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New Cytotoxic Agents, BE-52440A and B, Produced by a Streptomycete

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New cytotoxic substances, designated BE-52440A and B, were isolated from the mycelium of *Streptomyces* sp. A52440. The active principles were extracted from the mycelium by methanol and purified by silica gel column chromatography. BE-52440A and B exhibited cytotoxic activity against murine and human tumor cell lines.

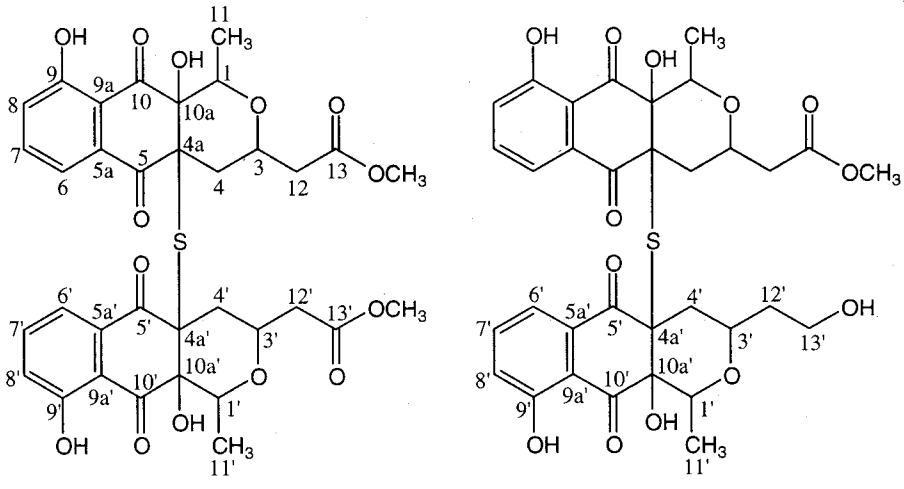
In the course of our screening program for new antitumor substances, *Streptomyces* strain A52440 isolated from a soil sample collected in Ibaraki prefecture, Japan, was found to produce active principles. These components, BE-52440A and B, were purified based on their inhibitory activity against the growth of the P388 murine leukemia cell line. BE-52440A and B inhibited not only the growth of murine tumor cell lines such as P388 leukemia, colon 26 cell lines but also human tumor cell lines such as DLD-1 colon, PC-13 lung and MKN-45 stomach cell lines. In this paper, the producing organism, fermentation, isolation, physicochemical properties, structure determination and biological properties of BE-52440A and B are described. The structure of BE-52440A and B are shown in Figure 1.

Materials and Methods

Taxonomic Studies

Characterization of the strain A52440 followed the method adopted by the International Streptomyces Project (ISP)[1]. Morphological observations were made with light

Fig. 1. Structures of BE-52440A and B.



BE-52440A BE-52440B

and scanning electron microscopes. Cell wall analysis was performed by the method of BECKER *et al.*[2] and YAMAGUCHI[3]. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[4].

Fermentation

The *Streptomyces* sp. A52440, cultured on a slant agar medium, was inoculated into 110 ml of a seed medium consisting of 0.2% glucose, 2.0% dextrin, 0.5% meat meal, 0.5% defatted rice bran, 0.1% defatted meat bone meal, 0.05% dry yeast, 0.025% magnesium sulfate, 0.025% sodium bromide, 0.25% sodium chloride, 0.05% potassium hydrogen phosphate, 0.0002% ferrous sulfate, 0.00004% cupric chloride, 0.00004% manganese chloride, 0.00004% cobalt chloride, 0.00008% zinc sulfate, 0.00008% sodium borate and 0.00024% ammonium molybdate (pH 7.2). The seed culture was incubated for 48 hours at 28°C on a rotary shaker (180 rpm). Two ml each of the culture broth was inoculated into 100 500-ml Erlenmeyer flasks containing 110 ml of medium and cultured on a rotary shaker (180 rpm) at 28°C for 120 hours.

Assay of Cytotoxic Activity

P388 Assay

The *in vitro* cytotoxicity assay using the P388 tumor cells was carried out essentially according to the method of KOJIRI *et al.*[5]. BE-52440A and B were first dissolved in dimethyl sulfoxide (DMSO). The solution was serially diluted with a cell culture medium containing 20% DMSO (20% DMSO-RPMI-1640 medium) with 2.5×10^4 tumor cells per 50 μl and the mixture was incubated under 5% CO_2 at 37°C for 72 hours. The viable cells were then counted with a Coulter counter.

DLD-1, PC-13, MKN-45 and Colon 26 Assay

The anti-proliferative activity of BE-52440A and B against DLD-1, PC-13, MKN-45 and colon 26 cell lines was measured according to the method of ARAKAWA *et al.*[6] with minor modification. BE-52440A and B were first dissolved in dimethyl sulfoxide (DMSO), and serially diluted with phosphate-buffered saline (PBS). The medium used for the culture of tumor 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 a test sample under 5% CO_2 at 37°C for 72 hours. After fixing with 50% trichloroacetic acid, tumor 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.

Fig. 2. Scanning electron micrograph of sporangia of *Streptomyces* sp. A52440.

Bar represents 3.0 μm

Results and Discussion

Taxonomy of the Producing Strain

The strain A52440 formed well developed and branching substrate mycelia and aerial mycelia, but fragmentation of the substrate mycelia was not observed. The spore long chains (<50) of the strain were spirals. The spore had rugose surface and was globular with a diameter of 1.0~1.2 μm (Fig. 2). Special morphology such as sclerotia and sporangia were not observed. The cultural characteristics of strain A52440 are summarized in Table 1. The whole-cell hydrolysate contained L,L-diaminopimeric acid. The physiological properties and carbon utilization of strain A52440 are shown in Table 2. The above-mentioned characteristics of strain A52440 revealed that it belonged to the genus *Streptomyces*. The strain A52440 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. A52440 under the accession No. FERM P-15296.

Isolation of BE-52440A and B

The mycelium was obtained by filtration of the whole broth (*ca.* 10 liters). Extraction was carried out from the

Table 1. Culture characteristics of strain A52440.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Abundant dark greenish yellow	Deep brown	Brown
Oatmeal agar (ISP-3)	Moderate	Moderate dark greenish yellow	Dark brown	Dark olive
Inorganic salts-starch agar (ISP-4)	Good	Abundant dark greenish yellow	Deep brown	Light olive
Glycerol-asparagine agar (ISP-5)	Moderate	Abundant dark greenish yellow	Dark brown	Brown
Tyrosine agar (ISP-7)	Moderate	Moderate dark greenish yellow	Dark brown	Brown
Nutrient agar	Moderate	Poor white	Orange yellow	None
Sucrose-nitrate agar	Moderate	Moderate	Deep brown	Light olive
Glucose-asparagine agar	Poor	Trace	Deep brown	None

Table 2. Physiological properties and carbon utilization of strain A52440.

Melanoid formation	
Tryptone-yeast broth (ISP-1)	−
Peptone-yeast extract-iron agar (ISP-6)	−
Tyrosine agar (ISP-7)	−
Coagulation of milk	−
Peptonization of milk	+
Liquefaction of gelatin	+
Hydrolysis of starch	+
NaCl tolerance	≦7%
Temperature range for growth	16～38℃
Carbon utilization	
D-Glucose	+
D-Xylose	+
L-Arabinose	+
L-Rhamnose	+
D-Fructose	+
Raffinose	−
D-Mannitol	+
i-Inositol	+
Sucrose	+
D-Galactose	+
Salicin	+

mycelium twice with 3 liters of methanol and the combined extracts were concentrated. The concentrated solution was extracted twice with 2 liters of ethyl acetate and the combined extracts were evaporated under reduced pressure. The residue was washed with 50 ml of ethanol. The residue was applied to a Silica gel column (Merck, 54×3.0 cm i.d.) and eluted with $CHCl_3$/MeOH (40 : 1). The fractions containing BE-52440A were concentrated *in vacuo*. The precipitate was filtered to afford BE-52440A (1.97 g) as a colorless powder. The fractions containing BE-52440B were concentrated *in vacuo*. Further purification by a TOYOPEARL HW-40S (TOSOH) column chromatography (50×2.0 cm i.d.) using CH_3OH as eluent yielded 27.5 mg of BE-52440B as a colorless powder.

Table 3. ^{13}C-NMR data for BE-52440A and B in $CDCl_3$.

	BE-52440A	BE-52440B
1	74.4	74.4
3	63.0	63.0
4	27.8	27.8
4a	61.3	61.2
5	189.4	189.5
5a	133.1	133.1
6	120.3	120.4
7	137.5	137.5
8	124.1	124.1
9	161.9	161.9
9a	114.5	114.5
10	195.7	195.8*
10a	76.1	76.1
11	14.2	14.2
12	39.6	39.6
13	170.6	170.6
OCH_3	51.8	51.8
1'	74.4	74.2
3'	63.0	65.3
4'	27.8	28.7
4'a	61.3	61.3
5'	189.4	189.3
5'a	133.1	133.3
6'	120.3	119.5
7'	137.5	137.3
8'	124.1	124.1
9'	161.9	161.9
9'a	114.5	114.7
10'	195.7	195.9*
10'a	76.1	76.2
11'	14.2	14.6
12'	39.6	36.9
13'	170.6	60.4

^{13}C NMR spectra were measured at 125 MHz.

Physico-chemical Properties

BE-52440A and B are soluble in DMSO but insoluble in hexane and water. Other properties are as follows: BE-52440A ($C_{34}H_{34}O_{14}S$); HRFAB-MS *m/z* 699.1755 $(M+H)^+$, calcd *m/z* 699.1748 $(M+H)^+$; $[\alpha]_D^{20}$ +252° (*c* 1.0, DMSO); UV λ_{max}^{MeOH} nm 233 (36,100), 356 (13,100); IR ν_{max} (KBr) cm^{-1} 3568, 3421, 1730, 1697, 1655, 1454, 1263, 1171, 1115, 1057. BE-52440B ($C_{33}H_{34}O_{13}S$) ; HRFAB-MS *m/z* 671.1791 $(M+H)^+$, calcd *m/z* 671.1798 $(M+H)^+$; $[\alpha]_D^{20}$ −215° (*c* 1.0, $CHCl_3$); UV λ_{max}^{MeOH} nm 233 (29,300), 356 (10,200); IR ν_{max} (KBr) cm^{-1} 3448, 1736, 1701, 1657, 1454, 1259, 1169, 1113, 1059, 1014.

Structure Determination

The ^{13}C- and ^{1}H-NMR data of BE-52440A and B are shown in Table 3 and 4, respectively. The molecular formula of BE-52440A was established as $C_{34}H_{34}O_{14}S$ from the result of HRFAB-MS spectral data and elemental analysis (Calcd: C 56.27, H 5.10, S 4.41 for $C_{34}H_{34}O_{14}S \cdot 1.5H_2O$, Found: C 56.36, H 5.10, S 4.41). The 17 proton atoms were observed by ^{1}H-NMR and 17 carbon atoms were observed by ^{13}C-NMR. The number of proton and carbon atoms observed by NMR was half of that by HRFAB-MS, data indicating that BE-52440A has a symmetrical structure. The ^{1}H-^{1}H COSY data of BE-52440A revealed 1,2,3- trisubstituted benzene, $-CH_2-CH-CH_2-$ and CH_3CH- moieties. In the ^{1}H-NMR spectrum, a D_2O-exchangeable proton signal at δ_H 11.3 was observed, which suggested the presence of a chelated phenolic proton. Based on the HMBC spectrum, the main part of the structure of BE-52440A was deduced (Fig. 3). The HMBC spectrum showed that 6-H (δ_H 7.73) was correlated to a

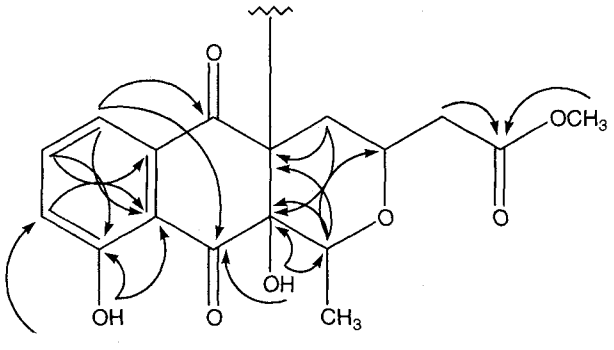
Table 4. ^{1}H-NMR data for BE-52440A and B in $CDCl_3$.

	BE-52440A	BE-52440B
1	4.10 (1H, q, J=7.3Hz)	4.09 (1H, q, J=7.0Hz)
3	3.92 (1H, m)	3.91 (1H, m)
4	2.25 (2H, m)	2.24 (2H, m)
6	7.73 (1H, dd, J=7.6, 1.6Hz)	7.73 (1H, dd, J=7.6, 1.5Hz)
7	7.76 (1H, t, J=7.6Hz)	7.76 (1H, t, J=7.6Hz)
8	7.28 (1H, dd, J=7.6, 1.6Hz)	7.28[a]
9-OH	11.3 (1H, s)	11.3 (1H, s)
10a-OH	3.65 (1H, s)	3.71 (1H, s)
11	1.16 (3H, d, J=7.3Hz)	1.16 (3H, d, J=7.0Hz)
12	2.51 (1H, dd, J=15.3, 9.4Hz)	2.50 (1H, dd, J=15.0, 9.2Hz)
	2.59 (1H, dd, J=15.3, 3.0Hz)	2.58 (1H, dd, J=15.0, 3.0Hz)
13-OCH_3	3.76 (3H, s)	3.76 (3H, s)
1'	4.10 (1H, q, J=7.3Hz)	4.12 (1H, q, J=7.3Hz)
3'	3.92 (1H, m)	3.67 (1H, m)
4'	2.25 (2H, m)	2.24 (2H, m)
6'	7.73 (1H, dd, J=7.6, 1.6Hz)	7.63 (1H, dd, J=7.8, 1.0Hz)
7'	7.76 (1H, t, J=7.6Hz)	7.70 (1H, t, J=7.8Hz)
8'	7.28 (1H, dd, J=7.6, 1.6Hz)	7.28[a]
9'-OH	11.3 (1H, s)	11.3 (1H, s)
10'a-OH	3.65 (1H, s)	3.78 (1H, s)
11'	1.16 (3H, d, J=7.3Hz)	1.20 (3H, d, J=7.3Hz)
12'	2.51 (1H, dd, J=15.3, 9.4Hz)	1.80 (2H, m)
	2.59 (1H, dd, J=15.3, 3.0Hz)	
13'		3.80 (2H, m)
13'-OCH_3	3.76 (3H, s)	
13'-OH		2.37 (1H, brs)

^{1}H NMR spectra were measured at 500 MHz.

a: overlapped signals.

Fig. 3. HMBC spectrum of BE-52440A.



carbonyl carbon (δ_C 189.4) strongly and another carbonyl carbon (δ_C 195.7) weakly. The D_2O-exchangeable proton (δ_H 3.65), which coupled to a carbonyl carbon (δ_C 195.7) in the HMBC spectrum, was shown to be a hydroxyl proton of a tertiary alcohol next to a carbonyl carbon. From these results and the molecular formula, the structure of BE-52440A was determined as shown in Figure 1. The relative stereochemistry was determined by the analysis of NOESY (in $CDCl_3$) and ROESY (in DMSO-d_6) spectra. In the ^{1}H-NMR spectrum of BE-52440 in $CDCl_3$, methylene protons (H-4) were observed at almost same chemical shifts. However in DMSO-d_6, methylene protons (H-4) were observed at δ_H 2.17 and δ_H 1.86, respectively (Table 5). In the ROESY experiment, NOE's were observed between 1-H and 10a-OH, between 3-H and 11-H and between 4-H (δ_H 2.17) and 10a-OH (Fig. 4). Based on the above results, the structure of BE-52440A was determined as shown in Figure 5.

The molecular formula of BE-52440B was established as $C_{33}H_{34}O_{13}S$ from HRFAB-MS and ^{13}C-NMR spectral data. The ^{1}H-NMR spectrum of BE-52440B has many signals with nearly the same chemical shifts as those of BE-52440A, as well as others with larger deviations. The ^{1}H-^{1}H COSY spectrum of BE-52440B again indicated 1,2,3-trisubstituted benzene and CH_3CH– moieties and also revealed the presence of a $-CH_2CHCH_2CH_2OH$ moiety.

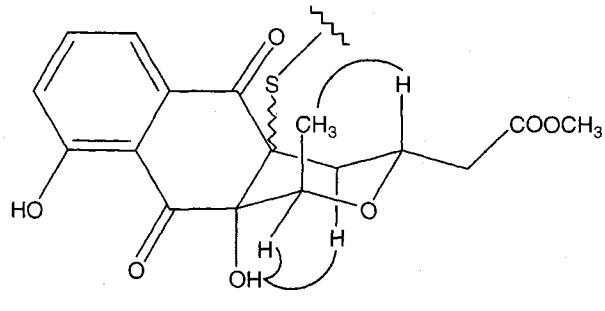
Table 5. ^{1}H-NMR and ^{13}C-NMR data for BE-52440A in DMSO-d_6.

	^{13}C	^{1}H
1	72.6	3.98 (1H, q, J=7.0Hz)
3	62.4	3.68 (1H, m)
4	27.4	2.17 (1H, dd, J=14.6, 12.2Hz)
		1.86 (1H, brd, J=14.6Hz)
4a	61.5	
5	187.7	
5a	132.9	
6	124.0	7.60 (1H, d, J=7.3Hz)
7	137.2	7.78 (1H, t, J=7.3Hz)
8	119.6	7.33 (1H, d, J=7.3Hz)
9	160.4	
9a	114.1	
9-OH		11.1 (1H, s)
10	197.0	
10a	75.9	
10a-OH		7.24 (1H, s)
11	14.7	1.08 (3H, d, J=7.0Hz)
12	39.6	2.38 (2H, m)
13	170.3	
OCH_3	51.3	3.65 (3H, s)
1'	72.6	3.98 (1H, q, J=7.0Hz)
3'	62.4	3.68 (1H, m)
4'	27.4	2.17 (1H, dd, J=14.6, 12.2Hz)
		1.86 (1H, brd, J=14.6Hz)
4'a	61.5	
5'	187.7	
5'a	132.9	
6'	124.0	7.60 (1H, d, J=7.3Hz)
7'	137.2	7.78 (1H, t, J=7.3Hz)
8'	119.6	7.33 (1H, d, J=7.3Hz)
9'	160.4	
9-OH		11.1 (1H, s)
9'a	114.1	
10'	197.0	
10'a	75.9	
10a-OH		7.24 (1H, s)
11'	14.7	1.08 (3H, d, J=7.0Hz)
12'	39.6	2.38 (2H, m)
13'	170.3	

^{13}C NMR spectrum was measured at 125 MHz.

^{1}H-NMR spectrum was measured at 500MHz at 50℃

Fig. 4. ROESY spectrum of BE-52440A.



Comparison of the ^{13}C-NMR spectrum of BE-52440B with that of BE-52440A again revealed strong similarities. However, one pair of carbonyl and methoxy carbons of BE-52440A was missing and an oxymethylene carbon was observed instead. From the HMBC spectrum and the molecular formula of BE-52440B, the structure of BE-52440B was determined as shown in Figure. 1. In the NOESY spectrum, correlations of BE-52440B were quite similar to those of BE-52440A.

Biological Activity

BE-52440A and B showed antiproliferative effects *in vitro* against murine tumor cell lines P388 leukemia and colon 26 colon cell lines and also against human tumor cell lines such as DLD-1 colon, PC-13 lung and MKN-45 stomach cell lines. The IC_{50} values against these cell lines are shown in Table 6. Antitumor activity of BE-52440A and B are under evaluation. BE-52440A is a dimmer of nanaomycin B methylester. Nanaomycins inhibit mainly Gram-positive bacteria and mycoplasmas[7~10]. But BE-52440A and B did not show antibacterial activity (data not shown). BE-52440A and B described in this report may be a new lead to antitumor agents.

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Fig. 5. Structure of BE-52440A.

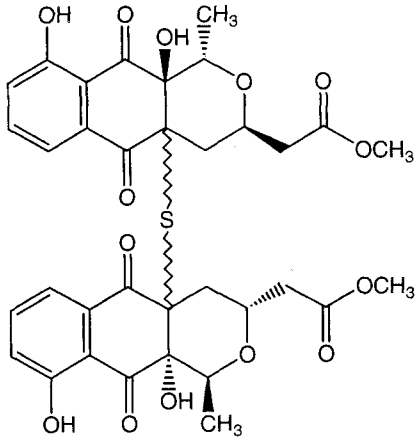

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Table 6. Growth inhibition of tumor cell lines by BE-52440A and B.

	IC_{50} (μg/ml)				
	P388	DLD-1	PC-13	MKN-45	Colon 26
BE-52440A	6.1	0.47	3.9	0.88	2.1
BE-52440B	1.1	0.69	2.6	2.9	3.4