RUBEOMYCIN, A NEW ANTHRACYCLINE ANTIBIOTIC COMPLEX

I. TAXONOMY OF PRODUCING ORGANISM, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF RUBEOMYCIN A, A_1, B AND B_1

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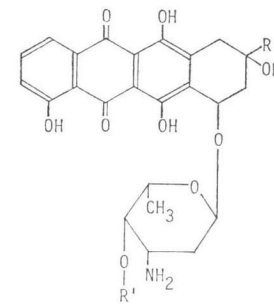
A new antibiotic complex has been obtained from the cultures of an actinomycete, strain FA-1180, isolated from a soil sample collected at lake side of Biwa in Japan.

On the basis of taxonomic studies the producing microorganism is designated as *Actinomadura roseoviolacea* var. *biwakoensis* nov. var. The antibiotic complex belongs to the class of anthracycline glycoside antibiotics. All components form deep red fine needles on crystallization; components are named rubeomycin A, A_1, B and B_1. These components exhibit activity against Gram-positive bacteria as well as Yoshida sarcoma cell *in vitro*. These components are also effective on P388 leukemia.

In the course of our screening program for new antibiotics produced by rare actinomycetes, we found that the *Actinomadura* strain FA-1180, isolated from a soil sample collected at lake side of Biwa in Japan, produced an anthracycline antibiotic complex which inhibited the growth of Gram-positive bacteria as well as Yoshida sarcoma cell *in vitro* but not of Gram-negative bacteria, yeasts and fungi. In addition to its *in vitro* activity the antibiotic increased the survival of CDF_1 mice bearing P388 leukemia. The antibiotic complex was isolated from the fermented mycelial cake of strain FA-1180 and separated into four related components named rubeomycin A, A_1, B and B_1. On the basis of their physicochemical properties, these antibiotics represent new anthracycline derivatives and are related to carminomycin[1] (Fig. 1).

Fig. 1. Structures of rubeomycin A, A_1, B and B_1.



	A and A_1	B and B_1
R:	$-\overset{\overset{O}{\|\|}}{C}-CH_3$	$-\overset{\overset{OH}{\|}}{C}H-CH_3$
R′:	(same in all components)	

$$-CH(CH_2OH)-O-CH(CH_2-CHOH-CH_3)-CH_3$$

In this paper, the taxonomy of the producing strain, production, isolation, preliminary investigation of physicochemical properties and biological activities of the antibiotics are presented. The chemical structure elucidation of these antibiotics will be reported in the next paper.

Taxonomy of Producing Organism

Most of the taxonomic studies on the culture were carried out in accordance with the methods adopted by the International Streptomyces Project[2]. Additional media recommended by WAKSMAN[3]

and NONOMURA *et al.*[4] were also used. Observation of the cultures was made after cultivation for 3 weeks at 27~28°C unless otherwise stated. The cell-wall components were analyzed with the pure cell-wall prepared in accordance with the method of YAMAGUCHI[5]. The cell-wall amino acids were detected by amino acid autoanalyzer Yanagimoto LC-5, sugars were detected by GLC-Mass LKB-9000 as their corresponding trimethylsilyl derivatives and stereoisomers of 2,6-diaminopimelic acid were determined by the paper chromatographic methods of HOARE and WORK[6]. The mycelium was cultivated for 7 days at 35°C in the yeast extract-malt extract broth (ISP No. 1).

Morphological Characteristics

The aerial mycelia were well developed, long, straight to wavy and monopodially branched on yeast extract - malt extract agar, oatmeal agar, oatmeal agar with added vitamin B mixture[4] and inorganic salts - starch agar with added vitamin B mixture (Fig. 2). The spore chains formed tightly closed spirals with 2~5 turns; pseudosporangia were also formed (Fig. 3). Electron microscopy revealed that the mature spores were about 0.8~1.1×0.6~1.0 μ, oval in shape, and with a smooth surface.

Fig. 2. Aerial mycelium of strain FA-1180 (ISP - 4 V, 14 days).

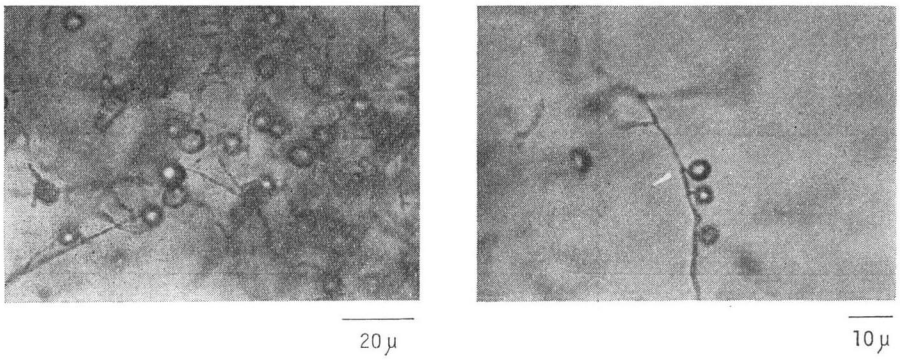
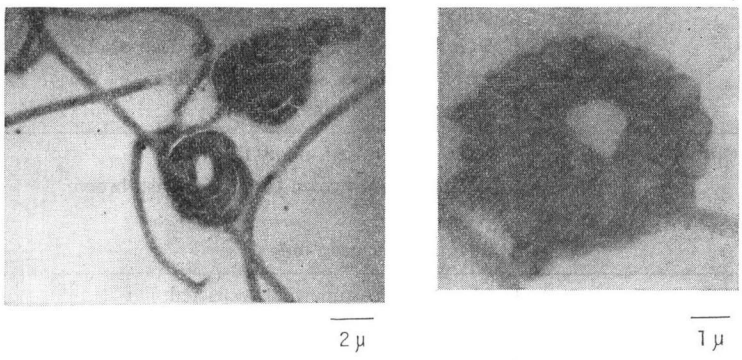


Fig. 3. Electron micrograph of strain FA-1180 (ISP - 3 V, 14 days).



Cell Wall Composition

The cell-wall of strain FA-1180 contains *meso*-diaminopimelic acid, glucose, mannose and madurose[7], but lacks glycine. The aforementioned cell-wall composition indicates the strain FA-1180 is an actinomycete species of the cell-wall type III B[8].

Cultural Characteristics

The cultural characteristics of strain FA-1180 are shown in Table 1. On most media, orange

Table 1. Cultural characteristics of strain FA-1180.

Medium	Characteristics
Tryptone - yeast extract broth (ISP No. 1)	GR: fair, pale yellowish flaky growth on bottom of tube, partially light orange to strong reddish orange ring on surface, in contact with glass AM: restricted, white to pale pink SP: slight, bright reddish orange
Yeast extract - malt extract agar (ISP No. 2)	GR: good, remarkably raised, many folds RC: strong orange to deep reddish orange, turning later into deep red AM: fair, pink to purplish pink SP: slight, strong reddish orange
Oat meal agar (ISP No. 3) and ISP No. 3 V*	GR: fair to somewhat restricted, flat, spreading RC: light yellowish brown AM: fair, powdery, white to pale pink, sometimes with drops formed on surface SP: strong reddish orange
Inorganic salts - starch agar (ISP No. 4)	GR: restricted, penetrating into medium RC: light yellowish brown AM: scant, white SP: none
ISP No. 4 V	GR: good, somewhat raised RC: strong reddish orange AM: fair to good, pale pink SP: strong orange zone around growth
Glycerol - asparagine agar (ISP No. 5)	GR: scant RC: light yellowish brown AM: none SP: none
ISP No. 5 V	GR: good, remarkably raised RC: strong reddish orange to deep orange AM: restricted, white to pale pink SP: slight, bright orange
Peptone - yeast extract - iron agar (ISP No. 6)	GR: fair, raised, many fine folds RC: grayish brown to dark grayish brown AM: none SP: slight, deep red
Tyrosine agar (ISP No. 7)	GR: fair to somewhat restricted, flat RC: dull red, turning later into deep red purple AM: none SP: none
ISP No. 7 V	GR: fair, flat RC: deep red purple AM: none SP: deep red purple

Table 1. (Continued)

Medium	Characteristics
Sucrose - nitrate agar (Waksman No. 1)	GR: fair RC: light orange to strong reddish orange AM: restricted, white to pale pink SP: none or slight, reddish orange
Waksman No. 1 V	GR: fair to good RC: bright orange to deep red purple AM: fair, pale pink to pale yellowish pink SP: deep red purple
Glucose - asparagine agar (Waksman No. 2)	GR: scant RC: pale yellow AM: none SP: none
Waksman No. 2 V	GR: fair to good, somewhat raised RC: bright reddish orange AM: scant, white to pale pink SP: bright orange
Bouillon agar (Waksman No. 8)	GR: fair RC: dull red to grayish red AM: scant, white SP: dull red
Glucose - bouillon agar	GR: very good, remarkably raised, many folds RC: dull red to dark red AM: scant, white SP: dull red
Calcium malate agar	GR: scant or none AM: none SP: none
Calcium malate agar V	GR: somewhat restricted, flat, spreading RC: vivid red purple AM: none SP: vivid red purple
Glycerol - calcium malate agar	GR: restricted to scant RC: bright orange AM: none SP: none
Glycerol - calcium malate agar V	GR: fair RC: dull red AM: none SP: none or slight, dull red
Bouillon - gelatin stab	GR: none

Table 1. (Continued)

Medium	Characteristics
Potato plug (Waksman No. 40)	GR: fair, somewhat raised RC: strong reddish orange to dull purplish red AM: restricted, white to grayish pink SP: grayish pink to grayish red
Löffler's blood serum	GR: fair to somewhat restricted RC: bright yellowish red, turning later into deep red purple AM: none SP: slight, deep red purple
Skim milk	GR: fair to somewhat restricted, ring present, bright orange to bright reddish orange AM: none SP: slight, light orange to bright orange

Symbols: GR, growth. RC, reverse color. AM, aerial mycelium. SP, soluble pigment.
* V: vitamin B mixture added[4]

Color names were assigned according to "Manual of Color Name", a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

to red or violet vegetative growth developed moderately and aerial mycelia were colored white to pink. The soluble pigments were orange to red or violet and behaved as pH indicators. The growth of this strain was enhanced by the addition of a vitamin B mixture in media.

Physiological Characteristics

The physiological characteristics of strain FA-1180 and its profile of carbohydrate utilization are shown in Table 2 and Table 3, respectively.

Table 2. Physiological properties of strain FA-1180.

Parameter observed	Results
Optimum temperature for growth	35~40°C
Liquefaction of gelatin	negative
Hydrolysis of starch	weakly positive
Milk peptonization	weakly positive
Milk coagulation	weakly positive
Nitrate reduction	positive
Production of H_2S	negative
Production of ammonia	negative
Melanoid pigment	negative

Table 3. Carbon utilization of strain FA-1180.

Carbon source	Response	Carbon source	Response
L-Arabinose	+	Melibiose	+
D-Xylose	+	Raffinose	−
D-Glucose	+	Trehalose	+
D-Galactose	+	Cellobiose	+
D-Fructose	+	*i*-Inositol	+
L-Rhamnose	+	D-Mannitol	+
D-Mannose	+	D-Sorbitol	±
D-Lactose	+	Salicin	+
Maltose	+	Inulin	−
Sucrose	+		

Symbols: +, utilization. ±, doubtful utilization. −, no utilization.

The microscopical and cultural studies and cell-wall analysis of FA-1180 indicate that this strain belongs to the genus *Actinomadura*; it closely resembles *Actinomadura roseoviolacea* Nonomura & Ohara. The comparison of cultural and physiological characteristics of strain FA-1180 with *Actinomadura roseoviolacea* Nonomura & Ohara KCC A-0145 was made by simultaneous cultivation and is shown in Table 4.

The differences, however, were not sufficient to designate strain FA-1180 as a new species and it

Table 4. Differences between strain FA-1180 and *A. roseoviolacea* KCC A-0145.

Medium	Strain FA-1180	*A. roseoviolacea* KCC A-0145
ISP No. 1	GR: fair, pale yellowish flaky growth on bottom of tube, partially light orange to strong reddish orange ring on surface, in contact with glass SP: slight, bright reddish orange	flaky growth on bottom of tube, pale yellow
ISP No. 4	GR: restricted, penetrating into medium RC: light yellowish brown AM: scant, white	restricted, spreading light reddish brown fair, white to pale pink
ISP No. 5 V	AM: restricted, white to pale pink	none
ISP No. 6	SP: slight, deep red	none
Waksman No. 1 V	GR: fair RC: deep red purple AM: fair, pale pink to pale yellowish pink	scant deep red purple none
Waksman No. 7 V	SP: none	vivid red purple
Carbon utilization		
Salicin	+	−
D-Sorbitol	+	−
Sucrose	+	−
i-Inositol	+	±
D-Mannitol	+	±
Hydrolysis of starch	weakly positive	negative

Symbols: Same as in Tables 1 and 3.

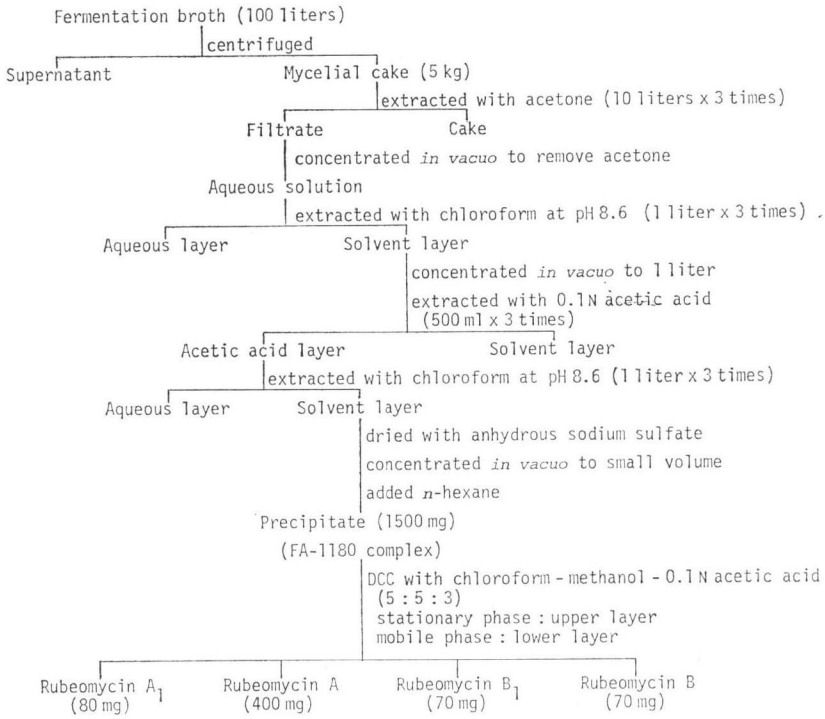
was named *Actinomadura roseoviolacea* var. *biwakoensis* nov. var. This strain has been deposited at the Institute for Fermentation, Osaka, Japan and Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, where it has been assigned the designations IFO 14092 and FERM-P 5155, respectively.

Production and Isolation

The producing organism, *A. roseoviolacea* var. *biwakoensis*, was grown in submerged culture in a 50-liter jar fermentor containing 35 liters of a medium consisting of 3% glucose, 1% corn steep liquor, 1% soy bean flour and 0.1% K_2HPO_4. The fermentor was inoculated with 700 ml of the seed culture which was prepared in 2-liter flasks each containing 350 ml of the same medium and incubated on a reciprocal shaker at 35°C for 3~5 days. Incubation was continued at 35°C for 7 days with aeration at 30 liters per minute and agitation at 250 rpm. The progress of fermentation was monitored by determination of the mycelial volume and the optical density at 496 nm of acetone extracts of mycelium, because the antibiotics were accumulated in the mycelium and not excreted in the filtrate under these culture conditions.

The isolation of antibiotics was accomplished using the general procedure for anthracycline antibiotics, as shown in Fig. 4. The fermentation broth (100 liters) was centrifuged and the antibiotics were extracted from the harvested mycelial cake (5 kg) three times with acetone (10 liters) at 50°C.

Fig. 4. Purification process of the antibiotics.



The extract was concentrated *in vacuo* to remove acetone and the concentrate extracted three times with 1 liter of chloroform at pH 8.6. The chloroform layer was concentrated *in vacuo* to 1 liter. Then the antibiotics were extracted three times with 0.1 N acetic acid (500 ml). The acetic acid layer was subsequently adjusted to pH 8.6 with a sodium bicarbonate saturated solution and extracted three times with 1 liter of chloroform. The combined extracts were dried with anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and then a large excess of *n*-hexane was added to precipitate the antibiotics (1500 mg).

Further separation of the complex into its components was achieved by droplet countercurrent chromatography (DCC) using chloroform - methanol - 0.1 N acetic acid (5: 5: 3) as the solvent system. A DCC apparatus containing 300 separation tubes was used and separation was accomplished by the down flow method. The effluents were monitored by silica gel TLC with a solvent system of chloroform - methanol - acetic acid (80: 20: 4). The fractions containing each component were pooled and adjusted to pH 8.6 with the sodium bicarbonate saturated solution. The separated solvent layer was washed with deionized water and dried with anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and the components were crystallized using solvent systems chloroform - *n*-hexane for A and A_1, and chloroform for B and B_1. Each component was obtained as deep red fine needle crystals.

Physicochemical Properties

All the components of rubeomycin were amphoteric and soluble in methanol, acetone, chloroform and acidic water, but barely soluble or insoluble in ethyl ether, *n*-hexane, petroleum ether and water. The physicochemical properties of the components are summarized in Table 5. The UV and visible spectra of all the components were very similar (Figs. 5A ~ 5D). The IR spectra were approximately

Table 5. Physicochemical properties of rubeomycin A, A_1, B and B_1.

	A	A_1	B	B_1
Melting point °C (dec.)	155~160	174~176	144~146	152~154
Elemental analysis (%)*	F C	F C	F C	F C
C :	58.7 60.1	58.7 60.1	59.7 59.1	58.4 59.1
H :	6.7 6.2	5.6 6.2	6.7 6.5	7.3 6.5
N :	2.0 2.1	1.9 2.1	1.7 2.1	1.9 2.1
O :	31.6 31.6	— 31.6	— 31.5	— 31.5
Molecular weight (FD-mass)	659	659	661	661
Molecular formula	$C_{33}H_{41}NO_{13}$	$C_{33}H_{41}NO_{13}$	$C_{33}H_{43}NO_{13}$	$C_{33}H_{43}NO_{13}$
λ_{max} nm ($E^{1\%}_{1cm}$) in MeOH	237 (595)	237 (605)	237 (562)	237 (582)
	255 (447)	255 (464)	255 (453)	255 (466)
	292 (141)	292 (161)	292 (133)	292 (137)
	493 (250)	493 (260)	493 (239)	493 (251)
	530 (174)	530 (196)	530 (173)	530 (182)
$[\alpha]^{20}_D$ in $CHCl_3$	+120.6 c 0.199	+170.4 c 0.053	+118.0 c 0.128	+151.0 c 0.090
Rf value on silica gel TLC a:**	0.57	0.74	0.34	0.47
b:	0.05	0.11	0.02	0.04
c:	0.31	0.40	0.23	0.27

* F: found, C: calculated.

** a: $CHCl_3$ - MeOH - acetic acid, 80: 20: 4.
b: $CHCl_3$ - MeOH, 10: 1.
c: $CHCl_3$ - MeOH - toluene, 7: 3: 3.

the same for all the components, except for the absoption at 1720 cm^{-1}, which indicates, in the spectra of A and A_1 components, the presence of a $-COCH_3$ group in the hydroaromatic ring; this band is lacking in the spectra of the B and B_1 components (Figs. 6A and 6B). This consideration was supported from the CMR spectra (Figs. 7A~7D). In the CMR spectra of all the components, the signals due to the 33 carbon atoms are present.

Biological Properties

Antimicrobial Activities

Antimicrobial activities of each component of rubeomycin are shown in Table 6. One loopful of test organism suspension was streaked on agar plates containing two-fold dilutions of the antibiotic acetic acid salt. The inoculated plates were incubated for 24 hours at 37°C for bacteria or 7 days at 30°C for fungi and yeasts. Brain heart infusion agar (Nissui) for bacteria and SABOURAUD's agar for fungi and yeasts were used. All the components showed selective antibacterial activity against Gram-positive bacteria, A and A_1 exhibiting higher activity (lower MIC) than B and B_1.

Growth Inhibitory Activities against Yoshida Sarcoma Cell

The acetic acid salts of the antibiotics were dissolved in the Fisher's medium containing 20% of horse serum; dilutions were made with the same medium. These media containing the antibiotics were inoculated with 5×10^4 cells/ml of Yoshida sarcoma and incubated for 2~3 days at 37°C. The growth inhibitory activity of the A_1 component was about 10 times larger than that of other components.

Fig. 5A. UV spectrum of rubeomycin A.

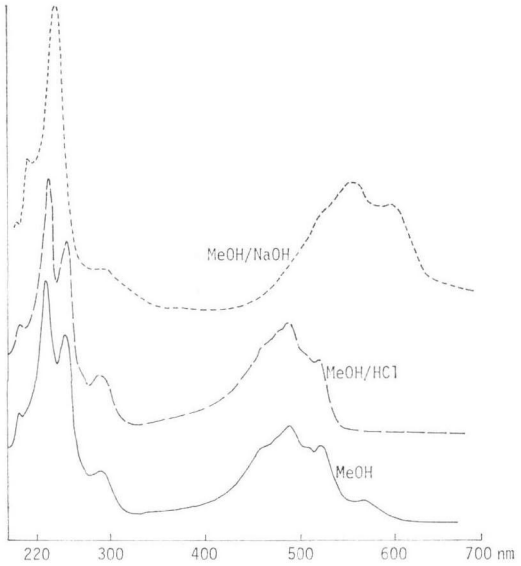


Fig. 5B. UV spectrum of rubeomycin B.

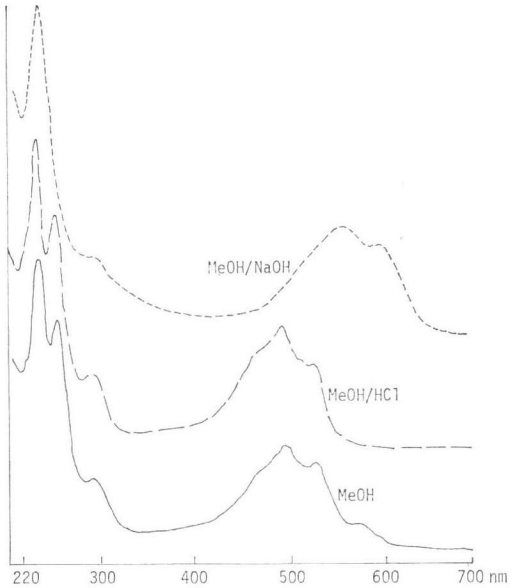


Fig. 5C. UV spectrum of rubeomycin A_1.

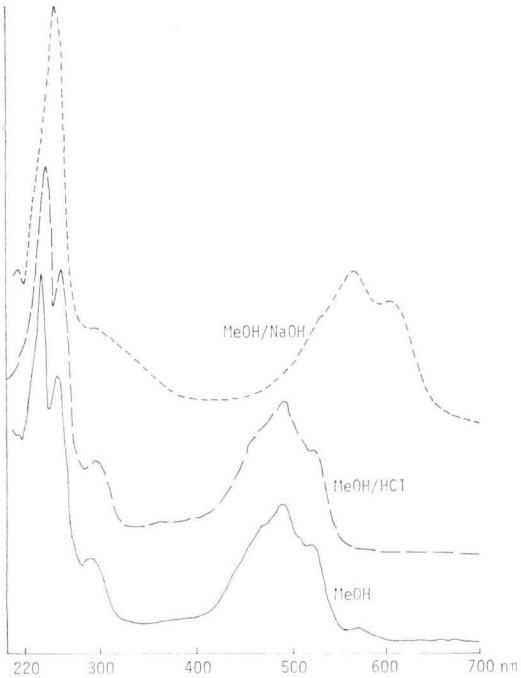
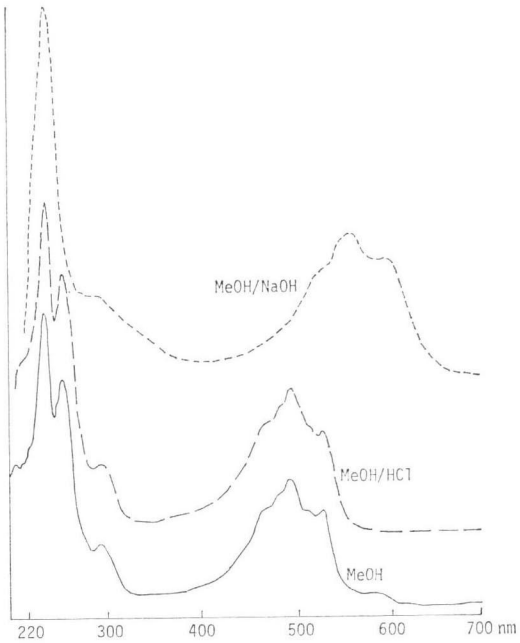


Fig. 5D. UV spectrum of rubeomycin B_1.



Antitumor Activities in Mice

The antitumor activities of the antibiotics were studied by prolongation of the median survival time (MST) of mice bearing P388 leukemia. For testing P388 leukemia was implanted intraperitoneally into CDF_1 mice using 10^6 cells/animal. Twenty-four hours after the implantation of tumor cells,

Table 6. Antimicrobial spectra of rubeomycin A, A_1, B and B_1.

Organism	Minimum inhibitory concentration (mcg/ml)			
	A	A_1	B	B_1
Bacillus subtilis PCI 219	<0.2	<0.2	0.8	0.4
Bacillus subtilis 17A	0.8	<0.2	1.56	1.56
Bacillus subtilis 45T	<0.2	<0.2	<0.2	<0.2
Staphylococcus aureus 209P	0.8	0.4	3.125	3.125
Staphylococcus aureus Terajima	1.56	1.56	12.5	12.5
Staphylococcus aureus MS353	<0.2	<0.2	3.125	3.125
Streptococcus faecalis	1.6	0.8	25.0	25.0
Micrococcus luteus ATCC 9341	<0.2	<0.2	0.8	1.56
Escherichia coli NIHJ-JC-2	>100	>100	>100	>100
Escherichia coli Keio	>100	>100	>100	>100
Salmonella typhimurium IID 971	>100	>100	>100	>100
Salmonella typhi 901	>100	>100	>100	>100
Serratia marcescens IAM 1184	>100	>100	>100	>100
Proteus vulgaris OX-19	>100	>100	>100	>100
Candida albicans	>100	>100	>100	>100
Saccharomyces cerevisiae IFO 1047	>100	>100	>100	>100
Trichophyton mentagrophytes IFO 5809	>100	>100	>100	>100
Trichophyton rubrum IFO 9185	>100	>100	>100	>100
Penicillium chrysogenum IFO 4626	>100	>100	>100	>100
Aspergillus fumigatus IFO 4057	>100	>100	>100	>100

Table 7. Activities of rubeomycin A, A_1, B and B_1 against P388 leukemia.

	Dose (mg/kg/day)	MST/range (days)	T/C* (%)	Body weight change (g)
Rubeomycin A	1.0	22.5/10~26	230	−1.3
	0.5	14.5/11~19	156	−0.7
	0.25	16.3/16~17	166	+1.0
	0.125	15.8/16~17	161	+1.5
Rubeomycin A_1	1.0	2.8/3~4	29	Toxic
	0.5	4.0/4	41	〃
	0.25	5.0/5	51	〃
	0.125	7.3/6~7	75	〃
Rubeomycin B	4.0	23.5/22~25	200	−1.4
	2.0	22.5/22~25	191	−1.7
	1.0	17.5/17~22	179	+0.9
	0.5	16.5/16~18	168	+0.8
	0.25	14.3/14~15	146	+1.7
	0.125	12.8/13~14	131	+2.0
Rubeomycin B_1	1.0	17.5/17~20	179	−0.8
	0.5	16.5/16~20	168	0
	0.25	14.8/15~16	151	+1.4
	0.125	13.8/14~15	141	+1.1

* T/C: The ratio of median survival time of the treated group divided by that of the control group.

Fig. 6A. Infrared absorption spectra of rubeomycin A and B (KBr).

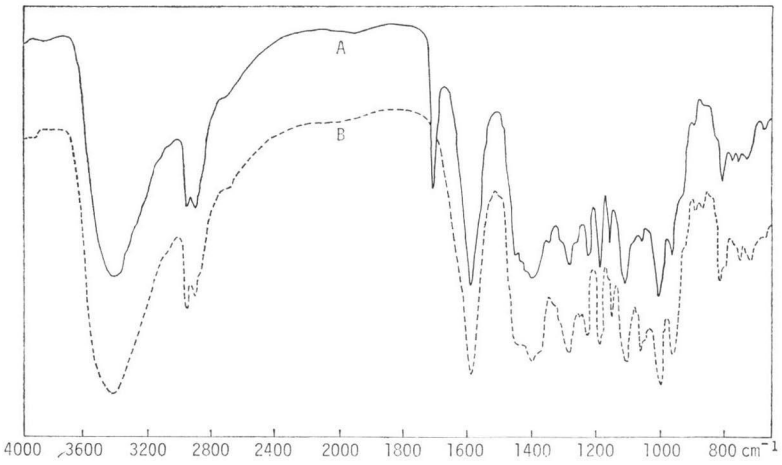


Fig. 6B. Infrared absorption spectra of rubeomycin A_1 and B_1 (KBr).

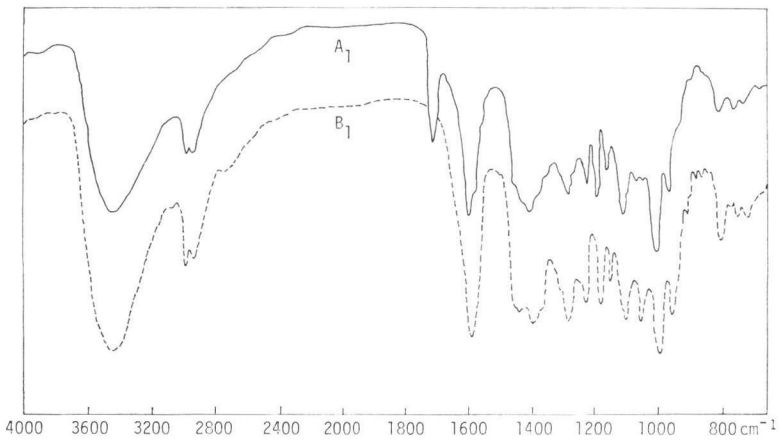


Fig. 7A. CMR spectrum of rubeomycin A in $CDCl_3$.

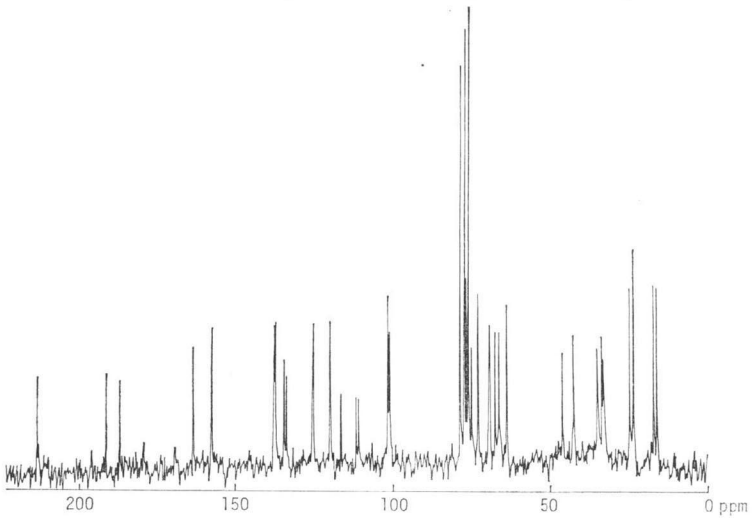


Fig. 7B. CMR spectrum of rubeomycin B in $CDCl_3$ - CH_3OH.

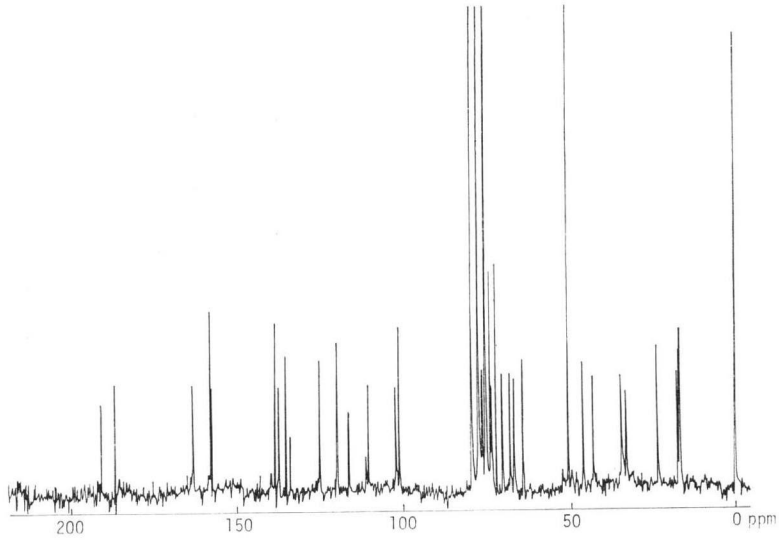
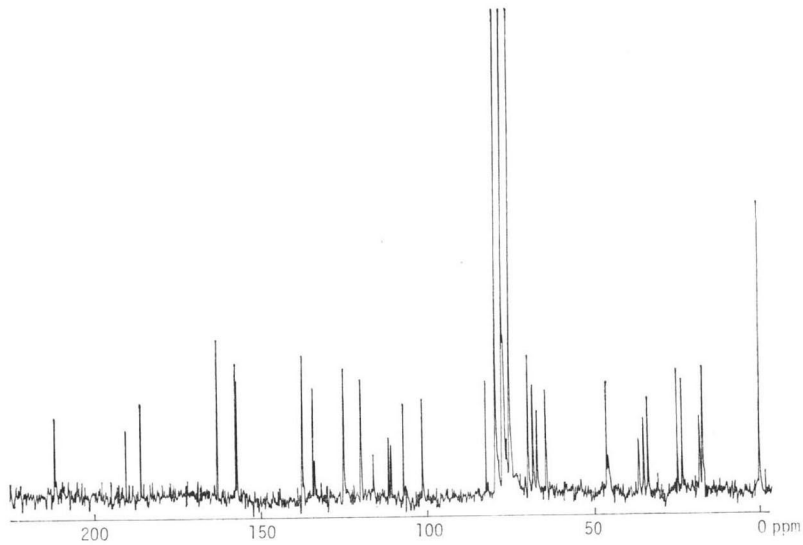


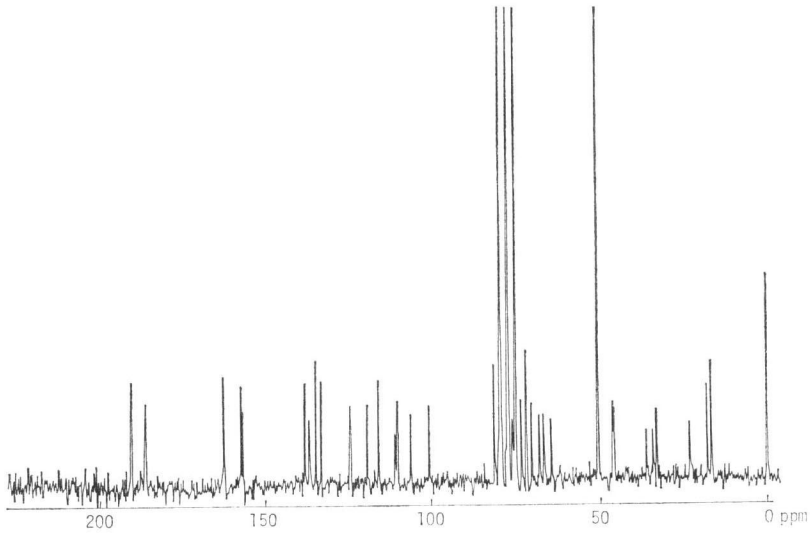
Fig. 7C. CMR spectrum of rubeomycin A_1 in $CDCl_3$.



graded doses of the antibiotics dissolved in saline were administered to mice intraperitoneally (0.1 ml per 10 grams of body weight). The treatment was given once a day for 5 days.

The results are shown in Table 7. Rubeomycin A, B and B_1 showed significant prolongation of life with doses in the range of 0.125~4.0 mg/kg/day, but the A_1 component was more toxic than the others; therefore the prolongation of life with A_1 was not observed under the experimental conditions used.

Fig. 7D. CMR spectrum of rubeomycin B_1 in $CDCl_3$ - CH_3OH.



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