STUDIES ON A NEW NUCLEOSIDE ANTIBIOTIC, DAPIRAMICIN

I. PRODUCING ORGANISM, ASSAY METHOD AND FERMENTATION

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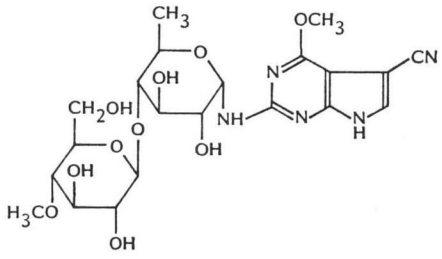
A new antibiotic, dapiramicin, has been isolated from the fermentation broth of *Micromonospora* sp. SF-1917. Since the dapiramicin exhibited essentially no *in vitro* activity by usual bioassays, two assay methods for dapiramicin were developed, HPLC and a new bioassay by growth inhibition of fungal mycelia on a slide glass. Fermentation of dapiramicin is also described.

In the course of our screening for new agricultural antibiotics, a novel nucleoside antibiotic named dapiramicin[1] (Fig. 1) was isolated from the culture filtrate of *Micromonospora* sp. SF-1917. The antibiotic was highly effective in the control of sheath blight, a destructive disease of rice plants caused by *Rhizoctonia solani*, in a pot test. In this paper, taxonomy of the producing organism, assay methods, and fermentation of the antibiotic are described. Isolation, physico-chemical and biological characterization will be described in subsequent papers[2,3].

Fig. 1. Structure of dapiramicin.



Characterization of the Producing Microorganism

The producing microorganism, strain SF-1917 was isolated from a soil sample collected at Nachi-Katsuura, Wakayama Prefecture, Japan. For the taxonomic characterization, the methods of the International Streptomyces Project (ISP)[4] were used, with additional media recommended by WAKSMAN[5].

Strain SF-1917 shows the following morphological characteristics. Vegetative mycelia are well developed and branched. Formation of true aerial mycelium is not observed on any of the agar media used. Spores are borne singly from the vegetative mycelia, usually on short sporophores but sometimes sessile. By electron microscopy the spores are spherical or oval in shape with smooth surface, and about 0.6~0.7 μm in diameter (Fig. 2).

Cultural characteristics of strain SF-1917 are summarized in Table 1. Physiological properties of strain SF-1917 are as follows: starch hydrolysis, nitrate reduction, gelatin liquefaction, milk peptonization and coagulation are positive, but production of melanoid pigment is negative. Strain SF-1917

Fig. 2. Scanning electron micrograph of spores of strain SF-1917.

a) ×3,000 b) ×10,000

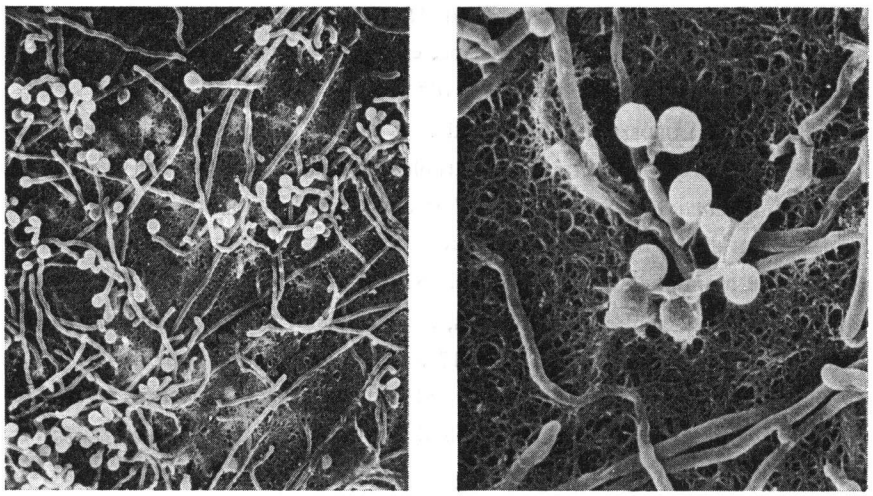


Table 1. Cultural characteristics of strain SF-1917.

Medium	Growth	Color of colony	Soluble pigment
Sucrose - nitrate agar	Moderate ~ good, flat	Light tan (3gc) ~ cork tan (4ie)	None
Glucose - asparagine agar	Moderate, flat	Dark covert gray (2ih)	None
Glycerol - asparagine agar (ISP medium 5)	Poor, flat	Light tan (3gc) turning black	None
Inorganic salts - starch agar (ISP medium 4)	Moderate ~ good, flat	Light brown (4ng) ~ beige brown (3ig) turning black	None
Oatmeal agar (ISP medium 3)	Moderate, flat	Beige brown (3ig) ~ beaver (3li)	None
Yeast extract - malt extract agar (ISP medium 2)	Moderate, wrinkled	Light orange (4ia) ~ beaver (3li) turning black	None
Tyrosine agar (ISP medium 7)	Poor	Pearl pink (3ca) turning black	Light grayish pink
Nutrient agar	Moderate	Light tan (3gc)	None
Bennett agar	Good, elevated, wrinkled	Cinnamon (3le) turning beaver (3li)	None
Potato plug	Good, elevated	Orange (3la) turning black	None

( ): Color number designations taken from Color Harmony Manual, 4th ed., Container Corporation of America, Chicago, 1958.

is aerobic and shows good growth at 26 ~ 34°C in a pH range of 6.4 ~ 8.4.

Utilization of carbon sources was performed by the method of LUEDEMANN[6]. L-Arabinose, D-fructose, D-glucose, D-melibiose, sucrose and D-xylose are utilized for growth, but cellulose, glycerol, *i*-inositol, D-mannitol, raffinose and L-rhamnose are not utilized. *meso*-Diaminopimelic acid was detected in the whole-cell hydrolysates.

From the taxonomic studies described above, strain SF-1917 was assigned to the genus Micromonospora Ørskov 1923. Differentiation of many species of the genus Micromonospora has been discussed by several workers[7~10], but no systematic method has been established to unequivocally

classify them. According to the criteria of Micromonospora taxonomy by SVESHNIKOVA[8], strain SF-1917 belonged to the *fusca-chalcea* group, based on mycelial pigments. *Micromonospora aurantiaca, M. carbonacea, M. chalcea, M. halophytica* and *M. purpureochromogenes*[11] belong to this group. However, as pointed out by SVESHNIKOVA, the species of this group including strain SF-1917 are very much alike and difficult to distinguish from each other.

On the other hand, LUEDEMANN[6] proposed that carbohydrate utilization was useful for characterizing species of Micromonospora. The carbohydrate utilization pattern of strain SF-1917 resembles that of *M. carbonacea*. Strain SF-1917 is also similar to *M. carbonacea* in production of mycelial pigments, but differs from it in sporulation morphology. Sympodial hyphae are observed in *M. carbonacea*[12] and monopodial hyphae in strain SF-1917.

Considering the above-mentioned properties, it was difficult to identify strain SF-1917 to species Therefore, we tentatively assigned strain SF-1917 to *Micromonospora* sp. SF-1917 until a systematic method for classification is established. Strain SF-1917 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession number of FERM-P 3559.

Assay Methods of Dapiramicin

Although dapiramicin showed good *in vivo* activity against the sheath blight of rice plant caused by *Rhizoctonia solani*, it was not detected by the usual agar-diffusion method using various bacteria and fungi as test organisms, because a clear inhibitory zone was not observed on agar plates. Therefore, the content of dapiramicin in the fermentation broth was assayed by the use of high performance liquid chromatography (HPLC) and a novel bioassay measuring the growth inhibition of fungal mycelia on slide glass (GIM).

HPLC Assay

For the HPLC assay of dapiramicin produced in the fermentation broth, the broth was pretreated as follows. The broth filtrate (10 ml) was adsorbed on a small column (2 ml) of Diaion HP-20 (Mitsubishi Chemical Industries, Ltd.). The column was washed with water (6 ml) and the antibiotic adsorbed on the resin was eluted with 10 ml of 50% aqueous ethanol. Each 20 μl of the eluate was injected to a column of μBondapak C_{18} (ID 3.9 mm $\times$ 30 cm, Waters Associates, Inc.). For analysis, a Shimadzu LC-3A instrument equipped with a UV detector at 254 nm was used. The mobile phase was a mixture of methanol, acetonitrile and water (25: 5: 70) and was flowed 0.6 ml per minute. The HPLC assay of dapiramicin in the fermentation broth is shown in Fig. 3. Dapiramicin showed retention time of 13.4 minutes, and was detectable less than 1 μg/

Fig. 3. HPLC assay of dapiramicin in fermentation broth.

Sample : Diaion HP-20 eluate of fermentation broth
Column : μBondapak C_{18} ID 3.9 mm $\times$ 30 cm
Solvent : MeOH - CH_3CN - H_2O (25: 5: 70)
Flow rate: 0.6 ml/minute
Detection: UV 254 nm

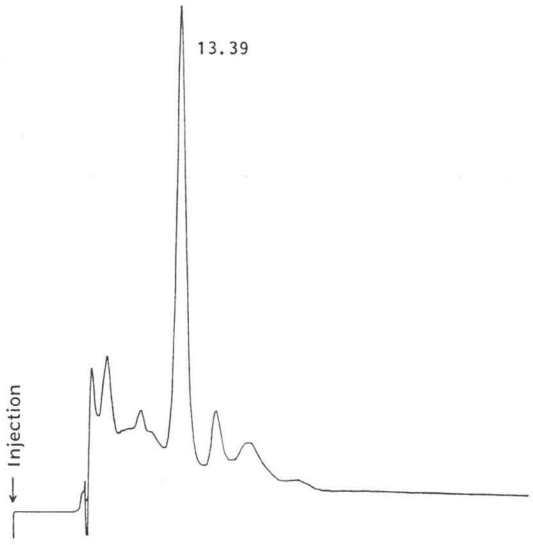
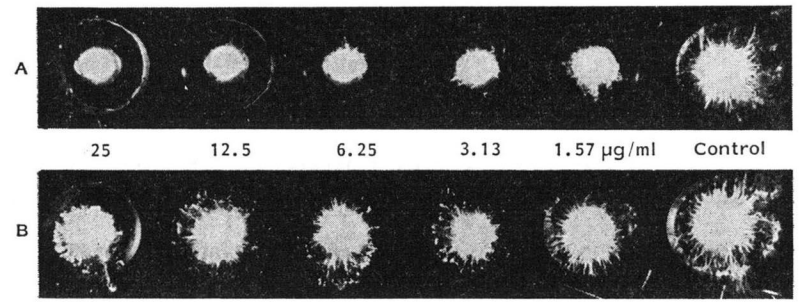


Fig. 4. Growth inhibition of mycelia of *R. solani* on slide glass.
A: Dapiramicin, B: validamycin.



ml in the fermentation broth by this HPLC assay method.

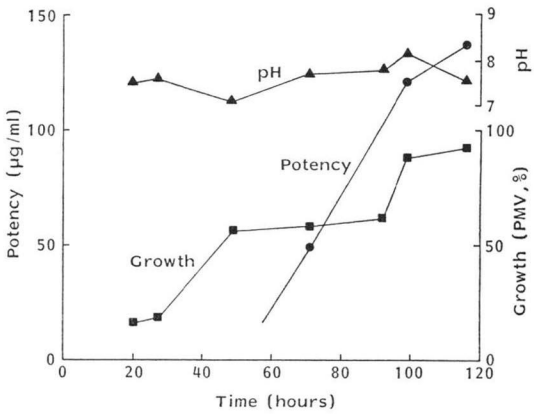
Growth Inhibition of Fungal Mycelia (GIM) Assay

Preincubated agar disks of the *Rhizoctonia solani* were put on a slide glass with sample solution in water of various concentrations, and incubated at 28°C for 15 hours in a moisture chamber. The concentration of dapiramicin in the sample was determined by comparing the minimum concentration of growth inhibition of mycelia of a sample with that of the standard solution of dapiramicin. A comparison of growth inhibition of mycelia between dapiramicin and validamycin[13~15] is shown in Fig. 4. Growth of mycelia was completely inhibited by 6.25 μg/ml of dapiramicin but was only slightly suppressed by validamycin in this method. The GIM assay method was the most sensitive bioassay for dapiramicin.

Fermentation of Dapiramicin

A well-grown agar slant of strain SF-1917 was inoculated into 20 ml of a seed culture medium consisting of 1.0% soluble starch, 1.0% glucose, 0.5% peptone, 0.3% yeast extract, 0.2% meat extract, 0.2% soybean meal and 0.1% $CaCO_3$ (pH 7.0) in a 100-ml Erlenmeyer flask. The inoculated flask was shaken on a rotary shaker (220 rpm) at 28°C for 3 days. Four milliliters of the first seed were inoculated into 80 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 28°C for 3 days, 30 ml of the second seed was transferred to 600 ml of the same medium in a 5-liter Erlenmeyer flask. It was shaken at 28°C for 3 days. The third seed culture (600 ml) was added to a 30-liter jar fermentor containing 20 liters of the following production medium: 4.0% maltose syrup, 0.3% soybean oil, 2.0% soybean meal, 0.5% distiller's solubles, 1.0% Pharmamedia, 0.3% $CaCO_3$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $NiCl_2 \cdot 6H_2O$ and 0 0001% $CoCl_2 \cdot 6H_2O$ in tap water. The medium was adjusted to pH 7 before sterilization. Silicon KM68-2F (Shin-Etsu Chemical Co., Ltd.) was used as an antifoam agent. The fermentation was carried out at 28°C with an air-flow rate of 20 liters per minute and an agitation rate of 270 rpm. The growth was measured as packed mycelia volume (PMV, %), and the antibiotic titer was assayed by HPLC and GIM.

Fig. 5 Time course of production of dapiramicin.



A typical time course of dapiramicin pro-

duction in a 30-liter fermentor is shown in Fig. 5. The production of dapiramicin started at about 50 hours after inoculation and reached over 130 μg/ml at 120 hours after inoculation.

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