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143. Tomoharu Okuda, Kokichi Ashino, Yoshiyuki Egawa, and Makoto Suzuki: Studies on Streptomyces Antibiotic, Cycloheximide. I. Isolation of Naramycin-A and its Identification with Cycloheximide

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In the course of the search for new antibiotics, a brownish gray, sporulating Streptomyces sp., designated TW 305-a, was isolated in this laboratory from a soil sample from Nara Prefecture in October, 1954. This culture produces two antifungal antibiotics, named Naramycin-A and -B, which have been isolated in crystalline form from the broth in which this organism was grown.

In the present paper, the authors would like to report on the taxonomic studies of Streptomyces TW 305-a, the isolation of Naramycin-A, and its identification with cycloheximide (Acti-dione). The isolation of Naramycin-B together with its physical and chemical properties will be reported in succeeding papers.

I. Taxonomic Studies on Streptomyces TW 305-a

Characters of the Naramycin-producing strain, Str. TW 305-a, on various media were studied. The mycelium, growing in agar substrate, is hyaline and branched. The aerial mycelium develops on most of media, and gives rise to sporophores which bear cylindrical conidia in chains. The aerial mycelium is white, later turns brownish gray, and numerous open spirals are observed. The cultural and physiological characters of this culture are listed in Tables I and II.

TABLE I. Cultural Characteristics of Streptomyces TW 305-a

Morphology: Branching mycelium; numerous spirals; spores cylindrical

Medium (Temp., °C)	Growth	Aerial mycelium	Soluble pigment
Synthetic agar (27)	Colorless, thin, spreading, colonies growing into medium, later turns brownish.	Brownish gray	None
Glucose-asparagine agar (27)	—ditto—	Brownish gray to gray with white patches, abundant, thick	None
Starch agar (27)	—ditto—	Brownish gray	None
Ca malate agar (27)	White to cream colored, glossy	Very scant, white to gray	None
Tyrosine agar (27)	Sand color, glossy, restricted	Brownish gray on a few portion of the surface	None
Plain agar (27)	Same as synthetic agar	Brownish gray	None
Nutrient agar (38)	Colorless to cream, glossy, slightly wrinkled	None	None
Yeast extract agar (38)	Whitish to light brownish gray, thick, spreading, elevated, glossy on the surface	Brownish gray	None
Blood agar (38)	Yellowish to dark brown, much wrinkled	None	None
Blood serum (38)	Cream colored, glossy, mycoid-like	None	None
Egg medium (38)	Pale yellow, glossy, much wrinkled	None	None
Gelatine (38)	Poor growth on the surface, cream-colored, flaky sediment on the bottom of the liquefied portion	None	None

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Potato plug (38)	Grayish to dark brown, much wrinkled	White to gray, thick velvety	Color of plug unchanged, black narrow zone appeared around the colony (15 days)
Carrot plug (38)	Dark brown, spreading, wrinkled	Whitish-gray, later turns dark brown with white patches	Color of plug unchanged
Synthetic solution (27)	Flaky sediment on the bottom of the tube, no growth on the surface	None	None
Glucose broth (38)	Colorless flaky sediment on the bottom of the tube (15 days), later surface ring, cream-colored (30 days)	Scant, white	None
Milk (38)	Poor growth on the surface, bulky sediment on the bottom of the tube	None	None

TABLE II. Physiological Properties of Streptomyces TW 305-a

1. Proteolytic action	Haemolysis: Positive Serum: Positive and strong Gelatine: Positive, 1 cm. in 30 days Milk: Rapid coagulation, followed by distinct peptonization
2. Effect on reaction	Milk: Unchanged (15 days), Alkaline (30 days). Glucose-bouillon: Alkaline (30 days)
3. Starch hydrolysis	Strong (35 mm. 12.5 mm.)
4. Nitrite production	Positive
5. Cellulose decomposition	None (filter paper method)
6. Tyrosinase production	Negative

The utilization of carbon sources studied by the method described by Pridham and Gottlieb[1] is shown in Table III. This culture utilizes most of hydrocarbons tested except dulcitol, mannitol, and inulin.

TABLE III. Utilization of Carbon Compounds

Xylose	++	Rhamnose	++	Raffinose	+	Arabinose	+
Lactose	++	Salicin	+	Mannitol	−	Sucrose	++
Inositol	+	Glucose	++	Maltose	+	Mannose	+
Glycerol	+	Dextrin	+	Fructose	++	Starch	++
Galactose	+	Sorbitol	++	Dulcitol	−	Inulin	−
Na acetate	+	Na citrate	+	Na succinate	+		

Note: 1. Synthetic agar except carbohydrate was used as the basal medium.
2. Observed on 10th day.

This strain does not produce any antibacterial antibiotic in the culture.

The classification of this culture was made after the keys described in Bergy's Manual.[2] From the morphological aspect, this strain belongs to the 3rd groups of Streptomyces (spiral formation in aerial mycelium; long, open spirals) and from cultural properties, to saprophytic, non-thermophilic, and non-chromogenic types.

1) T. G. Pridham, D. Gottlieb: J. Bacteriol., **56**, 107 (1948).
2) R. S. Breed, E. G. D. Murray, N. R. Smith: "Bergy's Manual of Determinative Bacteriology," 7th Ed., Williams & Wilkins Co., Baltimore, 744 (1957).

Among the Streptomyces described in the Bergy's Manual, *Str. griseolus*, *Str. fasciculus*, *Str. griseus*, and *Str. longisporoflavus* resemble Str. TW 305-a in some cultural properties. As reported by Leach, *et al.*[3] and by others, *Str. griseus* is a representative strain which produces cycloheximide as a by-product of streptomycin. In addition to *Str. griseus*, *Str. noursei*[4] and Str. No. ETH 7796[5] are also said to produce cycloheximide.

In the distinctive character of Str. TW 305-a in forming numerous open spirals on the synthetic agar, this species is distinguished from *Str. griseolus*, *Str. fasciculus*, *Str. griseus*, and Str. ETH 7796. As emphasized by Pridham, *et al.*[6] and as discussed in the recent symposium on the classification of Streptomycetes,[7] morphological characteristics of sporulating hyphae are the primary guide to classification or grouping of the Streptomycetes. Besides this characteristic, following differences are to be noted; *Str. griseolus* produces gray to pallid, neutral gray aerial mycelium on synthetic agar and gives thick, brown ring with abundant ash-gray aerial mycelium on the surface of glucose broth. *Str. griseus* produces water-green aerial mycelium on conventional media. Furthermore, it is not reported that *Str. griseus* produces cycloheximide as a sole antibiotic produced. Str. ETH 7796 produces greenish to yellowish pigment in synthetic and protein media. *Str. fasciculus* has a strong ability to decompose cellulose for the growth, but its ability to reduce nitrate is limited.

Str. longisporoflavus and *Str. noursei* form typical or atypical spirals, but *Str. noursei* produces shell-pink aerial mycelium on the glucose-asparagine agar, and purple to pomegranate-purple aerial mycelium on some media. *Str. longisporoflavus* most closely resembles TW 305-a in morphological and cultural characteristics in that the former produces long open spirals with cylindrical spores and gives yellowish vegetative mycelia which produce yellow to brownish yellow aerial mycelia, but the physiological properties differ from that of Str. TW 305-a, especially in its weak ability to hydrolyze starch and slow power of peptonizing milk.

Consequently, this strain was considered to be a new species of Streptomycetes and it was proposed to name it *Streptomyces naraensis* nov. sp. after the place where it was found.

II. Isolation and Identification of Naramycin-A

From the fermentation broth in which this strain was grown, active component was concentrated by means of solvent-extraction method as illustrated in Chart 1 or by carbon-adsorption procedure. By treating the enriched concentrates with warm isoamyl acetate, Naramycin-A predominatingly crystallized out. Crude Naramycin-A was purified by decolorizing with activated alumina (H-form) and by repeated recrystallization from isoamyl acetate.

Naramycin-A comes as colorless, optically active crystals melting at 116~116.5°, and has a formula $C_{15}H_{23}O_4N$. From the physical and chemical studies of Naramycin-A it was found to be identical with cycloheximide (Acti-dione) reported by Leach, *et al.*[3] Details are given in the Experimental Section.

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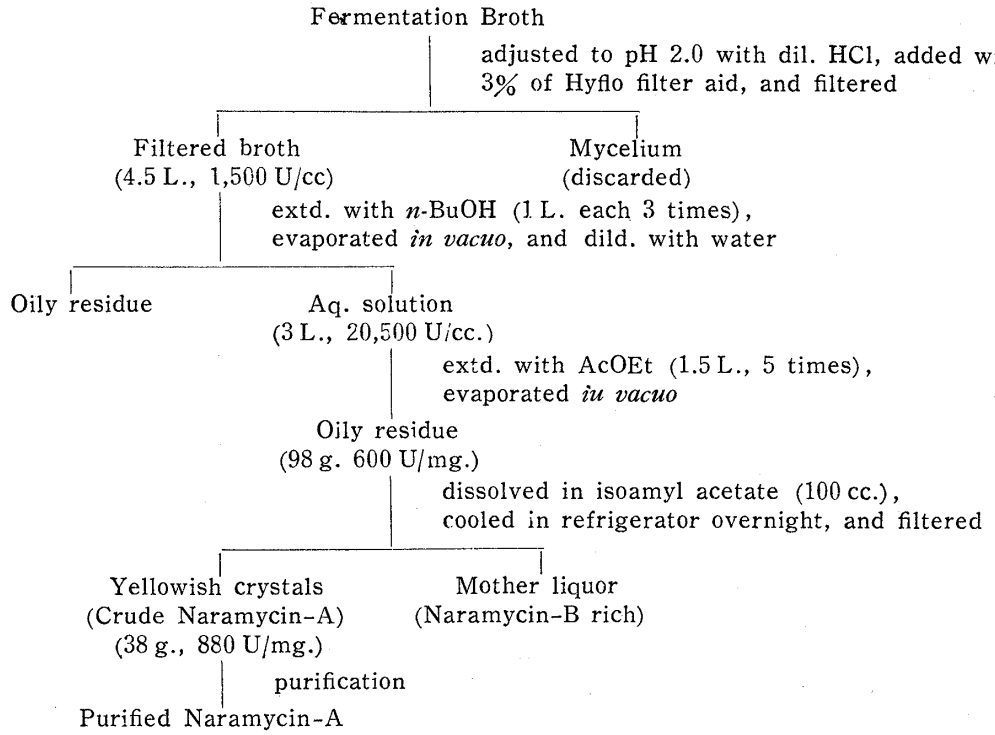
3) F. E. Leach, J. H. Ford, A. F. Whiffen: J. Am. Chem. Soc., **69**, 474(1947).
4) R. Brown, E. L. Hazen: "Antibiotic Annual," 1955~1956, 245.
5) R. Corbaz, L. Ettlinger, E. Gäumann, W. Keller-Schierlein, F. Kradolfer, L. Neipp, V. Prelog, H. Zähner: Helv. Chim. Acta, **38**, 1445(1955).
6) T. G. Pridham, C. W. Hesseltine, R. G. Benedict: Appl. Microbiol., **6**, 52(1958).
7) Abstract of Papers, Meeting of the Japanese Agricultural Chemical Society, 109(1958).

Chart I. Isolation of Naramycin-A



Experimental

(All m.p.s are not corrected)

Culture and Assay Methods—Antibiotics assay followed the familiar two-dose, cup-plate, agar diffusion method (ratio of doses, 4 : 1), using Naramycin-A (m.p. 116~116.5°) as a standard. *Saccharomyces sake* was employed as the test organism. No difference was observed between the activities of Naramycin-A and authentic Acti-dione (Upjohn Co. Ltd.; m.p. 115.5~116°.).

Streptomyces naraensis was grown at 28° in shaking cultures, using the medium consisting of 3% glycerol, 1% meat extract, 1% peptone, 0.5% NaCl, and 0.35% $CaCO_3$ (pH. 7.0), when the culture gave the yield as high as 1000~1500 γ/cc. Details of cultural conditions will be published in another paper.

Isolation of Naramycin-A—Isolation of Naramycin-A from the fermentation broth was easily carried out as illustrated in Fig. 1 by modifying the procedure reported by Leach, *et al.*[3] and others. Naramycin—A crystallized out from isoamyl acetate solution of concentrated antibiotics.

Purification of Naramycin-A—Thirty-eight grams of crude Naramycin-A (880 γ/mg.), dissolved in 80 cc. of benzene containing 3% of MeOH was poured into a column filled with 40 g. of activated alumina (H-form) and the column was developed with the same solvent. The eluate was collected

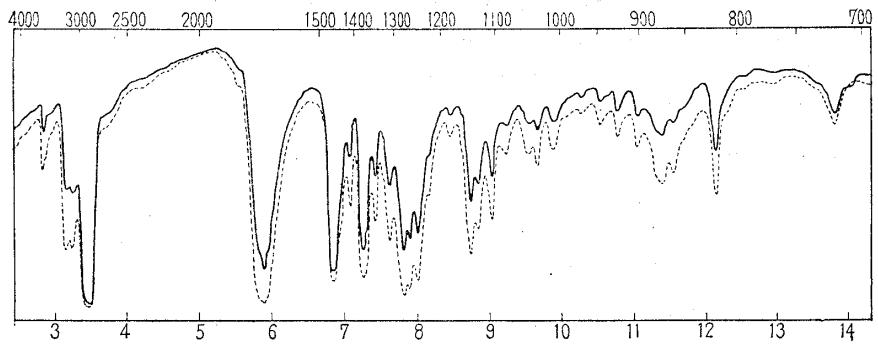


Fig. 1. Infrared Spectra of Naramycin-A and Acti-dione (in Nujol Mulls)

———: Naramycin-A
----------: Acti-dione

in 100-cc. fractions. The first 300 cc. of the eluate, which contained 93% of activity, was evaporated *in vacuo*. Colorless syrup thus obtained was crystallized from isoamyl acetate. After three recrystallizations from isoamyl acetate, 30 g. of colorless needles were obtained; m.p. 116~116.5°, $[\alpha]_D^9$ +8° (c=2, H_2O). This sample showed no depression in m.p. on admixture with the authentic sample of Acti-dione (Upjohn Co. Ltd.) and their infrared spectra agreed well as illustrated in Fig. 1. *Anal.* Calcd. for $C_{15}H_{23}O_4N$: C, 64.02; H, 8.24; N, 4.98. Found: C, 64.30; H, 8.46; N, 5.02.

Derivatives of Naramycin-A—For further studies and for the proof of identification, following compounds were derived from Naramycin—A and compared with those derived from authentic Acti-dione. Each procedure was carried out as described in the literature.[8,9]

Naramycin-A acetate: Colorless plates, m.p. 147~147.5°; $[\alpha]_D^{12.5}$ +24.6° (c=2, dehyd. MeOH).

Dehydronaramycin-A: Colorless leaflets, m.p. 176~177°.

Anhydronaramycin-A: Colorless prisms, m.p. 133~134°.

These products showed no depression in m.p. on admixture with the authentic samples derived from Acti-dione.

Anhydronaramycin-A was obtained by treating Naramycin-A with BF_3-ether complex in dehyd. benzene containing a small amount of glacial AcOH, instead of treating Naramycin-A with P_2O_5. The product melted at 134.5~135.5°, showing no depression on admixture with Anhydroacti-dione.

Summary

From the fermentation broth of *Streptomyces naraensis nov. sp.* two antiyeast antibiotics, named Naramycin-A and -B, were obtained. Of these antibiotics, Naramycin-A was identified with cycloheximide (Acti-dione) reported by Leach, *et al.* Taxonomic studies of *Streptomyces naraensis* were described.

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8) J. Ford, B. E. Leach; J. Am. Chem. Soc., **70**, 1223 (1948).
9) E. C. Kornfeld, R. G. Jones, T. V. Parke: *Ibid.*, **71**, 150 (1949).