NOVEL ANTIBIOTICS, FURAQUINOCINS A AND B

TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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Two novel antibiotics, furaquinocins A and B were isolated from the culture broth of *Streptomyces* sp. KO-3988. These antibiotics possess cytocidal activities against HeLa S3 cells *in vitro* at concentrations of 3.1 μg/ml for A and 1.6 μg/ml for B. Neither substance possessed 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 activities, a fraction of the fermentation broth of *Streptomyces* sp. KO-3988, which had been isolated from a soil sample collected in Shizuoka Prefecture, Japan, showed potent cytocidal activity. Two active components designated as furaquinocins A and B were obtained from the culture broth of this microorganism.

The present paper deals with the taxonomic studies of the producing strain, and the production, isolation and physico-chemical properties of the new antibiotics. The preliminarily biological activities of furaquinocins against HeLa S3 cells and various microorganisms are also described.

Materials and Methods

General Experimental Procedures

MP's were determined using a Yanagimoto MP-3 hot stage microscope and are uncorrected. UV spectra were recorded on a Shimadzu model UV-200S spectrophotometer. IR spectra were recorded on a Jasco model A-102 interferometer. Mass spectra were obtained with a Jeol model DX-300 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-400 instrument. Kieselgel 60 (Merck), Diaion HP-20 (Mitsubishi Chemical Industries) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis and for preparative TLC.

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 SHIRLING 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.

Anti HeLa S3 Tests

HeLa S3 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.

Table 1. Cultural characteristics of strain KO-3988.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar[a]	G: Very poor R: Colorless AM: None SP: None	Tyrosine agar[a]	G: Good, mustard brown (2ni) R: Mustard tan (2lg) AM: Good, white-natural (a-2dc) SP: Covert brown (2li)
Oatmeal agar[a]	G: Moderate, bamboo (2gc) R: Bamboo (2gc) AM: None SP: None	Sucrose-nitrate agar[b]	G: Moderate, alabaster tint (13ba) R: Alabaster tint (13ba) AM: Moderate, white-alabaster tint (a-13ba) SP: None
Inorganic salts-starch agar[a]	G: Good, pearl pink-nude tan (3ca-4ge) R: Pearl pink-light spice brown (3ca-4lg) AM: Moderate, white-beige gray (a-3ih) SP: Peach tan (5gc)	Glucose-nitrate agar[b]	G: Good, light tan (3gc) R: Camel (3ie) AM: None SP: Light tan (3gc)
Glycerol-asparagine agar	G: Good, nude tan (4gc) R: Nude tan (4gc) AM: Moderate (5cb) SP: Dusty peach (5ec)	Glycerol-calcium malate agar[b]	G: Good, camel (3ie) R: Camel (3ie) AM: None SP: Dusty coral (6gc)
Glucose-asparagine agar	G: Good, light tan (3gc) R: Light amber (3ic) AM: Very poor, white (a) SP: None	Glucose-peptone agar[b]	G: Good, cork tan-tile red (4ie-5ne) R: Henna (5pg) AM: None SP: Tile red (5ne)
Peptone-yeast extract-iron agar[a]	G: Moderate, natural (3dc) R: Natural (3dc) AM: None SP: Sepia brown (3pn)	Nutrient agar[b]	G: Moderate, bamboo (2gc) R: Bamboo (2gc) AM: None SP: Bamboo (2gc)

[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, HeLa S3 cells (5×10^4) in 2 ml of medium were plated in a 30-mm Petri dish and incubated for 48 hours at 37°C in a 5% CO_2-95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of the antibiotic. After 72 hours incubation, the HeLa S3 cells were trypsinized to form a single cell suspension. Cells were counted with a hemocytometer.

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-3988

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 tyrosine agar and sucrose-nitrate agar but grow poorly on other media. The mature sporophores were of the *Rectiflexibiles* type and had more than 20 spores per chain. The spores were cylindrical in shape and 1.4×0.9 μm in size. They had a smooth surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. KO-3988 grown on PRIDHAM and GOTTLIEB's medium containing 1% mannitol agar for 14 days.

Bar represents 1.0 μm.

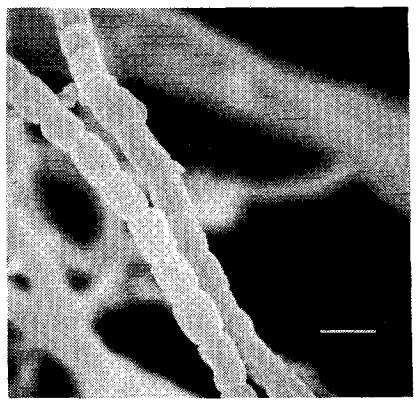


Table 2. Physiological properties of strain KO-3988.

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Liquefaction of gelatin (21~22°C)	−
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	15~35°C

+: Active, −: inactive.

Table 3. Utilization of carbon sources by strain KO-3988.

Utilized:	D-Glucose, D-fructose, D-mannitol, L-arabinose, *i*-inositol, D-xylose
Weakly utilized:	L-Rhamnose, melibiose
Not utilized:	Raffinose, sucrose

The cultural characteristics and the utilization of carbon sources of KO-3988 are shown in Tables 1, 2 and 3, respectively.

The strain exhibits the following properties. Sporophore, *Rectiflexibiles*; spores, cylindrical and smooth surface; color of vegetative mycelia, brown; color of aerial mycelia, white or gray; soluble pigment, brown; DAP isomer in cell wall, LL-type.

Based on the taxonomic properties described above, strain KO-3988 is considered to belong to the genus *Streptomyces*; and to be a strain of the gray series of the PRIDHAM and TRESNER's system[6]. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. KO-3988. The accession No. is FERM P-10369.

Fermentation and Isolation of the Active Components

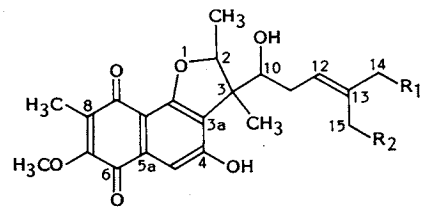
A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 100 ml seed medium consisting of starch 2%, soy bean meal 1%, NaCl 0.3% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). The flasks were inoculated at 27°C for 72 hours on a reciprocal shaker. Then 300 ml 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 72 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters/minute.

The culture broth of *Streptomyces* sp. KO-3988 (20 liters) was filtered and the mycelium mass was extracted with MeOH (20 liters). The MeOH extract was evaporated *in vacuo* to about 1 liter and the concentrated solution was extracted with EtOAc (1 liter). The filtrate of the culture broth was adsorbed on a chromatographic column of Diaion HP-20. The column was washed with water and eluted with 50 and 80% aqueous MeOH (each 2 liters) and then with 100% MeOH (2 liters).

The EtOAc layer of the MeOH extract of the mycelium and the 100% MeOH fraction of the Diaion HP-20 column, both of which showed cytocidal activity against HeLa S3 cells, were combined to give 9.7 g of residue after evaporation under reduced pressure.

This residue was subjected to a silica gel column chromatography (3.5 × 33 cm) eluted successively with $CHCl_3$, $CHCl_3$-MeOH (19:1), $CHCl_3$-MeOH (9:1) and MeOH (each 2 liters). The active fraction

eluting from 3 to 4 liters (4.1 g) was then separated by a second silica gel column chromatography (3.5 × 33 cm) with $CHCl_3$ (300 ml) and 1,500 ml of $CHCl_3$ - MeOH (19 : 1) to give a crude fraction containing furaquinocins A and B in the 450～800 ml fraction. The fraction was further separated by a third silica gel column chromatography (3.5 × 33 cm) with 2.7 liters of $CHCl_3$ - MeOH (19 : 1). The fraction eluting from 300 to 800 ml (390 mg) was purified by a Sephadex LH-20 column chromatography (1.5 × 21 cm) with MeOH to give furaquinocin A (**1**, 88 mg) as yellow needles. The fraction from the third silica gel column chromatography eluting from 1,200 to

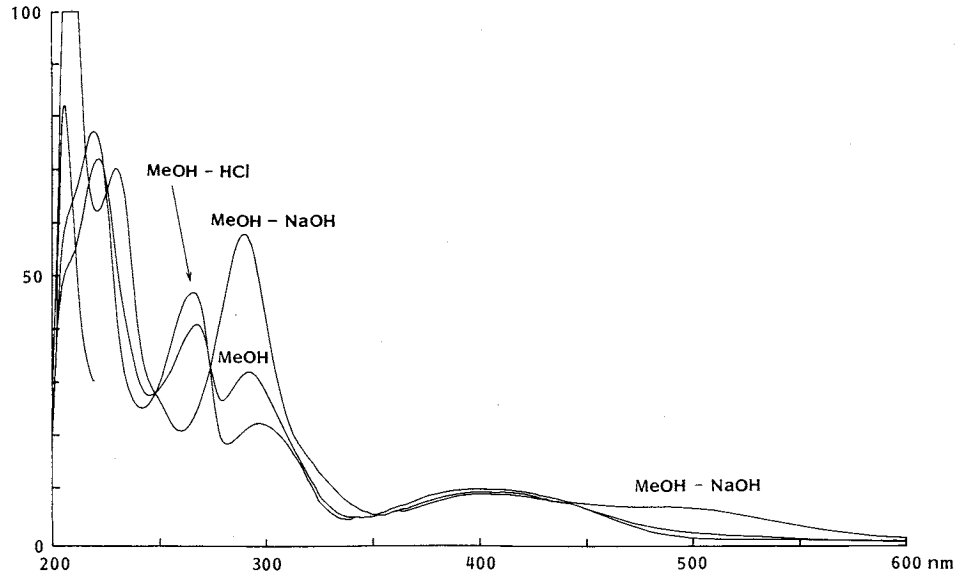


1 R_1=H R_2=OH
2 R_1=OH R_2=H

Table 4. Physico-chemical properties of furaquinocins A (**1**) and B (**2**).

	1	**2**
Appearance	Yellow needles	Yellow powder
MP (°C)	182～183	101～104
Optical rotation		
$[\alpha]_D^{19}$	−46° (*c* 0.58, $CHCl_3$)	−132° (*c* 0.57, $CHCl_3$)
TLC (silica gel)		
$CHCl_3$ - MeOH (100 : 1)	0.14	0.07
$CHCl_3$ - MeOH (19 : 1)	0.59	0.49
Benzene - acetone (2 : 1)	0.53	0.40
Molecular formula	$C_{22}H_{26}O_7$	$C_{22}H_{26}O_7$
MW	402	402
UV λ_{max}^{MeOH} nm	222, 268, 292, 408	223, 269, 294, 410
IR ν_{max} (KBr) (cm^{-1})	3450, 1672, 1643, 1381, 1115	3450, 1659, 1635, 1368
Color reaction		
Positive	50% $H_2SO_4+\Delta$, iodine, $FeCl_3$	50% $H_2SO_4+\Delta$, iodine, $FeCl_3$
Negative	Ninhydrin reagent, DRAGENDORFF's reagent	Ninhydrin reagent, DRAGENDORFF's reagent

Fig. 2. UV spectrum of furaquinocin A (**1**).



2,200 ml (337 mg) was purified by a Sephadex LH-20 (3.0×45 cm) with MeOH to afford furaquinocin B (**2**, 286 mg) as yellow powder.

Physico-chemical Properties of Furaquinocins A (**1**) and B (**2**)

The physico-chemical properties of furaquinocins A (**1**) and B (**2**) are summarized in Table 4. The UV and IR absorption spectra of **1** are shown in Figs. 2 and 3, respectively. These antibiotics are soluble in $CHCl_3$, EtOAc and MeOH but practically insoluble in H_2O. Furaquinocins A (**1**) and B (**2**) gave positive color reaction with iodine, 50% sulfuric acid and $FeCl_3$ and was negative to DRAGENDORFF's reagent and ninhydrin.

Fig. 3. IR spectrum of furaquinocin A (**1**).

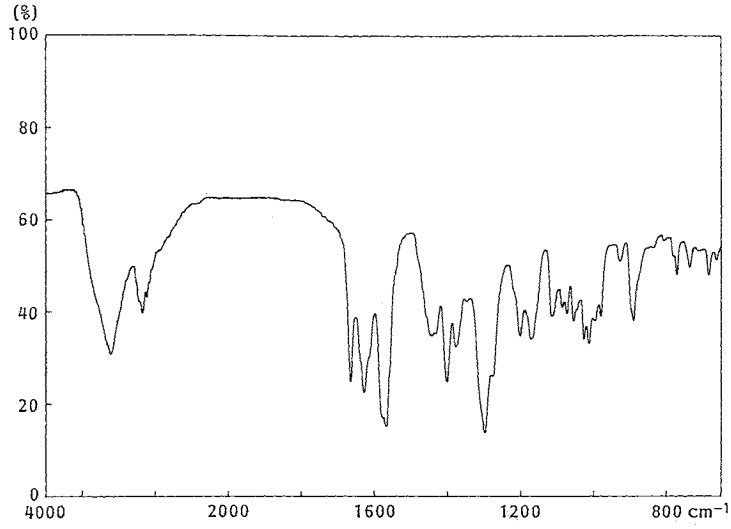


Fig. 4. ^1H NMR spectrum of furaquinocin A (**1**).

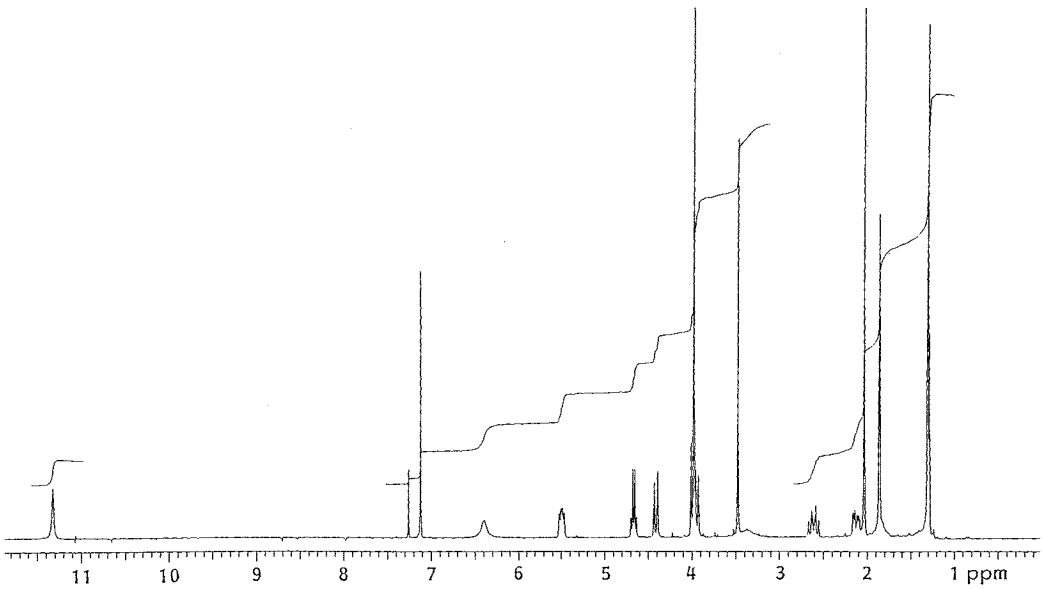
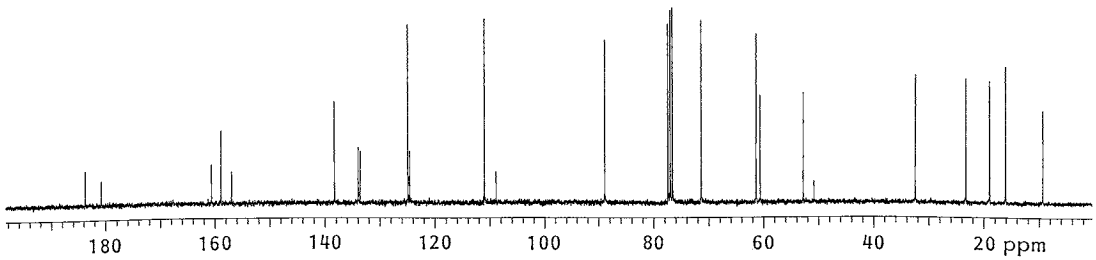


Fig. 5. ^{13}C NMR spectrum of furaquinocin A (**1**).



Studies on the structure elucidation of these antibiotics will be reported in a separate paper[7].

Biological Activity Tests of Furaquinocins A (**1**) and B (**2**)

Furaquinocins A (**1**) and B (**2**) 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 activities (MIC) of furaquinocins A (**1**) and B (**2**) against HeLa S3 cells are 3.1 and 1.6 μg/ml, respectively.

We are now investigating the biological activities of these compounds further and the results will be reported elsewhere.

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