NEW ANTIBIOTIC, ISOHEMATINIC ACID

I. TAXONOMY OF PRODUCING ORGANISM, FERMENTATION AND ISOLATION

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New antibiotic, isohematinic acid, was found in the culture broth of an actinomycete strain SANK 61681, which was identified as a strain of *Actinoplanes philippinensis*. Fermentation of isohematinic acid was performed by conventional submerged culture in a 600-liter fermentor. Isolation of isohematinic acid was performed by adsorption of the antibiotic from the culture filtrate on a column of Diaion HP-20 followed by elution with aqueous acetone and extraction with ethyl acetate. Isohematinic acid was finally crystallized from hot methanol.

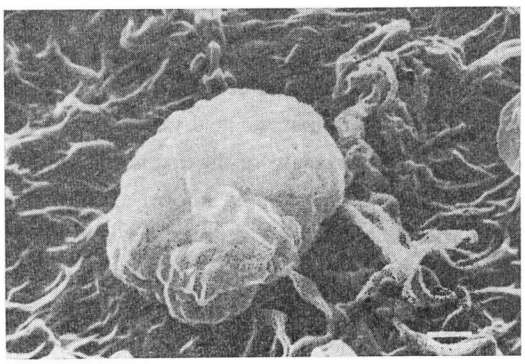
Isohematinic acid was obtained from the culture broth of strain SANK 61681 isolated from a soil sample collected at Shioya-gun, Tochigi Prefecture, Japan. Taxonomic studies on the producing organism revealed its identity as *Actinoplanes philippinensis*, and physico-chemical properties as well as biological characterization of the antibiotic showed it to be different from known antibiotics.

The present paper deals with the taxonomy of the producing organism, fermentation and isolation of the antibiotic. Physico-chemical properties, structural elucidation and biological activities of isohematinic acid will be presented in the subsequent paper.

Taxonomic Studies of Strain SANK 61681

Morphological and physiological properties of strain SANK 61681 were determined by media and methods described by SHIRLING and GOTTLIEB[1] and those recommended by WAKSMAN[2] along with several supplementary tests. Observations of the cultures were made after incubation at 28°C for 2 weeks unless otherwise stated. Color names were assigned according to "Guide to Color Standard" (Nippon Shikisai Kenkyusho, Tokyo, Japan). The characteristics of strain SANK 61681 were compared with those of the known species of actinomycetes described in "The Actinomycetes, Vol. 2" by WAKSMAN[2], "BERGEY's Manual of Determinative Bacteriology (8th ed.)"[3] and other recent literature concerning taxonomy of the family *Actinoplanaceae*[4].

Plate 1. Scanning electron micrograph of sporangia of strain SANK 61681 (Potato extract - carrot extract agar, 28°C, 14 days, A mark equals 1 μm).



Strain SANK 61681 formed sporangia abundantly only on potato extract - carrot extract agar. The sporangia were spherical to subspherical in shape and 5×16 μm in size as shown in plate 1. The surface of the sporangia was

Table 1. Cultural characteristics of strain SANK 61681.

Yeast extract - malt extract agar (ISP 2)	G	: Abundant, raised, ridged, yellowish orange
	AM	: None
	SP	: None
	SPG	: None
Oatmeal agar (ISP 3)	G	: Abundant, raised, ridged, yellowish orange
	AM	: None
	SP	: None
	SPG	: None
Inorganic salts - starch agar (ISP 4)	G	: Abundant, raised, wrinkled, dull yellowish orange
	AM	: None
	SP	: None
	SPG	: None
Glycerol - asparagine agar (ISP 5)	G	: Moderate, flat, pale orange
	AM	: None
	SP	: None
	SPG	: None
Tyrosine agar (ISP 7)	G	: Abundant, raised, wrinkled, light brown
	AM	: None
	SP	: Light brown
	SPG	: None
Sucrose - nitrate agar	G	: Moderate, flat, pale brown
	AM	: None
	SP	: None
	SPG	: None
Glucose - asparagine agar	G	: Moderate, flat, brownish white
	AM	: None
	SP	: None
	SPG	: None
Nutrient agar (Difco)	G	: Moderate, flat, yellowish brown
	AM	: None
	SP	: None
	SPG	: None
Water agar	G	: Poor, flat, yellowish brown
	AM	: None
	SP	: None
	SPG	: None
Potato extract - carrot extract agar	G	: Moderate, flat, yellowish brown
	AM	: None
	SP	: None
	SPG	: Scant
Soil extract agar	G	: Poor, flat, pale yellowish brown
	AM	: None
	SP	: None
	SPG	: None

G: Growth. AM: Aerial mycelium. SP: Soluble pigment. SPG: Sporangium.

smooth. An agar plug cut from typical mature growth was placed on a slide glass and one drop of sterilized distilled water was dropped on the surface of mycelium. After putting a cover glass on it, motility of the spores was observed with a light microscope. The zoospores with polar flagella were observed and were spherical to rod shape and $0.8 \sim 1.5 \times 1.4 \sim 2.0$ μm in size.

The cultural characteristics of strain SANK 61681 on various media are shown in Table 1. The development of aerial mycelium was not observed on the majority of media tested. The color of the substrate mycelium was usually yellowish orange to yellowish brown and a soluble pigment, light brown in color, was detected only in tyrosine agar (ISP 7). Physiological properties and utilization of carbon sources are summarized in Tables 2 and 3, respectively. Cell wall analysis of strain SANK 61681 was performed by the method described by BECKER *et al.*[5] and *meso*-diaminopimelic acid and glycine but not hydroxydiaminopimelic acid were detected as major constituents. This is in accordance with cell wall type II. The results of the taxonomic studies mentioned above show that SANK 61681 belongs to genus *Actinoplanes*. Among known species of genus *Actinoplanes*[6], characteristics of strain SANK 61681 are closely related to those of *Actinoplanes philippinensis*[3,7] except utilization of inositol and casein decomposition. Strain SANK 61681 did not utilize inositol nor decompose casein while *A. philippinensis* did. These differences were not sufficient to consider strain SANK 61681 as a new species. It was, therefore, concluded that strain SANK 61681 belonged to the species *A. philippinensis* and it was de-

Table 2. Physiological properties of strain SANK 61681.

Nitrate reduction	negative
Starch hydrolysis	positive
Gelatin liquefaction	positive
Milk peptonization	negative
Milk coagulation	negative
Melanin formation*	negative
Casein decomposition	negative
Tyrosine decomposition	negative
Xanthine decomposition	negative
Growth temperature range**	9.5~33°C
Optimum temperature range**	17.0~31.5°C

* Tryptone - yeast extract broth (ISP 1)
Peptone - yeast extract - iron agar (ISP 6)
Tyrosine agar (ISP 7)
** Yeast extract - malt extract agar (ISP 2)

Table 3. Carbon utilization pattern of strain SANK 61681.

D-Glucose	+	D-Mannose	+
L-Arabinose	+	Sucrose	+
D-Xylose	+	Raffinose	±
D-Fructose	+	D-Mannitol	+
Inositol	−	Salicin	+
L-Rhamnose	+	Cellulose	−
D-Galactose	+	Control	−

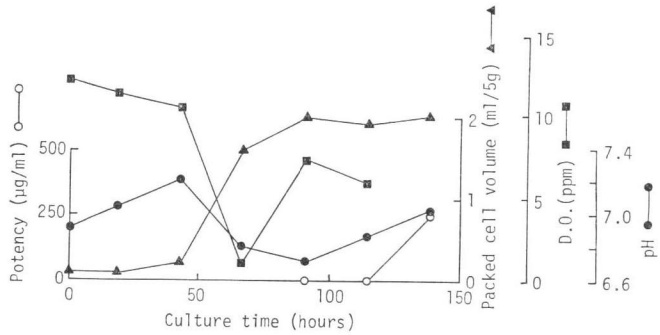
+: Positive utilization.
±: Weakly positive utilization.
−: Negative utilization.

signated as *A. philippinensis* SANK 61681. Progeny of the type strain of *A. philippinensis* SANK 61681 have been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaragi Prefecture, Japan with accession number of FERM P-6045.

Fermentation

Strain SANK 61681 was maintained on agar slants of a yeast extract - malt extract medium. One loopful of mycelial growth of strain SANK 61681 on the agar slant was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of the medium composed of glucose 1.0%, glycerol 1.0%, sucrose 1.0%, pressed yeast 1.0%, soybean meal 2.0%, oatmeal 0.5%, Casamino Acids 0.5%, $CaCO_3$ 0.1% and Nissan Disfoam CB-442 (Nissan Chemical Co., Ltd., Japan) 0.01%. The pH of the medium was adjusted to pH 7.0 before sterilization. The flasks were incubated on a rotary shaker at 28°C for 120 hours. A 35 ml aliquot of the culture from the Erlenmeyer flask was inoculated into several 2-liter Erlenmeyer flasks each containing 700 ml of the medium described above. The flasks were incubated at 28°C for 48 hours as a seed culture. After inoculation of 3 liters of the seed culture into a 600-liter fermentor containing 300 liters of a medium composed of glucose 2.0%, soluble starch 1.0%, pressed yeast 0.9%, meat extract 0.5%, Polypeptone 0.5%, NaCl 0.5%, $CaCO_3$ 0.3% and Nissan Disfoam CB-442 0.01%. Fermentation was carried out for 139 hours with agitation (190 rpm) and aeration (150 liters/minute) at 28°C. Mycelial growth was expressed as the packed cell volume (ml) after centrifugation of 5 g of the culture broth at 3,000 rpm for 10 minutes. Antibiotic production during fermentation was monitored by

Fig. 1. Fermentation of isohematinic acid in 600-liter fermentor.



paper-disc agar diffusion method using *Bacteroides fragilis* SANK 71176 as the test organism. An example of a typical time course of the fermentation in 600-liter fermentor is shown in Fig. 1. The maximal potency of the antibiotic, approximately 250 μg/ml, was obtained after 139 hours of fermentation.

Isolation

The culture broth from a 600-liter fermentor was filtered with the aid of infusorial earth (Celite 545, John-Manville Products Co., U.S.A.). The filtrate (350 liters) thus obtained was adjusted to pH 3.0 with diluted hydrochloric acid and further filtered with the aid of Celite 545. The filtrate (350 liters, pH 3.0) was adsorbed on a column of 60 liters of Diaion HP-20 (Mitsubishi Chemical Ind. Ltd.). The column was washed with 180 liters of deionized water and the antibiotic was eluted with 30% aqueous acetone. The active fraction (120 liters) was concentrated under reduced pressure to 4-liters. A 400-g aliquot of NaCl was added to the concentrate and the antibiotic was extracted twice with 4-liter batches of ethyl acetate. The extracts were pooled and concentrated to 300 ml under reduced pressure. The concentrate was applied to a column consisting of 2 liters of Sephadex LH-20 equilibrated with a solvent mixture composed of chloroform - ethyl acetate (1: 1 v/v) and the column was developed further with the same solvent system. The eluate was collected in 500 ml fractions and fractions No. 11 through No. 15 were pooled and the solvent removed to yield 20.3 g of crude pale yellowish crystals of isohematinic acid. The crude crystals were recrystallized from hot methanol to give 8.1 g of isohematinic acid, mp 138°C, $C_8H_9NO_4$, as colorless crystals.

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