NEW ANTITUMOR ANTIBIOTICS, OS-4742 A_1, A_2, B_1 AND B_2 PRODUCED BY A STRAIN OF *STREPTOMYCES*

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New antibiotics, OS-4742 A_1, A_2, B_1 and B_2, were isolated from the culture broth of the strain OS-4742, which was designated as *Streptomyces matensis* subsp. *vineus*. These compounds have anthracycline chromophores and sugar moieties, but do not contain nitrogen. They possess antimicrobial activities against Gram-positive bacteria and antitumor activities against S-180 solid tumor on mice.

In the course of our screening program of antibiotics from actinomycetes, new antibiotics, OS-4742 A_1, A_2, B_1 and B_2, effective against Gram-positive bacteria and Sarcoma-180 solid tumor were obtained from the culture broth of an actinomycete (strain OS-4742) which had been isolated from a soil sample collected at Takada-city in Niigata Prefecture, Japan. In the present paper, taxonomy of the producing strain, production, isolation, characterization and biological properties of the antibiotics are described.

Characteristics of the Producing Strain

Morphological Characteristics

The morphology of the strain cultured on salts-starch agar for 14 days at 27°C was observed microscopically (Plates 1 and 2). The aerial mycelium of the strain is abundant on either synthetic or organic agar medium. It forms no whorls, but extends aerial hyphae forming open spirals or occasionally loops. The spores are oval or cylindrical and are $0.5 \sim 0.8\ \mu \times 0.8 \sim 1.0\mu$ in size. Their surfaces are spiny.

Cultural and Physiological Characteristics

The strain OS-4742 was cultivated on various media described by WAKSMAN[1] and International *Streptomyces* Project (ISP)[2] at 27°C, and the changes of growth, aerial mycelium and soluble pigment were observed after a period of 7, 14 and 21 days. Utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[2] containing 1% of various carbon sources. Color names and hue numbers indicated were those of the Color Harmony Manual (4th edition)[3].

The cultural and physiological characteristics of the strain OS-4742 are listed in Tables 1 and 2, respectively. The utilization of carbon sources by the strain is shown in Table 3. The cultural and physiological characteristics can be summarized as follows: growth is moderate or good, and is colorless to yellow brown, pale orange or red brown on either synthetic or organic agar media; aerial mass color is white to brownish gray; soluble pigment is yellow brown to red brown or red yellow; no melanoid pigment is produced. The cell wall preparation of strain OS-4742 was found to contain

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Plate 1. Photomicrograph of the sporophores of strain OS-4742.

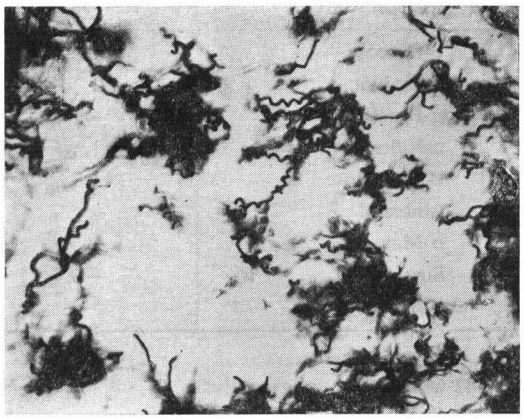


Plate 2. Electronmicrograph of the spores of strain OS-4742.

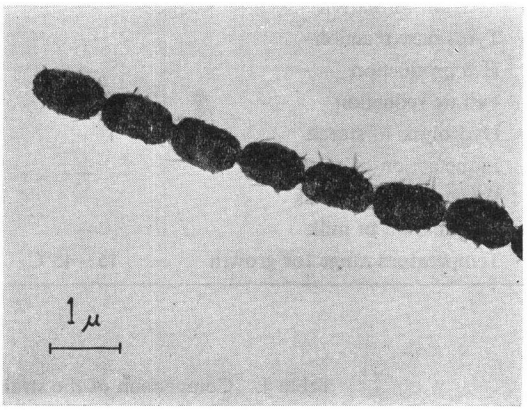


Table 1. Cultural characteristics of strain OS-4742

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	colorless	moderate, 2 ca (Light Ivory)	2 la (Bright Yellow)
Glucose-nitrate agar	3 lg (Light Brown)	moderate, 3 ge (Camel)	3 pi (Golden Brown) to 5 pl (Deep Brown)
Glycerol-asparagine agar	colorless	abundant, 3 ec (Light Beige)	4 ng (Light Brown) to 6 pi (Brown Mahogany)
Glucose-asparagine agar	3 ng (Yellow Maple)	moderate, 3 ca (Pearl Pink) to 5 ec (Dusty Peach)	6 lg (Dark Redwood)
Glycerol-calcium malate agar	2 lc (Gold)	poor, 3 ca (Pearl Pink)	3 ic (Light Amber)
Inorganic salts-starch agar	2 ea (Light Wheat)	abundant, white to 3 dc (Natural)	3 gc (Bamboo)
Tyrosine agar	3 lg (Light Brown)	mossy, 3 ca (Pearl Pink) to 3 dc (Natural)	3 ng (Yellow Maple) to 3 ie (Camel)
Glucose-peptone agar	2 ic (Honey Gold)	—	3 pc (Amber)
Yeast extract-malt extract agar	colorless	abundant, 2 ec (Oatmeal Sand)	4 ne (Luggage Tan) to 4 pi (Oak Brown)
Oatmeal agar	colorless	poor, 3 cb (Sand)	2 ne (Mustard Gold)
Peptone-yeast extract iron agar	2 ea (Light Wheat)	—	—
Peptone-beef extract agar	2 ea (Light Wheat)	moderate, white	—

LL-diaminopimelic acid but no meso-isomer.

From the above results, the strain is nonchromogenic and belongs to gray or red series of the genus *Streptomyces* classified by PRIDHAM and TRESNER[4]. Among known *Streptomyces* species described in "BERGEY's Manual of Determinative Bacteriology", 8th ed.[4], "The Actinomycetes" Vol. II by WAKSMAN[1] and ISP reports by SHIRLING and GOTTLIEB[5~8], *Streptomyces matensis* MARGALITH, BERETTA and TIMBAL[9] was closely related to the strain OS-4742. In comparison of the strain OS-4742 with *S. matensis* KCC S-0651 (IFO 12,889), all of the morphological and physiological characteristics and most of the cultural characteristics of the former were in agreement with those of the latter. However, as shown in Table 4, such characteristics of the former as growth, aerial mycelium and formation of soluble pigment on some media were different from those of the latter. Furthermore,

Table 2. Physiological characteristics of strain OS-4742

Melanin formation	—
Tyrosinase reaction	—
H_2S production	—
Nitrate reduction	—
Hydrolysis of starch	+
Liquefaction of gelatin	±
Peptonization of milk	+
Coagulation of milk	—
Temperature range for growth	15~45°C

Table 3. The utilization of carbon sources by strain OS-4742

Carbon source	Response
D-Glucose	+
L-Arabinose	+
D-Fructose	+
D-Xylose	+
L-Rhamnose	+
i-Inositol	+
D-Mannitol	+
Sucrose	—
Raffinose	—

Table 4. Comparison of the strain OS-4742 with *Streptomyces matensis*

		Strain OS-4742	*S. matensis* KCC S-0651 (IFO 12,889)
Cultural characteristics			
1) Yeast extract-malt extract agar	G*	Colorless	3 ig (Camel)
	AM*	Abundant, 2 ec (Oatmeal Sand)	Powder, 3 fe (Silver Gray)
	SP*	4 ne (Luggage Tan) to 4 pi (Oak Brown)	—
2) Oatmeal agar	G	Colorless	Colorless
	AM	Poor, 3 cb (Sand)	Poor, white
	SP	2 ne (Oak Brown)	—
3) Inorganic salts-starch agar	G	2 ea (Light Wheat)	3 ge (Beige)
	AM	Abundant, white to 3dc (Natural)	Poor, gray
	SP	3 gc (Bamboo)	3 ec (Light Beige)
4) Glycerol-asparagine agar	G	Colorless	3 lg (Light Brown)
	AM	Abundant, 3 ec (Light Beige)	Moderate, white to 3 dc (Natural)
	SP	4 ng (Light Brown) to 6 pi (Brown Mahogany)	—
Production of the antibiotics		+	—

* G, growth; AM, aerial mycelium; SP, soluble pigment.

the former produced the new antibiotics OS-4742 A_1, A_2, B_1 and B_2, but the latter did not.

Therefore, the strain OS-4742 should be assigned to a new sub-species of *S. matensis*, and was designated as *Streptomyces matensis* subsp. *vineus* AWAYA subsp. nov. because of wine color of the soluble pigment. The strain OS-4742 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan, with the accession number FERM-P No. 3430.

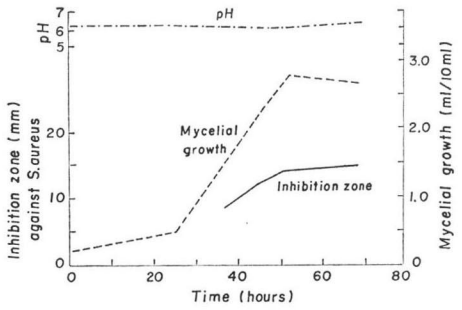
Production of the Antibiotics

The stock culture of strain OS-4742 (*S. matensis* subsp. *vineus*) was maintained as agar slant (KRAINSKY's agar medium). A 7-day culture of the agar slant was inoculated into a seed medium (100 ml) in a 500-ml SAKAGUCHI's flask and incubated for 2 days at 27°C. The composition of the seed medium was 2.0% glucose, 0.5% peptone, 0.3% dry yeast, 0.5% meat extract, 0.5% sodium chloride and 0.3% calcium carbonate (the pH value was adjusted to 7.0 with 6 N sodium hydroxide before sterilization). The culture (1st seed culture, 400 ml) was transferred into 20 liters of a seed

medium in a 30-liter jar fermentor and incubated for 2 days at 27°C (agitation, 300 rpm; aeration, 10 liters per minute) to give a 2nd seed culture. The 2nd seed culture (20 liters) was transferred into a 400-liter tank fermentor containing 200 liters of a production medium (1.0% glucose, 2.0% starch, 0.5% yeast extract, 0.5% peptone, 0.4% calcium carbonate, pH 7.0 before sterilization), and incubated for 3 days at 27°C (agitation, 200 rpm; aeration, 100 liters per minute). In the cultivation using a jar or a tank fermentor, Adekanol LG-109 (Asahi Electro-Chemical Co., Ltd.) was used as an antifoam agent. The antibiotic activity of the culture broth was assayed by the paper disc method using *Staphylococcus aureus* FDA 209P as a test organism.

Fig. 1. Time course of production of antibiotics OS-4742 by *Streptomyces matensis* subsp. *vineus*.

Cultivation was performed using a 400-liter tank containing 200 liters of a production medium. The medium and culture conditions are described in the text.



A typical time course of the fermentation is shown in Fig. 1. The antibiotic production started one day after the inoculation and the total amount of the antibiotics OS-4742 A_1, A_2, B_1 and B_2 ac-

Fig. 2. Isolation of antibiotics OS-4742 A_1, A_2, B_1 and B_2 from culture broth of *Streptomyces matensis* subsp. *vineus*.

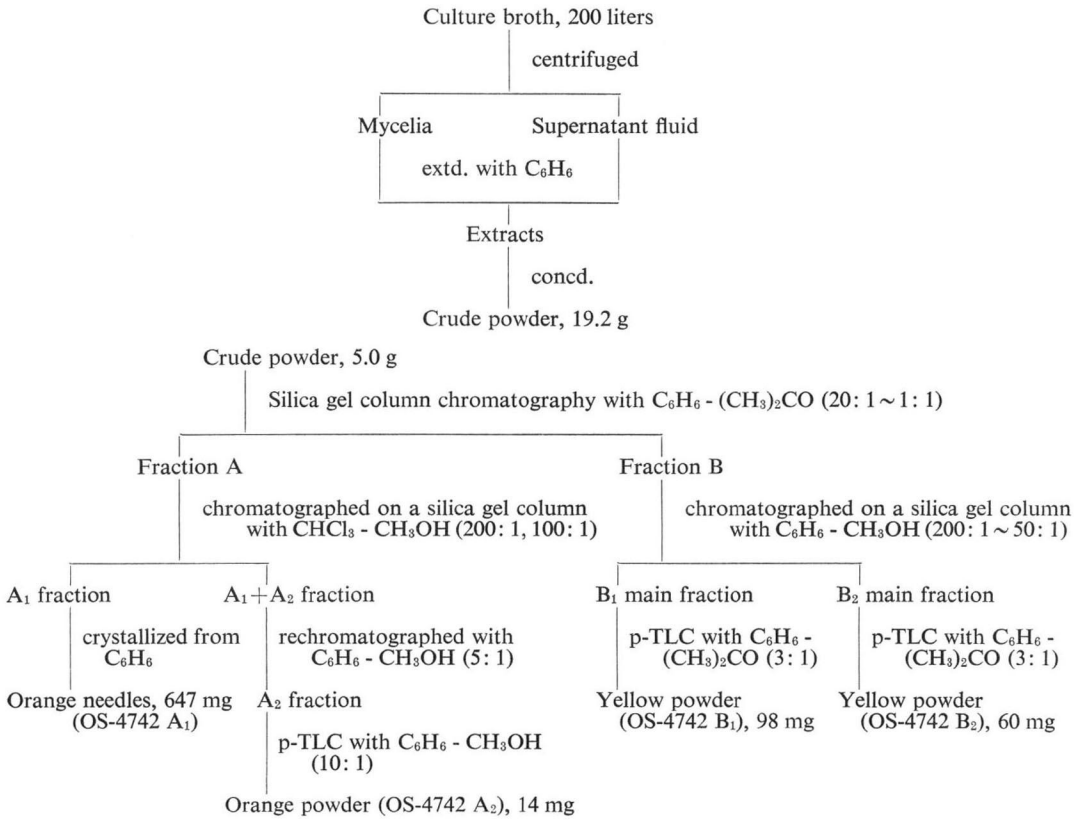


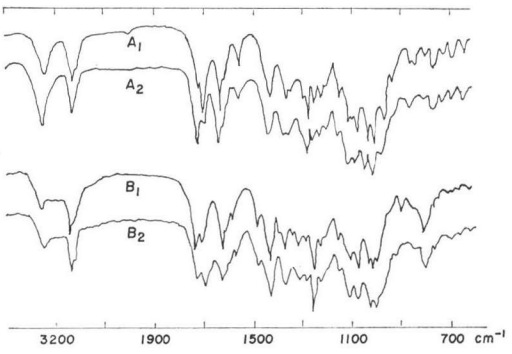
Table 5. TLC of antibiotics OS-4742 A_1, A_2, B_1 and B_2

Plate	Developing solvent	Rf values			
		A_1	A_2	B_1	B_2
Silica gel	$CHCl_3$-MeOH (10:1)	0.77	0.77	0.56	0.55
	C_6H_6-$(CH_3)_2CO$ (3:1)	0.56	0.56	0.06	0.03
Acid-treated silica gel*	C_6H_6-MeOH (5:1)			0.36	0.36
	C_6H_6-$(CH_3)_2CO$ (4:1)			0.50	0.12
Avicel SF plate	C_6H_6-MeOH (150:1)	0.83	0.81	0.63	0.25

* Prepared with 0.2% H_2SO_4 instead of H_2O.

cumulated at the 3rd day was about 30 μg/ml calculated in the antimicrobial activity of OS-4742 A_1.

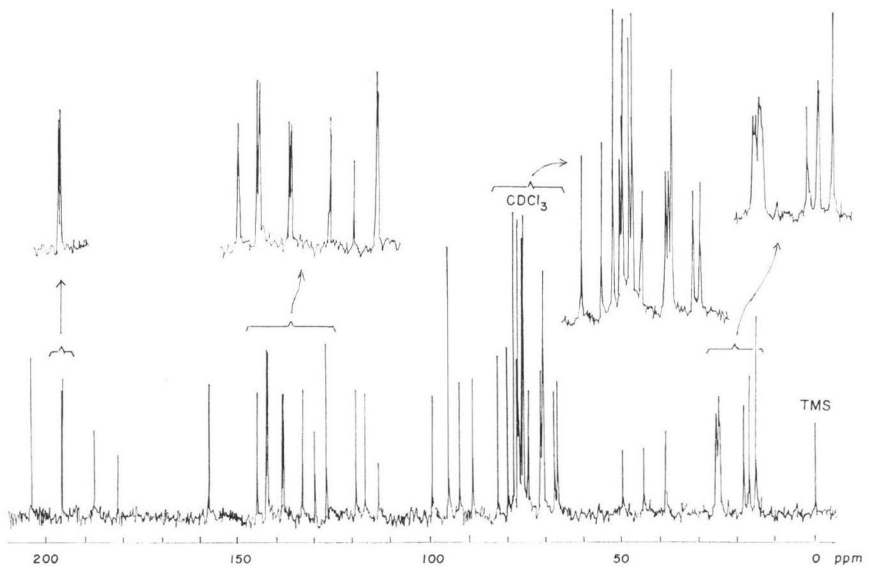
Fig. 3. IR spectra of antibiotics OS-4742 A_1, A_2, B_1 and B_2 (KBr method).



Isolation of the Antibiotics

Culture broth (200 liters) of *S. matensis* subsp. *vineus*, obtained by incubation in a 400-liter tank, was used as a starting material for the isolation of the antibiotics. The antibiotics were detected by their antibacterial activities against *Staphylococcus aureus* and silica-gel thin-layer chromatography. The culture broth was centrifuged to separate mycelia and filtrate. Both the mycelia and the filtrate were extracted with benzene (18 liters and 54 liters, respectively), and the extracts were combined and concentrated

Fig. 4. CMR spectrum of OS-4742 A_1 (in $CDCl_3$).



in vacuo to give a yellow brown crude powder (19.2 g). From the crude powder (5.0 g), as shown in Fig. 2, OS-4742 A_1, A_2, B_1 and B_2 were isolated.

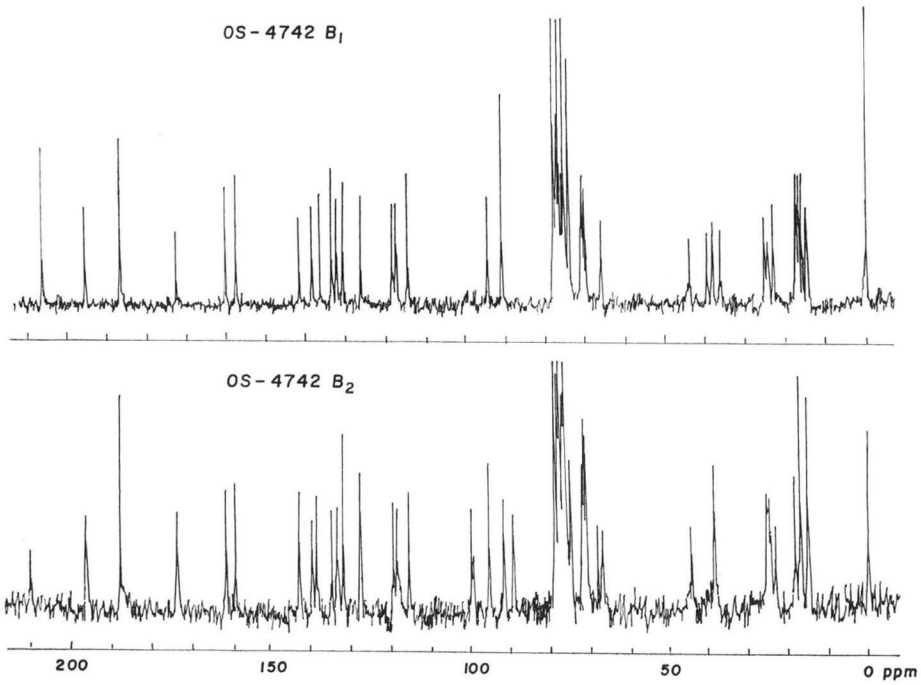
Fractions A and B were separated by silica-gel column chromatography with benzene and acetone (20: 1 ~ 1: 1). After silica-gel column chromatography of fraction A with chloroform and methanol (200 : 1 and 100 : 1), orange needles of OS-4742 A_1 (yield: 647 mg) were obtained from the eluate containing OS-4742 A_1 alone, and an orange powder of OS-4742 A_2 (yield: 14 mg) was obtained by rechromatography of the eluate containing OS-4742 A_1 and A_2 with benzene and methanol (5: 1) and then preparative thin-layer chromatography on silica gel with benzene and methanol (10 : 1).

From the fraction B, a yellow powder of OS-4742 B_1 (yield: 98 mg) and a yellow powder of OS-4742 B_2 (yield: 60 mg) were obtained by silica-gel column chromatography with benzene and methanol (200: 1 ~ 50: 1) and then preparative thin-layer chromatography with benzene and acetone (3: 1). Rf values of these compounds in thin-layer chromatography on silica gel and microcrystalline cellulose are listed in Table 5.

Physico-chemical Properties of OS-4742 A_1, A_2, B_1 and B_2

Each component obtained as described above is neutral in nature, and is soluble in lower alcohols, lower alkyl acetates, acetone, chloroform and benzene, but insoluble in water, petroleum ether, *n*-hexane and cyclohexane. These compounds are positive to ferric chloride and formaldehyde-*o*-dinitrobenzene reactions, but negative to ninhydrin and EHRLICH reactions. The solutions of the antibiotics in conc. sulfuric acid are blue. They contain no nitrogen and no halogen. The other physico-chemical properties of the antibiotics are listed in Table 6. The IR and CMR spectra are given in Figs. 3 ~ 5.

Fig. 5. CMR spectra of OS-4742 B_1 and B_2 (in $CDCl_3$).



The UV-absorptions of the antibiotics show that they have anthracycline chromophores. The IR and CMR spectra suggest that the compounds contain sugar moieties. Because no molecular ion peak was observed in the mass spectra, the molecular weights (Table 6) were estimated from the results of elemental analysis and CMR spectrometry.

Biological Properties of OS-4742 A_1, A_2, B_1 and B_2

The antimicrobial activities of OS-4742 A_1, A_2, B_1 and B_2 were determined by conventional agar dilution method using nutrient agar for bacteria (37°C, 24 hours) and glucose-potato agar for fungi

Table 6. Physico-chemical properties of antibiotics OS-4742 A_1, A_2, B_1 and B_2

	A_1	A_2	B_1	B_2
Nature	Neutral Orange needles	Neutral Orange powder	Neutral Yellow powder	Neutral Yellow powder
mp (°C)	162~163	173~176	132~135	128~131
$[\alpha]_D^{26}$ (in $CHCl_3$)	+92° (*c* 0.5)	+3° (*c* 0.4)	+76.8° (*c* 0.5)	+30.8° (*c* 0.5)
Anal. Found C	62.51	61.60	59.53	62.37
H	6.23	6.44	5.93	6.21
N	0	0	0	0
Formula	$C_{47\sim50}H_{58\sim60}O_{16\sim18}$		$C_{39\sim44}H_{46\sim52}O_{17\sim19}$	$C_{42\sim48}H_{50\sim58}O_{16\sim18}$
MW	880~950		786~872	810~922
$UV\lambda_{max}^{MeOH}$ nm($E_{1cm}^{1\%}$)	218(441) 318(59) 438(61)	216(364) 315 sh(107) 425(50)	230(325) 258(185) 295(60) 427(89) 440(87)	230(421) 258(226) 294(76) 427(107) 440(107)

Table 7. Antimicrobial spectra of antibiotics OS-4742 A_1, A_2, B_1 and B_2

Test organism	MIC (μg/ml)*			
	A_1	A_2	B_1	B_2
Staphylococcus aureus FDA 209P	0.78	12.5	1.56	1.56
S. aureus FS-1227 (Penicillin resistant)	0.39	3.12	6.25	3.12
Bacillus subtilis PCI 219	3.12	12.5	6.25	12.5
B. cereus T	12.5	50	6.25	25
Sarcina lutea PCI 1001	0.78	12.5	12.5	50
Mycobacterium smegmatis ATCC 607	>100	100	>100	>100
Escherichia coli NIHJ	>100	>100	>100	>100
Salmonella typhimurium	>100	>100	>100	>100
Shigella sonnei E-33	>100	>100	>100	>100
Pseudomonas aeruginosa P-3	>100	>100	>100	>100
Candida albicans	>100		>100	>100
Saccharomyces sake	>100		>100	>100
Piricularia oryzae	12.5		100	>100
Aspergillus niger	>100		>100	>100
Microsporum gypseum	25		>100	>100
Trichophyton interdigitale	100		>100	>100
T. mentagrophytes	100		>100	>100

* Agar dilution method using nutrient agar for bacteria (37°C, 24 hours) and glucose-potato agar for fungi (27°C, 72 hours).

Table 8. Antitumor activities of antibiotics OS-4742 A_1 and B complex(B_1+B_2)

Tumor : Sarcoma-180 solid (ddy mouse)
Treatment : Suspension of an antibiotic in 0.3% carboxymethyl cellulose was administered intraperitoneally.
Single shot : 1 day after transplantation of tumor
Multiple shot : 1, 2, 4, 5 and 6 day
Judgement : A tumor size ($ab^2/2$) at 7th day after transplantation of tumor was compared with that of control: a, length; b, width.

Antibiotic	Dose (mg/kg)	Tumor size (T/C*)	No. of death	Body weight change (g)
OS-4742 A_1	50	0.13	0/7	−3.0
	100	0.31	3/7	−0.6
	4.4×5	0.72	0/7	−0.7
	6.7×5	0.38	0/7	−2.0
OS-4742 B**	50	0.32	0/7	−0.3
	100	0.22	3/7	−0.8
	11.1×5	0.37	0/7	−2.0
	16.7×5	0.40	0/7	−2.1
Adriamycin	7.0	0.34	0/7	−1.9
	2.3×5	0.68	0/7	−2.4

* Treated/Control
** Fraction B given in Fig. 2 was used.

(27°C, 72 hours). As shown in Table 7, the antibiotics inhibited mainly Gram-positive bacteria. Among them, OS-4742 A_1 has the strongest activity.

As shown in Table 8, antibiotic OS-4742 A_1 exhibited an antitumor activity against Sarcoma-180 solid tumor on ddy mice. When 50 mg/kg of OS-4742 A_1 was injected intraperitoneally once at 1 day after transplantation of tumor, the tumor size (treated/control) at the 7 th day was 0.13, and when 6.7 mg/kg/day was injected once daily for 6 days, the tumor size at the 7th day was 0.38. Also a complex of OS-4742 B_1 and B_2 exhibited an antitumor activity against Sarcoma-180 solid tumor. From the results shown in Table 8, the acute toxicities (LD_{50}, ip) of OS-4742 A_1 and B complex (OS-4742 B_1+B_2) in mice were estimated to be 100~150 mg/kg.

Discussion

From the results described above, the antibiotics OS-4742 A_1, A_2, B_1 and B_2 isolated from the culture broth of *S. matensis* subsp. *vineus* were found to be antitumor antibiotics which had anthracycline chromophores and sugar moieties but contained no nitrogen. Among known anthracycline antibiotics containing no nitrogen, ayamycin A_2[10] (mp 202~204°, anal. found: C, 62.29; H, 7.09, MW 560, $[\alpha]_D^{23}$ −39.8±2° (*c* 0.835, dioxane)) and TA-435 A[11] (mp 153~157° (decomp.), anal. found: C, 61.90; H, 6.90, $[\alpha]_D^{22}$ +120° (*c* 0.115, $CHCl_3$)) resemble the antibiotics OS-4742 A_1 and A_2 in respect to UV-absorption. However, OS-4742 A_1 and A_2 are different from ayamycin A_2 in melting point, molecular weight and thin-layer chromatography (Rf values of ayamycin complex in thin-layer chromatography on silica-gel with chloroform-methanol (10: 1) are under 0.53, but that of OS-4742 A_1 or A_2 is 0.77) and from TA-435 A in elementary analysis, IR-absorption and antimicrobial activity. OS-4742 B_1 and B_2 resemble ryemycin A_2[12] (mp 218~220°C, $C_{22}H_{20}O_7$, $[\alpha]_D^{24}$ +160.9±10° (*c* 0.128, $CHCl_3$)) in respect to UV-absorption, but these differentiate from ryemycin A_2 in melting point, molecular formula and molecular weight.

Therefore, the antibiotics OS-4742 A_1, A_2, B_1 and B_2 were considered to be new antibiotics. Further investigations on their chemical structures and antitumor activities are in progress.

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