DENAMYCIN, A NEW ANTIBIOTIC

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Denamycin is a new antibiotic obtained from the cultured filtrate and mycelium of *Streptomyces* sp. No. 8756-CC_2. It is purified on silica gel column chromatography followed by crystallization from organic solvents. It shows inhibitive activities against the nucleic acid syntheses in the bacterial cells and against the growth of ascites or solid form of tumor in mice. It forms yellow needles melting at 226~228°C, and has an optical rotation of $[\alpha]_D^{20}$ +41° in methanol. The antibiotic shows ultraviolet absorption maximum at 311 mμ ($E_{1cm}^{1\%}$ 520) in ethanol, and is very unstable and degraded into several compounds under the atmospheric condition and ultraviolet light.

In the course of screening studies of search for the inhibitory substance of nucleic acid biosyntheses, the strains of *Streptomyces* whose cultured broth did not show antibacterial inhibition zones by the usual cup plate assay method of antibiotics were selected. The inhibitory effect of these cultured broths on the biosyntheses of DNA and RNA and on the bacterial growth was measured using *Bacillus subtilis* Marburg strain 168 (thymine-less, indole-less) according to the method described by TANAKA and others[1].

The antibiotic was first detected in a cultured broth of *Streptomyces* sp. No. 8756-CC_2, which exhibited no antimicrobial inhibition zones on a usual cup plate method, but inhibited the incorporations of ^{3}H-thymine and ^{14}C-uracil into the bacterial cells of *Bacillus subtilis* 168 and the growth of the EHRLICH ascites carcinoma in mice. The antibiotic named denamycin was extracted with organic solvents from the filtrate and mycelium, and was purified by column chromatography and recrystallization. The isolation and characterization and biological properties of denamycin are described in the present paper.

Producing Organism

Streptomyces sp. No. 8756-CC_2, the denamycin-producing strain was isolated from a soil sample collected at Bōzu-chō, Kawabe-gun, Kagoshima, Japan.

Aerial mycelium elevates spherically, forms guttation drops, and its color is brownish gray to reddish gray. The soluble pigment is brownish black. Six different types of colony were observed on agar media by the single colony isolation. Four types of them counterchanged one another, but the remaining two types were not changed. The details of physiological differences and the antibiotic-producing abilities

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of these six different types will be reported in another paper.

Production of Denamycin

Streptomyces No. 8756-CC_2 was grown on a slant of BENNETT's agar medium for five days at 27°C. Spores from the slant culture were incubated by shaking for 48 hours at 27°C in a medium for seed culture consisting of 1.0 % glycerin and 1.0 % meat extract. The antibiotic was produced in shake-flask fermentation in a medium composed of 1.5 % glucose, 1.0 % glycerin, 1.0 % meat extract, 1.0 % peptone and 0.04 % $CaCO_3$, pH adjusted at 7.2 before sterilization. Maximum yield was obtained by incubation for 72 hours at 27°C on a rotary shaker in 500-ml flasks containing 100 ml of the medium and 2 ml of the seed culture.

The large scale fermentation was attempted to produce the antibiotic in jar fermentor. It was difficult to obtain a good yield in usual cultural conditions on account of its instability to aeration. A successful result was obtained as follows: Six hundred ml of the seed culture was inoculated to the 30-liter jar fermentor containing 15 liters of the production medium. Aeration was maintained for 24 hours at a rate of 1/1 vol/min with 400 rpm agitation at 27°C. Thereafter, aeration was decreased to a rate of 0.5/1 vol/min with agitation of 200~300 rpm at the same temperature. Maximum potency of the antibiotic was obtained after 72 hours.

Assay Methods of Denamycin

A method described by TANAKA and others[1] has been applied to determine the inhbitivei activity of denamycin against the bacterial growth and the nucleic acid synthesis.

The *Bacillus subtilis* Marburg strain 168, a thymineless and indoleless strain, was inoculated into Penassay broth (17.5 g/liter) containing 2.5×10^{-5} M EDTA and incubated at 37°C by shaking until the optical density reached 0.3. The above cell suspension (0.2 ml) was transferred to shaking tube containing 0.8 ml of minimal medium M9[1] supplemented with 0.5 % Casamino acids, 2.5×10^{-5} M EDTA, 45 μc ^{3}H-thymine and 0.004 μc ^{14}C-uracil, and then 0.5 ml of cultured broth or sample solution to be tested was added. The bacterial growth was measured by optical density, and the radioactivity of dried doubly labeled cell on the Millipore filter was counted by scintillation counter.

The paper disk agar diffusion method can be applied to determine the quantities of denamycin with *Bacillus subtilis* 168 as test organism at high concentrations of more than 100 mcg/ml.

Denamycin gave purple color when it was sprayed with ferric chloride solution (10 % in ethanol) on the plates of thin-layer chromatograms. This test was used together with the microbiological methods to detect the active fractions during purification processes of the substance.

Isolation and Purification of Denamycin

Twenty liters of the cultured broth (pH 7.6) were filtered by centrifugal machine to give filtrate and mycelial cake. The moist mycelium was steeped in 5 liters of

80 % aqueous acetone for one night. The extracted aqueous acetone was concentrated *in vacuo* until acetone was removed. One liter of the aqueous residue was extracted twice with equal volumes of ethyl acetate. In addition, the filtrate of cultured broth was extracted twice with equal volumes of ethyl acetate. The combined extracted ethyl acetate solution was concentrated *in vacuo* to an oily residue. Twenty grams of oily residue was dissolved in 150 ml of chloroform and introduced on silica gel column (4 cm × 50 cm, Wako gel C 300), followed by development with the mixed solvent of chloroform and acetone at a volume ratio of 20 : 1. The active eluates were collected and concentrated to dryness. Five grams of brownish yellow solid was dissolved in 100 ml of chloroform and introduced on the same column and developed with the same mixed solvent system. Two grams of active yellow solid was obtained, it was rechromatographed on column (2.5 cm × 40 cm) of Mallinckrodt Silicic acid (100 mesh) in the same manner as above. The active eluate was concentrated to small volume and poured into *n*-hexane. The resulting yellowish crystals were recrystallized with the mixed solvent of acetone-hexane at a ratio of 1 : 10. Thirty milligrams of yellowish fine needle crystals of denamycin were obtained.

Physical and Chemical Properties of Denamycin

Denamycin occurs as yellow fine needle crystals having a weakly acidic property probably due to a phenolic hydroxy group and melts at 226~228°C.

It is moderately soluble in methanol, ethanol, acetone, ethylacetate, benzene, chloroform, carbon tetrachloride and ether, while insoluble in hexane and water. It is stable over a wide pH range (pH 2~9) at 100°C for 5 minutes in aqueous solution, but very unstable for ultraviolet irradiation and air oxidation.

Elementary analysis of denamycin gave the results of C 68.26, H 8.71, O 22.70 %. Mass spectrometry was applied to determine the molecular weight of denamycin, although the observed maximum peak at m/e 502 did not seem to be molecular ion peak from its isotope ratio. When the N.M.R. spectrum of denamycin was taken by

Fig. 1. Visible and ultraviolet absorption spectra of denamycin

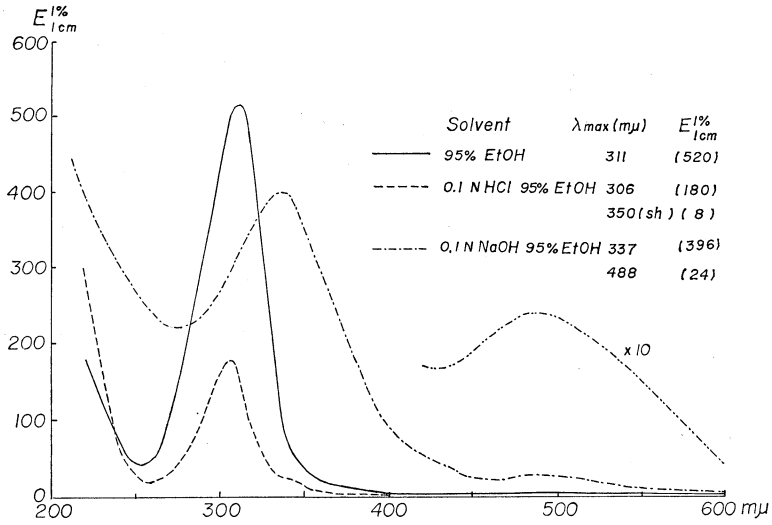
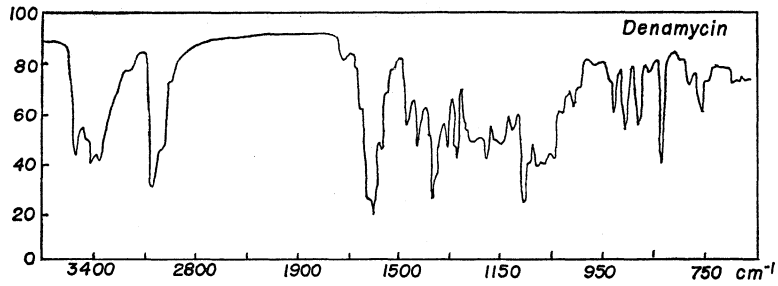


Fig. 2. Infrared absorption spectrum of denamycin (KBr)



accumulation technique, a singlet of methoxy group was assigned at 3.35 ppm. The quantitation of the methoxy group of denamycin chemically gave the value of 5.53 %. From the results of these data the molecular weight of denamycin is suggested to be more than 502.

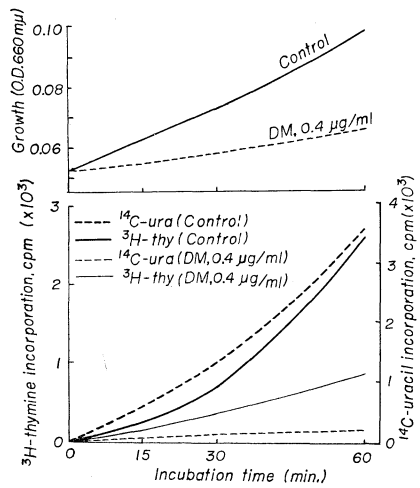
Denamycin shows negative reactions with TOLLENS, FEHLING, and ninhydrin, and positive reactions with LEMIUX, ferric chloride, LIEBERMANN, MILLON, diazo and sodium nitroprusside. The following color reactions may suggest the presence of the quinoid structure: color change from yellow in acidic solution to reddish-purple in alkaline solution; positive reactions with rhodamine and ammonia, and with 1-phenyl-3-methylpyrazol-5-one.

Optical rotation of denamycin is $[\alpha]_D^{20}$ +41° (*c* 1, methanol). Ultraviolet absorption spectra of denamycin shows maxima at 311 mμ ($E_{1cm}^{1\%}$ 520) in 95 % aqueous ethanol, 306 mμ ($E_{1cm}^{1\%}$ 180) and a shoulder at 350 mμ ($E_{1cm}^{1\%}$ 8) in acidic aqueous ethanol, 337 mμ ($E_{1cm}^{1\%}$ 396) and 488 mμ ($E_{1cm}^{1\%}$ 24) in alkaline aqueous ethanol, respectively, as shown in Fig. 1.

The infrared absorption spectrum in KBr tablet is shown in Fig. 2, which shows characteristic bands at 3550 cm^{-1} corresponding to hydroxyl group; 3440 cm^{-1} and 3370 cm^{-1} to phenolic hydroxyl group; 1715, 1645 and 1610 cm^{-1} to carbonyl.

The catalytic hydrogenation of denamycin was carried out in methanol with platinum dioxide at atmospheric pressure. It consumed a reasonable amount of hydrogen, and the product was also unstable to ultraviolet light and aeration.

Fig. 3. Time courses of ^{3}H-thymine and ^{14}C-uracil incorporations into *B. subtilis* 168 cells and the bacterial growth in the presence of 0.4 μg/ml of denamycin



Biological Properties of Denamycin

A method described by TANAKA and others[1] has been applied to test the effects of denamycin on the bacterial growth and on the nucleic acid syntheses of *Bacillus subtilis* 168. The results are shown in Fig. 3. Denamycin inhibited about 93 % of the incorporation of ^{14}C-uracil into the

bacterial cells at a concentration of 0.4 mcg/ml, whereas the inhibition of cell growth at that time was about 66%. From these results denamycin seems to be a stronger inhibitor of RNA synthesis rather than that of DNA synthesis in the cell of *B. subtilis* Marburg strain 168.

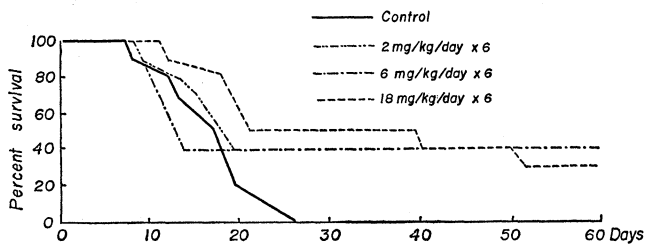
The tumor cell system in mice was used to determine the antitumor activity of denamycin which may show some parallelism with the inhibitory effects of nucleic acid biosyntheses. EHRLICH ascites or solid types of tumor were used for the antitumor tests using ten or seven mice in each test group.

Table 1. Effect of denamycin on EHRLICH solid tumor

Sample	Dose (mg/kg)		Weight difference (g)	Survivors	Tumor weight (mg)	T/C (%)*
	Daily	Total				
Denamycin	5	30	−0.37	7/7	252±48	66
	10	60	−0.47	7/7	198±68	52
	20	120	−1.50	7/7	147±14	38
Control	—	—	+0.75	7/7	308±60	100

* $T/C = \frac{\text{treated tumor weight}}{\text{control tumor weight}} \times 100$

Fig. 4. Effect of denamycin on EHRLICH ascites tumor



About two million cells of EHRLICH carcinoma were inoculated into mice intraperitoneally or subcutaneously 24 hours before beginning administration of the antibiotic. The results are shown in Fig. 4 and Table 1. In the case of intraperitoneal treatments of ascites cells, denamycin prolonged the survival periods of tumor-bearing mice more than two months, whereas most of the control mice died within three weeks. On the other hand, in the case of solid form of tumor the surviving mice were sacrificed after nine days and grown tumors were weighed

Table 2. Antimicrobial spectrum of denamycin (1)

Test organism	M. I. C. (mcg/ml)
Aerobacter aerogenes	>100
Escherichia coli	>100
Klebsiella pneumoniae	>100
Pseudomonas fluorescens	>100
Pseudomonas solanacearum	100
Serratia marcescens	>100
Serratia marcescens var. *kilensis*	>100
Shigella sonnei	25
Xanthomonas oryzae	>100
Bacillus subtilis	50
Bacillus subtilis 168 (thymine⁻, indole⁻)	0.4*
Bacillus cereus	25
Bacillus circulans	25
Corynebacterium xerosis	100
Sarcina lutea	>100
Staphylococcus aureus	50
Mycobacterium phlei	>100
Mycobacterium smegmatis	>100

* liquid dilution method

Table 3. Antimicrobial spectrum of denamycin (2)

Test organism	M. I. C. (mcg/ml)
Candida utilis	>100
Cryptococcus neoformans	>100
Rhodotorula glutinis	>100
Saccharomyces cerevisiae	>100
Alternaria kikuchiana	>100
Aspergillus oryzae	100
Botrytis cinerea	100
Botrytis fabae	>100
Cladosporium fulvum	>100
Fusarium lini	>100
Gibberella fujikuroi	>100
Glomerella cingulata	>100
Helminthosporium sesamum	100
Mucor ramannianus	>100
Ophiobolus miyabeanus	>100
Penicillium chrysogenum	100
Piricularia oryzae	100
Trichophyton mentagrophytes	100

to determine the T/C values (Table 1). The resulting average T/C values was observed in the range of 38 % to 66 %.

The toxicity of denamycin in mice was determined by intraperitoneal injection with emulsion containing 0.2 % carboxymethyl cellulose. Administration of 200 mg/kg of denamycin showed no toxic reaction for 2 weeks.

Antimicrobial activities of denamycin were examined by the agar streak dilution method. The results are shown in Tables 2 and 3. Denamycin exhibits the activity against gram-positive bacteria such as *Bacillus subtilis*, *Bacillus cereus* at the concentration of 25~50 mcg/ml, and weak activity against fungi, *Aspergillus oryzae*, *Botrytis cinerea*, *Helminthosporium sesamum*, *Penicillium chrysogenum* and *Piricularia oryzae* at the concentration of 100 mcg/ml. Gram-negative bacteria and yeast were not inhibited at 100 mcg/ml.

Acknowledgement

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Reference

1) TANAKA, T.; K. SAKAGUCHI, N. ŌTAKE & H. YONEHARA : An improved screening method for inhibition of nucleic acid synthesis. Agr. Biol. Chem. 32 : 100~103, 1968