A NEW ANTIBIOTIC, OKICENONE

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL CHARACTERISTICS

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(Received for publication March 1, 1991)

A new antibiotic, okicenone was isolated from the culture broth of *Streptomyces* sp. KO-3599. The antibiotic possesses cytocidal activity against mammalian tumor cells *in vitro* at concentrations of 0.53~11.0 μg/ml whereas the antibiotic showed no antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi or yeast at a concentration of 1,000 μg/ml.

In the course of a screening program for novel antibiotics showing cytocidal activity, okicenone was isolated from the culture broth of *Streptomyces* sp. KO-3599 which had been isolated from a soil sample collected in Okinawa Prefecture, Japan. The antibiotic exhibited cytocidal activity against HeLa S_3 cells *in vitro*, but did not show any antimicrobial activity against bacteria, fungi and yeasts.

The present paper deals with the taxonomic studies of the producing strain, and the production, isolation and physico-chemical properties of the new antibiotic. The preliminarily biological activities of okicenone against HeLa S_3 cells and various microorganisms are also described.

Materials and Methods

General Experimental Procedures

Kieselgel 60 (Merck) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis.

Taxonomic Studies

Type of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[1].

To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media recommended by SHIRILING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for 2 weeks. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th Ed.)[4]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[5] containing 1% carbon source at 27°C.

Cytotoxic Activity Tests

HeLa S_3, B16 melanoma and H69 human lung carcinoma cells were maintained in monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and an antibiotic (60 μg/ml of kanamycin) at 37°C. Mouse leukemia P388 and P388 doxorubicin-resistant cells (P388/ADM^R) were maintained in static culture in the same medium.

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Table 1. Cultural characteristics of strain KO-3599.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract - malt extract agar[a]	G: Good, dark brown (5pn) R: Dark brown (5pn) AM: Abundant, gray (f) SP: None	Tyrosine agar[a]	G: Good, dark brown (4pn) R: Dark brown (4pn) AM: Poor, aqua gray (19dc) SP: Chestnut brown (4ni)
Oatmeal agar[a]	G: Moderate, yellow maple (3ng) R: Yellow maple (3ng) AM: Very poor, dusk pewter (15fe) SP: None	Sucrose - nitrate agar[b]	G: Good, camel (3ie) R: Yellow maple (3ng) AM: Very poor, white (a) SP: None
Inorganic salts - starch agar[a]	G: Good, deep brown (4pl) R: Deep brown (3pl) AM: Poor, aqua gray (19fe) SP: None	Glucose - nitrate agar[b]	G: Good, bamboo (2gc) R: Bamboo (2gc) AM: None SP: None
Glycerol - asparagine agar	G: Moderate, chestnut brown (4ni) R: Chestnut brown (4ni) AM: Very poor, aqua gray (19fe) SP: Chestnut brown (4ni)	Glycerol - calcium malate agar[b]	G: Good, light spice brown (4lg) R: Light spice brown (4lg) AM: Very poor, white (a) and gray (e) SP: Camel (3ie)
Glucose - asparagine agar	G: Moderate, chestnut brown (4ni) R: Chestnut brown (4ni) AM: Poor, aqua gray (19fe) SP: Chestnut brown (4ni)	Glucose - peptone agar[b]	G: Good, honey gold (2ic) R: Topaz (3ne) AM: Very poor, white (a) SP: Old gold (2le)
Peptone - yeast extract - iron agar[a]	G: Good, Beaver (3li) R: Light brown (3lg) AM: None SP: Deep brown (3pl)	Nutrient agar[b]	G: Good, bamboo (2gc) R: Bamboo (2gc) AM: None SP: None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.
Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

To determine the cytotoxicity of the test materials, cells in 200 μl of medium were plated in 96-well cultue plate (Falcon) and incubated for 24 hours at 37°C in a 5% CO_2 - 95% air atmosphere. To each well was added 5 μl of medium containing a different concentration of the test material. After 72 hours incubation, the cell growth was evaluated by the method of ALLEY *et al.*[6].

Antimicrobial Activity Test

The antimicrobial spectra of the test materials were determined using 6 mm paper discs (Toyo Seisakusho Co., Ltd.). Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeast were grown on potato - broth agar medium. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results and Discussion

Taxonomy of the Producing Strain KO-3599

The vegetative mycelia grow abundantly on both synthetic and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The aerial mycelia grow abundantly on yeast extract - malt extact agar, but poorly on other media. The mature sporophores formed spiral spore chains and had more than 20 spores per chain. The spores were oval in shape, 0.7×0.4 μm in size and had a spiny surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. KO-3599 grown on glucose-asparagine agar for 14 days.

Bar represents 1.0 μm.

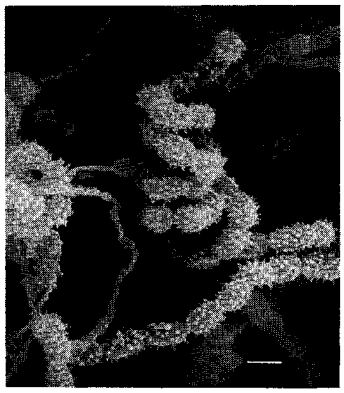

Table 2. Physiological properties of strain KO-3599.

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Liquefaction of gelatin (20°C)	+
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	15~42°C

+: Active, −: inactive.

Table 3. Utilization of carbon sources by strain KO-3599.

Utilized:	D-Glucose, D-fructose, L-rhamnose, D-mannitiol, *i*-inositol, L-arabinose, raffinose, D-xylose, sucrose, melibiose

The DAP in cell wall of strain KO-3599 was determined to be LL-type. The cultural characteristics and the utilization of carbon sources are shown in Tables 1, 2 and 3, respectively.

The strain exhibits the following properties. Sporophore, spirals; spores, oval and spiny surface; color of vegetative mycelia, brown or beige; color of aerial mycelia, gray or white; melanoid pigment are produced; DAP isomer in cell wall, LL-type.

Based on the taxonomic properties described above, strain KO-3599 is considered to belong to the genus *Streptomyces*[7]. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. KO-3599 and the accession No. is FERM P-10779.

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 100 ml seed medium consisting of starch 2%, soy bean meal 1%, NaCl 0.3% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). The flasks were inoculated at 27°C for 96 hours on a reciprocal shaker. Then 400 ml of the resulting culture were transferred to a 30-liter fermenter containing 20 liters of the same medium as described above. The fermentation was carried out at 27°C for 96 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters/minute.

The fermentation broth of *Streptomyces* sp. KO-3599 (20 liters) was extracted with EtOAc (18 liters) and the EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown syrup (21.7 g). This brown syrup was subjected to silica gel column chromatography (5.4 × 25 cm) using $CHCl_3$-CH_3OH as solvent. Fractions exhibiting cytocidal activity against HeLa S_3 cells were collected. Further separation of the active fractions (2.0 g) over silica gel column chromatographies eluted with $CHCl_3$-CH_3OH (9:1) and hexane-acetone (2:1) gave a crude fraction containing okicenone (**1**). Final purification with Sephadex LH-20 column chromatography (1.5 × 90 cm) eluted with CH_3OH afforded okicenone (**1**, 18.1 mg) as pale yellow needles.

Structure of Okicenone (**1**)

Studies on the structure elucidation of these antibiotics will be reported in a separate paper[8].

Fig. 2. Structure of okicenone (**1**).

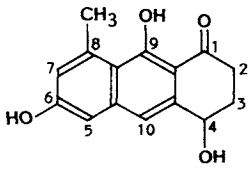

Table 4. Cytotoxicities of okicenone (**1**) against tumor cells.

Cultured cell	IC_{50} (μg/ml)
HeLa S_3	0.53
B16 melanoma	0.66
P388 leukemia	2.9
P388/ADM^R	11.0
H69 human lung carcinoma	>12.5

Biological Activity Tests of Okicenone (**1**)

Okicenone (**1**) showed no antimicrobial activities at the concentration of 1,000 μg/ml against *Xanthomonas oryzae* KB 88, *Candida albicans* KF 1, *Saccharomyces sake* KF 26, *Mucor racemosus* KF 223 (IFO 4581), *Piricularia oryzae* KF 180, *Aspergillus niger* KF 103 (ATCC 6275), *Staphylococcus aureus* KB 34 (FDA 209P), *Bacillus subtilis* KB 27 (PCI 219), *Escherichia coli* KB 8 (NIHJ), *E. coli* KB 176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB 105 (P3), *Micrococcus luteus* KB 40 (PCI 1001), *Bacteroides fragilis* KB 169, *Mycobacterium smegmatis* KB 42 (ATCC 607) and *Acholeplasma laidlawii* PG 8 KB 174.

Cytocidal activity of okicenone (**1**) was examined against mammalian tumor cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the IC_{50} values were 0.53~11.0 μg/ml as shown in Table 4. Among the known compounds structurally similar to okicenone, germichrysone[9,10] isolated from *Cassia occidentals* showed both cytotoxic and antitumor activity against mouse leukemia P388[11]. Okicenone (**1**) showed cytotoxic activity against various tumor cells in the present study. We are now expanding the biological evaluation of this antibiotic and the results will be reported elsewhere.

Acknowledgment

This work was supported in part by Grants-in-Aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and by funds from Japan Keirin Association. The authors would like to thank Mr. M. FURUKANE, the Kitasato Institute, for his assistance with the fermentation of *Streptomyces* sp. KO-3599.

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