STUDIES ON A NEW ANTIBIOTIC PIGMENT, AQUAYAMYCIN

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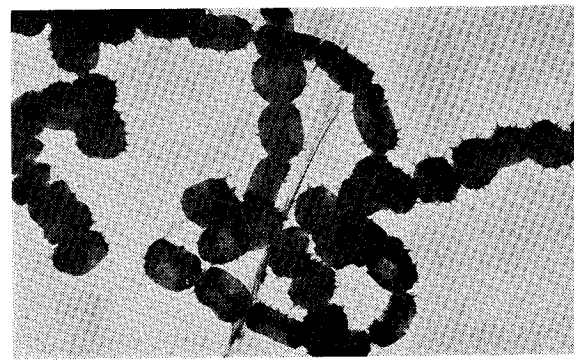
A new antibiotic pigment named aquayamycin, which inhibits growth of Gram positive bacteria, EHRLICH carcinoma in mice, and YOSHIDA rat sarcoma cells in tissue culture, was isolated from *Streptomyces misawanensis* nov. sp. HAMADA *et* OKAMI. It was obtained as orange yellow crystals having the molecular formula $C_{30\sim31}H_{34\sim40}O_{12}$. From the physical and chemical properties, aquayamycin was concluded to be a new antibiotic pigment of hydroxyquinone structure.

In a course of screening studies, applying multiple tests of biological activities to cultured broths of isolated microorganisms, we found a new antibiotic pigment produced by a streptomyces. The properties of the pigment are most closely related to ayamycin among known microbial products. However, it is more soluble in water than the latter and was named aquayamycin. The morphological characteristics of the streptomyces, the isolation procedure, chemical and physical properties and some biological activities of the pigment are reported in this paper.

Aquayamycin-producing Strain

An actinomyces was isolated from a soil collected at Misawa City of Aomori Prefecture in 1966 and numbered as MA 944-A 5 in the authors' laboratory. This strain was found to produce a new antibiotic, aquayamycin, and showed the characteristics described below on taxonomic examination.

Plate 1. Electron-micrograph of spores of *S. misawanensis* strain No. MA 944-A 5 (×11,200)



Microscopically, the strain shows well-branched substrate mycelia. Aerial mycelia which develop from substrate mycelia bear open spirals at their tips but do not form whorls. Spiny structure is observed on the surface of the spore under the electron-microscope as shown in Plate 1.

The following characteristics are observed on various media:

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(1) Glycerol CZAPEK's agar (glycerol nitrate agar, incubated at 27°C) : Pale reddish brown to grayish red brown growth. White aerial mycelium change to pink. Brown to grayish red brown soluble pigment. Agar medium around the growth is colored dark brown.

(2) KRAINSKY's glucose asparagine agar (incubated at 27°C) : Orange yellow, yellowish brown to brown colored growth. Cottony, white aerial mycelium changing to light gray or light brownish gray. Yellow to orange yellow or light brown soluble pigment. Distinguishable orange yellow zone around the growth.

(3) Calcium malate agar (incubated at 27°C) : Colorless growth with white to light grayish aerial mycelium. No soluble pigment. Calcium malate around the growth is quickly solubilized and becomes transparent after 3 days incubation.

(4) Peptone solution containing 1.0 % $NaNO_3$ (incubated at 27°C) : Colorless growth without aerial mycelium. Small amount of blackish soluble pigment. Indistinct reduction of nitrate.

(5) Potato plug (incubated at 27°C) : Pale yellowish brown and wrinkled growth which changes to dark yellowish brown or dark brown. White to brownish white aerial mycelium on the upper part of agar slant. Brownish black soluble pigment.

(6) Starch agar plate (incubated at 27°C) : Colorless to pale yellowish brown growth. White aerial mycelium shaded with light gray or light brownish gray. Yellow brown soluble pigment. Strong hydrolysis.

(7) Nutrient agar (incubated at 37°C) : Colorless growth without aerial mycelium. Slightly brownish soluble pigment.

(8) Nutrient agar (incubated at 27°C) : Colorless to pale brown growth without aerial mycelium. Brown soluble pigment.

(9) LOEFFLER's coagulated serum medium (incubated at 37°C) : Pale yellowish brown wrinkled growth. No aerial mycelium. Dark brown color around the growth. No liquefaction of coagulated serum.

(10) Gelatin stab (incubated at 20°C) : Pale yellow or pale yellowish brown growth without aerial mycelium. Light brown to yellowish brown soluble pigment. No or weak liquefaction of gelatin.

(11) Skim milk (incubated at 37°C) : Ring growth colored with yellowish brown which changes to brown or dark brown. No aerial mycelium. Pale brownish soluble pigment. Weak coagulation and peptonization of milk.

(12) Tyrosine agar (incubated at 27°C) : Growth colored with blackish tinge. Light grayish to light brownish gray aerial mycelium. Blackish soluble pigment. Positive tyrosinase reaction.

(13) Cellulose (incubated at 27°C) : No decomposition of filter paper.

(14) Carbohydrate utilization on PRIDHAM-GOTTLIEB's basal medium (incubated at 27°C) : Positive growth with inositol, lactose, galactose, glucose, fructose, starch, mannitol, dextrin, glycerol, dulcitol, xylose and maltose. Doubtful growth with sorbitol.

Summarizing the above, the strain MA 944-A 5 shows characteristics of the genus *Streptomyces* as follows: no whorl formation; formation of spiral; spiny surface of spores; yellowish brown to brown growth on various media; no aerial mycelium on media containing natural organic substances and white to light gray or light brownish gray aerial mycelium on synthetic media; yellowish brown to brownish black soluble pigment; significant orange yellow or dark brown color around the growth on KRAINSKY's glucose asparagine agar and glycerol nitrate agar, respectively; chromogenic type; weak proteolytic activity; strong hydrolysis of starch.

Among known species, *Streptomyces aureus* (WAKSMAN *et* CURTIS, 1916) WAKSMAN *et* HENRICI[3] and *Streptomyces phaeochromogenes* (CONN, 1917) WAKSMAN *et* HENRICI[3] resemble the strain MA 944-A 5. Those species, however, can be clearly differentiated from the strain MA 944-A 5 in that the surfaces of the conidial spores are smooth, while that of the strain MA 944-A 5 is spiny. On the other hand, the antibiotic from the strain MA 944-A 5 resembles ayamycin[1,2] and TA-435 A[4] which are produced by two resembling strains, O-80 and TA-435, isolated independently in different

laboratories. Therefore, comparative studies were made with these two strains.

In addition, *Streptomyces flaveolus* (WAKSMAN) WAKSMAN *et* HENRICI[8] was added to the comparative study, because this species was referred to in studies of the strains producing ayamycin. The comparative study revealed that the strain MA 944-A 5 differs from the strains O-80 and TA-435 A in the spiny surface of conidia of the former and smooth one of the latter two strains, and MA 944-A 5 differs from *S. flaveolus* in the hairy surface of conidia of the latter species. As described above, the strain MA 944-A 5 is differentiated from known species and is properly named by a new epithet. Therefore, the strain was given the name of *Streptomyces misawanensis* n. sp. HAMADA *et* OKAMI from the place where the soil was collected.

Fermentation, Isolation and Purification

After a survey of a proper medium for the antibiotic production, a medium containing 1 % starch, 1 % glucose, 1.5 % soy bean meal, 0.3 % NaCl, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7\,H_2O$ (pH 7.0) was found to be suitable for the production. Therefore, the strain MA 944-A 5 cultivated on an agar slant of glucose asparagine medium for 2~3 weeks was used to inoculate this glucose soy bean medium. The inoculated medium of 125 ml in a 500 ml flask was shaken on a reciprocating shaking machine with 8 cm amplitude, 130 strokes per minute, at 27°C for 48 hours. The pH of the broth changed to 6.8. Two ml of this broth were transferred to 125 ml of the same medium described and shake-cultured in the way described above for 72 hours. The fermented broth (pH 6.7) was adjusted to pH 2.4 with dilute hydrochloric acid and filtered to remove mycelial mass. The filtrate contained 195 mcg of the antibiotic per ml. Twenty-two liters of this filtrate was extracted twice with 7,000 ml of *n*-butanol each, and the solvent layer was concentrated *in vacuo* at 40°C. Reddish brown colored crude powder (4.17 g) containing 215 mcg of active principle per mg was obtained. This crude powder was placed on the top of a column filled with 90 g of silicic acid (Silicic acid A.R., 100 mesh, Mallinckrodt) moistened with 18 ml of water and slurried with water-saturated butyl acetate. The charged column was developed with water-saturated butyl acetate and the eluted fractions having antibacterial activity were collected. The active fraction in 2,100 ml was concentrated *in vacuo* at 40°C to 100 ml. To this concentrated solution, 100 ml of *n*-hexane were added to yield a precipitate. The precipitate was dried *in vacuo* and yielded 790 mg of orange yellow powder containing 600 mcg of the active principle per mg. The powder was extracted with water (240 ml), and a reddish brown crystalline powder (480 mg) containing 900 mcg of the active principle per mg was obtained after the concentration of the extract *in vacuo* at 40°C. This crystalline powder was again applied to the column filled with 100 g of the silicic acid. The active fractions developed with water-saturated *n*-butyl acetate were collected. From 340 ml of the fractions, 107 mg of reddish brown crystalline powder were obtained. On recrystaliziation with butyl acetate, 54 mg of orange yellow crystals were obtained.

In these experiments, activity of samples was assayed by a disc plate method using *Micrococcus flavus* as the test organism and pure aquayamycin crystals as the standard.

For qualitative examination of samples, the thin-layer chromatography using silica gel G and water-saturated ethyl acetate - butyl acetate (1 : 1) was employed.

Physical and Chemical Properties of Aquayamycin

Aquayamycin forms orange to reddish orange fine crystals with weakly acidic properties and melts at 189~190° with decomposition. The specific rotation $[\alpha]_D^{20}$ is +160° (c=1, in dioxane).

The result of elemental analysis were calculated for $C_{30\sim31}H_{34\sim40}O_{12}$: C 60.80~62.20, H 5.73~6.80, O 31.75~32.73. M. W. 586.60~604.66; found: C 61.57, H 6.28, O 32.06, no nitrogen; M. W. 584 (± 1.5 %) by vapor pressure osmometer in 95 % ethanol.

Electrometric titration showed one ionizable group with pKa' 9.2 in water and neutral equivalent 585.

Aquayamycin is soluble in methanol, ethanol, butanol, acetone, N,N-dimethylformamide, dimethyl sulfoxide, dioxane, pyridine, glacial acetic acid and alkaline water. It is sparingly soluble in water, ethyl acetate, butyl acetate and chloroform. It is insoluble in ethyl ether, benzene and hexane.

As shown in Fig. 1, ultraviolet absorption maxima of aquayamycin are observed as follows: 220 mμ ($E^{1\%}_{1cm}$ 675), 320 mμ ($E^{1\%}_{1cm}$ 140) and 430 mμ ($E^{1\%}_{1cm}$ 124) in methanol solution; 220 mμ ($E^{1\%}_{1cm}$ 575), 320 mμ ($E^{1\%}_{1cm}$ 110) and 430 mμ ($E^{1\%}_{1cm}$ 118) in 0.02 N HCl-methanol solution; 230 mμ ($E^{1\%}_{1cm}$ 482), 280 mμ ($E^{1\%}_{1cm}$ 240), 320 mμ ($E^{1\%}_{1cm}$ 170), 395 mμ ($E^{1\%}_{1cm}$ 76) and 540 mμ ($E^{1\%}_{1cm}$ 88) in 0.02 N NaOH-methanol solution.

As shown in Fig. 2, the infrared absorption spectrum of aquayamycin shows the following characteristic bands; 3450 cm^{-1} (hydroxyl group), 1730 cm^{-1} (ester carbonyl), 1640 cm^{-1} (hydrogen-bonded quinone).

The nuclear magnetic resonance spectrum of aquayamycin in deuterochloroform is shown in Fig. 3.

Aquayamycin is reddish orange in acidic solution and blue-violet in alkaline solution. The blue-violet color in alkaline solution is decolorized by hydrogen

Fig. 1. Absorption spectra of aquayamycin

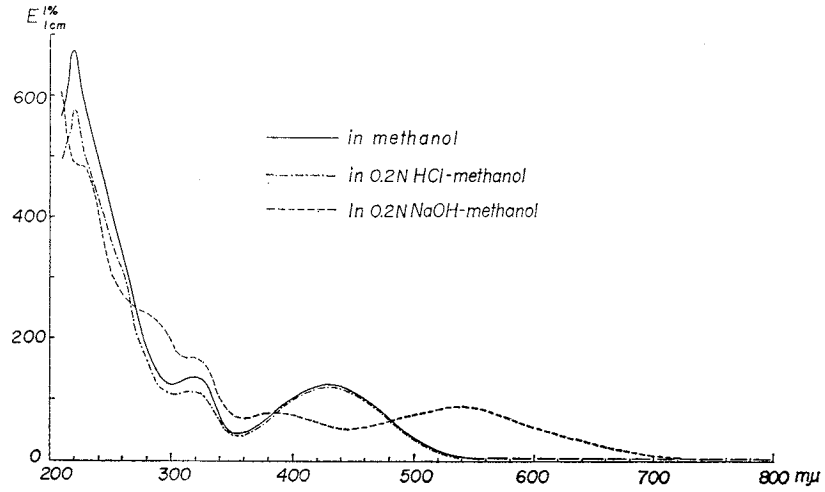
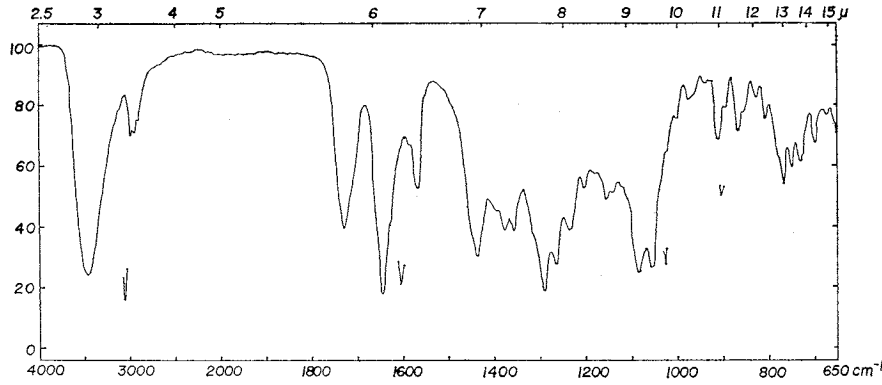
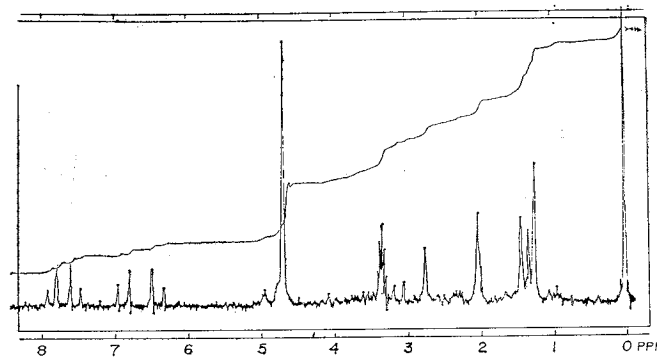


Fig. 2. Infrared absorption spectrum of aquayamycin in KBr



peroxide. It gives a brown color with ferric chloride and a blue-violet color (λ_{max}; 235 and 575 mμ) with magnesium acetate in methanol solution (a hydroxyquinone reaction[5]). In concentrated sulfuric acid, it is purple (λ_{max}; 275, 555, 600 mμ). It gives negative MOLISCH and FEHLING reactions.

Fig. 3. N.m.r. spectrum of aquayamycin in $CDCl_3$, ppm from TMS, 60 Mc



It is rather more stable in acidic solution than in neutral or alkaline solution. In aqueous solution containing 500 mcg/ml, 82 % or 45 % of activity remains after boiling for 30 or 60 minutes at pH 2.0, 74 % or 50 % at pH 5.0, 4 % or 0 % at pH 7.0, and 4 % or 0 % at pH 9.0, respectively.

Thin-layer chromatography using Silica gel G (E. Merck) gives a purple spot of the antibiotic, and Rf values are as follows; 0.41 with water-saturated ethyl acetate, 0.83 with water-saturated butanol, 0.10 with water-saturated butyl acetate, 0.19 with water-saturated ethyl acetate - butyl acetate (1 : 1), 0.09 with ethyl acetate - chloroform

Table 1. Comparison of Rf values on thin-layer chromatographies

Adsorbent	Solvent system	Aquaya-mycin	Ayamycin A_2	TA-435 A	Julimycin B-II
Silica gel G	water-saturated butyl acetate	0.41	0.60	0.85	0.68
〃	ethyl acetate-chloroform (3 : 2)	0.09	0.09	0.22	0.23
〃	chloroform-methanol (10 : 1)	0.30	0.90	0.90	0.50
〃	benzene-methanol (10 : 1)	0.06	0.19	0.57	0.06
〃	water	0.70	0	0	0
MN-Cellulose powder 300	water	0.66	0	0	0

(3 : 2), 0.30 with chloroform - methanol (10 : 1), 0.05 with benzene - methanol (10 : 1) and 0.70 with water. On thin-layer chromatography using aluminium oxide (Acidic, M. WOELM), aquayamycin remains at origin with methanol or with ethyl acetate. On thin-layer chromatographies using MN-cellulose powder 300 (MACHEREY and NAGEL), it gives Rf values of 0.66 with water, 0.98 with ethyl acetate - chloroform (3 : 2) and 0.95 with water-saturated ethyl acetate. It remains at the origin on high voltage electrophoresis (3300 V, 1.5 mA/cm, 15 minutes) using Toyo filter paper No. 51 and buffer consisting of formic acid - acetic acid - water (25 : 75 : 900).

A comparison of Rf values of aquayamycin, ayamycin A_2, TA-435 A and julimycin B-II[6] on thin-layer chromatographies in various solvent systems is shown in Table 1.

Biological Activity of Aquayamycin

As shown in Table 2, aquayamycin inhibits Gram positive bacteria except mycobacteria.

When 6.25, 12.5, 25 and 50 mcg/mouse of aquayamycin per day were administered daily to mice inoculated with EHRLICH ascites tumor for 10 days, prolongation of the survival period was observed at doses of more than 12.5 mcg per day. Aquayamycin inhibits multiplication of YOSHIDA rat sarcoma cells in tissue-culture as described by HORI *et al.*[7] At concentrations of 100, 10 and 1 mcg/ml of aquayamycin, inhibition of 85.2, 85.2 and 51.9 % was observed respectively.

Table 2. Antimicrobial spectrum of aquayamycin (Agar dilution method)

Organisms	Minimum inhibitory concentration (mcg/ml)
Sarcina lutea PCI 1001	3.12
Staphylococcus aureus FDA 209-P	12.5
S. aureus TERAJIMA	6.25
S. aureus 193	12.5
S. aureus 52-34	12.5
S. aureus actinomycin resistant	12.5
Bacillus subtilis NRRL B-558	25.0
Bacillus subtilis PCI 219	25.0
Bacillus anthracis	12.5
Escherichia coli NIHJ	50
Proteus vulgaris OX-19	100
Shigella flexneri 1 a, Ew 8	50
Salmonella enteritidis	50
Mycobacterium 607	100
Candida albicans 3147	100
Micrococcus flavus M-16	6.25
Pseudomonas aeruginosa A 3	100
Klebsiella pneumoniae PCI 602	25

Acute toxicity, LD_{50} in mice by intravenous injection was 12.5~25 mg/kg. Mice which were injected with lethal doses showed comatose-like state and died on the next day.

Detailed studies on the biological effects of aquayamycin will be published in another paper.

Discussion

Color reactions and spectroscopic properties of aquayamycin described above are characteristic for a hydroxyquinone structure. Many colored antibiotics which have been shown to have hydroxyquinone structures and are produced by actinomycetes contain nitrogen. Aquayamycin contains no nitrogen. Aquayamycin, therefore, was compared with known antibiotics lacking nitrogen and having similar ultraviolet spectra. As shown by the data in Table 1, aquayamycin is differentiated from ayamycin A_2, TA-435 A and julimycin B-II by the behaviors on thin-layer chromatography. The differences are also shown in physical and chemical properties, such as infrared spectra

and elemental analyses. Though the description of ayamycin A_1 and A_3 is insufficient, aquayamycin can be differentiated from these antibiotics by the differences in the region of 9.5~11 μ of the infrared spectra and from A_1 also by the ultraviolet spectra[2]. On paper chromatography ayamycin B has been described to move with the solvent front and it is differentiated from aquayamycin[1]. We could not find antibiotics identical with ayamycin A_2, TA-435 A or julimycin B-II in cultured broth of the aquayamycin-producing strain. We also could not find other antibiotic pigments in the cultured broth of this strain. Thus, it is certain that aquayamycin is a new antibiotic. The aquayamycin-producing strain can be differentiated from known species of streptomyces.

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