibited specific *in vivo* antitumor effect against B16 melanoma. Structural studies assigned (4*S*)-1,2-epoxy-2-hydroxymethyl-4-(*N*-isooctanoyl-L-serylamino)-6-methylhept-6-ene-3-one to eponemycin which is unrelated to the known antitumor antibiotics.

In our continuing search for new microbial metabolites with antitumor activity, *Streptomyces hygroscopicus* No. P247-71, isolated from a soil sample from the Philippines, was found to produce a novel antibiotic with specific activity against B16 melanoma. The active principle, eponemycin, was recovered from the fermentation broth with ethyl acetate extraction and purified by chromatography. Structural studies revealed it to be a novel molecule composed of isooctanoic acid, L-serine and an aminoepoxyketone. 9-Methylstreptimidone was isolated as a co-product from the crude extract.

Eponemycin showed potent growth inhibition against various tumor cells, but no inhibitory activity against bacteria and fungi. It induced significant prolongation of survival time of mice transplanted with B16 melanoma, while no antitumor activity was observed against P388 leukemia.

In this paper, we describe the production, isolation, physico-chemical characterization, structural elucidation and biological properties of eponemycin.

Taxonomy of the Producing Strain

Source of Organism

Strain P247-71 was isolated from a soil sample collected near the root of a tamarind at Mt. Apo, Davao, Mindanao Island, Philippines.

Morphology

Both substrate and aerial mycelia are formed. They are long, well-branched and not fragmented into short filaments. Chains of arthrospores are born on the aerial hyphae. The spore chain and spore morphology are as follows: 1) Spiral spore chains with 2 to 8 turns, 2) monopodially branched sporophores, 3) spores, oval or barrel-shaped (0.5 to 0.7 by 0.5 to 1.2 μm), and 4) spore ornamentation, rugose or smooth. Sporangium, motile spore and sclerotium are not observed.

Cultural and Physiological Characteristics

Strain P247-71 grows well in most descriptive media. Gray aerial mycelium with hygroscopic black patches is observed on International Streptomyces Project (ISP) agar media except for ISP No. 6 medium.

[†] Eponemycin was originally designated BMY-28647 or BU-3862T.

White to pale yellowish-gray aerial mycelium is formed on CZAPEK's sucrose-nitrate agar. The substrate mycelium is colorless or yellowish brown to grayish yellow. Melanin and other diffusible pigments are not produced. Most sugars are utilized for growth. The cultural and physiological characteristics are shown in Tables 1 and 2, respectively.

The morphological, cultural and physiological characteristics of strain P247-71 indicate that the strain belongs to the genus *Streptomyces*. According to the descriptions of PRIDHAM and TRESNER[1], the major characteristics of the strain are summarized as follows: 1) Gray aerial mycelium, 2) spiral spore chain, 3) absent melanoid, and 4) smooth spore wall ornamentation. The hygroscopic change of sporulated aerial mycelium is a distinct property of the strain. The major characteristics and those shown in Tables 1 and 2 of strain P247-71 place it in *S. hygroscopicus*.

Antibiotic Production

A well grown agar slant of *S. hygroscopicus* strain No. P247-71 was used to inoculate a vegetative medium consisting of soybean meal (Nikko Seiyu) 3%, Pharmamedia (Traders Protein) 0.5%, glucose 3%, yeast extract (Oriental) 0.1% and $CaCO_3$ 0.3%, the pH being adjusted to 7.0 before sterilization. The vegetative medium was incubated at 28°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the growth was transferred into a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium having the same composition as the vegetative medium. The fermentation was carried out at 28°C for 4 to 5 days with shaking on a rotary shaker.

The antitumor antibiotic production in the fermentation broth was determined by *in vitro* cytotoxic activity against B16 melanoma cells. The fermentation was also carried out in a tank fermenter. A 2-liter portion of the vegetative culture prepared by flask fermentation was transferred into a 200-liter tank

Table 1. Cultural characteristics of strain P247-71.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-Dox agar)	Moderate	Moderate; white to pale yellowish gray	Strong yellow brown (74)	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate; not turbid, floccose	None	Colorless	None
Yeast extract-malt extract agar (ISP No. 2)	Good	Scant; light gray (264) to black. Hygroscopic	Pale orange yellow (70)	None
Oatmeal agar (ISP No. 3)	Poor	Poor; light gray (264)	Greenish gray (155)	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Moderate; light gray (264)	Colorless	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	Moderate; brownish black (65). Hygroscopic	Colorless	None
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	None	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Moderate; dark gray (266)	Dark grayish yellow (91)	None
Glucose-asparagine agar	Moderate	Moderate; brownish black (65). Hygroscopic	Colorless	None
BENNETT's agar	Moderate	Moderate; black. Hygroscopic	Grayish yellow (90)	None

Observation after incubation at 28°C for 3 weeks.
Color and number in parenthesis follow ISCC-NBS designation.

Table 2. Physiological characteristics of strain P247-71.

Hydrolysis of:		L-Arabinose	+
Gelatin	+	D-Xylose	±
Soluble starch	+	D-Ribose	+
Potato starch	+	L-Rhamnose	+
Milk coagulation	−	D-Glucose	+
Milk peptonization	+	D-Galactose	+
Production of:		D-Fructose	+
Nitrate reductase	−	D-Mannose	−
Tyrosinase	−	L-Sorbose	−
Tolerance to:		Sucrose	+
Lysozyme, 0.01% (w/v)	−	Lactose	+
0.001% (w/v)	−	Cellobiose	+
NaCl, 1~6% (w/v)	+	Melibiose	+
7% (w/v)	−	Trehalose	±
pH 5.5~10.5	+	Raffinose	+
5.0 and 11.0	−	D-Melezitose	−
Temperature:		Soluble starch	+
Growth range	20~39°C	Cellulose	−
No growth	17 and 41°C	Dulcitol	−
Optimal growth	37~39°C	Inositol	+
Utilization of[a]:		D-Mannitol	+
Glycerol	+	D-Sorbitol	−
D-Arabinose	±	Salicin	+

[a] Basal medium: PRIDHAM-GOTTLIEB's inorganic salts medium (ISP No. 9 medium).
±: Weakly positive.

fermenter containing 120 liters of the fermentation medium. Fermentation was run at 28°C with agitation at 250 rpm and aeration rate of 120 liters per minute. The antitumor antibiotic level reached a maximum of 50 μg/ml after about 90 hours fermentation.

Isolation and Purification

The fermentation broth (23 liters, pH 7.4) was separated into the mycelial cake and the supernate by use of a Sharples-type centrifuge. The mycelial cake was extracted with methanol (6 liters); the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the broth supernate and extracted with ethyl acetate (20 liters). The organic layer was evaporated under reduced pressure to yield a crude antibiotic complex (21 g). This solid was dissolved in methylene chloride and applied to a column of silica gel (4.0 i.d. × 75 cm) which was developed with methylene chloride - methanol (100 : 0~90 : 10). The eluate was monitored by the cytotoxicity against B16 melanoma and also by color reaction with iodine on TLC plate. The first iodine-positive fractions eluted with 2% methanol were concentrated to give 9-methylstreptimidone[2] which was identified by its spectral data. The second iodine-positive fractions, eluted with 5% methanol, were collected, evaporated *in vacuo* and charged on a new silica gel column (3.5 i.d. × 45 cm) using ethyl acetate - methanol (50 : 1) as an eluant. The active fractions collected were concentrated and further purified by Sephadex LH-20 chromatography with methanol elution. The semi-pure solid (341 mg) obtained was then chromatographed on a reversed phase silica gel column (YMC-GEL ODS-A, 2.2 i.d. × 50 cm). Elution was performed with methanol - H_2O (1 : 1) and the combined active fractions were concentrated to an aqueous solution which was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and evaporated *in vacuo* to yield a homogeneous solid of eponemycin (175 mg).

Physico-chemical Properties

The physico-chemical properties of eponemycin are summarized in Table 3. It is readily soluble in chloroform, ethyl acetate, ethanol, *N,N*-dimethylformamide and dimethyl sulfoxide, but practically insoluble in water, *n*-hexane and benzene. Eponemycin showed positive reaction to iodine, ammonium molybdate-sulfuric acid and Rydon-Smith reagent, while it was negative to ninhydrin, anthrone and ferric chloride tests.

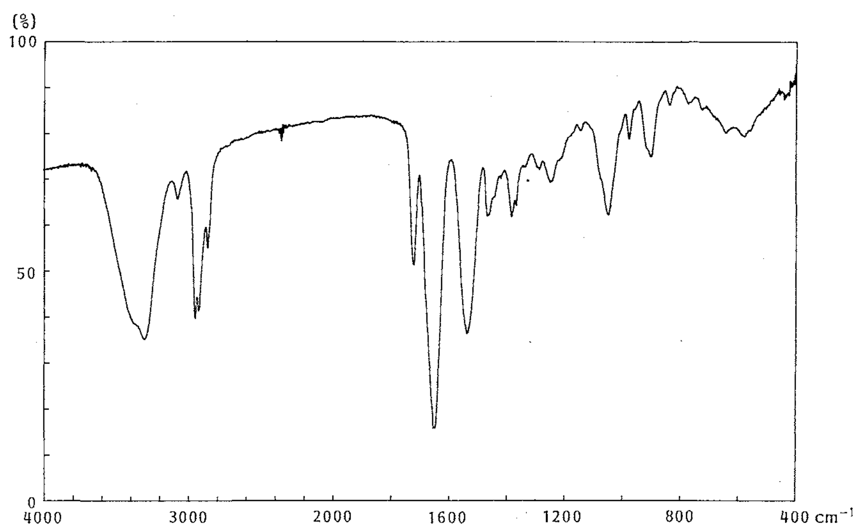
Its molecular formula was established as $C_{20}H_{34}N_2O_6$ on the basis of microanalysis and HREI-MS spectrum, (MH^+ *m/z* 399.2511). It did not exhibit characteristic UV absorption. In the IR spectrum (Fig. 1), eponemycin showed strong absorption bands at 3300 (OH and/or NH), 1720 (ketone or ester) and 1650 and 1530 cm^{-1} (amide). The ^1H NMR spectrum (Table 4) in $CDCl_3$ revealed the presence of three methyls (δ 0.86, d×2 and 1.75, s), one exomethylene (δ 4.79, br s and 4.83, br s) and two amide protons (δ 6.48, d and 7.03, d) in addition to 11 other methylene or methine protons at around δ 1.0~4.7. When the ^1H NMR was determined in DMSO-d_6, the presence of two other protons (δ 4.75, t and 5.07, dd) assignable to the hydroxyl protons of primary alcohols were also identified. The ^{13}C NMR spectrum demonstrated 20 carbons which were assigned to two CCH_3, one $=CCH_3$, eight CH_2, three $-\overset{|}{\underset{|}{C}}H$, one $>C<$, one $>C=CH_2$ and three C=O carbons.

Table 3. Physico-chemical properties of eponemycin.

Nature	Colorless sticky solid
$[\alpha]_D^{24}$	$+32\pm2°$ (*c* 0.5, MeOH)
Microanalysis	Calcd for $C_{20}H_{34}N_2O_6$: C 60.28, H 8.60, N 7.03
	Found: C 60.18, H 8.82, N 6.60
HREI-MS	MH^+, Found *m/z* 399.2511 (Calcd for $C_{20}H_{35}N_2O_6$: 399.2493)
TLC[a] (Rf)	0.37 (CH_2Cl_2-MeOH, 9:1)
	0.59 (EtOAc-MeOH, 4:1)
	0.35 (Methyl ethyl ketone-xylene-MeOH, 10:10:2)
HPLC[b] (Rt)	5.4 minutes

[a] SiO_2 (Merck F_{254}).
[b] Column: YMC packed ODS, A-301-3: MeOH-H_2O (65:35), 1.0 ml/minute.

Fig. 1. IR spectrum of eponemycin (KBr).

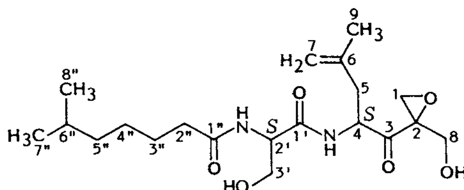


Structural Studies

The IR and NMR spectral data (Table 4) suggested a peptide structure for eponemycin (**1**). Two primary alcohols deduced by the ^{1}H NMR in DMSO-d_6 were substantiated by the fact that **1** afforded a di-*O*-acetyl derivative (**2**, MH^{+} *m/z* 483) upon acetylation in pyridine. The two AB type methylenes (δ 3.58 and 4.02, and 3.75 and 4.21) in **1** showed an acylation shift to δ 4.18 and 4.37, 4.01 and 4.87, respectively in **2**. Hydrogenation of **1** over palladium carbon gave two reduction products, dihydroeponemycin (**3**, MH^{+} *m/z* 401) and tetrahydroeponemycin (**4**, MH^{+} *m/z* 403). The ^{1}H NMR spectrum of **3** contained a newly produced methyl (δ 0.95, d) with concomitant disappearance of the exomethylene protons and an upfield shift of a methyl in **1** (from δ 1.75, s to 0.95, d). **4** exhibited a similar ^{1}H NMR pattern as that of **3**, and in addition, an AB type methylene observed in **1** (δ 3.12, d and 3.35, d) shifted in **4** to δ 3.81, dd and 3.90, m which were coupled to each other and a newly produced methine proton (δ 3.15, m). This result indicated that **1** contained an epoxide group which yielded a hydroxymethyl group upon catalytic reduction. Taking into consideration the functional groups assigned above, analysis of the ^{1}H and ^{13}C NMR and ^{1}H-^{1}H COSY spectra of **1** identified the following five structural units (A, B, C, D and E) which accounted for all the structural units of **1**.

In order to confirm fragments A and B, **1** was heated with 6 N HCl at 105°C for 16 hours. An acidic lipophilic product was extracted from the reaction mixture with diethyl ether and reacted with excess diazomethane. The methyl ester (**5**, M^{+} *m/z* 158) obtained was identified as methyl isooctanoate by the

Table 4. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$ of eponemycin).



Carbon No.	^{1}H NMR
1	3.12 (d, 5.0), 3.35 (d, 5.0)
2	—
4	4.61 (ddd, 6.6, 10.3, 3.6)
5	2.08 (dd, 10.3, 14.1), 2.59 (dd, 3.6, 14.1)
6	—
7	4.79 (br s), 4.83 (br s)
8	3.75 (d, 12.6), 4.21 (d, 12.6)
9	1.75 (s)
4-NH	7.03 (d, 6.6)
2′	4.48 (ddd, 7.3, 5.5, 3.5)
3′	3.58 (dd, 5.5, 11.4), 4.02 (dd, 3.5, 11.4)
2′-NH	6.48 (d, 7.3)
2″	2.21 (t, 7.7)
3″	1.60 (m)
4″	1.28 (m)
5″	1.16 (m)
6″	1.52 (m)
7″	0.86 (d, 6.6)
8″	0.86 (d, 6.6)

Carbon No.	^{13}C NMR
1	49.3 (t)
2	62.5 (s)
3	207.3 (s)
4	51.3 (d)
5	38.1 (t)
6	140.0 (s)
7	114.6 (t)
8	61.3 (t)
9	21.6 (q)
1′	171.2 (s)
2′	53.7 (d)
3′	62.7 (t)
1″	174.0 (s)
2″	36.4 (t)
3″	25.7 (t)
4″	26.9 (t)
5″	38.6 (t)
6″	27.7 (d)
7″	22.5 (q)
8″	22.5 (q)

GC-MS analysis. The aqueous hydrolysate was chromatographed on Dowex 50WX4 (H^+ type) with an increasing concentration of hydrochloric acid elution to yield L-serine (**6**).

In fragment C, long range ^1H-^1H correlations were observed between methylene protons, exomethylene protons and methyl protons. The epoxide methylene protons of fragment D and hydroxymethyl protons of fragment E showed only geminal couplings and no correlation with any other proton. The connectivities of these fragments were solved by clear correlations observed in the ^{13}C-^1H long range COSY spectrum of **1** (Fig. 3). The ketone carbon (fragment F) displayed contours with C-1 epoxide methylene protons (fragment D) and C-4 methine

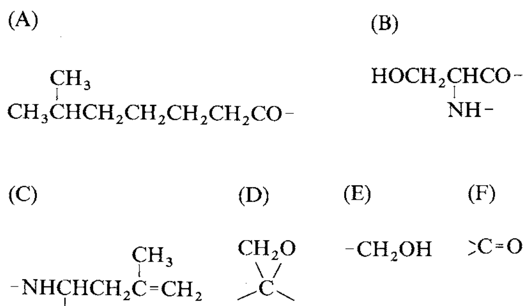


Fig. 2. Structures of eponemycin (**1**), dihydroeponemycin (**3**) and tetrahydroeponemycin (**4**).

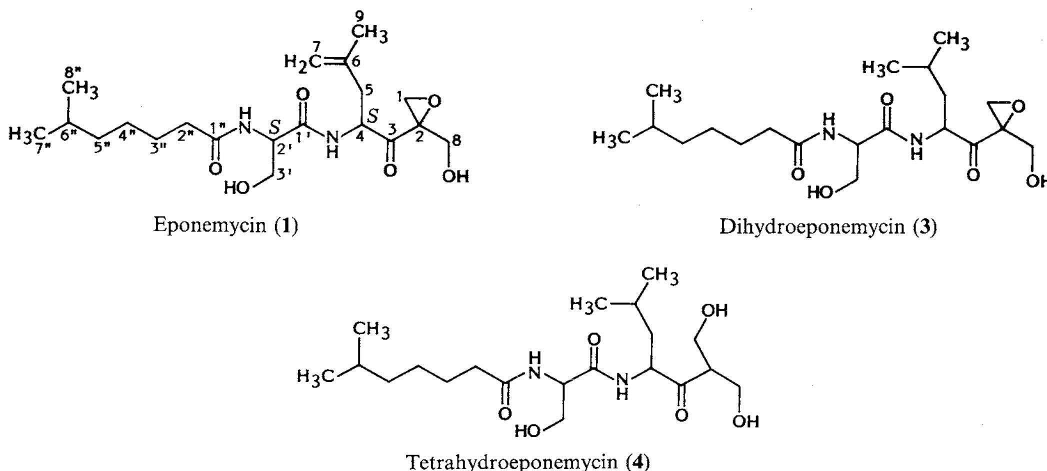


Fig. 3. ^{13}C-^1H Long range COSY (a) and HREI-MS (b) of eponemycin.

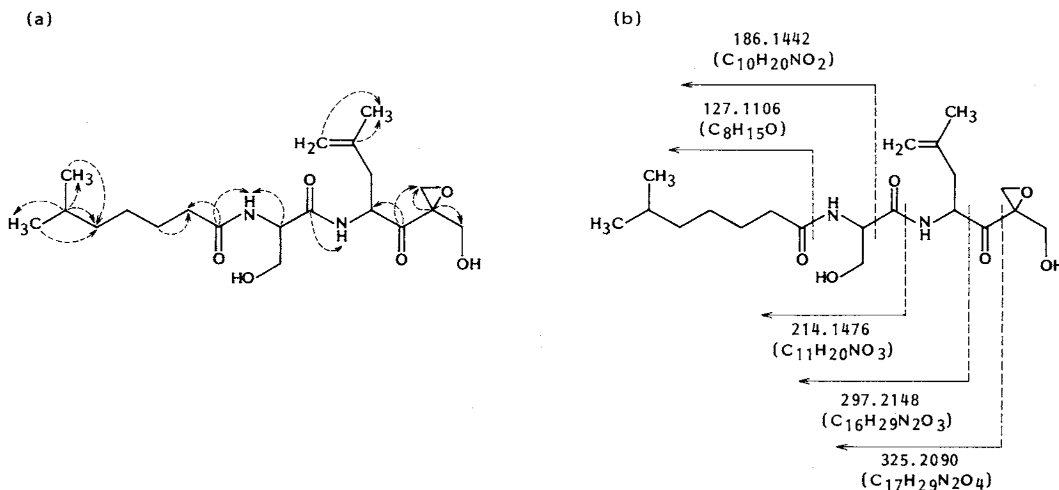


Table 5. *In vitro* cytotoxicity.

Compound	IC_{50} (μg/ml)				
	B16-F10	P388	L1210	Moser	HCT-116
Eponemycin (**1**)	0.0017	0.031	0.01	0.016	0.0097
Diacetyleponemycin (**2**)	0.0030	ND	ND	0.044	0.017
Dihydroeponemycin (**3**)	0.0032	ND	ND	0.038	0.013
Tetrahydroeponemycin (**4**)	0.53	ND	ND	>1.0	>1.0
Mitomycin C	0.50	ND	ND	1.2	0.80

ND: Not determined.

proton (fragment C), and C-2 tertiary carbon (fragment D) contours with C-8 methylene protons (fragment E) and C-1 epoxide protons (fragment D) establishing the structure of the epoxy-β-aminoketone moiety in **1**. The configuration of C-4 was determined to be "*S*" by the following process: Modified Baeyer-Villiger oxidation[3] followed by hydrolysis with 6 N hydrochloric acid. The products were chromatographed on an ion-exchange column to isolate two amino acids, leucine and serine. HPLC analysis using a chiral column established that they were L-leucine and L-serine.

Table 6. Inhibition of macromolecule biosynthesis in B16 melanoma cells.

Compound	IC_{50} (μg/ml)		
	DNA	RNA	Protein
Eponemycin	0.11	6.2	>100
Mitomycin C	0.41	19	28

The ^{13}C-^{1}H long range COSY spectrum also pointed out that the amide carbon C-1′ and C-1″ showed cross peaks to the 4-NH and 2′-NH, respectively, leading to the total structure of eponemycin (Fig. 3). The EI-MS of **1** provided further evidence for the structure (Fig. 3). The spectrum exhibited abundant ions derived by rupture of the CONH or COC bond with their molecular formula established by the HR spectrum.

Antimicrobial Activity

Antimicrobial activity of **1** was assessed by the serial 2-fold agar dilution method using Nutrient Agar (Eiken) for bacteria and Sabouraud Dextrose Agar (Difco) for fungi. Eponemycin did not show inhibitory activity against Gram-positive and Gram-negative bacteria and fungi up to 100 μg/ml.

Antitumor Activity

Eponemycin (**1**) and its derivatives (**2**~**4**) were tested for their *in vitro* cytotoxicity and inhibition of macromolecule biosynthesis by the method described in the Experimental section with the results summarized in Tables 5 and 6. **1** showed much more potent cytotoxicity than mitomycin C against both murine and human cells. The potency of **1** was approximately 80~300 times greater than that of mitomycin C in terms of IC_{50} values. **2** and **3** also showed similarly potent cytotoxicity with potency being approximately half that of **1**. On the other hand, **4** was significantly less active than these compounds.

The inhibitory effects of **1** on macromolecule biosynthesis were determined in cultured B16-F10 melanoma cells. As shown in Table 6, **1** inhibited DNA synthesis predominantly with an effect approximately 60 times higher than against RNA synthesis in terms of IC_{50} value. This compound did not inhibit protein synthesis at 100 μg/ml.

The *in vivo* antitumor activity was determined in tumor-bearing mice. As shown in Table 7, **1** demonstrated excellent therapeutic activity against B16 melanoma. When administered by the Q4D × 3

Table 7. Antitumor activity of eponemycin and its derivatives against B16 melanoma (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST (day)	T/C (%)	Average weight change on day 5 (g)
Expt 1					
Eponemycin (**1**)	8.0	Q4D × 3	20.0	167	−2.3
	4.0	Q4D × 3	19.0	158	−1.3
	2.0	Q4D × 3	18.0	150	−0.5
	1.0	Q4D × 3	17.0	142	+0.3
	0.5	Q4D × 3	15.5	129	+0.5
	0.25	Q4D × 3	14.0	117	+0.5
Mitomycin C	2.0	Q4D × 3	29.0	242	+0.3
	1.0	Q4D × 3	18.5	154	+0.5
	0.5	Q4D × 3	15.0	125	+0.5
	0.25	Q4D × 3	13.0	108	+0.3
Vehicle	—	Q4D × 3	12.0	—	+1.1
Expt 2					
Diacetyleponemycin (**2**)	8.0	Q4D × 3	24.0	141	−2.0
	4.0	Q4D × 3	23.0	135	+0.5
	2.0	Q4D × 3	20.5	121	+1.0
	1.0	Q4D × 3	20.0	118	+0.3
	0.5	Q4D × 3	19.0	112	+1.3
Dihydroeponemycin (**3**)	8.0	Q4D × 3	22.5	132	−1.0
	4.0	Q4D × 3	20.0	118	−0.5
	2.0	Q4D × 3	21.0	124	+0.5
	1.0	Q4D × 3	19.5	115	+0.5
	0.5	Q4D × 3	19.0	112	+0.5
Mitomycin C	2.0	Q4D × 3	≧33.0	≧194	0.0
	1.0	Q4D × 3	23.0	135	+1.0
	0.5	Q4D × 3	22.0	129	+0.3
	0.25	Q4D × 3	20.0	118	+0.3
Vehicle	—	Q4D × 3	17.0	—	+0.8

Table 8. Antitumor activity of eponemycin against L1210 leukemia (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST (day)	T/C (%)	Average weight change on day 5 (g)
Eponemycin	4.0	Q3D × 3	Toxic	Toxic	—
	2.0	Q3D × 3	11.5	144	−3.5
	1.0	Q3D × 3	10.0	125	−2.8
	0.5	Q3D × 3	9.5	119	−0.8
	0.25	Q3D × 3	9.5	119	0.0
Mitomycin C	2.0	Q1D × 3	13.0	163	−0.5
	1.0	Q1D × 3	11.0	138	−0.3
	0.5	Q1D × 3	10.5	131	0.0
	0.25	Q1D × 3	10.0	125	+0.8
Vehicle	–	Q3D × 3	8.0	—	+1.1

schedule, the compound provided good maximum T/C (%) and wide activity range, and was as potent as mitomycin C in terms of minimum effective dose. Both **2** and **3** also exhibited significant anti-B16 melanoma activity by the Q4D × 3 treatment schedule but they seemed to be approximately 8- and 16-fold less potent than the parent compound, respectively (Table 7). **1** gave moderate antitumor activity against L1210 leukemia with maximum T/C value of 144% (Table 8) but no significant prolongation of life span in P388 leukemia-bearing mice.

Discussion

The structural study established that eponemycin is (4*S*)-1,2-epoxy-2-hydroxymethyl-4-(*N*-isooctanoyl-L-serylamino)-6-methylhept-6-ene-3-one. It is a unique molecule not related to the known antitumor antitibiotics so far reported. It is interesting to note that the di-*O*-acetyl derivative and the dihydro derivative (reduced at the exomethylene moiety) are bioactive while the tetrahydro derivative having no epoxide function is bio-inactive. This indicates that the epoxide moiety is essential for antitumor activity. Eponemycin exhibited antitumor effect against B16 melanoma implanted in mice but no inhibitory effect against P388 leukemia. This might be explained by the difference of susceptibility of the two cells to eponemycin. Eponemycin showed 10-fold stronger *in vitro* cytotoxicity against B16 melanoma than against P388 leukemia. It should be noted that the anti-B16 *in vivo* activity of eponemycin is greatly dependent on the dosing schedule. Our antitumor fermentation screening directed to the slow growing tumors resulted in the discovery of a novel antitumor antibiotic eponemycin.

Experimental

Physico-chemical and Spectral Analysis

TLC was performed on precoated silica gel plate (Kieselgel $60F_{254}$, Merck). IR and UV spectra were recorded on a Jasco IR-810 IR spectrophotometer and a Jasco UVIDEC-610C spectrophotometer. EI-MS and SI-MS spectra were measured on a Hitachi M80B, GC-MS on a Jeol JMS-DX 300 and HREI-MS on a Hitachi M-2000 mass spectrometer operated in fourier transform mode. Amino acid analysis was carried out using the Waters PICO-TAG system.

Acetylation of Eponemycin (1)

Eponemycin (**1**, 10 mg) was stirred with acetic anhydride (0.1 ml) and dry pyridine (0.5 ml) for 18 hours at room temperature. The reaction mixture was diluted with ethyl acetate (10 ml), and the solution was washed successively with dil HCl (10 ml) and water (10 ml). The organic solution was dried over sodium sulfate and evaporated *in vacuo* to give di-*O*-acetyleponemycin (**2**, 13 mg). **2**: Colorless sticky solid; SI-MS *m/z* 483 $(M+H)^+$; EI-MS *m/z* 423 $(M-COOCH_3)$, 340, 256, 127; IR ν_{max} (KBr) cm^{-1} 3300, 3070, 2950, 1750, 1650, 1550, 1240, 1040; ^1H NMR (400 MHz, $CDCl_3$) δ 0.86 (6H, d, $J=6.6$ Hz), 1.18 (2H, m), 1.28 (2H, m), 1.52 (1H, m), 1.59 (2H, m), 1.76 (3H, s), 2.05 (3H, s), 2.07 (1H, dd, $J=10.2$ and 13.9 Hz), 2.09 (3H, s), 2.21 (2H, t, $J=6.6$ Hz), 2.60 (1H, dd, $J=3.7$ and 13.9 Hz), 3.09 (1H, d, $J=5.0$ Hz), 3.38 (1H, d, $J=5.0$ Hz), 4.01 (1H, d, $J=12.1$ Hz), 4.18 (1H, dd, $J=5.5$ and 11.4 Hz), 4.37 (1H, dd, $J=5.9$ and 11.4 Hz), 4.61 (1H, ddd, $J=6.6$, 10.2 and 3.7 Hz), 4.70 (1H, ddd, $J=6.9$, 5.5 and 5.9 Hz), 4.80 (1H, br s), 4.87 (1H, d, $J=12.1$ Hz), 4.88 (1H, br s), 6.22 (1H, d, $J=6.9$ Hz), 6.53 (1H, d, $J=6.6$ Hz).

Hydrogenation of 1

1 (30 mg) dissolved in methanol (10 ml) was hydrogenated under atmospheric pressure in the presence of 20% Pd-C (15 mg) for 20 hours. After the catalyst was removed by filtration, the filtrate was evaporated *in vacuo* to yield an oily residue (27 mg). This solid was purified by preparative TLC (SiO_2, CH_2Cl_2 - MeOH 9 : 1) followed by Sephadex LH-20 chromatography to afford dihydroeponemycin (**3**, 13.4 mg) and tetrahydroeponemycin (**4**, 4.7 mg). **3**: Colorless oil; SI-MS *m/z* 401 $(M+H)^+$; EI-MS *m/z* 370 $(M+H-CH_2OH)$, 299, 214, 127; IR ν_{max} (KBr) cm^{-1} 3300, 3070, 2950, 1720, 1640, 1530, 1050; ^1H NMR (400 MHz, $CDCl_3$) δ 0.85 (6H, d, $J=7.6$ Hz), 0.94 (3H, d, $J=6.2$ Hz), 0.95 (3H, d, $J=6.2$ Hz), 1.16 (2H, m), 1.28 (4H, m), 1.52 (1H, m), 1.60 (2H, m), 1.66 (1H, m), 2.22 (2H, t, $J=7.5$ Hz), 3.10 (1H, d, $J=4.9$ Hz), 3.31 (1H, d, $J=4.9$ Hz), 3.58 (1H, dd, $J=5.8$ and 11.6 Hz), 3.73 (1H, d, $J=12.6$ Hz), 4.03 (1H, dd, $J=2.7$ and 11.6 Hz), 4.21 (1H, d, $J=12.6$ Hz), 4.49 (1H, ddd, $J=7.3$, 5.8 and 2.7 Hz), 4.51 (1H, m), 6.51 (1H, d, $J=7.3$ Hz), 7.10 (1H, d, $J=7.0$ Hz). **4**: Colorless oil; SI-MS *m/z* 403 $(M+H)^+$; EI-MS *m/z* 372 $(M+H-CH_2OH)$, 299, 214, 127; IR ν_{max} (KBr) cm^{-1} 3300, 3070, 2950, 1710, 1640, 1530, 1050; ^1H NMR (400 MHz, $CDCl_3$) δ 0.85 (6H, d, $J=7.6$ Hz), 0.94 (3H, d, $J=6.2$ Hz), 0.95 (3H, d, $J=6.2$ Hz), 1.16 (2H, m), 1.29 (4H, m), 1.52 (1H, m), 1.61 (2H, m), 1.70 (1H, m), 2.24 (2H, t, $J=7.0$ Hz), 3.15 (1H, m), 3.59 (1H, dd, $J=5.9$ and 11.7 Hz), 3.74 (1H, dd, $J=4.7$ and 11.0 Hz), 3.81 (1H, dd, $J=7.3$ and 11.4 Hz), 3.90 (2H, m), 4.06 (1H, dd, $J=3.3$ and 11.7 Hz), 4.54 (1H, m), 4.58 (1H, m), 6.52 (1H, d, $J=6.9$ Hz), 7.31 (1H,

d, $J=5.5$ Hz).

Acid Hydrolysis of **1**

1 (40 mg) in 6 N HCl (2 ml) was heated in a sealed tube at 105°C for 16 hours. The reaction mixture was diluted with water (20 ml) and extracted twice with diethyl ether (20 ml). The ether extracts were combined, dried over Na_2SO_4 and concentrated to give an oil (6.4 mg) which was treated with diazomethane to afford the methyl ester (**5**, 6.9 mg). **5**: Yellow oil, GLC Rt 8.92 minutes (Column: OV-17, 4 i.d. mm × 2 m, temperature was programmed at 5°C/minute from 50 to 150°C); CI-MS *m/z* 159 $(M+H)^+$; EI-MS *m/z* 158 (M^+), 143, 115, 109. Identified as methyl isooctanoate.

The aqueous solution was evaporated *in vacuo* and the residue (30 mg) was chromatographed on a column of Dowex 50WX4 (H^+ form, 5 ml) which was developed with an increasing concentration of HCl (0.01 ~ 1.0 N). The eluates from 0.1 N HCl were pooled, evaporated *in vacuo* and lyophilized to yield a sticky solid (**6**, 9.2 mg), which was identified as serine by TLC, IR, ^{1}H NMR and amino acid analysis.The configuration of serine was determined to be L-form (Rt 8.25 minutes) by chiral HPLC (column: MCI GEL ODS 1HU, 4.6 × 150 mm, Mitsubishi Chemical Industries Limited, elution: *N*,*N*-Dipropyl-L-alanine 2 mM, copper acetate 1 mM, pH 5.7, flow rate: 0.5 ml/minute, detection: UV 230 nm, Rt: L-Ser 8.25 minutes, D-Ser 7.00 minutes, **6**, 8.25 minutes).

Determination of C-4 Configuration

A mixture of **3** and **4** (68 mg) was dissolved in methylene chloride (1.0 ml) and stirred vigorously with disodium hydrogen phosphate (120 mg). A peroxytrifluoroacetic acid solution prepared from trifluoroacetic anhydride (0.2 ml) and 90% hydrogen peroxide (0.03 ml) in cold methylene chloride (0.2 ml) was then added slowly to the stirred sample mixture. After addition, the mixture was heated under reflux for 30 minutes and then filtered. The filtrate was diluted with methylene chloride (10 ml), washed successively with 10% sodium carbonate solution (10 ml) and water (10 ml), dried over sodium sulfate and evaporated to yield an oily residue (46 mg). This material was heated with 6 N hydrochloric acid (5 ml) at 105°C for 16 hours under N_2 gas. The hydrolysate was diluted with water (20 ml), washed with ether and evaporated *in vacuo* to afford a pale yellow solid (28 mg). It was chromatographed on a column of Dowex 50WX4 (H^+ form, 1.1 i.d. × 20 cm) developed with a stepwise increasing concentration of hydrochloric acid (0.05 ~ 1 N). The first ninhydrin-positive fractions eluted with 0.1 N hydrochloric acid were pooled, concentrated and lyophilized to give a sticky solid of serine (13.4 mg). The second ninhydrin-positive fractions eluted with 0.3 N hydrochloric acid were similarly treated to yield a white powder (**7**, 3.6 mg) which was identified as leucine by TLC and amino acid analysis. Upon development under the condition shown below, **7** was eluted at Rt of 14.46 minutes which was identical with that of standard L-leucine. Condition: Column MCI GEL CRS10W, 4.6 × 50 mm, Mitsubishi Chemical Industries Limited, elution: $CuSO_4$ 2 mM, flow rate: 1.0 ml/minute, detection: UV 254 nm, Rt: L-Leucine 14.49 minutes, D-leucine 8.15 minutes.

In Vitro Cytotoxicity and Inhibition of Macromolecule Biosynthesis

B16-F10 (murine melanoma), P388 (murine leukemia), L1210 (murine leukemia) and Moser (human colorectal carcinoma) cells were grown in EAGLE's minimum essential medium (Nissui) supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), and HCT-116 (human colon carcinoma) cells were grown in McCOY's 5A Medium (Gibco) supplemented with FCS (10%), benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C under humidified atmosphere in a CO_2 incubator. The above exponentially growing cells were harvested, counted and suspended in the culture media at 1.5×10^5, 1.2×10^4, 1.2×10^4, 2.5×10^5 and 3.0×10^5 cells/ml, respectively. After planting into wells of the 96- or 24-well tissue culture plate with the test materials, they were incubated for 72 hours. The cytotoxic activities against B16-F10, Moser and HCT-116 cells were colorimetrically determined at 540 nm after staining viable cells with neutral red solution. Activity against P388 and L1210 cells was determined by counting the number of viable cells. The inhibition of macromolecule biosynthesis was assessed using B16-F10 melanoma cells. The cells (1×10^5 cells/ml) were incubated with the test materials at 37°C for 4.5 hours (for DNA synthesis) or 4 hours (for RNA and protein synthesis). Isotopically labeled precursor, [^{3}H]thymidine, [^{14}C]uridine or [^{3}H]leucine was added to the cultured mixture and further incubated for 30 minutes (for DNA synthesis) or 60 minutes

(for RNA and protein synthesis). After washing with chilled 5% TCA solution, the radioactivity incorporated into the acid-insoluble fraction of the tumor cells was determined with a liquid scintillation counter.

In Vivo Antitumor Activity

B16 melanoma, L1210 leukemia and P388 leukemia were intraperitoneally inoculated with 0.5 ml of 10% tumor brei, 10^5 and 10^6 cells per mouse, respectively. The test materials were intraperitoneally administered to the mice by the following three different treatment schedules: Once daily on days 1 to 3 (Q1D × 3), once a day on days 1, 4 and 7 (Q3D × 3) and on days 1, 5 and 9 (Q4D × 3).

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