STUDIES ON A NEW PIGMENT ANTIBIOTIC, CHROTHIOMYCIN

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A new pigment antibiotic named chrothiomycin, which inhibits the growth of some Gram-positive bacteria and the enzymic activities of oxygenases involved in catecholamine biosynthesis *in vitro*, was isolated from fermentation broths of *Streptomyces pluricolorescens*. It is obtained as blackish purple crystals, $C_{27}H_{31\sim33}O_{13}NS$.

Chrothiomycin inhibits reactions of tyrosine hydroxylase[1] and dopamine β-hydroxylase[2,3]. It also exhibits weak antibacterial activities. It is obtained as blackish purple crystals containing sulfur and named chrothiomycin. In this paper, characters of chrothiomycin-producing *Streptomyces* are described together with the chemical and physical properties of the antibiotic and some of the biological properties.

Table 1. Comparative studies on the strain MB445-A1 and *Streptomyces pluricolorescens*

	Streptomyces pluricolorescens	MB445-A1
Surface of spore	smooth	smooth
Whorl and spiral formation	—	—
Color of aerial mycelium	white to olive or pinkish	white to pale pink
Soluble pigment	slightly yellowish brown or light wine color	purple to brownish purple or yellowish brown
Color of growth	colorless to yellowish brown with reddish tinge	colorless to pale yellowish brown
Melanin formation	—	—
Liquefaction of gelatin	+	+
Coagulation of milk and peptonization	+	+
Hydrolysis of starch	weak to medium	weak to medium
Carbohydrate utilization		
Arabinose	+	—
Maltose	+	—
Inositol	— (doubtful)	+
Lactose	— (doubtful)	+
Production of antibiotics	pluramycin[4] A and B	chrothiomycin

Using an arginine-glycerol-salt medium an actinomyces was isolated from a soil sample collected at Kumamoto City in Japan in 1967 and designated MB445-A1 in the authors' laboratory. This strain which belongs to the genus *Streptomyces* has the following characteristics: no whorl nor spiral formation; smooth spore surface: colorless to pale yellowish brown growth on various media; no aerial mycelium on natural organic media except potato; white or brownish white to pale pink or pale orange aerial mycelium on synthetic media; purple to brownish purple, pale yellowish brown

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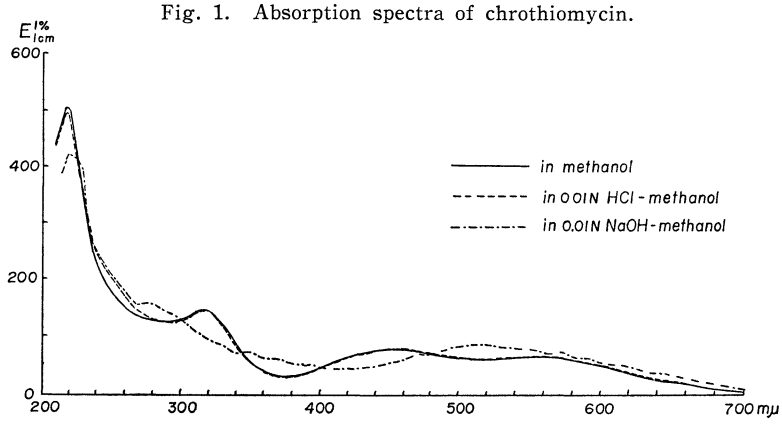
to yellowish brown or brown soluble pigment; dark yellow to yellowish brown around the growth on KRAINSKY glucose asparagine agar; purple to brownish purple around the growth on glycerol CZAPEK agar; nonchromogenic type; proteolytic activity in medium strength; positive reduction of nitrate; weak to medium hydrolysis of starch. These properties are common with *Streptomyces pluricolorescens*[5,6] and therefore, the strain MB445-A1 was compared with a type culture of *Streptomyces pluricolorescens*. As shown in Table 1, color of aerial mycelium of *Streptomyces pluricolorescens* is white to pink or olive, while that of the strain MB445-A1 is white to pale pink. *Streptomyces pluricolorescens* utilizes arabinose and maltose, but the result of the test on inositol and lactose is not clear, while the strain MB445-A1 does not utilize arabinose and maltose, but does inositol and lactose. However, with this latter exceptions, there are no marked differences between the two cultures and therefore the strain MB445-A1 can be assigned to *Streptomyces pluricolorescens*.

The strain MB445-A1 was grown in shake-flask culture in a medium containing 1 % starch, 1 % glucose, 0.75 % meat extract, 0.75 % peptone, 0.3 % NaCl, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$ and 0.0002 % $ZnSO_4 \cdot 7H_2O$ (pH 7.0~7.2). The antibiotic in the filtrate of the cultured broth was extracted with *n*-butanol under acid condition. The solvent extract was concentrated and the antibiotic then precipitated by addition of *n*-hexane. It was purified by repetition of a silicic acid column chromatography using a solvent system composed of methanol and benzene (1 : 20), and crystallized from a mixed solvent containing methanol and benzene (1 : 20). In all experiments, the concentration of the antibiotic was determined by a disc plate method using *Sarcina lutea* as a test organism and by a test of inhibition of enzymic activity of tyrosine hydroxylase.

Chrothiomycin crystals thus obtained were shown by n.m.r. to contain benzene as the crystal solvent. To remove benzene from the crystals, the crystals were dissolved in methanol and concentrated *in vacuo* to dryness. By repetition of the same procedure, chrothiomycin was obtained as an amorphous powder. Crystalline chrothiomycin melts at 193~195°C and chrothiomycin in the amorphous powder melts at 218~222°C under decomposition. A tentative molecular formula $C_{27}H_{31\sim33}O_{13}NS$ was calculated from the elemental analysis of chrothiomycin and the molecular weight (615) determined

Fig. 1. Absorption spectra of chrothiomycin.



by vapor pressure osmometer in 95% ethanol. Electrometric titration showed two ionized groups with pKa' 9.20 and 11.6 in 66% N,N-dimethylformamide, and the equivalent was 632. Chrothiomycin is soluble in methanol, ethanol, butanol, acetone, dimethylformamide, dimethylsulfoxide, dioxane, pyridine, glacial acetic acid and alkaline water, sparingly soluble in water, acid water, ethyl ether, ethyl acetate, butyl acetate and chloroform, and insoluble in benzene, bromobenzene, *n*-hexane and carbon tetrachloride. As shown in Fig. 1, chrothiomycin shows ultraviolet absorption maxima at the following wave lengths : 220 mμ ($E^{1\%}_{1cm}$ 505), 320 mμ ($E^{1\%}_{1cm}$ 145), 445 mμ ($E^{1\%}_{1cm}$ 80) and 560 mμ ($E^{1\%}_{1cm}$ 65) in methanol solution ; 220 mμ ($E^{1\%}_{1cm}$ 495), 320 mμ ($E^{1\%}_{1cm}$ 195), 445 mμ ($E^{1\%}_{1cm}$ 80) and 560 mμ ($E^{1\%}_{1cm}$ 65) in 0.01 N HCl-methanol ; 225 mμ ($E^{1\%}_{1cm}$ 420), 280 mμ ($E^{1\%}_{1cm}$ 155) and 510 mμ ($E^{1\%}_{1cm}$ 90) in 0.01 N NaOH-methanol. The infrared absorption spectrum of crystalline chrothiomycin in KBr tablet is shown in Fig. 2. The n.m.r. spectrum of crystalline chrothiomycin in tetradeuteromethanol using tetramethylsilane as internal reference at 100 MHz is shown in Fig. 3. Chrothiomycin shows reddish purple color

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

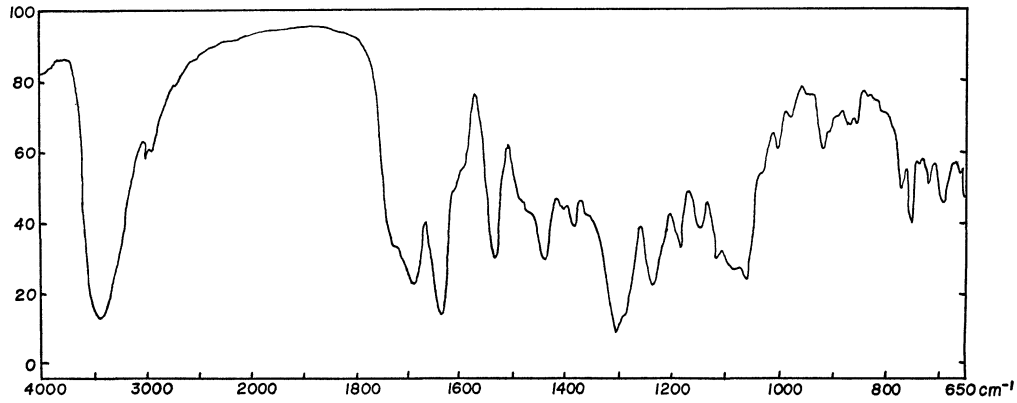
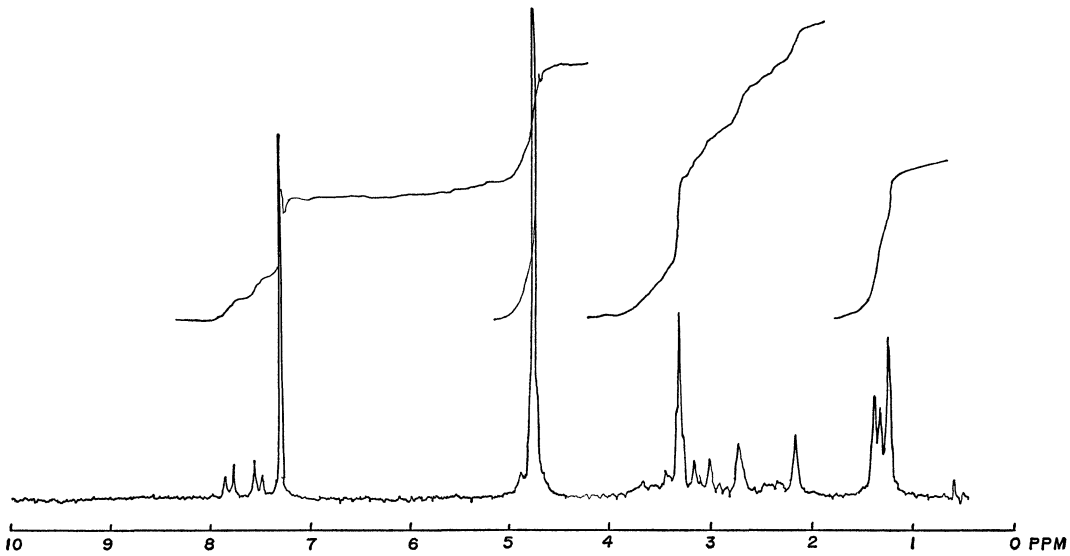


Fig. 3. N.m.r. spectrum of chrothiomycin in tetradeuteromethanol.



in neutral and acidic solution, and blue in alkaline solution, which is readily decolorized by hydrogen peroxide. It is also blue in concentrated sulfuric acid. Chrothiomycin shows positive hydroxyquinone reaction with magnesium acetate. It gives negative BRADY, MOLISCH-UDRANSKY and FEHLING reactions. It is more stable in acidic solution than in neutral or alkaline solution. In an aqueous solution containing 500 mcg/ml, 100 % of the activity remained after heating at 90°C for 30 minutes at pH 2.0, 90 % at pH 5.0, 82 % at pH 7.0 and 76 % at pH 9.0. On thin-layer chromatography using a silica gel plate, chrothiomycin gives a purple spot of the following Rf values: 0.22 with water-saturated ethyl ether, 0.77 with water-saturated butanol, 0.09 with ethyl acetate, 0.03 with butyl acetate, 0.50 with methanol, 0.45 with butanol, 0.72 with water and 0 with benzene and with chloroform.

The following minimum inhibitory concentrations were observed by the agar dilution methods:

Staphylococcus aureus TERAJIMA	50 mcg/ml
Staphylococcus aureus SMITH	100 mcg/ml
Staphylococcus aureus 193	100 mcg/ml
Staphylococcus aureus 308-A1	50 mcg/ml
Bacillus anthracis	100 mcg/ml
Micrococcus flavus 16	50 mcg/ml
Sarcina lutea	25 mcg/ml

However, at 100 mcg/ml no inhibition was observed against *Staphylococcus aureus* FDA 209P, *Bacillus cereus* ATCC 10702, *Bacillus subtilis* PCI 219, *Bacillus subtilis* NRRL B-558, *Klebsiella pneumoniae* PCI 602, *Proteus vulgalis* OX 19, *Escherichia coli* NIHJ, *Escherichia coli* K-12, *Pseudomonas aeruginosa* A3, *Shigella flexneri* la Ew 8, *Salmonella enteritidis* G 14, *Serratia marcescens*, *Pseudomonas fluorescens*, *Mycobacterium smegmatis* ATCC 607, *Candida albicans* 3147.

Table 2. Tyrosine hydroxylase inhibition by chrothiomycin

Chrothiomycin concentration mcg/ml	Percent inhibition	
	Fe^{++}-free system*	2.5×10^{-3} M Fe^{++}-added system**
50.0	99.0	80.0
25.0		79.0
12.5		74.0
6.25		65.0
3.13		64.0
1.56		52.0
0.78		38.0
0.39	99.0	30.0
0.20	97.0	
0.10	98.0	
0.05	97.0	
0.03	87.0	
0.01	67.0	
0.006	43.0	
0.003	29.0	
0.002	21.0	
0.001	17.0	

* The reaction mixture contained 200 μmoles of acetate buffer (pH 6.0), 0.1 μmoles of L-tyrosine containing 1.1×10^5 c.p.m. of L-tyrosine-C^{14}, 100 μmoles of mercaptoethanol, 1 μmole of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, tyrosine hydroxylase (0.4 mg protein) and distilled weter to 1 ml. Aqueous solution of chrothiomycin was added in the indicated concentration (mcg/ml). Incubation was continued for 15 minutes at 30°C.

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Chrothiomycin inhibits reactions of tyrosine hydroxylase[1], and the result of the test is shown in Table 2. The chrothiomycin concentration required for 50 % inhibition of tyrosine hydroxylase is 0.007 mcg/ml in Fe^{++}-free system, while it is 1.5 mcg/ml in 2.5×10^{-3} M Fe^{++}-added system[7]. Chrothiomycin inhibits also another oxygenase, dopamine β-hydroxylase and the inhibitory activity is shown in Table 3. The chrothiomycin concentration required for 50 % inhibition of dopamine β-hydroxylase is

0.1 mcg/ml. Chrothiomycin inhibits multiplication of YOSHIDA rat sarcoma in tissue culture. When tested by a method described by HORI *et al.*[8], 87.0, 71.8, 60.8, 50.9, 23.2 and 0.7 % of inhibition were observed at 50, 10, 8, 6, 4 and 2 mcg/ml of chrothiomycin. The acute toxicity (LD_{50}) to mice by intravenous injection was 25 mg/kg.

The data presented above serve to characterize chrothiomycin and it can readily be distinguished from known antibiotics because it is a quinone type antibiotic containing sulfur.

Table 3. Dopamine β-hydroxylase inhibition by chrothiomycin

Chrothiomycin concentration mcg/ml	Percent inhibition
10.0	97.0
1.0	92.0
0.1	52.0
0.01	11.0
0.001	7.0

Reaction mixture contained 200 μmoles of potassium phosphate buffer (pH 6.5), 10 μmoles of tyramine, 10 μmoles of fumarate, 10 μmoles of ascorbate, 250 mcg of catalase, 10 μmoles of N-ethylmaleimide, dopamine β-hydroxylase (40 mcg protein) and distilled water to 1 ml. Aqueous solution of chrothiomycin was added in the indicated concentrations (mcg/ml). Incubation was continued for 30 minutes at 37°C.

Experimental

Production of chrothiomycin :

Fermentation studies showed the following medium to be suitable for chrothiomycin production : 1 % starch, 1 % glucose, 0.75 % meat extract, 0.75 % peptone, 0.3 % NaCl, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$ and 0.0002 % $ZnSO_4 \cdot 7H_2O$ (pH 7.0~7.2 after sterilization). The strain MB445-A1 was grown on KRAINSKY glucose asparagine agar slant for 2 or 3 weeks at 27°C and then used to inoculate 125 ml medium in 500 ml flasks which were then shaken on a reciprocating shaking machine (8 cm amplitude, 130 strokes per minute) at 27°C for 48 hours. Two ml amount of this broth were then used to inoculate the final fermentation stage using 125 ml of the same medium in 500 ml flasks with the same conditions of temperature and agitation. Activity was assayed by a disc plate method using *Sarcina lutea* as a test organism or by testing for inhibition of tyrosine hydroxylase[1] reaction.

Isolation and purification :

The broth (pH 8.2) harvested after 72 hours cultivation was adjusted to pH 2.0 with dilute hydrochloric acid and filtered to remove mycelium. The filtrate contained 32 mcg/ml of chrothiomycin. The filtrate (3.39 liters) was extracted twice with *n*-butanol (2 liters ×2). The extract (4.2 liters), which contained 24 mcg/ml of the antibiotic, was concentrated *in vacuo* at 40°C to 150 ml and 750 ml of *n*-hexane was added to precipitate 2.1 g of a brownish purple crude powder containing 60 mg/g of chrothiomycin. The crude powder (1.8 g) was dissolved in a small volume of methanol and adsorbed on to 9.2 g of silicic acid (Silicic acid, A. R., 100 mesh, Mallinckrodt), which was then placed on the top of a column of the same adsorbent (92.0 g). The chromatography was developed using a solvent system composed of methanol and benzene (1 : 20), and the effluent cut in 10 g fractions. Active fractions (fraction Nos. 161 to 373, 2.1 liters) were concentrated *in vacuo* at 40°C to dryness. The residue (93.8 mg) was charged again on a column of silicic acid (9.0 g) in the manner described above, and chromatography again developed by the same solvent system. The antibiotic was crystallized from active fractions (fraction Nos. 19 to 30, 120 ml). The crystals were filtered, washed with benzene and dried to obtain 12.8 mg crystalline chrothiomycin, m. p. 193~195°C. The yield from the fermented broth was 11.9 %.

Anal. Calcd. for $C_{27}H_{31}O_{13}NS$, (m. w. 609.71): C 53.19, H 5.14, O 34.11, N 2.30, S 5.26
$C_{27}H_{33}O_{13}NS$, (m. w. 611.63): C 53.16, H 5.44, O 34.01, N 2.28, S 5.24
Found : C 52.92, H 5.07, O 30.84, N 2.10, S 5.46

The tests of chlorine and phosphor were negative.

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