STUDIES ON AUROMOMYCIN

TOSHIAKI YAMASHITA, NORIYUKI NAOI, TAKAYOSHI HIDAKA
and KIYOSHI WATANABE

Biochemical Research Laboratory, Kanegafuchi Chemical Industry Co., Ltd.,
Takasago, Hyogo, Japan

YOSHIKI KUMADA, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan

(Received for publication October 5, 1978)

A new antitumor antibiotic, named auromomycin, was isolated from the culture broth of *Streptomyces macromomyceticus*, a macromomycin-producing strain. The antibiotic was recovered from the culture filtrate by salting out with ammonium sulfate and further purified by successive application of ion-exchange chromatography on Amberlite IRA-93 (Cl form) and DEAE-Sephadex (OH form), Gel filtration on Sephadex G-50 and hydrophobic chromatography on Octyl-Sepharose CL-4B. The antibiotic is an acidic polypeptide with a molecular weight of 12,500 and an isoelectric point of pH 5.4 and consists of 16 different amino acids. It has characteristic absorption maxima at 273 nm and 357 nm in the ultraviolet spectrum and two minima at 280 nm and 350 nm in the optical rotatory dispersion spectrum. Auromomycin exhibits antibacterial activity not only against Gram-positive bacteria, but also Gram-negative bacteria. Antitumor activities of auromomycin were revealed against EHRLICH ascites carcinoma, ascites sarcoma 180, L1210 leukemia and LEWIS lung carcinoma. Auromomycin was found to be converted into macromomycin by adsorption chromatography on Amberlite XAD.

Macromomycin, a polypeptide antibiotic produced by *Streptomyces macromomyceticus*[1] has been shown to be active against P388 leukemia, L1210 leukemia, B16 melanoma and LEWIS lung carcinoma[2]. Macromomycin was reported to inhibit DNA synthesis by binding to the cell membrane by KUNIMOTO *et al.*[3,4], LIPPMAN[5] and WINKELHAKE *et al.*[6] Recently, SUZUKI *et al.*[7] demonstrated that macromomycin caused inhibition of DNA synthesis and mitosis, and DNA strand scission.

During the course of our studies for the production and purification of macromomycin, one active fraction purified from the culture broth of *S. macromomyceticus* often had a yellow color, while macromomycin was colorless. Therefore we tried to isolate the yellow substance from the culture broth of *S. macromomyceticus* and obtained a new antibiotic, designated auromomycin, which showed a wider antibacterial spectrum than macromomycin.

In this paper, the production, isolation and physicochemical and biological properties of auromomycin, and the conversion of auromomycin into macromomycin are described.

Fermentation

The procedures used are similar to those employed for the production of macromomycin as described previously[1,8].

Streptomyces macromomyceticus ATCC 29816 was inoculated into 100 ml of a culture medium containing 1% glucose, 1% soluble starch, 1.5% soybean powder, 0.1% dipotassium phosphate, 0.3% sodium chloride, 0.1% magnesium sulfate, 0.0007% copper sulfate, 0.0001% ferrous sulfate,

0.0008% manganese chloride, 0.0002% zinc sulfate and 0.05% antifoamer Disfoam CC-118 (Nippon Oils and Fats Co.) in a 500-ml Sakaguchi flask. The pH was adjusted to 7.2 before sterilization. The seed inoculum was grown at 28°C for 2 days on a reciprocal shaker. The cultured broth (90 ml) was transferred to 18 liters of the same medium in a jar fermentor (30 liters volume). The fermentation was continued under aeration of 18 liters/min. at 28°C for 3 days with stirring at 500 rpm. Maximal production of the antibiotic was attained after 60~80 hours.

The activity of auromomycin was determined by the cylinder agar plate method using *Sarcina lutea* and *Escherichia coli* as the assay organisms. The nutrient agar medium employed for seed and base layers contained 0.6% peptone, 0.3% yeast extract, 0.15% meat extract, 0.1% glucose and 1.5% agar, and the pH was adjusted to 6.7 before sterilization. The diameters of the inhibition zone at the concentration of 0.5 mg/ml aqueous solution of auromomycin were about 20.0 mm and 19.5 mm against *S. lutea* and *E. coli*, respectively. During purification, fractions were monitored by the inhibition zone against *E. coli*, comparing with that of the broth filtrate.

Isolation and Purification

Sixty-four liters of the harvested broth collected from 4 jar fermentors were filtered with filter aid (Dicalite). The filtrate was saturated with ammonium sulfate and allowed to stand at 5°C for 4 hours. Six hundred ml of an aqueous solution containing 265 g of anhydrous sodium carbonate and immediately thereafter, 600 ml of an aqueous solution containing 265 g of ferric chloride hexahydrate as a coagulant were added. The resulting precipitate was collected by continuous centrifugal separation at 5°C and 10,000 rpm. The precipitate (2.2 kg) was dissolved in 13 liters of deionized water. Insoluble material was removed by centrifugal separation and 12.1 liters of the supernatant containing auromomycin were obtained.

The solution was passed through a column (7×78 cm, The size of column is represented by column bed size.) of Amberlite IRA-93 (3 liters, Cl form, Rohm and Haas Co.) to afford 13.2 liters of the effluent. The effluent was ultrafiltered by using Ultra Filter UH-1 membrane (M.W. 1,000, Toyo Filter Paper Co.) and Ultrafilter apparatus (MC-6, Bioengineering Co.) under a pressure of 3.5 kg/cm^2 of N_2 gas to concentrate to 200 ml. The concentrate was placed on a column (8.5×88 cm) of Sephadex G-50 (Pharmacia Fine Chemicals) and chromatographed with deionized water at a flow rate of 750 ml/hours. The eluate was collected in fractions of 15 ml with a fraction collector (7000 Ultrorac Fraction Collector, LKB). Based on the ultraviolet absorptions at 280 nm and 360 nm and antibacterial activities against *S. lutea* and *E. coli* of each fraction, the fractions (tube No. 163~189) containing auromomycin were combined. To 400 ml of the active fraction obtained, 80 g of ammonium sulfate (35% saturation) were added, and the solution was applied to a column (3×42.5 cm) of Octyl-Sepharose CL-4B (Pharmacia Fine Chemicals). The column was subsequently eluted with ammonium sulfate 35% saturation and fractions of tube No. 76~105 (10 ml/tube) were combined. Whereby 300 ml of a yellow fraction containing auromomycin was obtained after a peak of impure proteins. This fraction was fully desalted by Ultra Filter UH-1 and Ultrafilter apparatus (MC-4, Bioengineering Co.) and applied to a column (3×42.5 cm) of DEAE-Sephadex A-25 (OH form, Pharmacia Fine Chemicals). The column was eluted with 0.2 M Tris-HCl buffer (pH 7.0) to afford 180 ml of the active fraction. The fraction was concentrated with Ultra Filter UH-1 to 70 ml and thereafter subjected to column chromatography on Sephadex G-50 (2 liters, 5.6×81 cm) using deionized water as an eluting solvent.

The eluate was collected in fractions of 15 ml and 150 ml of the active fraction (tube No. 63~72) were obtained and concentrated to 20 ml by Ultra Filter UH-1. To concentrate, 2.7 g of ammonium sulfate was gradually added. The solution was allowed to stand in a dark place at 5°C for 4 days, whereupon yellow plate-like crystals of auromomycin precipitated. The mother liquor was almost colorless.

The crystals were collected on a glass filter, washed with 10 ml of a 15% aqueous solution of ammonium sulfate and dissolved in 10 ml of deionized water. The solution was desalted by column chromatography on Sephadex G-25 (200 ml, 2.4×44 cm, Pharmacia Fine Chemicals), and 15 ml of the active fraction containing auromomycin was lyophilized to afford 430 mg of yellow powder.

It was shown to be homogeneous, displaying a single band on polyacrylamide gel electrophoresis according to the method of ORSTEIN-DAVIS[9,10].

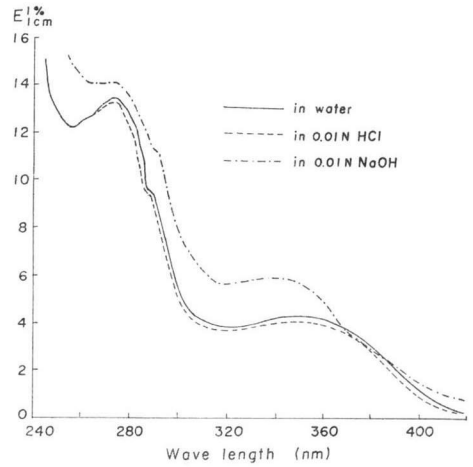
Physico-chemical Properties

Auromomycin formed plate-like yellow crystals and was obtained as a lyophilized yellow powder.

It is a weakly acidic protein, which shows an isoelectric point of pH 5.4 as determined by electrofocusing using LKB Amploline 8101 (pH of the carrier ampholite is in the range of 5~8). It does not show a definite melting or decomposition point and carbonizes completely at 260°C. It is soluble in water but insoluble in organic solvents such as methanol, ethanol, acetone and ethyl acetate.

Auromomycin exhibits an optical rotation of $[\alpha]_D^{20}$ −280 in 1% aqueous solution. The ultraviolet absorption shows $E_{1cm}^{1\%}$ 13.3 at 273 nm and $E_{1cm}^{1\%}$ 4.6 at 357 nm in aqueous solution, $E_{1cm}^{1\%}$ 14.0 at 270~274 nm and $E_{1cm}^{1\%}$ 5.9 at 340 nm in 0.01 N sodium hydroxide solution, and $E_{1cm}^{1\%}$ 13.2 at 272 nm and $E_{1cm}^{1\%}$ 4.4 at 356 nm in 0.01 N hydrochloric acid solution, with a shoulder at 290 nm in all three solvents. The optical rotatory dispersion spectrum measured in an aqueous solution (2 mg/ml) shows two troughs with minima at 280 nm and 350 nm. The ultraviolet absorption, infrared and optical rotatory dispersion spectra are shown in Fig. 1, Fig. 2 and Fig. 3, respectively.

Fig. 1. Ultraviolet absorption spectra of auromomycin.



The antibiotic decolorizes potassium permanganate solution, gives positive FOLIN-LOWRY, xanthoprotein, EHRLICH, biuret and ninhydrin reactions, and negative phenol-sulfuric acid, anthrone, orcinol, ELSON-MORGAN and MOLISCH reactions.

The molecular weight of auromomycin was estimated to be 12,500, since it was eluted in the same

Fig. 2. Infrared absorption spectrum of auromomycin in KBr.

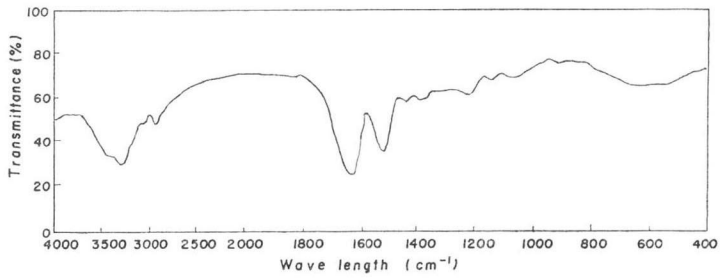


Fig. 3. Optical rotatory dispersion spectra of auromomycin, macromomycin and white powder. Measurement of the spectrum was made on aqueous solution of auromomycin (2 mg/ml), and macromomycin and white powder (3 mg/ml), respectively.

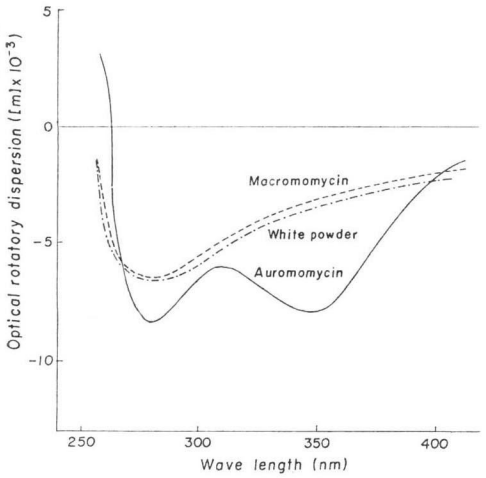


Fig. 4. Ultraviolet absorption spectra of macromomycin and white powder.

The spectra of macromomycin and white powder showed the same pattern.

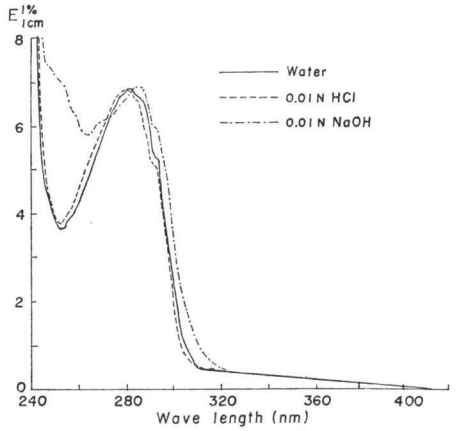


Table 1. Amino acid composition of auromomycin, macromomycin and white powder[a].

Amino acid	Auromomycin % (W/W)	Auromomycin (No. of residues)	Macromomycin % (W/W)	Macromomycin (No. of residues)	White powder % (W/W)	White powder (No. of residues
Aspartic acid	8.04	(8)	7.80	(8)	7.80	(8)
Threonine	15.29	(18)	15.54	(18)	14.86	(18)
Serine	7.44	(10)	7.73	(10)	7.16	(10)
Glutamic acid	8.79	(8)	8.64	(8)	8.28	(8)
Proline	4.26	(5)	4.34	(5)	4.32	(5 or 6)
Glycine	10.76	(20)	10.99	(20)	10.47	(20)
Alanine	12.14	(19)	12.44	(19)	12.04	(19)
Half-cystine	3.52	(4)	3.57	(4)	3.42	(4)
Valine	14.01	(16 or 17)	13.58	(16)	13.50	(16 or 17)
Methionine	0	(0)	0	(0)	0	(0)
Isoleucine	2.99	(3)	2.79	(3)	2.70	(3)
Leucine	4.73	(5)	4.55	(5)	4.51	(5)
Tyrosine	1.51	(1)	1.44	(1)	1.42	(1)
Phenylalanine	2.86	(2)	2.69	(2)	2.63	(2)
Tryptophan	2.03	(1)	1.64	(1)	2.01	(1)
Lysine	3.78	(3 or 4)	3.71	(3 or 4)	3.60	(3 or 4)
Histidine	2.47	(2)	2.36	(2)	2.28	(2)
Arginine	0	(0)	0	(0)	0	(0)

a) White powder is a polypeptide antibiotic obtained from auromomycin by Amberlite XAD-7 chromatography.

b) The proteins were hydrolyzed with 6 N HCl at 110°C for 22 hours.

c) Cystine and tryptophan were measured after performic acid oxidation and barium hydroxide hydrolysis, respectively.

d) Amino acids were determined by using Hitachi Amino Acid Analyzer KLA-5 (Hitachi Ltd.).

fractions of tube No. 29~31 (3 ml/tube) as cytochrome C, when chromatographed with blue dextran 2000 (Seikagaku Kogyo Co.) and human albumin, chymotrypsinogen and cytochrome C (M.W. 45,000 25,000 and 12,500, respectively, Boehringer Mannheim Co.) as standards on a column (1.5×90 cm) of Sephadex G-50 (160 ml) using 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.0) as an eluting buffer at a flow rate of 35 ml/hour. As shown in Table 1, it consists of 16 different amino acids, with methionine and arginine absent. One tryptophan residue was found in the auromomycin molecule as determined by N-bromosuccinimide oxidation[11]. The reduced form of auromomycin was also shown to possess four sulfhydryl groups by *p*-chloromercuribenzoic acid titration[12]. This amino acid composition supports a molecular weight of approximately 12,000. The N-terminal amino acid of the antibiotic was identified as alanine by dinitrophenylation[13].

Auromomycin is relatively stable in the pH range of 5~9, but loses its antibacterial activity rapidly in strong acid or alkaline solution. On standing at room temperature both the solid form and aqueous solution of auromomycin gradually lose activity. Both the solid and aqueous solution of the antibiotic are rapidly inactivated by ultraviolet irradiation.

Biological Properties

The antimicrobial spectrum of auromomycin is shown in Table 2. The antibiotic inhibited both

Table 2. Antimicrobial spectrum of auromomycin (AUR), macromomycin (MCR) and white powder (WP).

Test organisms	Minimum inhibitory concentration (μg/ml)			Media*
	AUR	MCR	WP	
Staphylococcus aureus FDA 209P	0.1	1.56	1.56	A
Staphylococcus aureus Smith	0.1	1.56	1.56	A
Micrococcus flavus FDA16	0.05	1.56	0.78	A
Sarcina lutea PCI 1001	0.1	1.56	0.78	A
Bacillus anthracis	0.2	3.12	3.12	A
Bacillus subtilis NRRL 558	0.2	1.56	1.56	A
Bacillus subtilis PCI 219	0.1	0.39	0.78	A
Bacillus cereus	0.2	6.25	6.25	A
Escherichia coli NIHJ	3.12	>100	>100	A
Escherichia coli K-12	6.25	>100	>100	A
Shigella dysenteriae	1.56	>100	>100	A
Shigella flexneri 46JS 11811	6.25	>100	>100	A
Salmonella enteritidis 1891	25	>100	>100	A
Proteus vulgaris OX-19	3.12	>100	>100	A
Proteus rettgeri GN 311	6.25	>100	>100	A
Proteus rettgeri GN 466	0.78	>100	>100	A
Serratia marcescens	50	>100	>100	A
Pseudomonas aeruginosa A3	>100	>100	>100	A
Klebsiella pneumoniae PCI 602	3.12	>100	>100	A
Mycobacterium 607	3.12	>100	>100	B
Mycobacterium phlei	3.12	>100	>100	B
Candida albicans 3147	>100	>100	>100	C

* A: Nutrient agar medium; B: 1% Glycerol nutrient agar medium; C: 1% Glucose nutrient agar medium.

Table 3. Effect of auromomycin on survival time of ascites sarcoma 180.

Dose (mg/kg/day)	MST (days)	ILS (%)	60-Day survivors
1	18.5	13.5	0/6
0.5	40.7	149.7	1/6
0.25	51.0	212.9	4/6
0.125	60.0	268.1	6/6
0.0625	48.8	199.4	4/6
0.0313	46.0	182.2	3/6
0.0156	49.0	200.6	4/6
0.0078	38.7	137.4	2/6
—	16.3	0	0/6

Ascites sarcoma 180 (2×10^6 cells) were intraperitoneally inoculated into ddY mice. Auromomycin was dissolved in physiological saline and intraperitoneally injected once daily for 5 days, starting 24 hours after tumor inoculation.

MST: Mean survival time. ILS: Increase in life span.

Table 4. Effect of auromomycin on survival time of EHRLICH ascites carcinoma.

Dose (mg/kg/day)	MST (days)	ILS (%)	60-Day survivors
1	25.2	65.8	0/6
0.5	43.7	187.5	3/6
0.25	59.0	288.2	5/6
0.125	55.3	263.8	4/6
0.0625	51.3	237.5	4/6
0.0313	45.0	196.1	3/6
0.0156	39.5	159.9	3/6
0.0078	23.7	55.9	1/6
—	15.2	0	0/6

EHRLICH ascites carcinoma (2×10^6 cells) were intraperitoneally inoculated into ddY mice. Auromomycin was dissolved in physiological saline and intraperitoneally injected once daily for 5 days, starting 24 hours after tumor inoculation.

MST: Mean survival time. ILS: Increase in life span.

Table 5. Effect of auromomycin on survival time of L1210 leukemia.

Dose (mg/kg/day)	MST (days)	ILS (%)	30-Day survivors
1	10.3	21.2	0/6
0.5	12.2	43.5	0/6
0.25	12.7	49.4	0/6
0.125	11.5	35.3	0/6
0.0625	11.5	35.3	0/6
0.0313	11.0	29.4	0/6
0.0156	9.7	14.1	0/6
—	8.5	0	0/6

L1210 leukemia (1×10^5 cells) were intraperitoneally inoculated into BDF_1 mice. Auromomycin was dissolved in physiological saline and intraperitoneally injected once daily for 5 days, starting 24 hours after tumor inoculation.

MST: Mean survival time. ILS: Increase in life span.

Table 6. Comparison of the antitumor activity of auromomycin, macromomycin and neocarzinostatin against LEWIS lung carcinoma subcutaneously implanted.

Agents	Dose (mg/kg/day)	Route	MST (days)	ILS (%)	90-Day survivors
AUR	0.25	ip	20.8	−37.9	0/6
	0.0625	ip	35.7	6.6	0/6
MCR	3.13	ip	26.8	−20.0	0/6
	0.78	ip	33.5	0	0/6
NCS	0.25	ip	29.5	−11.9	0/6
	0.0625	ip	31.2	−6.9	0/6
AUR	0.25	sc	32.0	−4.5	0/6
	0.0625	sc	50.3	50.1	2/6
MCR	3.13	sc	70.5	110.4	3/6
	0.78	sc	63.7	90.1	3/6
NCS	0.25	sc	65.5	95.5	3/6
	0.0625	sc	36.5	9.0	0/6
	—		33.5	0	0/6

BDF_1 mice were subcutaneously inoculated with 2×10^6 cells of LEWIS lung carcinoma. Auromomycin (AUR), macromomycin (MCR), and neocarzinostatin (NCS) were dissolved in physiological saline and were injected intraperitoneally (ip) or subcutaneously (sc) at the site of tumor inoculation once daily for 10 days, starting 24 hours after tumor inoculation.

MST: Mean survival time. ILS: Increase in life span.

Gram-positive and Gram-negative bacteria, although it did not inhibit *Pseudomonas aeruginosa*. Also, it did not effect *Candida albicans*.

Treatment with proteolytic enzymes such as trypsin, chymotrypsin, papain, thermolysin and pronase (pH 7.5, 30°C for 1 hour) failed to reduce antibacterial activity.

As shown in Tables 3 and 4, auromomycin was markedly active against ascites sarcoma 180 and

EHRLICH ascites carcinoma in a dose range of 0.0078~0.5 mg/kg/day when administered intraperitoneally once daily for 5 days, starting one day after tumor inoculation. Most of the mice treated with 0.0156~0.25 mg/kg/day survived for more than 60 days. As shown in Table 5, it was also active against L1210 leukemia in a dose range of 0.03~0.5 mg/kg/day when administered intraperitoneally once daily for 5 days, starting 24 hours after tumor inoculation. The maximal increase of life span was about 49%.

The antitumor activity of auromomycin was compared with macromomycin and neocarzinostatin (Yamanouchi Pharmaceutical Co.) against LEWIS lung carcinoma as shown in Table 6 and Table 7. All of the drugs were inactive when administered intraperitoneally against LEWIS lung carcinoma implanted subcutaneously, but were active when administered subcutaneously at the site of tumor inoculation (Table 6). In the latter case macromomycin was more effective than auromomycin and neocarzinostatin. As shown in Table 7, a single intravenous injection of auromomycin possessed marked activity against LEWIS lung carcinoma implanted intravenously. Treatment with 1.5 mg/kg of auromomycin resulted in survival of all the mice for over 60 days after tumor inoculation. Macromomycin and neocarzinostatin were also effective but their activity was less than that of auromomycin.

Table 7. Comparison of the antitumor activity of auromomycin, macromomycin and neocarzinostatin against LEWIS lung carcinoma intravenously implanted.

Agents	Dose (mg/kg)	MST (days)	ILS (%)	60-Day survivors
AUR	1.5	60.0	105.5	6/6
MCR	20.0	40.3	38.0	3/6
NCS	1.0	33.7	15.4	1/6
	—	29.2	0	0/6

BDF_1 mice were intravenously inoculated with 1×10^5 cells of LEWIS lung carcinoma. Auromomycin (AUR), macromomycin (MCR), and neocarzinostatin (NCS) were dissolved in physiological saline and were intravenously injected once 24 hours after tumor inoculation. Each dose used was 1/2 of the LD_{50} (AUR: 3 mg/kg, MCR: 40 mg/kg, NCS: 2 mg/kg) by intravenous injection.

MST: Mean survival time. ILS: Increase in life span.

The LD_{50} by a single intravenous injection of auromomycin to ddY mice was 3 mg/kg.

Conversion of Auromomycin into Macromomycin

A variety of methods such as adsorption chromatography using silica gel, alumina and Amberlite XAD (Rohm and Haas Co.), ion-exchange chromatography, treatment with acid, alkaline or heat, and irradiation with ultraviolet light were studied for the conversion of auromomycin into macromomycin. Consequently, adsorption chromatography on Amberlite XAD-7 was found to be most suitable. The procedures used are as follows:

Auromomycin (500 mg) was dissolved in 200 ml of an 8% aqueous solution of ammonium sulfate. The solution was applied to a column (3×21 cm) of Amberlite XAD-7 (150 ml). The column was washed with 200 ml of the 8% aqueous solution of ammonium sulfate at a flow rate of 450 ml/hour and followed immediately by elution with 30% ethanol collecting fractions of 5 ml. The fractions of tube No. 13~32 which showed antibacterial activity against *S. lutea* and strong ultraviolet absorption at 280 nm were combined to afford 100 ml. The fraction obtained was concentrated to 60 ml using a rotary evaporator at 40°C under reduced pressure to remove ethanol. The concentrate was placed on a column (5.6×81 cm) of Sephadex G-50 (2 liters), and developed with deionized water to obtain 150 ml of an active fraction. This fraction was lyophilized to yield 140 mg of a white powder.

The physicochemical and biological properties of the white powder obtained were compared

with those of auromomycin and macromomycin (authentic sample produced by Kanegafuchi Chemical Ind., Co.). The same 16 amino acids were detected in the hydrolysate of the powder as in hydrolysates of auromomycin and macromomycin, as shown in Table 1. Similarly, the mobility of the protein on a column of Sephadex G-50 was similar to that of cytochrome C (M.W. 12,500) under the same condition as the molecular weight of auromomycin was determined, suggesting that its molecular weight was approximately 12,500. Its isoelectric point as measured with LKB Ampholine 8101 was also found to be pH 5.4. By contrast, as shown in Fig. 4, the ultraviolet absorption showed $E^{1\%}_{1cm}$ 6.7 at 280 nm in aqueous solution, $E^{1\%}_{1cm}$ 6.7 at 280~284 nm in 0.01 N sodium hydroxide solution, and $E^{1\%}_{1cm}$ 6.7 at 280 nm in 0.01 N hydrochloric acid solution, with a shoulder at 290 nm in all three solvents. It is noted that the characteristic maximum at 340~360 nm in the ultraviolet absorption spectrum of auromomycin disappears in that of the powder and the maximum shifts from 273 nm to 280 nm with the

Fig. 5. Elution patterns from chromatography of the mixture of auromomycin and macromomycin, and white powder alone on Octyl-Sepharose CL-4B.

One hundred mg of auromomycin and 100 mg of macromomycin were dissolved in 30 ml of first eluting buffer (0.1 mM phosphate buffer, pH 7.0, of 40% saturation with ammonium sulfate) and mixed. The solution was added to a column (2×38 cm) of Octyl-Sepharose CL-4B (120 ml).

The column was eluted with the same buffer until peak A was eluted, which required approximately 600 ml of the first eluting buffer, and thereafter, with second eluting buffer (0.1 mM phosphate buffer, pH 7.0, of 20% saturation with ammonium sulfate) until peak B was eluted. The volume of a fraction collected by a fraction collector was 10 ml.

The absorbance at 280 nm (○) and the antibacterial activities against *S. lutea* (△) and *E. coli* (◇) of the fractions in the eluate were measured. Independently, one hundred and fifty mg of white powder obtained in the present investigation was dissolved in 30 ml of the first eluting buffer, and chromatographed with the same procedures as described above. The absorbance at 280 nm (●) and the antibacterial activities against *S. lutea* (▲) and *E. coli* (no activity showed) were similarly measured.

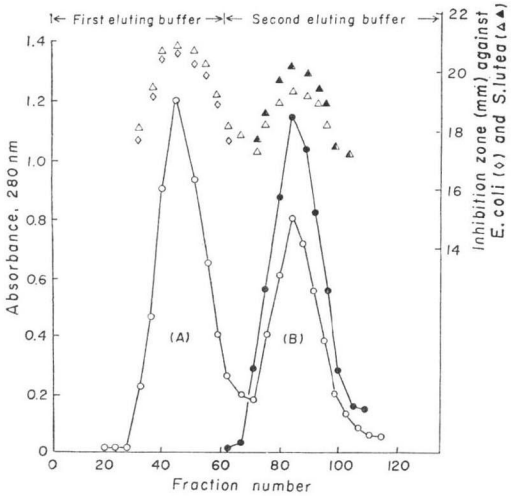


Table 8. Comparison of the characteristic properties between auromomycin, macromomycin and white powder.

	Auromomycin	Macromomycin and white powder
Property of purified product	yellow plate-like crystals	white powder
Molecular weight (the method of gel filtration)	12,500	12,500
Isoelectric point	pH 5.4	pH 5.4
No. of constitutive amino acids	16 (Met and Arg are absent)	16 (Met and Arg are absent)
λ_{max} ($E^{1\%}_{1cm}$) in the UV absorption spectrum (in water)	273 nm (13.3) and 357 nm (4.6)	280 nm (6.7)
Minimum in ORD[a] spectrum	280 nm and 350 nm	280 nm
Antibacterial activity	Inhibition of Gram-positive and Gram-negative bacteria	Inhibition of Gram-positive bacteria
Antitumor activity: effective dose (mg/kg/day) against L1210	0.03~0.5 mg/kg/day	0.2~6 mg/kg/day
and max. ILS[b] (%)	49%	>70%
LD_{50} (iv injection to mice)	3 mg/kg	35 mg/kg
Affinity on Octyl-Sepharose CL-4B	weaker	stronger

a) ORD: Optical rotatory dispersion.
b) ILS: Increase in life span.

conversion of auromomycin into the white powder *i.e.*, macromomycin (Fig. 1,4). As shown in Fig. 3, the optical rotatory dispersion spectrum of the powder showed only a single trough with a minimum at 280 nm whereas the minimum at 350 nm in auromomycin disappeared. The ultraviolet absorption and the optical rotatory dispersion spectra of the powder agreed completely with those of macromomycin. There was no difference in the infrared spectra between authentic auromomycin, macromomycin and the powder. As observed with macromomycin, the powder inhibited Gram-positive bacteria but exhibited no activity against Gram-negative bacteria, as shown in Table 2. It was active against L1210 leukemia when 0.2~6 mg/kg/day was administered intraperitoneally once daily for 5 consecutive days starting 24 hours after the tumor inoculation. The maximal increase of life span was over 70%. The LD_{50} after a single intravenous injection administered to mice was 35 mg/kg. Fig. 5 shows the elution pattern from chromatography of the mixture of auromomycin and macromomycin and the white powder alone on a column (2×38 cm) of Octyl-Sepharose CL-4B (120 ml). Fractions (10 ml) were collected with the elution of two peaks (A and B). The first peak (A) and the second peak (B) contained auromomycin and macromomycin, respectively, which were confirmed by examining the antibacterial activities of the fractions against *S. lutea* and *E. coli*. These auromomycin and macromomycin can be separated by Octyl-Sepharose CL-4B chromatography and the powder exhibits the same behavior as macromomycin.

The characteristic differences in physicochemical and biological properties between auromomycin, macromomycin and white powder obtained in the present investigation are summarized in Table 8. These properties of the white powder differed from those of auromomycin and were the same as those of macromomycin which were determined at the same time and reported previously[1,8,14]. Therefore, it was identified as macromomycin.

Discussion

While carrying out studies for the purification of macromomycin from culture filtrates of *Streptomyces macromomyceticus* ATCC 29816, we often experienced that lyophilized macromomycin powders purified by conventional procedures[1,8] such as salting out with ammonium sulfate, gel filtration and ion exchange, had a yellow color. The fact suggested that the strain could produce macromomycin and at the same time another antibiotic. It had been difficult to separate macromomycin and auromomycin, but we succeeded in their separation using Octyl-Sepharose CL-4B (Fig. 5).

Auromomycin forms plate-like yellow crystals, but macromomycin is not crystallized. Auromomycin is very similar to macromomycin in the following properties: molecular weight, isoelectric point and constitutive amino acids. But as summarized in Table 8, auromomycin is clearly different from macromomycin in the following properties: ultraviolet absorption spectrum, optical rotatory dispersion spectrum, antibacterial activity, effective dose range against L1210 leukemia and the maximal increase in life span, and acute toxicity to mice. Auromomycin is also observed to be different from neocarzinostatin[15]. For example, neocarzinostatin does not contain histidine which is present in auromomycin, but contains arginine which is absent in auromomycin. The isoelectric point of neocarzinostatin is pH 3.5, whereas that of auromomycin is pH 5.4. Neocarzinostatin does not inhibit Gram-negative bacteria but auromomycin inhibits these bacteria.

Auromomycin was confirmed to be converted into macromomycin by adsorption chromatography on Amberlite XAD-7. This method is useful for preparation of macromomycin. The application of this chromatography to culture filtrates of *Streptomyces macromomyceticus* which contains both auromomycin and macromomycin can also give homogeneous macromomycin. The mechanism of the conversion is not well defined, except the chromophore present in the auromomycin molecule having a maximum ultraviolet absorption at 350~360 nm is removed by Amberlite XAD-7 chromatography.

References

1) CHIMURA, H.; M. ISHIZUKA, M. HAMADA, S. HORI, K. KIMURA, J. IWANAGA, T. TAKEUCHI & H. UMEZAWA: A new antibiotic, macromomycin, exhibiting antitumor activity and antimicrobial activity. J. Antibiotics 21: 44~49, 1968
2) LIPPMAN, M. M.; W. R. LASTER, B. J. ABBOTT, J. VENDITTI & M. BARATTA: Antitumor activity of macromomycin B (NSC-170105) against murine leukemia, melanoma and lung carcinoma. Cancer Res. 35: 939~945, 1975
3) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Reversible binding of macromomycin, a macromolecular peptide antibiotic, to cell membranes. J. Antibiotics 24: 203~205, 1971
4) KUNIMOTO, T.; M. HORI & H. UMEZAWA: Macromomycin, an inhibitor of the membrane function of tumor cells. Cancer Res. 32: 1251~1256, 1972
5) LIPPMAN, M. M.: *In vitro* studies on macromomycin (NSC-170105). Effects on cultured TA3Ha and L1210 cells. Cancer Chemoth. Rept. 58: 181~187, 1974
6) WINKELHAKE, J. L. & F. L. A. BUCKMIRE: Radioimmune assay and characteristics of antibodies to macromomycin (NSC-170105). Cancer Res. 37: 1197~1204, 1977
7) SUZUKI, H.; T. NISHIMURA, K. MUTO & N. TANAKA: Mechanism of action of macromomycin: DNA strand scission, inhibition of DNA synthesis and mitosis. J. Antibiotics 31: 875~883, 1978
8) YAMASHITA, T.; N. NAOI, K. WATANABE, T. TAKEUCHI & H. UMEZAWA: Further purification and characterization of macromomycin. J. Antibiotics 29: 415~423, 1976
9) ORSTEIN, L.: Disc electrophoresis. I. Background and theory. Ann. New York Acad. Sci. 121: 321~349, 1964
10) DAVIS, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. New York Acad. Sci. 121: 404~427, 1964
11) SPANDE, T. F. & B. WITKOP: Determination of the tryptophan content of protein with N-bromosuccinimide. Methods Enzymol. 11: 498~506, 1967
12) BOYER, P. D.: Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. J. Am. Chem. Soc. 76: 4331~4337, 1954
13) SANGER, F.: Free amino groups of insulin. Biochem. J. 39: 507~515, 1945
14) IM, W. B.; C. CHIANG & R. MONTGOMERY: Studies on macromomycin, an antitumor protein. J. Biol. Chem. 253: 3259~3264, 1978
15) MAEDA, H.; K. KUMAGAI & N. ISHIDA: Characterization of neocarzinostatin. J. Antibiotics, Ser. A 19: 253~259, 1966