A NEW ANTIBIOTIC, MACROMOMYCIN, EXHIBITING ANTITUMOR AND ANTIMICROBIAL ACTIVITY

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A macromolecular peptide was isolated from a culture filtrate of *Streptomyces macromomyceticus* n. sp. which is described in this paper. Macromomycin was isolated as a white powder by precipitation with ammonium sulfate, followed by dialysis, Dowex 1X2 column chromatography and Sephadex chromatography. It is a peptide with Sw 20 value of 1.27 and a molecular weight of about 15,000. It inhibits sarcoma 180 and mouse leukemia L-1210. It inhibits *S. aureus*, *M. flavus*, *S. lutea*, *B. subtilis*, and *B. anthracis*.

Culture filtrates of actinomycetes tested against L-1210, give much lower frequency of positive results than when tested on EHRLICH carcinoma. However, a culture inhibiting both EHRLICH ascites carcinoma and L-1210 was found and the active agent was isolated. It is a macromolecular peptide which also inhibits the growth of Gram positive bacteria. This antibiotic was differentiated from neocarzinostatin and other known macromolecular peptides showing antitumor activity and was named macromomycin. Characteristics of the strain producing this antibiotic, processes of production and isolation, and properties of the antibiotic are reported in this paper.

Characteristics of the Strain No. M480-M1

The strain was isolated from a soil sample collected at Setagaya, Tokyo and was numbered M480-M1. Colonies on CZAPEK's agar medium containing glycerol as the carbon source were examined microscopically. Long branched substrate mycelium of about 1 μ in width and aerial hyphae were observed. Neither whorls nor spirals were observed. The surface of the spore was shown to be smooth by electron microscopy, as shown in Plate 1. The characteristics on various media are described below. The description in parenthesis follows the color standard published by Container Corporation of America.

Plate 1. Spore surface of *Streptomyces macromomyceticus* n. sp. (×8500)

1. On glycerol CZAPEK's agar (27°C): Abundant greenish gray (Mistletoe Green 24 1/2 li) growth; white to gray aerial mycelium; no soluble pigment.
2. On KRAINSKY's glucose asparagine agar (27°C): Grayish growth; no or white aerial mycelium; no soluble pigment.
3. On calcium malate agar (27°C): Colorless growth; white to brownish white aerial mycelium slightly; no soluble pigment; transparent zone around the growth.
4. In peptone water with 1.0 % sodium nitrate (27°C): Colorless growth; no aerial mycelium; no soluble pigment; reduction of nitrate to nitrite.
5. On starch plate (27°C): Colorless to grayish growth; abundant brownish gray (Pussywillow Gray 5 dc to Mauve Gray 8 ig) aerial mycelium; no soluble pigment; positive hydrolysis of starch.
6. On tyrosine agar (27°C): Dark brownish gray growth; white to brownish white aerial mycelium; black soluble pigment; positive tyrosinase reaction.
7. On potato plug (27°C): Abundant, wrinkled, greenish gray (Mistletoe Green 24 1/2 li) to grayish yellowish brown (Mustard Tan 2 1 g) growth; no or brownish white aerial mycelium; brownish soluble pigment.
8. On nutrient agar (27°C): Colorless to olive-gray growth; no aerial mycelium; no or slightly brown soluble pigment.
9. On nutrient agar (37°C): Colorless growth; no aerial mycelium; no soluble pigment.
10. On LOEFFLER's coagulated serum (37°C): Wrinkled colorless growth; no aerial mycelium; brownish soluble pigment; no liquefaction of coagulated serum.
11. On gelatin stab (20°C): Colorless to brownish growth; no aerial mycelium; brown soluble pigment; liquefaction of gelatin in medium strength.
12. On skimmed milk (37°C): Colorless growth; no aerial mycelium; no soluble pigment; coagulation and peptonization of milk.
13. On cellulose (27°C): Poor growth; no hydrolysis.
14. Utilization of carbon sources on PRIDHAM-GOTTLIEB basal medium (27°C): Abundant growth with glycerol, xylose, galactose, glucose, maltose, mannose, dextrin, starch, salicin; no or slight growth with fructose, lactose, sucrose; no growth with arabinose, rhamnose, dulcitol, inositol, mannitol, sorbitol, raffinose, inulin.

As shown by the characteristics described above, strain M 480-M 1 belongs to *Streptomyces* and to the chromogenic type, though the production of brown soluble pigment is absent or slight in nutrient agar. It forms neither whorls nor spirals. The surface of the spores is smooth. The growth on various media is greenish gray, and the aerial mycelium is colored white to gray or white to brownish gray. No soluble pigment is formed in synthetic media but brown pigment occur frequently in organic media. Nitrate is reduced to nitrite. The proteolytic action is relatively weak and it hydrolyzes starch. Among known species, *Streptomyces lydicus* DE BOER *et al.* has many characteristics in common with strain M 480-M 1. However, the property of the spore surface of this species has not been described. According to the description, *S. lydicus* forms buff to olive-tan growth, but no melanin pigment. In these points *S. lydicus* is different from strain M 480-M 1. *S. lydicus* is known to produce streptolydigin. As described later, macromomycin is related to neocarzinostatin in antibacterial effect, antitumor effect and macromolecular nature. *Streptomyces carzinostaticus*[1,8] producing neocarzinostatin forms spores which have hairy projections. The strain No. M 480-M 1 was thus found to be different from known species and was designated *Streptomyces macromomyceticus* n. sp. HAMADA *et* OKAMI.

Production, Isolation and Characters of Macromomycin

Shaking cultures were fermented at 130 strokes per minute with 8-cm in amplitude and 120 ml of medium in 500-ml flask. The strain M 480-M 1 was shake-cultured for 48 hours and 2 ml of the culture broth was inoculated to new medium. Tank fermentation was carried out in a 70 L stainless steel tank, which contained 40 L of medium. Eight hundred ml of 48-hours shaking culture was inoculated. The tank was stirred at 300 r.p.m. and 40 L of sterile air was passed per minute. Soybean oil and silicon oil were used for antifoam.

Macromomycin was determined by a cylinder plate method using *Micrococcus flavus* as the test organism. *M. flavus* was shake-cultured at 28°C for 48 hours in a medium containing 2.0 % glucose, 1.0 % meat extract, 1.0 % peptone, and 0.3 % sodium chloride. The pH was adjusted to 7.0 after sterilization. The medium employed for preparation of the plate contained 0.1 % glucose, 0.6 % peptone, 0.4 % casamino acid, 0.15 % meat extract, 0.3 % yeast extract, and 1.0 % agar and the pH was adjusted to 7.0.

When strain M 480-M 1 was shake-cultured in a medium containing 1.0% glucose, 1.0 % starch, 1.5 % soybean meal, 0.1 % K_2HPO_4, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.3 % NaCl, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$, and 0.0002 % $ZnSO_4 \cdot 5H_2O$ (pH was adjusted to 7.0), then the culture filtrate after 4 days showed an inhibition zone of 24 mm diameter. A strain obtained by selection of productive spores produced 800 mcg/ml of macromomycin after 4 days of shaking culture in a medium similar to that described above except with 2.0 % soybean meal. Macromomycin was precipitated by saturation with ammonium sulfate and separated from the salt by dialysis against water. The impurities could be separated from macromomycin by their adsorption on a cation exchange resin such as Dowex 1 X 2 or IRA-400 in Cl^- form. Active carbon was also useful to separate impurities. Macromomycin was purified by column chromatography using Sephadex G-50 or G-100. An example of the isolation is described below.

Nine liter of the cultured broth from shake flasks was filtered. The filtrate contained 2,700 mg of macromomycin. To 8.5 L of the filtrate, 850 ml of 50 % $ZnCl_2$ aq. solution was added and filtered, and to 8.0 L of the filtrate, 5.5 kg of ammonium sulfate was added. After 3 hours at 5°C with occasional stirring, the precipitate containing macromomycin was separated by centrifugation at 4~5°C. The precipitate was dissolved in 120 ml of distilled water.

The solution, which contained 2,436 mg of macromomycin, was placed in cellophane tubes and dialyzed against water for 12 hours at 5~10°C. The solution thus obtained contained 1,950 mg of macromomycin in 260 ml. The non-dialyzable solution was passed through a column (4.0 cm × 60 cm) of Dowex 1 X 2 resin (50~100 mesh, 700 ml) in Cl^- form. Then 2 L of distilled water was passed. The effluent from the column was fractionated into 50 ml portions and those showing activity against *M. flavus* were collected. To the active solution (700 ml) containing 1,260 mg of macromomycin, 490 g of ammonium sulfate was added. The precipitate was collected and dissolved in 52 ml of distilled water and dialyzed. The dialyzed solution (100 ml) containing

1,200 mg of macromomycin was subjected to column chromatography (5.3 cm × 100 cm) on Sephadex G-25 (2.0 L). The fractions containing macromomycin were collected, rapidly frozen, and lyophilized, yielding 600 mg of powder of 60 % purity. This powder was further purified by column chromatography (3.5 cm × 170 cm) with Sephadex G-50 (1.0 L) and the active effluent (270 ml) was diluted with distilled water to 810 ml, rapidly frozen, and lyophilized, yielding 340 mg of pure macromomycin. Sephadex G-100 was also useful for purification of macromomycin.

Macromomycin is obtained as a white powder. It becomes brown at 240°C, and decomposes with bubbling at 255~258°C. It gives $[\alpha]_D^{20}+119°$ (c 1, H_2O). It is soluble in water but insoluble in methanol, ethanol, butanol, acetone, ethyl acetate and benzene. The ultraviolet absorption shows $E_{1cm}^{1\%}$ 8.2 at 280 mμ in H_2O, $E_{1cm}^{1\%}$ 9.2 at 284 mμ in 0.1 N NaOH and $E_{1cm}^{1\%}$ 8.5 at 278 mμ in 0.1 N HCl, with a shoulder at 290 mμ in all cases. The ultraviolet spectrum is shown in Fig. 1, and the infrared spectrum in Fig. 2.

It decolorizes permanganate and gives positive FOLIN-LOWRY, EHRLICH, SAKAGUCHI and biuret reactions. Ninhydrin reaction is weakly positive. MOLISCH, ferric chloride, anthrone, FEHLING, TOLLEN's and BENEDICT reactions are negative.

Pure macromomycin obtained by Sephadex G-50 this chromatography was subjected to the analysis by ultracentrifugation[2,3] at 59,780 r.p.m. The solution contained 0.93 % of macromomycin. The pattern is shown in Plate 2, indicating purity. The Sw 20 value was calculated to be 1.27 and analysis by ARCHIBALD's method indicated about 15,000 for the molecular weight.

Elementary analysis: C 43.89, H

Fig. 1. Ultraviolet absorption spectrum of macromomycin

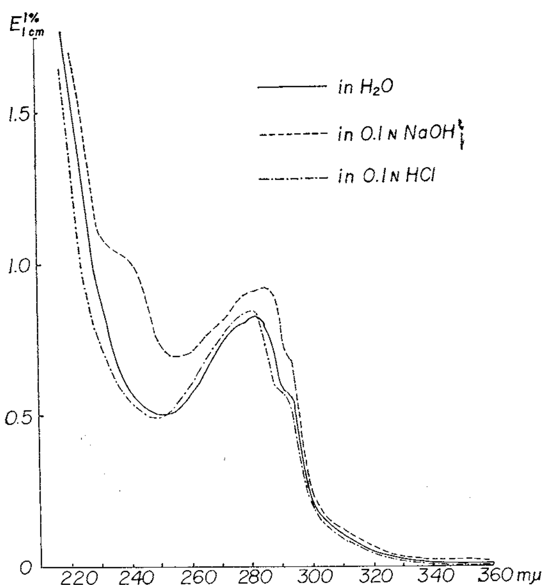


Fig. 2. Infrared absorption spectrum of macromomycin in KBR

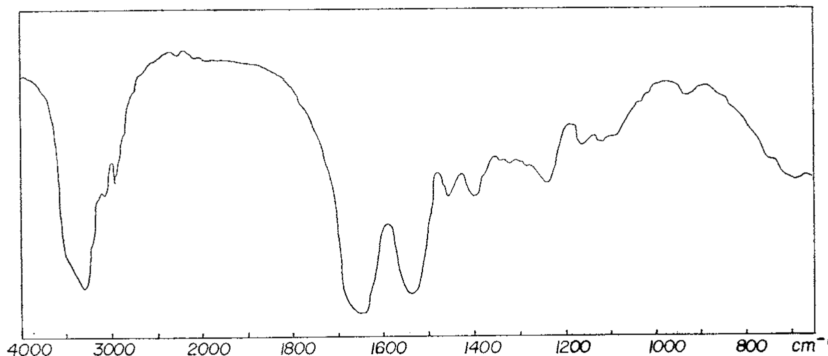
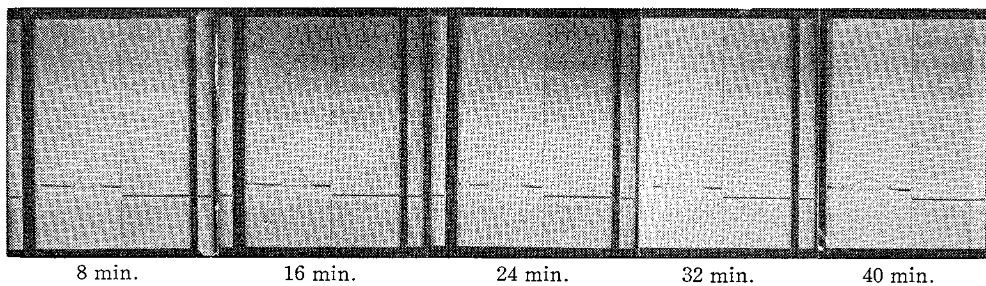


Plate 2. Ultracentrifuge pattern of macromomycin



8 min. 16 min. 24 min. 32 min. 40 min.

11.55, N 13.46, S 2.39, O 23.76. Tests for halogen and phosphor were negative. The following formula can be calculated for macromomycin : $(C_{49}H_{153}O_{24}N_{13}S)_{8\sim10}$.

The amino acid analysis of the hydrolysate of macromomycin (6 N HCl, 105°C, 15 hours) indicated at least 15 amino acids. They are lysine, histidine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, isoleucine, leucine, tyrosine and phenylalanine.

On paper electrophoresis at 450 V for 4.5 hours in pH 8.6 barbital buffer (μ=0.05) macromomycin moves toward the cathode 2.0 cm. In 0.06 M phosphate buffer at pH 6.8, it moves 1.2 cm to cathode.

Macromomycin is not inactivated by trypsin and chymotrypsin in barbital buffer at pH 8.6 (μ=0.05) at 27°C for 24 hours. It is not inactivated by bacterial acid protease in 0.06 M phosphate buffer at pH 6.8 for 24 hours at 27°C. Macromomycin is inactivated by ultraviolet irradiation.

Table 1. Antibacterial spectrum of macromomycin

	Minimal inhibitory concentration in mcg/ml	Media*
Staphylococcus aureus 209 P	1.56	A
〃 Smith	0.2	〃
Sarcina lutea PCI 1001	0.01	〃
Micrococcus flavus	<0.05	〃
Bacillus subtilis NRRL 558	0.39	〃
Bacillus anthracis	0.2	〃
Salmonella enteritidis	>100	〃
Shigella flexneri la EW 8	>100	〃
Proteus vulgaris OX 19	>100	〃
Klebsiella pneumoniae PCI 602	>100	〃
Escherichia coli NIHJ	>100	〃
〃 K 12	>100	〃
Serratia marcescens	>100	〃
Pseudomonas aeruginosa A 3	>100	〃
Pseudomonas fluorescens	>100	〃
Xanthomonas oryzae	100	B
Pyricularia oryzae	>100	〃
Cryptococcus neoformans	12.5	〃
Torula utilis	1.56	〃
Candida albicans Yu-1200	>100	〃
Mycobacterium 607	>100	C
Mycobacterium phlei	>100	〃

* A : Nutrient agar medium, B : 1 % glucose nutrient agar medium, C : 1 % glycerin nutrient agar medium.

The antimicrobial spectrum is shown in Table 1. It inhibits *Staphylococcus aureus*, *Micrococcus flavus*, *Sarcina lutea*, *B. anthracis*, *B. cereus* and *B. subtilis* at 0.05～1.56 mcg/ml and *Xanthomonas oryzae* at 100 mcg/ml, but showed no inhibition of other bacteria in Table 1 at 100 mcg/ml except *Torula utilis*.

Mice tolerated the intravenous injection of 62.5 mg/kg. The minimum effective dose against sarcoma 180 by daily intraperitoneal injection was 3.9～7.8 mcg/mouse/day and the daily injection of 250 mcg/mouse/day showed no toxicity. The daily injection of 40 mcg/mouse/day prolonged the survival of mice intraperitoneally inoculated with

L-1210 mouse leukemia; the survival time was 150 % of the control.

Among known high molecular weight antitumor substances, neocarzinostatin has antibacterial activity and has been reported to be active against mouse leukemia L-1210. Neocarzinostatin (Lot T-6-24) was easily differentiated from macromomycin by paper electrophoresis. Neocarzinostatin moved faster than macromomycin to cathode: 6.5 cm for neocarzinostatin and 2.0 cm for macromomycin under the conditions described above. Amino acid analysis of neocarzinostatin was reported[4,5] to show no histidine, but macromomycin contains this amino acid. The Sw 20 value of neocarzinostatin has been reported to be 1.44 while macromomycin has a value of 1.27. Marinamycin[6,7] was reported to be macromolecular and to show antibacterial activity. Marinamycin gives no peak in the ultraviolet spectrum, is not adsorbed on strongly acidic resin, and is precipitated with $ZnCl_2$. Macromomycin shows maximum absorption at 280 mμ in H_2O, is not observed in the effluent from a strongly acidic resin, and is not precipitated with $ZnCl_2$. Thus, macromomycin is differentiated from the known antitumor substances.

References

1) SHOJI, J.: Preliminary studies on the isolation of carzinostatin complex and its characteristics. Studies on the streptomyces antibiotics. XXXXIII. J. Antibiotics, Ser. A 14(1): 27~33, Jan. 1961.
2) SCHACHMAN, H. K.: Ultracentrifugation and related techniques (diffusion, viscosity) for molecular size and shape. Methods in Enzymology 4: 52~65, 1957.
3) TOMITA, S.: On ultracentrifugation. Kagaku-Kogyo-Siryo 30(2): 68~82, 1961.
4) ISHIDA, N.; K. MIYAZAKI, K. KUMAGAI & M. RIKIMARU: Neocarzinostatin, an antitumor antibiotic of high molecular weight. Isolation, physicochemical properties and biological activities. J. Antibiotics, Ser. A 18(2): 68~76, Mar. 1965.
5) MAEDA, H.; K. KUMAGAI & N. ISHIDA: Characterization of neocarzinostatin. J. Antibiotics, Ser. A 19(6): 253~259, Nov. 1966.
6) SOEDA, M.: Studies on marinamycin, an antitumor antibiotic substance. J. Antibiotics, Ser. B 12: 300~304, Aug. 1959.
7) SOEDA, M. & M. MITOMI: Chemical studies on marinamycin. II. Fundamental chemical studies on marinamycin. J. Antibiotics, Ser. A 15(4): 182~186, July 1962.
8) The Society For Actinomycetes, Japan: Electron-micrograms of Actinomycetes, No. 1, 1965.