ISOLATION AND CHARACTERIZATION OF A NEW ANTIBIOTIC, NEOPLURAMYCIN

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A new crystalline antibiotic named neopluramycin, which inhibits growth of Gram-positive bacteria, leukemia L-1210 in mice and YOSHIDA rat sarcoma cells in tissue culture, has been isolated from the cultured broth of a strain of *Streptomyces pluricolorescens*. It was obtained as orange crystals, and analytical data and molecular weight determination are consistent with the empirical formula $C_{40}H_{50}N_2O_{10}$. Neopluramycin resembles pluramycin A, but is differentiated by its antibacterial spectrum, toxicity, thin-layer chromatography, and infrared absorption spectrum.

An antibiotic produced by a strain of *Streptomyces pluricolorescens* was isolated from the cultured broth by solvent extraction. In exhibits inhibition against Gram-positive bacteria, YOSHIDA rat sarcoma cells and leukemia L-1210 in mice. Taxonomic studies of the streptomyces, isolation and characterization of neopluramycin are discribed in this paper. The antibiotic was differentiated from known antibiotics and named neopluramycin.

Taxonomy of the Neopluramycin-Producing Strain

The strain was isolated from a soil sample collected in the garden of our institute, Shinagawa-ku, Tokyo, Japan. The laboratory number MB760-MG1 was given to this strain. The strain showed the following characters:

1. Microscopic observation

Aerial mycelium is developed from fine branched vegetative mycelium. Aerial mycelium forms neither whorls nor spirals. The spore surface is smooth (electron-microscopy).

2. Cultural characteristics on various media

The description in parenthesis follows the color standard published by Container Corporation of America.

(1) On glycerol CZAPEK's agar (27°C): Colorless to pale yellow to yellowish brown [Luggage Tan, 4 ne] growth; reddish purple [Old Wine, 7½ ng] reverse; light olive gray aerial mycelium; pale vinaceous soluble pigment.

(2) On KRAINSKY's glucose asparagine agar (27°C): Yellow to dull yellow growth; powdery yellowish white to olive gray [Lt Olive Gray, 1½ ge] aerial mycelium; yellowish soluble pigment.

(3) On calcium malate agar (27°C): Colorless growth; purple [Orchid Gray, 9 ig] reverse; light olive gray [Cream, 1½ ca~Oatmeal, 2 ec] aerial mycelium; faint brownish

soluble pigment; transparent zone around the growth.

(4) In peptone water with 1.0% sodium nitrate (27°C): Colorless to pale yellow growth; white aerial mycelium; yellow to pale brown soluble pigment; reduction of nitrate to nirite.

(5) On potato plug (27°C): Colorless to pale yellow growth; white to light olive gray aerial mycelium; yellowish brown to dark yellowish brown soluble pigment.

(6) On starch plate (27°C): Yellow to dull yellow [Mustard Gold, 2 ne] growth; white to yellowish white to light olive gray [Oatmeal, 2 ec] aerial mycelium; yellowish soluble pigment; strong hydrolysis of starch.

(7) On nutrient agar (27°C): Colorless to pale yellow to pale brown growth; brownish purple reverse; yellowish white to brownish white aerial mycelium; brownish soluble pigment.

(8) On nutrient agar (37°C): Wrinkled colorless growth; thin white aerial mycelium; no soluble pigment.

(9) On LOEFFLER's coagulated serum (37°C): Wrinkled colorless to pale yellow growth; no aerial mycelium; yellowish brown soluble pigment; strong liquefaction of coagulated serum.

(10) On gelatin stab (20°C): Colorless to pale yellow to dull yellow growth; white aerial mycelium; light brown soluble pigment; medium to strong liquefaction of gelatin.

(11) On skimmed milk (37°C); Colorless to pale yellow growth; no aerial mycelium; pale yellowish brown soluble pigment: no coagulation; peptonization is observed after about 14 days culture and relatively strong.

(12) On tyrosine agar (27°C): Colorless to pale brown growth; light olive gray aerial mycelium; pale reddish brown soluble pigment; negative tyrosinase reaction.

(13) On cellulose (27°C): Poor growth; no hydrolysis.

(14) Utilization of carbon sources on PRIDHAM-GOTTLIEB basal medium (27°C): Xylose, rhamnose, glucose, mannose, galactose, fructose, maltose, lactose, starch, dextrin, glycerol and mannitol are utilized; arabinose, saccharose, raffinose, inulin, sorbitol, dulcitol, inocitol are not utilized.

The characters of the strain MB760-MG1 described above can be summarized as follows: it belongs to nonchromogenic type of streptomyces; it forms neither whorls nor spirals; surface of spores is smooth; growth is pale yellow to yellowish brown on

Table 1. Comparison of the strain MB760-MG1 with *S. pluricolorescens*

	The strain MB760-MG1	The type culture of *S. pluricolorescens* ISP No. 5019*
Surface of spores	smooth	smooth
Whorl	none	none
Spiral	none	none
Color of growth	plae yellow to yellowish brown; reverse is reddish purple	pale yellow to pale yellowish brown to light brown; reverse is purplish
Color of aerial mycelium	light olive gray	grayish white to light gray to light olive gray
Soluble pigment on nutrient agar (27°C culture)	brownish	none
Melanoid pigment	none	none
Hydrolysis of starch	medium to strong	medium to strong
Liquefaction of gelatin	medium to strong	medium to storng
Peptonization of milk	strong	strong
Liquefaction of serum	strong	strong
Utilization of saccharose	probably not utilized	probably utilized

* ISP: International Streptomyces Project by SHIRLING and GOTTLIEB.

various media; reverse of growth is reddish purple on various media; aerial mycelium is light olive gray on various media; soluble pigment is yellow to brownish; it has strong proteolytic activity and strong hydrolytic activity of starch.

Among known species of *Streptomyces*, the strain MB760-MG1 was closely related to pluramycin-producing strain, *Streptomyces pluricolorescens*[1,2] described by OKAMI and UMEZAWA, 1956. Although some minor differences are observed in the utilization of saccharose and soluble pigment on nutrient agar as shown in Table 1, the strain MB760-MG1 can be assigned to *S. pluricolorescens*.

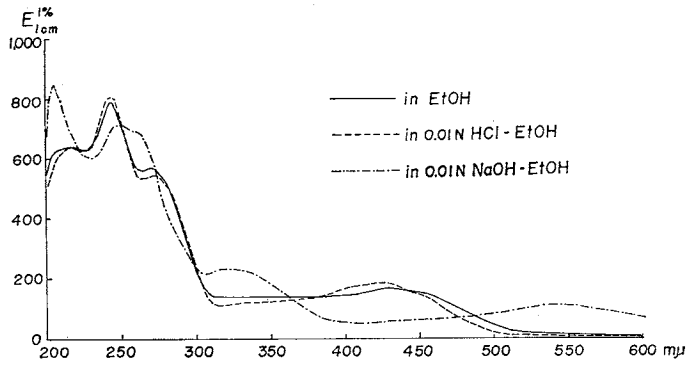
Production and Isolation

Neopluramycin was produced by submerged culture at 27°C in a medium containing 1.0 % glucose, 1.0 % starch, 1.5 % soybean meal, 0.3 % NaCl, 0.1 % K_2HPO_4, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MgCl_2 \cdot 4H_2O$ and 0.0002 % $ZnSO_4 \cdot 7H_2O$ (adjusted to pH 7.0). The broth was harvested at 2~4 days, and contained about 50 mcg/ml of neopluramycin and the lesser amounts of two other antibiotics which were closely related to the iyomycin B group[3] and pluramycin B[4].

The results of testing the activities of the cultured broth against *Micrococcus flavus*, *Mycobacterium smegmatis* ATCC 607 and YOSHIDA rat sarcoma cells in tissue culture[5] and of bioautography of the thin-layer chromatogram indicated that neopluramycin existed mainly in the culture filtrate and the mycelial cake contained less than 10 % of the antibiotic in the filtrate.

Neopluramycin in the filtered broth (pH 7~8) was extracted with *n*-butanol, ethyl acetate, *n*-butyl acetate or chloroform, and re-transferred into acidic water (pH 2.0). On countercurrent distribution with ethyl acetate-1 % phosphate buffer at pH 6.5, the distribution coefficient of neopluramycin was 12.0 and that of iyomycin B was 0.08, and at pH 5.3, those of neopluramycin and pluramycin B were 0.06 and 8.5, respectively. According to the results of the countercurrent distribution studies, separation of neopluramycin from other antibiotics was accomplished by ethyl acetate extraction at pH 7.0 from the aqueous extract, followed by re-extraction with water at pH 5.0. Neopluramycin in aqueous extract was re-transferred into ethyl acetate at pH 7.0~7.4. After repeating the same extraction procedure, the ethyl acetate extract was concentrated to dryness under reduced pressure yielding reddish orange crystalline powder of neopluramycin, which was recrystallized from ethyl acetate as orange crystals.

Fig. 1. Ultraviolet absorption spectrum of neopluramycin.



Physical and Chemical Properties

Neopluramycin forms orange crystals melting at 180~184°C under decomposition. $[\alpha]_D^{26}$ +362° (*c* 1.05, chloroform).

Fig. 2. Infrared absorption spectrum of neopluramycin (KBr).

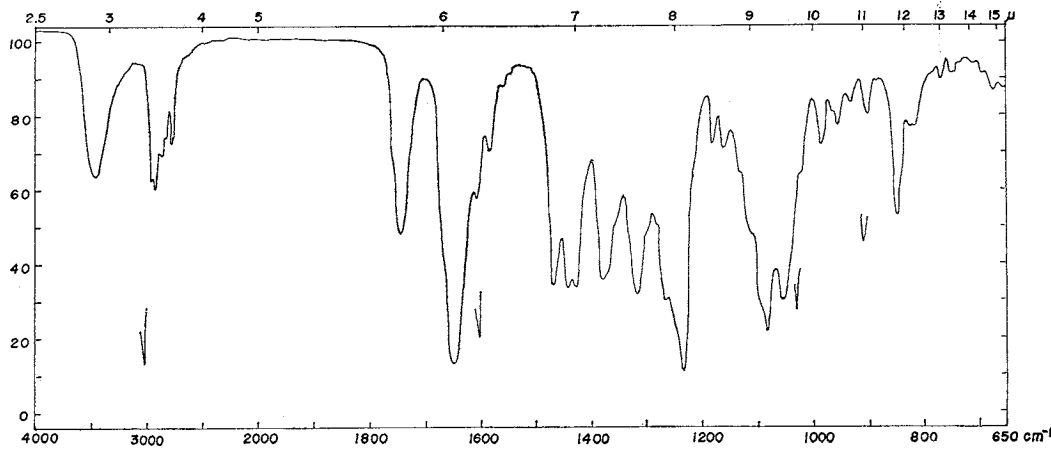
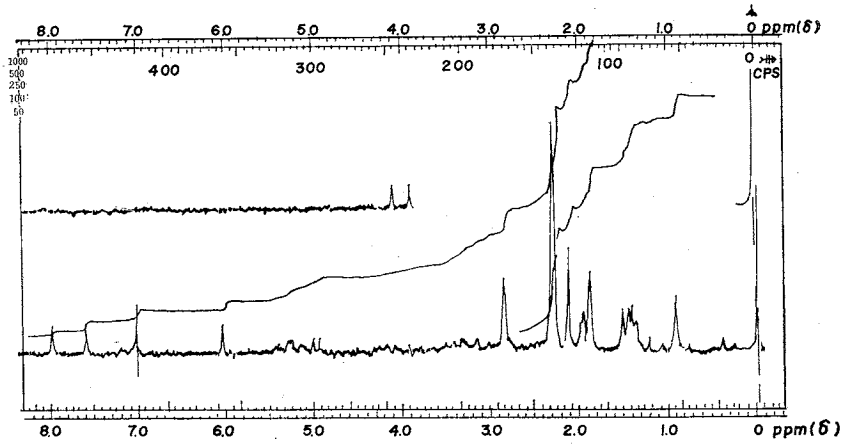


Fig. 3. NMR spectrum of neopluramycin in deuterochloroform (60 MHz).



Elemental analysis gave C 66.80 %, H 7.03 %, N 3.93 %, O 22.00 %, no halogen or sulfur. The molecular weight determination by vapor pressure osmometer in chloroform gave 750±35. The values calculated for $C_{40}H_{50}N_2O_{10}$ are C 66.83 %, H 7.01 %, N 3.90 %, O 22.26 %, mol. wt. 718.82.

The ultraviolet absorption spectrum (Fig. 1) shows maxima as follows: 216 mμ ($E^{1\%}_{1cm}$ 640), 243 mμ ($E^{1\%}_{1cm}$ 790), 270 mμ ($E^{1\%}_{1cm}$ 564) and 430 mμ ($E^{1\%}_{1cm}$ 164) in ethanol; 218 mμ ($E^{1\%}_{1cm}$ 648), 243 mμ ($E^{1\%}_{1cm}$ 808), 272 mμ ($E^{1\%}_{1cm}$ 564) and 426 mμ ($E^{1\%}_{1cm}$ 184) in 0.01 N HCl-ethanol; 206 mμ ($E^{1\%}_{1cm}$ 816), 248 mμ ($E^{1\%}_{1cm}$ 714), 260 mμ (shoulder, $E^{1\%}_{1cm}$ 682), 320 mμ ($E^{1\%}_{1cm}$ 222) and 546 mμ ($E^{1\%}_{1cm}$ 96) in 0.01 N NaOH-ethanol. The infrared absorption spectrum (Fig. 2) shows bands at 1745, 1645, and 1605 cm^{-1}, characteristic to carbonyl groups. The nuclear magnetic resonance spectrum in deuterochlorform is shown in Fig. 3.

Neopluramycin is soluble in methanol, ethanol, *n*-butanol, ethyl acetate, *n*-butyl acetate, acetone, benzene, chloroform, carbon tetrachloride and acidic water, but insoluble or scarcely soluble in ethyl ether, *n*-hexane and water.

Neopluramycin turns to yellow from orange in acidic solution and to purple in

alkaline solution, and the purple color is decolorized by hydrogen peroxide. It gives a reddish purple color with magnesium acetate in methanol (a reaction for hydroxyquinone[6]).

On thin-layer chromatography using Silica gel G (E. Merck), the antibiotic gives a yellowish spot and Rf values are as follows: 0.05 with acetone, 0.03~0.09 with acetone - ethanol (4 : 1), 0.06~0.18 with acetone - ethanol (1 : 1), 0~0.10 with acetone - ethanol (1 : 4), 0.05~0.10 with acetone - methanol (4 : 1), 0~0.10 with acetone - methanol (9 : 1), 0.05~0.20 with methanol, 0 with chloroform, 0~0.05 with chloroform - methanol (9 : 1), 0.80~0.90 with ethanol - 28 % ammonia - water (8 : 1 : 1) and 0.25~0.30 with *n*-butanol - acetic acid - water (4 : 1 : 2).

Biological Properties

The antimicrobial spectrum of the antibiotic observed by agar dilution method is shown in Table 2, and neopluramycin inhibits growth of Gram-positive bacteria.

Table 2. The antimicrobial spectrum of neopluramycin

Organisms	Minimum inhibitory concentration (mcg/ml)
Staphylococcus aureus FDA 209P	100
Staphylococcus aureus Terajima	50
Staphylococcus aureus Smith	100
Staphylococcus aureus 193	50
Staphylococcus aureus 52-34	100
Micrococcus flavus 16	6.25
Sarcina lutea PCI 1001	3.12
Bacillus cereus ATCC 10702	100
Bacillus anthracis	12.5
Bacillus subtilis PCI 219	50
Bacillus subtilis NRRL B-558	100
Corynebacterium bovis 1810	1.56
Escherichia coli NIHJ	>100
Escherichia coli K-12	>100
Shigella flexneri 1a Ew8	>100
Salmonella typhosa	>100
Salmonella enteritidis	100
Pseudomonas aeruginosa A3	>100
Pseudomonas fluorescens	>100
Klebsiella pneumoniae PCI 602	>100
Proteus vulgaris OX19	>100
Mycobacterium smegmatis ATCC 607	25
Mycobacterium phlei	6.25
Candida albicans 3147	>100

Significant prolongation in the survival period of mice inoculated with lymphatic leukemia L-1210 was observed by treatment with neopluramycin intraperitoneally. At daily doses of 3.12, 1.56 and 0.78 mcg of neopluramycin per mouse for 10 days, prolongation rates of the survival period were 190 %, 167 % and 161 %, respectively. Neopluramycin inhibited multiplication of YOSHIDA rat sarcoma cells in tissue culture[5], and at concentrations of 0.1, 0.02 and 0.004 mcg/ml, inhibitions of 85.7, 62.1 and 22.7 % were observed, respectively. Acute intravenous LD_{50} of neopluramycin in mice was 12.5~25 mg/kg. Evaluation was made at 12 days because mice dosed at 25 mg/kg died between 7~11 days after the injection.

Comparison with Other Antibiotics

Neopluramycin resembles pluramycin A[4] which is produced by *Streptomyces pluricolorescens*, but it is distinguished from other hydroquinone antibiotics containing nitrogen, such as anthracidins[7], hedamycin[8], iyomycin B group[3], rubiflavin[9] and kidamycin[10] by ultraviolet and infrared absorption spectra. Ultraviolet absorption spectra of neopluramycin and pluramycin A are almost similar, but infrared absorptions of neopluramycin (1745, 1645 and 1605 cm^{-1}) and pluramycin A (1745, 1660 and 1630

cm^{-1}) at carbonyl region are different. Furthermore, with thin-layer chormatography on Silica gel G using acetone-ethanol (1:1), neopluramycin exhibits the Rf 0.06~0.18, whereas pluramycin A exhibits Rf 0.20~0.30. Neopluramycin is weaker than pluramycin A in antimicrobial activity, and the toxicity of neopluramycin is lower than that of pluramycin A.

Experimental

Fermentation: *Streptomyces pluricolorescens* MB760-MG1 was cultured in a 2,000-liter fermenter (dispense volume: 1,000 liters). The medium contained 1.0% glucose, 1.0% starch, 1.5% soybean meal, 0.3% NaCl, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0008% $MgCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$, and was adjusted to pH 7.0. It was sterilized at 120°C for 30 minutes. Vegetative mycelia contained in 5% volume of the shake-cultured broth (26 hours) was used as inoculum. The temperature was maintained at 27°C, the air-flow rate was held constant at 1,000 liters per minute and the agitation was operated at 165 r. p. m. The cultured broth (pH 7.75) was harvested after 42-hour fermentation.

Isolation: The cultured broth was filtered using filter aid, which was washed with 285 liters of water. Neopluramycin was extracted with 250 liters of *n*-butanol from the combined filtrate (1,100 liters, pH 8.1) and retransferred into 125 liters of acidid water (pH 2.0 with hydrochloric acid). The antibiotic in the aqueous extract was transferred into 70 liters of ethyl acetate at pH 7.0, and re-extracted with 50 liters of water at pH 5.0, and then re-transferred into 20 liters of ethyl acetate at pH 7.2. The solvent extract was concentrated to dryness under reduced pressure yielding 2.47 g of the crude reddish powder.

The crude powder (2.41 g) was dissolved in 400 ml of ethyl acetate and the insoluble impurities were removed by filtration. The filtrate was washed with 400 ml of water at pH 7.0, and extracted with 400 ml of acidic water (pH 5.0), and the aqueous extract was re-extracted with 400 ml of ethyl acetate at pH 7.0. The extract was concentrated to dryness under reduced pressure yielding the reddish orange crystalline powder of neopluramycin (1.36 g). The crystalline powder was dissolved in 100 ml of hot ethyl acetate, and the solution was concentrated to about 30 ml under reduced pressure to obtain orange crystals (790 mg). By two recrystallizations from ethyl acetate, 567 mg of noepluramycin was obtained. m. p. 180~184°C (decomp.), $[\alpha]_D^{26}$ +362° (*c* 1.05, chloroform).

References

1) Shirling, E. B. & D. Gottlieb: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Inter. J. Syst. Bact. 18: 159, 1968
2) Waksman, S. A.: The Actinomycetes, Vo. II, pp. 259~260, The Williams & Wilkins Co., Baltimore, 1961
3) Sano, Y.; N. Kanda & T. Hata: Iyomycin, a new antitumor antibiotic from *Streptomyces*. III. Isolation and properties of iyomycin B. J. Antibiotics, Ser. A 17: 117~123, 1964
4) Maeda, K.; T. Takeuchi, K. Nitta, K. Yagishita, R. Utahara, T. Osato, M. Ueda, S. Kondo, Y. Okami & H. Umezawa: A new antitumor substance, pluramycin. Studies on antitumor substances produced by *Actinomycetes*. XI. J. Antibiotics, Ser. A 9: 75~81, 1956.
5) Hori, M.; E. Ito, T. Takeuchi & H. Umezawa: Inhibitory effects of antitumor substances on growth and glycolysis of Yoshida rat sarcoma cells. J. Antibiotics, Ser. A 16: 1~6, 1963
6) Shibata, S.: Rhodocladonic acid, a pigment produced by a lichen of genus *Cladonia*. J. Pharmaceut. Soc. Jap. 61: 320~325, 1941.
7) Yoshida, T. & K. Katagiri: Anthracidins A and B, new antibiotics. Antimicr. Agents & Chemoth.-1963: 63~67, 1964
8) Schmitz, H.; K. E. Crook, Jr. & J. A. Bush: Hedamycin, a new antitumor antibiotic. I. Production, isolation, and characterization. Antimicr. Agents & Chemoth.-1966: 606~612, 1967
9) Aszalos, A.; M. Jelinek & B. Berk: Rubiflavin, a toxic antitumor antibiotic. Antimicr. Agents & Chemoth.-1964: 68~74, 1965
10) Hata, T.; I. Umezawa, K. Komiyama, K. Asano, N. Kanda, H. Fujita & M. Kono: Studies on a new antitumor antibiotic, kidamycin. Progress in Antimicr. & Anticancer Chemoth., Vol. I, pp. 80~86, Univ. of Tokyo Press, Tokyo, 1970