STUDIES ON MARINE MICROORGANISMS. V

A NEW ANTIBIOTIC, APLASMOMYCIN, PRODUCED BY A STREPTOMYCETE ISOLATED FROM SHALLOW SEA MUD

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A new antibiotic, aplasmomycin, which inhibits growth of Gram-positive bacteria including mycobacteria *in vitro*, and plasmodia *in vivo* was obtained from a strain of *Streptomyces griseus* isolated from shallow sea sediment in Sagami Bay. The antibiotic forms colorless needle-like crystals and has a molecular formula of $C_{41}H_{60}O_{14}Na$. Based on its physical and chemical properties, aplasmomycin was concluded to be a new antibiotic. The antibiotic was produced in selected media devised to relate to a marine environment.

As shown in a previous paper[1], it is expected that marine Actinomycetales may represent a good source of new metabolites. In the course of screening for antibiotics produced only in specially devised media relating to marine environments, an Actinomycetales produced a new antimicrobial product, SS-228 Y[2,3] having inhibitory activities against tumors and dopamine β-hydroxylase as well. In this paper, another new antibiotic, aplasmomycin, will be described including taxonomy of its producer, cultural conditions for production of aplasmomycin, and isolation and properties of the active principle.

Identification of the Aplasmomycin-producing Strain

An Actinomycetales was isolated from shallow sea mud collected at Koajiro inlet of Sagami Bay in 1971 and designated as SS-20. Characteristics are described below:

Morphology

Branched substrate mycelium develops aerial mycelium, about 1 μ in width with tips straight to flexuous in tufts, as shown in Plate 1. Under an electron microscope, the spores showed smooth surfaces without spiny or hairy structures. The spores are oval to oblong and measure $0.6\sim0.8\times1.0\sim1.2$ μ. Typical chains of spores consisting of more than 10 spores per chain are shown in Plate 2. The morphology of sporophores categorizes this organism in Section *Rectiflexibiles*. Special organs such as sclerotia were not observed.

Cultural characteristics on various agar media

Table 1 shows the cultural characteristics of strain SS-20 on various media suggested by SHIRLING and GOTTLIEB[4] or by WAKSMAN[5], with addition of media that had been used for isolation of marine Actinomycetales[2].

Color designations are those of the Japan Color Standard[6] and Color Harmony Manual[7].

Physiological properties

Utilization of carbon sources was examined by using the PRIDHAM-GOTTLIEB basal medium (ISP. Med. No. 9). The results are shown in Table 2. Other physiological characteristics are described in

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Plates 1 and 2. Photographs of *Streptomyces griseus* strain SS-20 (14 days culture on salts-starch agar)

Plate 1 ×100 Plate 2 ×3,000

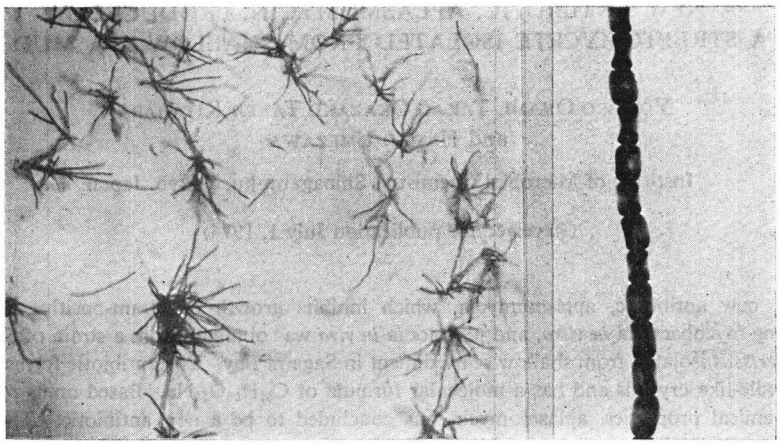

Table 1. Cultural characteristics of *Streptomyces griseus* strain SS-20

Agar medium	Appearance of growth		
	Aerial mycelium (AM)	Growth (G)	Diffusible pigment (DP)
Starch-casein-seawater[2]	light olive gray (1 gc)	fair, colorless	none
Glycerol-glycine[2]	white (a)	fair, pale yellow (1 1/2 ca)	none
Maltose-yeast ext.[2]	olive gray (1 1/2 ig)	fair, colorless	none
Dilute yeast ext.-glucose (DilYE)*	yellowish gray (1 1/2 ca)	poor, colorless	none
Sucrose-nitrate[5]	grayish white (3 ba)	fair, brownish white (2 db)	none
Glucose-asparagine[5]	grayish white (3 ba)	good, pale yellowish brown (2 gc)	none
Glycerol-asparagine[4]	grayish white (3 ba)	fair, pale yellowish brown (2 gc)	none
Inorganic salts-starch[4]	light olive gray (1 le)	excellent, dull yellow (2 ne)	none
Tyrosine[4]	brownish white (2 cb)	good, dull yellow orange (3 gc)	slightly, brownish white
Nutrient[5]	brownish white (2 cb)	fair, pale yellow orange (2 fb)	slightly, pale yellow-orange
Yeast-malt ext.[4]	light olive gray	excellent, pale yellowish brown (3 gc)	none
Oatmeal[4]	yellowish gray (1 1/2 gc)	good, pale yellow orange (2 fb)	none
Peptone-yeast ext. iron[4]	brownish white (2 cb)	fair, pale yellow (2 fb)	none
Calcium malate[5]	grayish white (3 ba)	fair, yellowish gray (2 db)	none

* DilYE: yeast extract 0.025%, malt extract 0.0625%, glucose 0.025%, NaCl 3.0% and agar 1.7%, pH 7.4 before sterilization

Table 3. Optimum temperature for growth on MYS agar (maltose 1.0%, yeast extract 0.4% and agar 1.7%, pH 7.2 before sterilization) appears to be about 27°C by the following results.

Temperature (°C)	4	8	15	20	24	27	32	37	42	45
Growth*	−	−	±	±	++	+++	++	++	±	−

*−: no growth ±: faint growth
+: fair growth ++: good growth
+++: excellent growth

Table 2. Utilization of carbon compounds by *Streptomyces griseus*, strain SS-20.

Carbon source	Utilization
No carbon source	−
D-Glucose	+
L-Arabinose	−
Sucrose	−
D-Xylose	+
i-Inositol	−
D-Mannitol	+
D-Fructose	+
L-Rhamnose	−
Raffinose	−
Cellulose	−

Table 3. Physiological characteristics of *Streptomyces griseus*, strain SS-20.

Test	Results
Hydrolysis of starch	Positive
Tyrosinase reaction	Negative
Solubilization of calcium malate	Weakly positive
Liquefaction of gelatin	Positive
Milk coagulation	Positive
Milk peptonization	Positive
Nitrate reduction	Positive

Summarizing the above, strain SS-20 belongs to the genus *Streptomyces* WAKSMAN and HENRICI, 1943. Among known species of *Streptomyces*, *Streptomyces griseus* (KRAINSKY) WAKSMAN and HENRICI most resembles strain SS-20 in morphological, cultural and physiological characteristics. Therefore, SS-20 is concluded to be a strain of *S. griseus*. Progeny of strain SS-20 has been deposited in the Fermentation Research Institute, Chiba, Japan and assigned accession number FERM-P No. 3448.

Production of Aplasmomycin

A wide variety of media was examined to define reproducible and suitable conditions for production of aplasmomycin by strain SS-20. Because this strain was isolated from marine sediment which is poor in nutrients and fairly high in salt concentration, the effect of NaCl and nutrient concentrations on the production of aplasmomycin was studied in basal medium YE broth, containing yeast extract 0.4%, malt extract 1.0% and glucose 0.4%, pH 7.4. When strain SS-20 was shake-cultured in YE broth, it did not produce aplasmomycin. However, it did produce the antibiotic in a medium with diluted nutrients and high salt concentration. The maximum yield (50 mcg/ml) of aplasmomycin was obtained in dilYE medium where basal medium YE was diluted to 1/16 with deionized water supplemented with 3.0% NaCl, as shown in Table 4.

Because Kobu-Cha medium containing the powdered tangled sea weed, *Laminaria* sp., as nutrient source was successful for the culture of marine Actinomycetales as described in the previous paper[2], the production of aplasmomycin in Kobu-Cha medium was examined. Table 5 shows the effect of NaCl on aplasmomycin production by strain SS-20 in medium containing Kobu-Cha* 1.0%, glucose 1.0%, pH 7.8 before sterilization. Maximum production (125 mcg/ml) was obtained on supplementation with 1.5% NaCl. After a survey of media in 500-ml SAKAGUCHI flasks for antibiotic production, a medium containing Kobu-Cha 1.0%, glucose 1.0%, NaCl 1.5%, pH 7.8 was found to be suitable. Spores of strain SS-20 cultivated on an agar slant of SC (soluble starch 1.0%, casein 0.1%, agar 1.7%, artificial sea water 500 ml+dist. water 500 ml, pH 7.4, before sterilization) medium at 27°C for 2~3 weeks were inoculated into 125 ml of liquid medium in a 500-ml flask and shaken on a reciprocal shaker with 8-cm amplitude, 130 strokes per minute at 27°C for 72 hours. Twenty ml of this broth were transferred to 1,250 ml of the same medium in a flask and shake-cultured as above for 120 hours. Anti-

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Table 4. Effect of nutrients and NaCl on aplasmomycin production by *Streptomyces griseus* strain SS-20.

Dilution	NaCl concentration and mcg/ml					
	0%	1%	2%	3%	4%	5%
YE*	0	0	0	0	tr**	tr
× 1/2	0	0	5	5	10	20
× 1/4	0	tr	15	15	20	30
× 1/8	0	tr	15	40	30	40
× 1/16	0	0	15	50	45	45
× 1/32	0	0	0	15	10	0

*Basal medium: yeast ext. 0.4%, malt ext. 1.0%, glucose 0.4%, pH 7.4, before sterilization

** less than 5 mcg/ml aplasmomycin

The medium (10 ml) was incubated in test tubes (18×150 mm) on a shaker (1.8-cm amplitude, 288 strokes/min) at 27°C for 5 days.

Table 5. Effect of NaCl on aplasmomycin production by *Streptomyces griseus*, strain SS-20.

NaCl (%)	Aplasmomycin (mcg/ml)	NaCl (%)	Aplasmomycin (mcg/ml)
0	70	2.5	110
0.25	105	3.0	120
0.5	100	3.5	80
1.0	110	4.0	35
1.5	125	4.5	tr*
2.0	110	5.0	0

* less than 5 mcg/ml aplasmomycin

Basal medium: Kobu-Cha 1.0%, glucose 1.0%. pH 7.8 before sterilization.

The medium (10 ml) was incubated in test tube (18×150 mm) on a shaker (1.8-cm amplitude, 228 strokes/min) at 27°C for 5 days.

biotic production was determined by a cylinder-plate microbiological assay using *Staphylococcus aureus* FDA 209P as the test organism. The maximum yield obtained was 60 mcg/ml at 120-hour incubation.

Isolation and Purification

The fermented broth (pH 6.5) was adjusted to pH 5.0 with dilute hydrochloric acid and filtered to remove the mycelium. The mycelium does not contain much of the antibiotics. Twenty liters of filtrate containing 50~60 mcg of aplasmomycin per ml were extracted with 10 liters of butyl acetate. The solvent layer was concentrated *in vacuo* at 40°C to give a yellowish colored syrup of aplasmomycin (420 mg). The syrup was placed on the top of column (1.5×20 cm) filled with 30 g of alumina (neutral, Woelm).

The charged column was developed with *n*-hexane and ethyl acetate (9:1) and the eluted fractions having antimicrobial activity were collected. The combined fraction was concentrated *in vacuo* at 40°C to give a pale yellowish powder (230 mg), which was 90% purified aplasmomycin. The powder was

Fig. 1. IR spectrum of aplasmomycin (KBr)

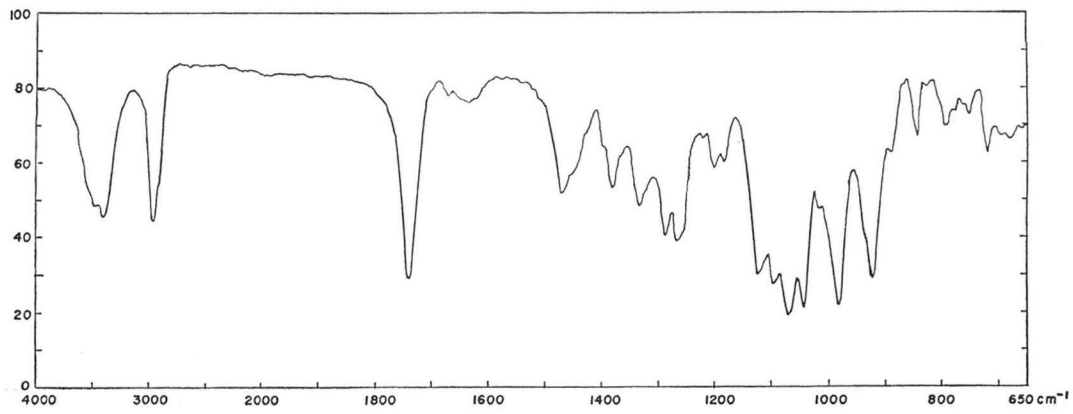
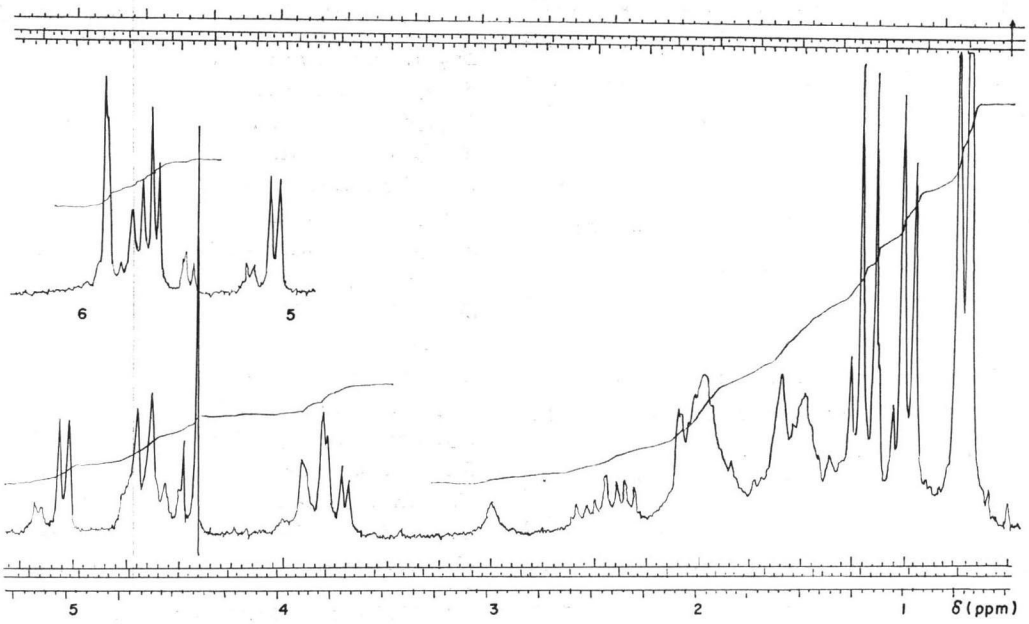


Fig. 2. PMR spectrum of aplasmomycin (100 MHz, $CDCl_3$)



again applied to the column (1.5×20 cm) filled with 30 g silicic acid (Silicic AR CC-7, Mallinckrodt). The charged column was developed with benzene and ethyl acetate (9:1), and eluted fractions having antimicrobial activity were collected. The active fractions were combined, then shaken with 2 N sodium hydroxide. The solvent layer was washed with water and dehydrated with anhydrous sodium sulfate. After the organic solvent was evaporated *in vacuo* at 40°C, aplasmomycin (120 mg) was crystallized from methanol-water.

Physicochemical Properties of Aplasmomycin

Aplasmomycin forms colorless needles, melting at 283~285°C with decomposition. Its sodium content could not be removed successfully because of acid lability. It does not contain nitrogen, sulfur, phosphorus or halogen. The result of elemental analysis was as follows: C 59.87, H 7.79, N 0.00, O 29.58 (by difference), Na 2.76 and the tentative formula is calculated as $C_{41}H_{60}O_{14}Na \cdot H_2O$ (C 60.22, H 7.58, O 29.38, Na 2.82). The high resolution mass spectrum showed $(M-1)^+$, *m/e* 798.3881 (calcd. for $C_{41}H_{59}O_{14}Na$, 798.3799). It is soluble in many of organic solvents but only slightly soluble in water. The uv spectrum showed only end absorption and $[\alpha]_D^{22}$ was +225° (*c* 1.24, $CHCl_3$). The mass spectrum showed peaks at *m/e* 814, 798 [$(M-1)^+$, base peak], 741, 726, 223, 205, 189, 179 and a peak at *m/e* 814 could be explained as a trace ion of K-containing aplasmomycin. As shown in Fig. 1, its infrared absorption spectrum shows a carbonyl band at 1740 cm^{-1} and no carboxylate band at 1600 cm^{-1} region. The pmr spectrum of aplasmomycin ($CDCl_3$) is shown in Fig. 2 and indicates absence of methoxyl groups. The ^{13}C-nmr spectrum (dioxane-d_8) showed 21 peaks. Twenty of them indicate two equivalent carbons by each signal from their intensities. The chemical shifts from TMS according to alkyl carbons will be as follows (off resonances): δ 13.2(q), 16.5(q), 19.4(q),21.4(q), 25.7(t), 29.4(t), 32.7(t), 33.6(d), 36.4(t) and 39.4(s). The carbons attached to oxygen will be as follows: δ 77.5(d), 78.6(d), 78.7

(d), 79.7(d), 80.2(d), 80.9(d) and 106.7(s). The olefinic carbons appear at δ 128.6(d) and 133.0 (d), and carbonyl carbon at δ 170.6(s). The residual small signal at δ 131.4(s) indicates one carbon and relatively low chemical shift will indicate that oxygen atoms attach to the carbon atom. From these data, a symmetric structure is suggested. The detailed structure will be presented in a subsequent paper. A positive color reaction was obtained by sulfuric acid-vanillin (violet), but negative by ferric chloride, periodate-benzidine and red tetrazolium. Rf values on silica gel thin-layer chromatography (Kiesel gel 60 F_{254}, Merck) with various solvents were as follows (detected with sulfuric acid-vanillin): benzene-ethyl acetate (1:1) 0.72; chloroform 0.08; and chloroform - methanol (9: 1) 0.97. The sodium atom of aplasmomycin was replaced by silver when excess silver nitrate was added to a methanol solution of aplasmomycin. The silver-aplasmomycin forms colorless needles (mp. 218~220°C) from methanol-water and the $[\alpha]_D^{23°}$ was +194° (*c* 0.34, $CHCl_3$). The mass spectrum showed peaks at *m/e* 884, 882 and 827.

Table 6. Biological activity of aplasmomycin

Organism	M.I.C. (mcg/ml)
Staphylococcus aureus FDA 209 P	1.56
Staphylococcus aureus Smith	1.56
Staphylococcus aureus 193	3.12
Staphylococcus aureus EMf	1.56
Staphylococcus aureus NBf	0.78
Staphylococcus aureus Terajima	3.12
Staphylococcus aureus MS 8800	3.12
Micrococcus flavus FDA 16	1.56
Sarcina lutea PCI 1001	1.56
Bacillus anthracis	0.78
Bacillus subtilis PCI 219	1.56
Corynebacterium bovis 1810	0.78
Mycobacterium smegmatis 607	6.25
Mycobacterium phlei	6.25
Escherichia coli K-12	>100
Salmonella typhi T-63	>100
Candida albicans	>100

* Medium: Nutrient agar dilution method for bacteria.
Nutrient agar containing 1% glycerol for mycobacteria and 1% glucose for yeast.

Biological Activity

As shown in Table 6, aplasmomycin inhibits Gram-positive bacteria including mycobacteria *in vitro* on the media indicated. Groups of 5 male mice of 4 weeks age were infected intraperitoneally by *Plasmodium berghei* (NK 65). In the control infected group, 3 of 5 mice died within 8 days and plasmodia were observed in more than half of the red cells in the 2 surviving mice. When aplasmomycin in peanut-oil (homogenized using glass beads) was administered orally in 2 doses of 100 mg/kg each by sonde, it decreased the number of plasmodium-containing red cells and all treated mice survived. Details of these experiments and results as well as other biological activities will be presented separately.

The acute toxicity (LD_{50}) in mice was 125 mg/kg by intraperitoneal injection.

Discussion

Two-hundred strains of marine Actinomycetales, which showed no antimicrobial activity when shake-cultured in ordinary media such as YE (yeast extract 0.4%, malt extract 1.0% and glucose 0.4%, pH 7.4) and PS (soluble starch 3.0%, Pharmamedia 1.5%, cornsteep liquor 2.0%, beef extract 1.0%, pH 7.4) were selected. Among these 200 strains, 7 showed antimicrobial activity against test microorganisms when they were cultured in dilYE or Kobu-Cha media. When strain SS-20 was shake-cultured in dilYE and Kobu-Cha media, it produced aplasmomycin. It is of especial interest that this strain produces aplasmomycin favorably in dilYE broth which is prepared by diluting YE medium to 1/16 with water and supplemented with 3.0% NaCl.

Aplasmomycin appears to belong to an ionophore antibiotic group based on its physicochemical properties. The ir spectrum, however, showed no carboxylate band and it differs from other polyether antibiotics hitherto reported, such as nigericin and monensins[8]. Some polyether antibiotics have been reported to be inhibitory to coccidia of chickens but not to plasmodia. A few, such as septamycin[9] and 31,559 RP[10] were found to be active against plasmodia, and they seem to be considerably toxic to animals (the former: 5 mg/kg i.v. in dogs, 10~30 mg/kg orally in rats, the latter: 150 mg/kg orally in chickens). In this regard, aplasmomycin is less toxic to animals than those above, and administration of multiple large doses is promising. Aplasmomycin is somewhat similar to macrotetrolide group antibiotics such as nonactin[11] but physicochemical properties of aplasmomycin differ from all the members in that group. The absolute structure by X-ray crystallography will be described by Y. IITAKA of Tokyo University in a separate paper. The structure contains methane tetroxy carbon $\left(\begin{smallmatrix}-O\\-O\end{smallmatrix}\!\!>\!C\!<\!\!\begin{smallmatrix}O-\\O-\end{smallmatrix}\right)$ and appears to represent the first discovery of this structural feature in nature.

Acknowledgement

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