A NEW ANTITUMOR SUBSTANCE, BE-18591, PRODUCED BY A STREPTOMYCETE

I. FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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New antitumor substance, designated BE-18591, was isolated from the culture broth of a streptomycete, strain BA18591. The active principle was extracted from mycelium by methanol and purified by silica gel chromatography. BE-18591 inhibited the growth of MKN-45 human stomach cancer cell line as well as P388 cell line. In *in vivo* experiments, BE-18591 inhibited the growth of Ehrlich ascites tumor.BE-18591 showed antimicrobial activity against Gram-positive and some Gram-negative bacteria.

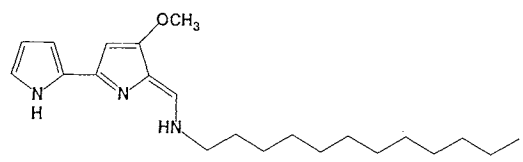
In the course of our screening program for new antitumor substances, a strain BA18591 isolated from a plant sample collected in Hamamatsu, Shizuoka Prefecture, Japan, was found to produce an active principle. This strain was classified as *Streptomyces* sp. The active principle was extracted from the mycelium of the strain with methanol and was purified by silica gel column chromatography. BE-18591 showed cytotoxic activity against a human tumor cell as well as a murine tumor cell line. BE-18591 also displayed antimicrobial activities against Gram-positive and some Gram-negative bacteria. This paper describes the isolation, physico-chemical properties and biological activities of BE-18591. The structure elucidation studies of this compound are described in an accompanying paper[1]. The structure of BE-18591 is shown in Fig. 1.

Materials and Methods

Fermentation

Spores of strain BA18591 were inoculated into 100 ml of a medium (pH 6.7) composed of 0.1% glucose, 2.0% dextrin, 1.0% corn gluten meal, 0.5% fish meal, 0.1% yeast extract, 0.1% sodium chloride, 0.05% magnesium sulfate, 0.05% calcium chloride, 0.0002% ferrous sulfate, 0.00004% cupric chloride, 0.00004% manganese chloride, 0.00004% cobalt chloride, 0.00008% zinc sulfate, 0.00008% sodium borate, 0.00024% ammonium molybdate and 0.5% 3-(*N*-morpholino)propane sulfonic acid in four 500-ml conical flasks and cultured at 28°C for 72 hours. Two ml of the seed culture was dispensed into each of one hundred of 500-ml conical flasks containing 100 ml of the above medium and cultured on a rotary shaker (180 rpm) at 28°C for 96 hours.

Fig. 1. Structure of BE-18591.



Biological Assays

P388 Assay

The *in vitro* cytotoxic activity of BE-18591 against P388 was measured according to the method

described previously[2].

MKN-45 Assay[3]

BE-18591 was first dissolved in dimethyl sulfoxide (DMSO). The solution was serially diluted with phosphate-buffered saline (PBS). The media used for the culture of MKN-45 human stomach cancer cells was RPMI-1640 medium containing 10% fetal bovine serum (FBS). The cell line was cultured in 96-well microplates (3×10^3 cells/well) with or without the test sample under 5% CO_2 at 37°C for 72 hours. After fixing with 50% trichloroacetic acid, cells were stained by 0.4% sulforhodamine B and the dye was extracted from the stained cells with 10 mM Tris(hydroxymethyl)-aminomethane solution. Absorbance of the extract was read at 540 nm.

In Vivo Antitumor Activity

Antitumor activity of BE-18591 in mice was examined against Ehrlich tumor cell and P388 leukemia cell (ascites type). Ehrlich and P388 cells were inoculated i.p. into CDF_1 mice at 10^6 cells per mouse. BE-18591 was given i.p. once a day from the 1st to 10th day. The effect of BE-18591 was evaluated on the basis of the mean survival time (MST) in days. The results are expressed as T/C (%); T/C (%) = MST in days of treated animals (T)/control animals (C) × 100.

Antimicrobial Assay

The antimicrobial activity of BE-18591 was determined by agar dilution method.

General Procedure

MP was taken with a Yanako MP-S3 melting point apparatus and was uncorrected. MS was carried out on a JEOL JMS-DX 300 spectrometer. UV and IR spectra were recorded on a Shimadzu UV-265FW spectrometer and a Hitachi 270-30 spectrometer, respectively. ^1H and ^{13}C NMR spectra were obtained using a Varian VXR 300 spectrometer at 300 MHz and 75 MHz, respectively. Chemical shifts were converted to values in ppm downfield from TMS as an internal standard.

Results

Isolation Procedure

Isolation was performed by using cytotoxic activity against P388 cell line as an index. The mycelium was obtained by filtration from the whole broth (*ca.* 10 liters). This mycelium was extracted twice with 5 liters of methanol and the extract was concentrated under reduced pressure to about 300 ml. The concentrated solution was extracted twice with 600 ml of ethyl acetate and the extract was concentrated *in vacuo* to give 5.9 g of a crude substance containing BE-18591. This crude substance was dissolved in 200 ml of *n*-hexane-ethyl acetate (10:1) and the solution was chromatographed on a silica gel column (3 × 32 cm) and developed with *n*-hexane-ethyl acetate (10:1) 400 ml, (5:1) 360 ml and (2:1) 1500 ml. Fractions containing BE-18591 were collected and concentrated *in vacuo* to give 1.8 g of BE-18591 as a yellowish-green amorphous solid.

Physico-chemical Properties

BE-18591 was found to be basic in nature, freely soluble in methanol, chloroform or dimethyl sulfoxide and slightly soluble in water. BE-18591 gave a positive color reaction with potassium permanganate and sulfuric acid. The other physico-chemical properties of BE-18591 are summarized in Table 1. The ^1H and ^{13}C NMR spectra are shown in Figs. 2 and 3, respectively.

Biological Activities

In the cytotoxic assay, the concentration of BE-18591 required to inhibit grown of the P388 and

Table 1. Physico-chemical properties of BE-18591.

Appearance	Yellowish green solid
MP (°C)	50～53
Molecular formula	$C_{22}H_{35}N_3O$
Rf	0.7
Kieselgel 60, Merck ($CHCl_3$-MeOH (10:1))	
FAB-MS	Found: m/z 358.2881 ($(M+H)^+$)
	Calcd: m/z 358.2903 for $C_{22}H_{36}N_3O$
UV λ_{max} nm (ε)	
0.1 N HCl-MeOH	257 (26,400), 285 (sh, 11,400), 325 (10,000), 406 (128,000)
0.1 N NaOH-MeOH	252 (28,400), 365 (61,300)
IR (KBr) cm^{-1}	3190, 2926, 1671, 1599, 1530, 1470, 1386, 1167, 1137, 1041, 1011, 969, 801, 738

Fig. 2. ^1H NMR spectrum of BE-18591 in $CDCl_3$ (300 MHz).

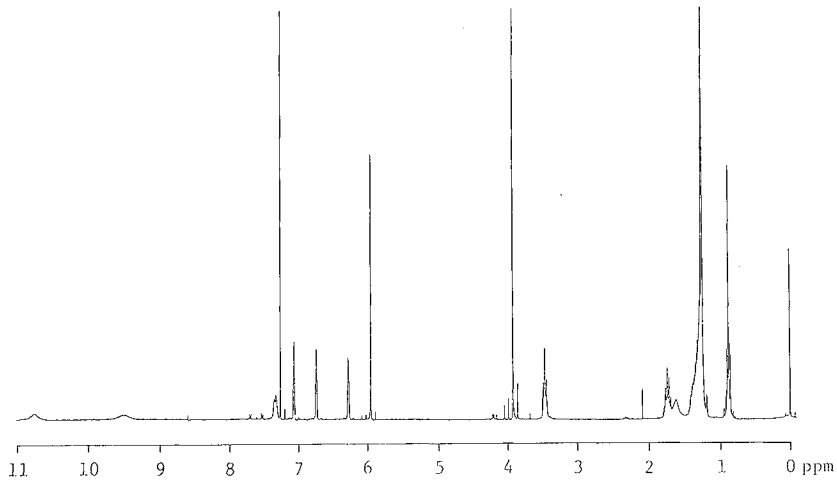


Fig. 3. ^{13}C NMR spectrum of BE-18591 in $CDCl_3$ (75 MHz).

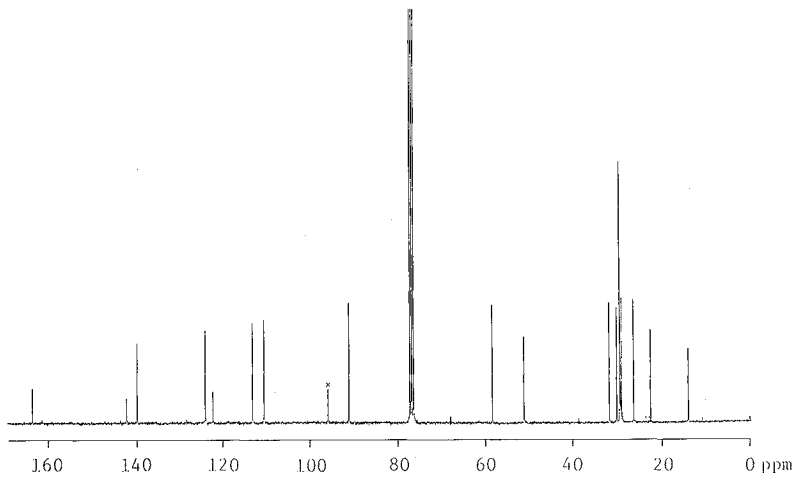


Table 2. Antitumor effect of BE-18591.

Substance	Dose mg/kg × 10 days	Ehrlich ascites T/C (%)	P388 ascites T/C (%)
Control		100	100
		(16.3±3.9)[a]	(10.2±0.42)[a]
BE-18591	0.3125	108	98
	1.25	99	98
	5	122	100
	20	152	104

[a] Mean survival time (days)±SD.

Table 3. Antimicrobial activities of BE-18591.

Test organism	MIC (μg/ml)
Bacillus subtilis ATCC 6633	3.13
B. cereus IFO 3001	3.13
Staphylococcus aureus FDA 209P	1.56
S. aureus Smith	3.13
Micrococcus luteus ATCC 9341	0.78
Enterococcus faecalis IFO 12580	3.13
Streptococcus thermophilus IFO 3535	1.56
Corynebacterium xerosis 53-K-1	3.13
Escherichia coli NIHJ JC-2	>100
Klebsiella pneumoniae ATCC 10031	6.25
Enterobacter cloacae IFO 13535	>100
Pseudomonas aeruginosa IFO 3445	>100
Flavobacter calcoaceticus IFO 12535	1.56
Torulopsis colliculosa IFO 1083	50
Wicherhamia fluorescens IFO 1116	12.5
Saccharomyces cerevisiae IFO 0283	>100
Candida albicans IFO 1270	100
Endomyces ovetensis IFO 1201	25

MKN-45 cell lines by 50% (IC_{50}) was 0.285 and 0.52 μg/ml, respectively. BE-18591 showed an antitumor effect on transplanted mouse Ehrlich tumor cells (ascites type). The results are summarized in Table 2. With regard to the acute toxicity of BE-18591 on CDF_1 mice, no death was found on the 5th day when 100 mg/kg was intraperitoneally administered. The antimicrobial activities of BE-18591 is shown in Table 3. BE-18591 displayed antibacterial activity against Gram-positive bacteria and some Gram-negative bacteria.

The BE-18591 producing strain was classified as a new species of Streptomyces, therefore the taxonomic studies will be reported in a separate paper. The strain has been deposited at the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, with the access No. FERM-P11437.

Discussion

We are continuing the screening of new leads to find more effective antitumor drugs. In the course of our screening, BE-18591 was isolated from *Streptomyces* sp. The structure of BE-18591 is related to the group of prodigiosin-like red pigments produced by *Serratia* and *Streptomyces*[4,5]. BE-18591 has only two conjugated pyrrole rings and its appearance is yellowish green, though all prodigiosins have three conjugated pyrrole rings. Tambjamines[6,7] isolated from marine source of ascidians and nudibranchs as defensive metabolites against their predators have two conjugated pyrrole rings, but their biological evaluation was entirely targeted for their defensive activity against predators except for their antimicrobial activity[6]. BE-18591 showed cytotoxic activity against MKN-45 human stomach cancer cell line and also, like prodigiosins, showed antimicrobial activities against Gram-positive and some Gram-negative bacteria[8]. Unfortunately, BE-18591 showed no antitumor effect on transplanted P388 tumor cells (ascites type). Recently, prodigiosin C-25 was found to be a potent immunosuppressant and its detailed mode of action was investigated[9,10]. Accordingly, it will be necessary to evaluate the immunosuppressive activity of BE-18591.

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