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New Cytotoxic Agents, BE-54238A and B, Produced by a Streptomycete

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New cytotoxic substances, designated BE-54238A and B, were isolated from the culture broth of *Streptomyces* sp. A54238. The active principles were extracted from the mycelium by methanol and purified by Diaion HP-20 and Sephadex LH-20 column chromatographies. BE-54238A 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 A54238 isolated from a soil sample was found to produce active principles. These components, BE-54238A and B, were purified based on their inhibitory activity against the growth of the P388 murine leukemia cell line. BE-54238A 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, physico-chemical properties, structure determination and biological properties of BE-54238A and B are described. The structures of BE-54238 A and B are shown in Fig. 1.

Materials and Methods

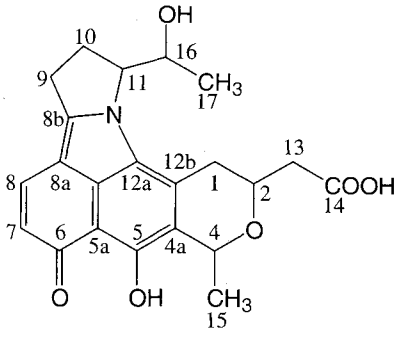
Taxonomic Studies

Characterization of the strain A54238 followed the method adopted by the International Streptomyces Project (ISP)[1]. Morphological observations were made with light 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].

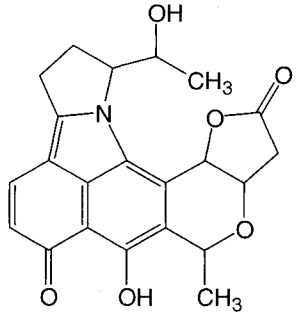
Fermentation

The *Streptomyces* sp. A54238, cultured on a slant agar

Fig. 1. Structures of BE-54238A and B.



BE-54238A



BE-54238B

medium, was inoculated into 110 ml of a seed medium consisting of 0.1% glucose, 2.0% dextrin, 0.5% fish meal, 1.0% corn gluten meal, 0.1% yeast extract, 0.05% magnesium sulfate, 0.1% sodium chloride, 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 and 0.00024% sodium molybdate (pH 7.0). 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 50 of 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-54238A 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 ml 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 antiproliferative activity of BE-54238A 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-54238A and B were first dissolved in dimethyl sulfoxide (DMSO), and serially diluted with phosphate-buffered saline (PBS). The media 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.

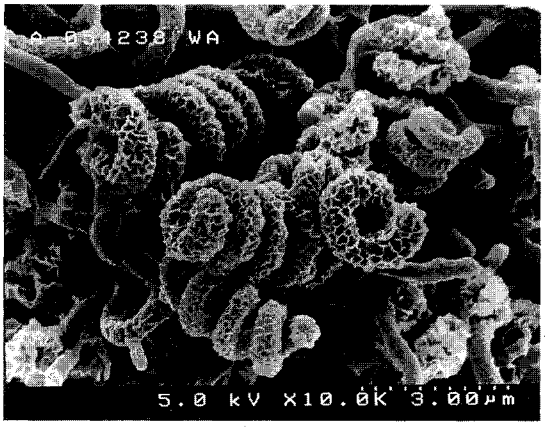
Results and Discussion

Taxonomy of the Producing Strain

The strain A54238 formed well developed and branching substrate mycelia and aerial mycelia, but fragmentation of the substrate mycelia was not observed. The spore long chains (30~50) of the strain were tight or compact spirals. The spore had rugose surface and was oval with a diameter of 1.3~0.3×1.1~0.9 μm (Fig. 2). Special morphology such as sclerotia and sporangia were not observed. The cultural characteristics of strain A54238 are summarized in Table 1. The aerial mycelia became black and moist, and showed hygroscopic appearance with maturity on the yeast extract-malt extract agar. The whole-cell hydrolysate contained L,L-diaminopimelic acid. This suggests the cell wall of this strain belongs to type I. The physiological properties and carbon utilization of strain A54238 are shown in Table 2. The above-mentioned characteristics of strain A54238 revealed that it belonged to the genus *Streptomyces*. The strain A54238 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. A54238 under the accession No. FERM P-15721.

Fig. 2. Scanning electron micrograph of sporangia of *Streptomyces* sp. A54238.

Bar represents 3.0 μm



Isolation of BE-54238A and B

The filtrate was obtained by filtration of the whole broth (*ca.* 5.5 liters). After the filtrate was adjusted to pH 3 with 1 N HCl, it was applied to a Diaion HP-20 column (1 liter). It was washed with water (5 liters) and eluted with MeOH (1 liter). The eluate containing BE-54238A and B was concentrated to about 1 liter. The concentrated solution was extracted with 1 liter of ethyl acetate and the extract was evaporated under reduced pressure. Further purification by

Table 1. Culture characteristics of strain A54238.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Abundant blue dark gray	Deep brown	Orange
Oatmeal agar (ISP-3)	Good	Abundant blue dark gray	Orange	Pale brown
Inorganic salts-starch agar (ISP-4)	Good	Abundant purplish dark gray	Deep brown	Deep brown
Glycerol-asparagine agar (ISP-5)	Good	Abundant purplish dark gray	Orange	Orange yellow
Peptone-yeast extract-iron agar (ISP-6)	Moderate	Moderate purplish gray	Orange	None
Tyrosine agar (ISP-7)	Good	Abundant dark grayish blue	Deep brown	Brownish orang
Nutrient agar	Good	Moderate white	Yellowish white	None
Sucrose-nitrate agar	Moderate	Moderate blue gray	Yellowish white	None
Glucose-asparagine agar	Good	Moderate white	Yellowish white	None

Table 2. Physiological properties and carbon utilization of strain A54238.

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	16～43℃
Carbon utilization	
D-Glucose	+
D-Xylose	+
L-Arabinose	+
L-Rhamnose	+
D-Fructose	+
Raffinose	+
D-Mannitol	+
i-Inositol	+
Sucrose	+
D-Galactose	+
Salicin	±

Table 3. ^{13}C NMR data for BE-54238A and B in DMSO-d_6.

	BE-54238A	BE-54238B
1	30.0	71.6
2	62.8	65.7
4	67.2	66.4
4a	119.5	120.7
5	155.4	154.3
5a	109.6	110.9
6	184.7	185.4
7	121.4	121.7
8	134.9	135.9
8a	103.6	103.6
8b	154.6	156.0
9	24.7	24.8
10	27.5	27.5
11	64.2	64.0
12a	121.8	123.1
12b	127.1	119.9
13	40.4	36.8
14	172.1	175.0
15	19.5	18.5
16	68.7	68.9
17	20.3	20.1

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

a Sephadex LH-20 column chromatography (80×3.0 cm i.d.) using EtOAc/CH_3CN/H_2O (10 : 5 : 1) as eluent yielded 65.2 mg of BE-54238A and 37.8 mg of BE-54238B as a yellow orange powders.

Physico-chemical Properties

BE-54238A and B are soluble in DMSO, but are insoluble in hexane and water. Other properties are as follows: BE-54238A ($C_{22}H_{23}NO_6$); HRFAB-MS *m/z* 398.1591 $(M+H)^+$, calcd *m/z* 398.1603 $(M+H)^+$; UV λ_{max}^{MeOH} nm 276 (16,700), 411 (27,700); IR ν_{max} (KBr) cm^{-1} 3444, 2976, 1709, 1645, 1527, 1489, 1464, 1381, 1259, 1223, 1176, 1122, 1076, 608. BE-54238B ($C_{22}H_{21}NO_6$) ; HRFAB-MS *m/z* 396.1446 $(M+H)^+$, calcd *m/z* 396.1447 $(M+H)^+$; UV λ_{max}^{MeOH} nm 278 (21,500), 420 (27,600); IR ν_{max} (KBr) cm^{-1} 3444, 2972, 1774, 1647, 1537, 1497, 1259, 1157, 1122, 608.

Structure Determination

The structure of BE-54238B was determined initially. The ^{13}C and ^1H NMR data of BE-54238A and B are shown in Table 3 and 4, respectively. The molecular formula of BE-54238B was established as $C_{22}H_{21}NO_6$ from the result of HRFAB-MS spectral and ^{13}C NMR data. The ^{13}C NMR data and DEPT experiment revealed the presence of two methyl carbons, five sp^3 methine carbons, three methylene carbons, two sp^2 methine carbons, eight sp^2 quaternary carbons and two carbonyl carbon signals. The molecular formula agreed well with these data. In the ^1H NMR spectrum, a D_2O-exchangeable proton signal at δ_H 12.5 was observed. This result indicated the presence of a chelated phenolic proton. ^1H-^1H COSY and HMBC experiments of BE-54238B revealed the structure as shown in Fig. 3. The ROE and $^{13}C\{^1H\}$ NOE difference experiments[7] gave support to the structure of BE-54238B (Fig. 4). In the difference ROE and ROESY experiments, the correlations of the methyl protons (δ_H 1.53) to 2-H (δ_H 5.01), of 1-H (δ_H 5.72) to 2-H, 11-H (δ_H 4.61) and 16-H (δ_H 4.17) and of 8-H (δ_H 8.03) to 9-H (δ_H 3.23) were observed. In the $^{13}C\{^1H\}$ NOE difference experiments, the correlations of 4-H (δ_H 5.25) to C-1 (δ_c 71.6), C-2 (δ_c 65.7), C-4a (δ_c 120.7) and C-5 (δ_c 154.3), of 1-H to C-4 (δ_c 66.4), C-12a (δ_c 123.1) and C-12b (δ_c 119.9), of 7-H (δ_H 6.47) to C-6 (δ_c 185.4) and of 8-H (δ_H 8.03) to C-8a (δ_c 103.6) were observed. For the confirmation of nitrogen, the FG-^{15}N-^1H HMBC experiment[8~10] was employed. The ^{15}N-^1H HMBC spectrum showed the correlations from 9-H (δ_H 3.23), 10-H (δ_H 2.70) and 16-H (δ_H 4.17) to the nitrogen atom (Fig. 5). From these results, the structure of BE-54238B was determined as shown in Fig. 1.

Table 4. ^{1}H NMR data for BE-54238A and B in DMSO-d_6.

	BE-54238A	BE-54238B
1	2.95 (1H, dd, J=17.2, 10.4Hz)	5.72 (1H, d, J=3.0Hz)
	3.20 (1H, m)	
2	4.46 (1H, m)	5.01 (1H, m)
4	5.15 (1H, q, J=6.4Hz)	5.25 (1H, q, J=6.7Hz)
5-OH	12.3 (1H, brs)	12.5 (1H, brs)
7	6.42 (1H, d, J=9.2Hz)	6.47 (1H, d, J=9.2Hz)
8	7.95 (1H, d, J=9.2Hz)	8.03 (1H, d, J=9.2Hz)
9	3.20 (1H, m)	3.23 (2H, m)
	3.12 (1H, dd, J=17.7, 7.3Hz)	
10	2.65 (2H, m)	2.70 (2H, m)
11	4.69 (1H, brd, J=7.9Hz)	4.61 (1H, brd, J=7.6Hz)
13	2.49 (overlapped with solvent)	2.52 (1H, d, J=17.7Hz)
	2.68 (1H, dd, J=15.6, 4.8Hz)	3.34 (1H, dd, J=17.7, 5.2Hz)
15	1.53 (3H, d, J=6.4Hz)	1.56 (3H, d, J=6.7Hz)
16	4.15 (1H, m)	4.17 (1H, brq, J=6.4Hz)
16-OH	4.90 (1H, d, J=5.2Hz)	4.95 (1H, brs)
17	1.20 (3H, d, J=6.4Hz)	1.20 (3H, d, J=6.4Hz)

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

Fig. 3. HMBC correlations for BE-54238B.

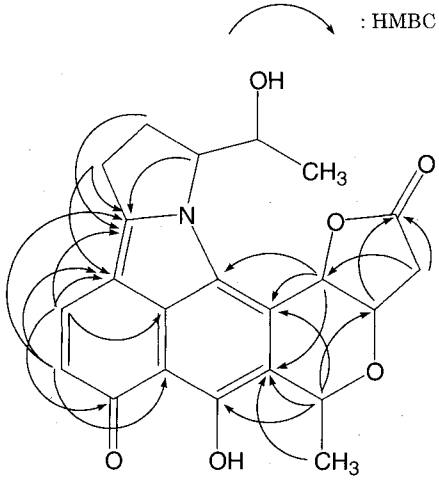
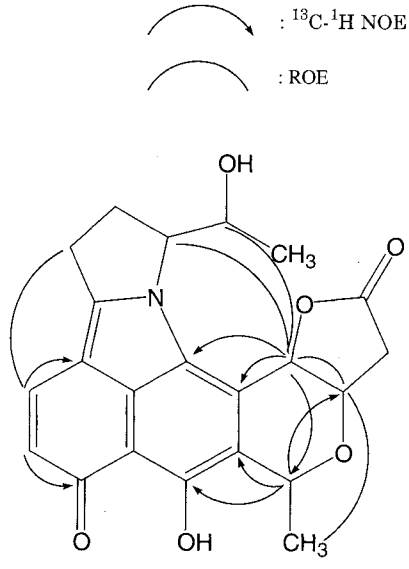


Fig. 4. DF-ROE, ROESY and ^{13}C-^{1}H NOE experiments of BE-54238B.



The molecular formula of BE-54238A was established as $C_{22}H_{23}NO_6$ from the result of HRFAB-MS spectral data and ^{13}C NMR data. BE-54238A was larger than BE-54238B by two protons. In the ^{1}H NMR spectra of BE-54238A, the proton of BE-54238B at δ_H 5.72 was absent and 2 extra protons at δ_H 2.95 and δ_H 3.20 were observed. In the ^{13}C NMR spectra of BE-54238A, the methine carbon of BE-54238B at δ_c 71.6 was absent and a new methylene carbon at δ_c 30.0 was observed. In a comparison of the ^{1}H NMR and ^{13}C NMR spectrum of BE-54238A and B, there is little difference between the chemical shifts of BE-54238A and B except for the data given above. In the HMBC spectra of BE-54238A, the correlations shown in Fig. 6 were observed. These results defined the structure of BE-54238A as shown in Fig. 6. This structure was supported by the data observed in ROE experiments (Fig. 7). From these results, the structure of BE-54238A was determined as shown in Fig. 1.

Biological Activity

BE-54238A and B showed antiproliferative effects *in vitro* against murine tumor cell lines such as 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 5. Acute toxicity of BE-54238A was tested at 30, 100 and 200 mg/kg (female CDF_1 mouse). The LD_{50} value of BE-54238A in mice by a single i.p. injection was >200 mg/kg. Acute toxicity of BE-54238B was tested at 10, 30 and 100 mg/kg (female CDF_1 mouse). The LD_{50} value of BE-54238B in mice by a single i.p. injection was >100 mg/kg. Antitumor activity of BE-54238A and B are under evaluation. BE-54238A and B described in this report represent the discovery of a novel pharmacophore which may be a new lead to antitumor agents.

Fig. 5. FG-^{15}N-^{1}H HMBC correlations for BE-54238B.

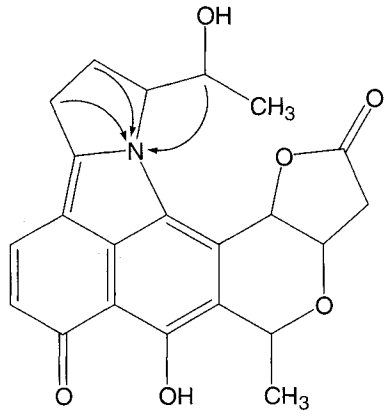


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Fig. 6. HMBC correlations for BE-54238A.

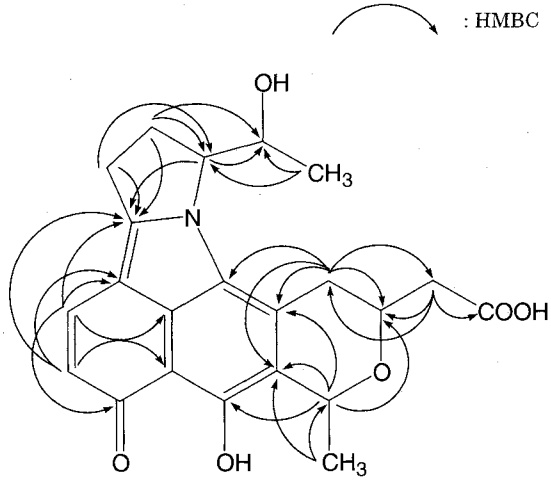

Fig. 7. DF-ROE and ROESY experiments of BE-54238A.

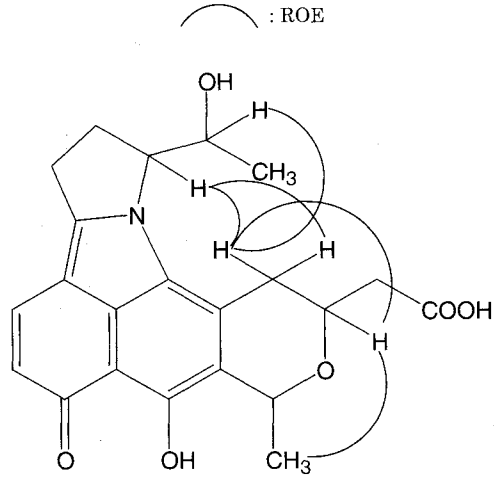

Table 5. Growth inhibition of tumor cell lines by BE-54238A and B.

	IC_{50} (μM)				
	P388	DLD-1	PC-13	MKN-45	Colon 26
BE-54238A	9.1	3.5	11.3	8.1	8.6
BE-54238B	58.2	10.1	53.2	45.6	20.5

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