A NEW ANTIBIOTIC, MULTHIOMYCIN

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Multhiomycin is a new antibiotic obtained from the mycelium of *Streptomyces antibioticus* 8446-CC_1. It is extracted with methanol and purified by silica gel chromatography. It forms yellow needle-shaped crystals, melts at above 300°C and has no or negligible optical activity. $C_{44}H_{45}O_{11}N_{11}S_5$ was suggested for its molecular formula by elemental analysis and molecular weight determination. It exhibits inhibitory activity against gram-positive bacteria but no activity against gram-negative bacteria, mycobacteria and fungi.

In recent years, a new screening method for inhibitors of nucleic acid syntheses using *Bacillus subtilis* 168 had been developed by T. TANAKA *et al.*[1] and succesively denamycin[2] and pentalenolactone[3] were isolated in our laboratory. In this screening program, a fermentation broth of *Streptomyces antibioticus* 8446-CC_1 was found to inhibit the growth of *B. subtilis* and ^{14}C-amino acids incorporations rather than ^{3}H-thymine and ^{14}C-uracil incorporations into the bacterial cells. The active substance was isolated in a pure form and was named as multhiomycin in relation to its high content of sulfur. In this report, we describe the production, isolation procedure, physicochemical and biological properties of multhiomycin.

Producing Organism

Streptomyces sp. 8446-CC_1 was isolated from a soil sample collected at Kagamihara, Gifu Prefecture, Japan.

Morphological characteristics of the strain were summarized as follows: The aerial mycelium branched monopodially as cluster and formed straight spore-chains which belong to "Rectus-flexibilis (RF)" according to PRIDHAM *et al.*[4] Spores were cylindrical to oval ($0.5 \sim 0.8 \times 1.0 \sim 1.8\ \mu$) with smooth surface.

Cultural and physiological characteristics of the strain 8446-CC_1 are shown in Tables 1 and 2. The strain was determined to belong to "Gray series" of TRESNER and BACKUS[5] by the fact that the color of aerial mycelium was light gray to light brownish gray. The substrate mycelium or reverse side of colony showed no distinctive colors (yellowish gray to yellowish brown) on all media. Soluble pigments were produced slightly and not distinctively (yellowish gray to pale yellowish brown) on synthetic or some organic media. Chromogenic pigments (yellowish brown to dark brown) were produced on most organic media and melanoid pigment on tyrosine agar.

Glucose, rhamnose, mannose, lactose, raffinose, mannitol, sucrose, glycerol and

Table 1. Cultural characteristics of *Streptomyces* sp. 8446-CC_1

Medium	Cultural characteristics
Sucrose-nitrate agar	G: thin, colorless to yellowish gray AM: abundant, powdery, white SP: very slight, yellowish gray
Glycerol-nitrate agar	G: thin, colorless to yellowish gray AM: poor, powdery, white SP: very slight, yellowish gray
Glucose-asparagine agar	G: moderate, yellowish gray with pale yellowish brown reverse AM: abundant, velvety, light gray to light brownish gray SP: slight, pale yellowish brown
Glycerol-calcium malate agar	G: moderate, spreading and penetrating into agar, yellowish gray AM: abundant, velvety, light brownish gray with whitish patch SP: very slight, yellowish gray
Starch agar	G: moderate, spreading and penetrating into agar, yellowish gray with light brownish gray reverse AM: abundant, velvety, light gray with brownish tinge SP: very slight, yellowish gray
Peptone-beef extract agar	G: moderate, pale yellowish brown with yellowish brown reverse AM: poor, powdery, white at margin of colony SP: yellowish brown
Glucose-peptone-beef extract agar	G: thick and wrinkled, yellowish gray AM: poor, powdery, white at margin of colony SP: yellowish brown
Glucose-peptone-agar	G: thin, pale yellow AM: none SP: slight, pale yellow
Glucose-casein digest-yeast-beef agar	G: moderate and wrinkled, pale yellowish brown reverse AM: moderate, velvety, light gray with brownish tinge SP: slight, pale yellowish brown
Oatmeal-yeast extract agar	G: moderate, spreading and penetrating into agar, yellowish gray AM: abundant, velvety, light gray with brownish tinge SP: very slight, yellowish
Potato plag	G: thick and wrinkled AM: abundant, light gray with brownish tinge SP: dark brown
LOEFFLER'S blood serum	G: moderate, dark brown AM: poor, powdery, white SP: dark brown
Gelatin	G: surface ring, pale yellowish brown AM: none SP: brown
Milk	G: surface ring, pale yellowish brown AM: abundant, powdery, white SP: brown to dark brown
Cellulose medium	G: thin, colorless AM: abundant, powdery, light gray SP: none

G: Growth, AM: Aerial mycelium, SP: Soluble pigments

salicin were utilized as carbon source for the growth, but arabinose, fructose and cellulose were not.

When the characteristics of the strain 8446-CC_1 were compared with those of *Streptomyces* species described by WAKSMAN[6], SHIRLING and GOTTLIEB[7,8,9] and others, there existed some similarities between 8446-CC_1 and *Streptomyces antibioticus*, *S. caiusiae*, *S. eurythermus*, *S. showdoensis* and *S. tanashiensis* as shown in Table 3. However, the latter four species related to *S. antibioticus*[10] were differentiated from the strain 8446-CC_1 in the following points. The substrate mycelium of *S. caiusiae*[6] showed distinctive citron-yellow color on most media. *S. eurythermus*[6,8] occasionally formed spiral spore-chains. *S. showdoensis*[10] developed white to yellowish gray aerial mycelium on synthetic media. *S. tanashiensis*[6,9] did not produce melanoid pigment on tyrosine agar. *S. antibioticus*[6,9] was closely related to the strain 8446-CC_1 in morphological and cultural characteristics except for carbon utilization (arabinose +, sucrose −, fructose + and raffinose −). The considerations outlined above lead to the conclusion that the strain 8446-CC_1 belongs to *Streptomyces antibioticus*.

Table 2. Physiological characteristics of *Streptomyces* sp. 8446-CC_1

Optimum growth temperature	25~30°C
Chromogenicity: deep brown pigment on organic media	+
melanine formation from tyrosine	+
Starch hydrolysis	+
Cellulose decomposition	−
Nitrate reduction	+
Proteolytic activities: gelatin liquefaction	+
milk peptonization	+
blood serum liquefaction	+
Carbon utilization: Glucose, Rhamnose, Mannose, Lactose, Raffinose, Mannitol	++
Sucrose, Glycerol, Salicin	+
Arabinose, Fructose, Cellulose	−

Table 3. Comparison of characteristics of *Streptomyces* sp. 8446-CC_1 and related species

	Streptomyces sp. 8446-CC_1	*S. antibioticus*	*S. caiusiae*	*S. eurythermus*	*S. showdoensis*	*S. tanashiensis*
Morphological section	RF	RF	RF	RF occasion S	RF	RF
Spore surface	Smooth	Smooth	?	Smooth	Smooth	Smooth
Color of aerial mycelium	Gray	Gray	Gray	Gray	Gray	Gray
Color of substrate mycelium	NDP	NDP	Yellow	NDP	NDP	NDP
Melanoid pigments on organic media	+	+	+	+	+	+
Melanoid pigments on tyrosine agar	+	+	+	+	+	−
Other soluble pigments	NDP	NDP	NDP	NDP	NDP	NDP
Resemble to			*S. antibioticus*	*S. antibioticus*	*S. antibioticus*	*S. antibioticus* & *S. aureus*

RF: Rectus-flexibilis S: Spira NDP: no distinctive pigments

Fermentation of Multhiomycin

The production of multhiomycin in jar fermentors was carried out as follows: Fifteen liters of a medium consisting of 2.5% dextrin, 2% dry yeast, 0.5% NaCl

and 0.4 % $CaCO_3$ were sterilized at 120°C for 20 minutes in a stainless steel fermentor of 30-liter volume.

Each jar was inoculated with 300 ml of a seed broth prepared in shaking flasks from a culture of *Streptomyces antibioticus* sp. 8446-CC_1 in the same medium for 48 hours at 27°C. Air was supplied at a rate of 1 volume per volume of broth per minute with agitation at 300 r.p.m. at 27°C. The fermentation was terminated at 96 hours after inoculation.

The same assay method described previously[1] was used except for ^{3}H-thymine and ^{14}C-uracil incorporations into *B. subtilis* 168 cells. A paper disc method was inadequate for this antibiotic assay, because sufficient diffusion was not observed even at a high concentration.

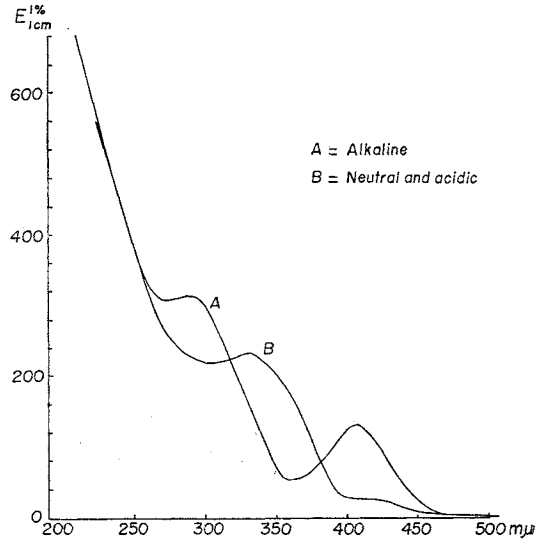
Isolation of Multhiomycin

Multhiomycin was extracted with 16 liters of methanol from the mycelial cake after filtration of the fermentation broth (30 liters). The methanolic solution was concentrated *in vacuo* to remove methanol and the residual aqueous solution was extracted with 1,000 ml of ethyl acetate. The ethyl acetate extract was evaporated *in vacuo* to 100 ml. *n*-Hexane was added to give crude precipitates of multhiomycin. The precipitates were collected on a glass filter and washed with 10 ml of methanol. Six grams of crude multhiomycin were obtained.

Further purification of multhiomycin was carried out by silica gel chromatography. One gram of crude multhiomycin suspended in 5 ml of ethyl acetate containing 10 % dimethylformamide was charged on a silica gel (Mallinckrodt Chemical Works) column (2.2×15 cm). Pure multhiomycin was eluted with the same solvent. The active fractions eluted were collected and left at room temperature for 2 or 3 days in test tubes to give yellow crystals of multhiomycin.

The crystals (60 mg) were pure enough for the physical and chemical characterization. The low yield by this procedure is due to the low solubility of multhiomycin in the solvent system used and 60 % of the activity was not eluted. This part of activity was eluted by dimethylformamide and added to the next lot.

Fig. 1. Ultraviolet spectrum of multhiomycin in methanol.

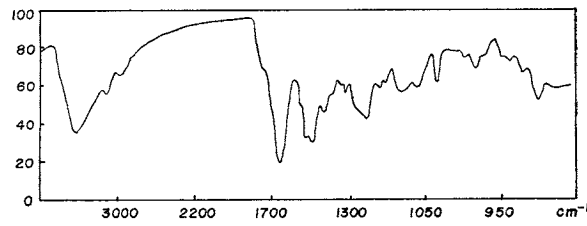


Properties of Multhiomycin

Multhiomycin forms yellow needle shaped crystals and melts at above 300°C (dec.). One percent solution of multhiomycin in dimethylformamide shows no or negligible optical activity. It is soluble in dimethylformamide,

pyridine, dimethylsulfoxide and slightly soluble in ethyl acetate, methanol, ethanol, dioxane and insoluble in water, glacial acetic acid and other organic solvents.

Fig. 2. Infrared spectrum of multhiomycin (KBr tablet)



Ultraviolet and visible spectra show maxima at 328 mμ ($E^{1\%}_{1cm}$ 220) and a shoulder at 420 mμ ($E^{1\%}_{1cm}$ 20) in neutral and acidic methanol, and at 292 mμ ($E^{1\%}_{1cm}$ 255) and at 406 mμ ($E^{1\%}_{1cm}$ 132) in alkaline methanol as presented in Fig. 1.

Its infrared absorption spectrum measured in KBr disc is shown in Fig. 2.

The molecular weight was shown to be 1043 by isothermal distillation and the elemental analysis suggests $C_{44}H_{45}O_{11}N_{11}S_5$ for its molecular formula.

Analysis: Calcd for $C_{44}H_{45}O_{11}N_{11}S_5$, molecular weight 1064
C 49.66, H 4.26, O 16.54, N 14.48, S 15.06
Found: C 49.74, H 4.17, O 16.74, N 15.13, S 15.03

Color reactions: positive — ferric chloride, FOLLIN, LEMIEUX.
negative — ninhydrin, FEHLING, PONCEAU-3R, biuret.

Table 4. Antimicrobial spectrum of multhiomycin

Organism	Minimal inhibitory concentration (mcg/ml)	Organism	Minimal inhibitory concentration (mcg/ml)
Agrobacterium tumefaciens B_6	>100	*Botrytis cinerea*	>100
Bacillus cereus IAM 1729	25	*Botrytis fabae* IAM-5125	>100
Bacillus circulans MSK	0.006	*Candida utitis* IAM-4215	>100
Bacillus subtilis PCI-219	0.2	*Cladosporium fulvum* NIAS	>100
Bacillus subtilis 168 (Marburg strain)	0.05	*Corticium sasakii* NIAS	>100
		Cryptococcus neoformans IAM-4514	>100
Sarcina lutea NIHJ	0.006	*Fusarium lini* NIAS	>100
Serratia marcescens IAM-1021	>100	*Gibberella fujikuroi* NIAS	>100
Serratia marcescens var. *kilensis* ATCC-9986	>100	*Gibberella saubinetii* NIAS	>100
		Gloeosporium kaki NIAS	>100
Staphylococcus aureus FDA-209P	0.013	*Glomerella cingulata* IAM-8050	>100
Corynebacterium xerosis IID	0.1	*Glomerella lagenarium*	>100
Aerobacter aerogenes IAM-1063	>100	*Helminthosporium sesamum*	>100
Escherichia coli NIHJ	>100	*Helminthosporium sigmoideum*	>100
Klebsiella pneumoniae	>100	*Macrosporium bataticola*	>100
Pseudomonas fluorescens IAM-1201	>100	*Mucor ramannianus* IAM-6128	>100
Pseudomonas solanacearum NIAS	0.006	*Ophiobolus miyabeanus* NIAS	>100
Pseudomonas tabaci NIAS	>100	*Penicillium chrysogenum* Q-176	>100
Shigella sonnei IID	0.025	*Piricularia oryzae* NIAS	>100
Xanthomonas oryzae NIAS	>100	*Saccharomyces cerevisiae* NIHJ-F-130	>100
Mycobacterium phlei IID Tim	>100		
Mycobacterium smegmatis 607	>100	*Trichophyton mentagrophytes* NIHJ-640	>100
Alternaria kikuchiana IAM-5005	>100		
Aspergillus oryzae	>100		

No reduction of activity was observed after heating for 5 minutes, 100°C at pH 2.0~5.0.

The minimal inhibitory concentration of multhiomycin is shown in Table 4.

The intravenous injection of 10 mg/kg to mice did not exhibit any toxicity and administration at higher concentration was difficult because of its low solubility. Studies on the mode of action of the antibiotic is now in progress. Details of the investigation will be published in the following papers.

Discussion

Multhiomycin, a sulphur-containing antibiotic, is related to known antibiotics such as siomycin[11], thiopeptin[12], thiostrepton[13], althiomycin[14] and taitomycin[15]. But from the view point of elemental analysis and UV spectrum, these antibiotics with the exception of taitomycin are differentiated from multhiomycin.

The difference between multhiomycin and taitomycin exists in their solubility in organic solvents. The former is only slightly soluble in methanol, ethanol, ethyl acetate and glacial acetic acid, but the latter is soluble in these solvents. Multhiomycin contains more sulphur than taitomycin by their microanalysis. Thus, taking account of the above data, it is concluded that multhiomycin is a new antibiontic.

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