NEW INHIBITORS OF MELANOGENESIS, OH-3984 K1 AND K2

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL CHARACTERISTICS

KANKI KOMIYAMA, SATOSHI TAKAMATSU, YŌKO TAKAHASHI, MAYUMI SHINOSE, MASAHIKO HAYASHI, HARUO TANAKA, YUZURU IWAI and SATOSHI ŌMURA*

The Kitasato Institute, and School of Pharmaceutical Sciences of Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

GENJI IMOKAWA

Kao Corporation, Biological Science Laboratories, 2606 Akabane, Ichikaimachi, Haga, Tochigi 321-34, Japan

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Melanogenesis inhibitors, OH-3984 K1 and K2 were isolated from fermentation broth of *Streptomyces* sp. OH-3984. OH-3984 K1 and K2 inhibited the melanogenesis of B16 melanoma cells at concentrations of 7.5 and 3.8 μg/ml, respectively, whereas inhibition of tyrosinase activity has not been observed. The microbial metabolites showed no antimicrobiological activities against Gram-positive and Gram-negative bacteria, fungi or yeast at a concentration of 1,000 μg/ml.

In our continuing search for melanin synthesis inhibitors, OH-3984 K1 (**1**) and K2 (**2**) were isolated from the culture broth of *Streptomyces* sp. OH-3984 which had been isolated from a soil sample, together with three known macrocyclic antibiotics, albocycline, 2,3-dihydroalbocycline and 2,3-dihydro-11-hydroxyalbocycline.

The present paper deals with taxonomic studies of the producing strain, fermentation and isolation of the new antibiotics. The preliminary biological activities of **1** and **2** against melanin synthesis of B16 melanoma cells are also described.

Materials and Methods

General Experimental Procedures

Kieselgel 60 (70~230 mesh, 230~400 mesh, Merck) was used for column chromatography and DC-Alufolien Kieselgel 60 F_{254} (Merck) was used for TLC analysis. Analytical HPLC was carried out with 60~70% MeOH-H_2O using a YMC-Pack R-ODS-5 (YMC Co., Ltd., 5 μm, i.d. 4.6×250 mm) column employing a UV monitoring system (220 nm) at a flow rate of 0.8~1.0 ml/minute. Preparative HPLC was performed using a YMC-Pack D-ODS-5 (5 μm, i.d. 20×250 mm) column with a solvent system of MeOH-H_2O (70:30) at 7 ml/minute.

Taxonomic Studies

The type of diaminopimelic acid (A_2pm) 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 from the Color Harmony Manuàl (4th Ed.)[4]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon at 27°C[5]. The morphological properties were observed with a scanning electron microscope (Model S-430, Hitachi Co., Ltd.).

Melanin Synthesis Inhibitory Activity Tests

Stock culture of B16 melanoma cells were suspended in a monolayer in EAGLE's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) and 0.1% glucosamine hydrochloride at 4.5×10^3 cells/ml. The cell suspension (6 ml) was poured into a 6-well culture plate (350 mm i.d., Corning) and incubated at 37°C in 5% CO_2 - 95% air atmosphere. After 5-days of incubation, each well was washed twice with HANK's solution (6 ml) and renewed with fresh medium containing 2 mM theophylline and a different concentration of the test materials. Then, after 3-days of incubation, the adherent cells were washed with phosphate-buffered saline (PBS, 1 ml) and scraped with a cell scraper (Coster). The cell suspension was centrifugated at 1,500 rpm for 10 minutes. The color and volume of resulting cells were compared with those of controls.

Tyrosinase Inhibitory Activity Tests

Inhibitory activities of OH-3984 K1 and K2 against tyrosinase were determined. The crude tyrosinase was prepared from cultured B16 melanoma cells by the method of AKIU *et al.*[6]. Briefly, after washing cultured B16 melanoma cells (5×10^7) with PBS, the cells were sonicated in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% Triton X-100. The cell homogenate was centrifuged at 11,000 × *g* for 20 minutes. The supernatant was used as an enzyme source for the assay.

Tyrosinase activity was measured by a slight modification of the method of POMERANTZ[7]. Experiments were carried out at 37°C with the following components: 0.5 ml of crude enzyme solution (600 μg protein/ml); 0.5 ml of 0.05% L-DOPA dissolved in 50 mM sodium phosphate buffer (pH 6.8) and 0.5 ml of a different concentration of an arbutin and the test sample, in a total volume of 1.5 ml. Optical density of 475 nm was measured at regular time intervals and the inhibitory activity was calculated from the change of the initial rate of O.D.

Antimicrobial Activity Tests

The antimicrobial spectra of the test materials were determined using 6 mm paper disks (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 of incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results and Discussion

Taxonomy of the Producing Strain OH-3984

The vegetative mycelia grew abundantly on both synthetic and complex agar media, and did not show fragmentation into coccoid or bacillary elements. The aerial mycelia grew abundantly on glucose - asparagine agar and inorganic salts - starch agar, but grew 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, 0.8×1.1 μm in size, and had a spiny surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

The cultural characteristics, physiological properties and the utilization of carbon sources of strain OH-3984 are shown in Tables 1, 2 and 3, respectively. The strain exhibited the following properties: spiral sporophores; oval spores with a spiny surface; ivory vegetative mycelia and white aerial mycelia. Melanoid pigment was produced and A_2pm isomer of the LL-type was present in the cell wall. Good utilization of most sugars was observed.

Based on the taxonomic properties described above, strain OH-3984 was considered to belong to the genus *Streptomyces*[8] and to be a strain of the white series of PRIDHAM and TRESNER's system[9]. The strain was deposited in the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OH-3984, the access number is FERMP-13068.

Table 1. Cultural characteristics of strain OH-3984.

Medium	Cultural characteristics		Medium	Cultural characteristics	
Yeast extract - malt extract agar[a]	G:	Good, bamboo (2gc)	Tyrosine agar[a]	G:	Moderate, light ivory (2ca) mustard brown (2ni)
	R:	Bright gold (2nc)		R:	Pearl pink (3ca), adobe brown (3lg)
	AM:	Abundant, alabaster tint (13ba)		AM:	Moderate, alabaster tint (13ba)
	SP:	None		SP:	Camel (3ie)
Oatmeal agar[a]	G:	Good, bamboo (2fb)	Sucrose - nitrate agar[b]	G:	Moderate, light wheat (2ea)
	R:	Light wheat (2ea)		R:	Light wheat (2ea)
	AM:	Abundant, alabaster tint (13ba)		AM:	Moderate, light ivory (2ca)
	SP:	None		SP:	None
Inorganic salts - starch agar[a]	G:	Good, bamboo (2gc)	Glucose - nitrate agar[b]	G:	Poor, light wheat (2ea)
	R:	Bright gold (2nc)		R:	Light wheat (2ea)
	AM:	Abundant, alabaster tint (13ba)		AM:	Poor
	SP:	None		SP:	None
Glycerol - asparagine agar	G:	Good, light ivory (2ca)	Glycerol - calcium malate agar[b]	G:	Moderate, light wheat (2ea)
	R:	Cream ($1\frac{1}{2}$ca)		R:	Light wheat (2ea)
	AM:	Moderate, alabaster tint (13ba)		AM:	Abundant, alabaster tint (13ba)
	SP:	None		SP:	None
Glucose - asparagine agar	G:	Good, light wheat (2ea)	Glucose - peptone agar[b]	G:	Moderate, bamboo (2gc)
	R:	Light mustard tan (2ia)		R:	Mustard gold (2ne)
	AM:	Abundant, alabaster tint (13ba)		AM:	Moderate, alabaster tint (13ba)
	SP:	None		SP:	None
Peptone - yeast extract - iron agar[a]	G:	Moderate, adobe brown (3lg)	Nutrient agar[b]	G:	Abundant, bamboo (2gc)
	R:	Adobe brown (3lg)		R:	Colonial yellow (2ga)
	AM:	Poor		AM:	Poor
	SP:	Yellow maple (3ng)		SP:	None

[a] Medium recommended by ISP.

[b] Medium recommended by S. A. WAKSMAN.

Abbreviations: G, growth of vegatative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. OH-3984 grown on inorganic salts-starch agar for 14 days.

Bar represents 1.0 μm.

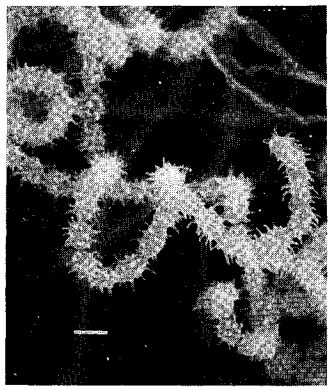


Table 2. Physiological properties of strain OH-3984.

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Reduction of nitrate	−
Liquefaction of gelatin (20°C)	−
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	12~46°C

+: Positive, −: negative.

Table 3. Utilization of carbon sources by strain OH-3984.

Utilized:	D-glucose, L-arabinose, D-xylose, raffinose, melibiose, D-mannitol, L-rhamnose, *i*-inositol, sucrose
Weakly utilized:	D-fructose

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a test tube (i.d. 2 × 20 cm) containing 10 ml of seed medium consisting of 2% glucose, 0.5% peptone, 0.3% dry yeast, 0.5% meat extract, 0.5% NaCl and 0.3% $CaCO_3$ (pH 7.0 before sterilization). The tube was incubated at 27°C for 72 hours on a reciprocal shaker. Then, 2 ml portions of the growth were transferred into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium. The flask was incubated at 27°C for 48 hours on a rotary shaker (210 rpm), and 400 ml of the resulting culture was transferred into a 50-liter fermentor containing 30 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 per minute.

The fermentation broth of *Streptomyces* sp. OH-3984 (30 liters) was extracted with EtOAc (25 liters) and the EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a brown syrup (7.8 g). This brown syrup was treated with *n*-hexane. The *n*-hexane soluble fraction (2.4 g) was subjected to silica gel column chromatography (70~240 mesh, i.d. 3 × 39 cm) using $CHCl_3$-$(CH_3)_2CO$ as the developing solvent. Fractions exhibiting melanin synthesis inhibitory activity on B16 melanoma cells were collected. Further separation of the active fractions (87 mg) by silica gel column chromatography (230~400 mesh, i.d. 1.5 × 13 cm) eluted with $CHCl_3$-EtOAc (9 : 1) and $CHCl_3$-$(CH_3)_2CO$ (9 : 1) gave rich fractions of **1** and **2**, respectively. Final isolations of **1** and **2** were performed using preparative HPLC. Pure **1** and **2** were obtained as colorless oil in yields of 36.6 mg and 92.7 mg, respectively.

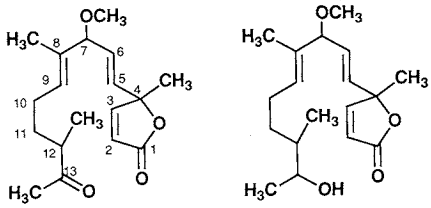
Structure of OH-3984 K1 (**1**) and K2 (**2**)

Structures of OH-3984 substances are shown in Fig. 2. Studies on the structural determination of these antibiotics will be reported in a separate paper.

Biological activity of OH-3984 K1 (**1**) and K2 (**2**)

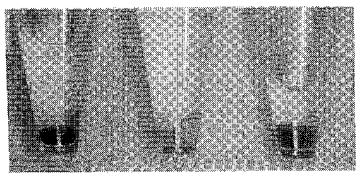
Antimicrobial activities of **1** and **2** against Gram-positive and Gram-negative bacteria, fungi or yeast were not observed at a concentration of 1,000 μg/ml (data not shown). Inhibitory effects of **1**, **2**,

Fig. 2. Structures of OH-3984 K1 (**1**) and K2 (**2**).



1 **2**

Fig. 3. Inhibitory effect of OH-3984 K1 on melanin synthesis of B16 melanoma cells.



Control 7.5 (μg/ml) 3.8 (μg/ml)

Table 4. Inhibitory effect on melanin synthesis of B16 melanoma cells.

Compound	MIC[a] (μg/ml)	Cytotoxicity[b] (μg/ml)
Arbutin	9.4	>100
Kojic acid	15.0	>100
Hydroquinone	0.94	0.94
OH-3984 K1 (**1**)	7.5	>100
OH-3984 K2 (**2**)	3.8	>100
Albocycline	0.12	0.24
2,3-Dihydroalbocycline	15	30
2,3-Dihydro-11-hydroxy-albocycline	30	60

[a] Minimal inhibitory concentration.
[b] Cell growth was suppressed at indicated concentration of the compound.

albocyclines, and known melanogenesis inhibitors on melanin synthesis of B16 melanoma cells are shown in Table 4. Compound **1** inhibited the melanogenesis of B16 melanoma cells with no inhibitory effect on cell growth at a concentration of 7.5 μg/ml (Fig. 3). Although albocyclines have been reported as antimicrobial agents, they inhibited melanogenesis of B16 melanoma cells at a low concentration of 0.12 μg/ml in the present experiment. However, albocyclines showed cytotoxicity on B16 melanoma cells at a concentration of approximately 2-fold the minimal inhibitory concentration for melanogenesis (Table 4).

In contrast, OH-3984 substances did not show any cytotoxicity at 100 μg/ml as in the cause of arbutin and kojic acid which are utilized clinically as a melanogenesis inhibitors. The inhibitory activity of OH-3984 substances on tyrosinase isolated from B16 melanoma cells was examined. Although the data are not given here, neither **1** nor **2** showed tyrosinase inhibitory activity at a concentration of 3.1 mg/ml, whereas arbutin used as a positive control showed inhibition at a concentration of 2.7 mg/ml.

There are many inhibitors of melanogenesis from chemical synthetic and natural sources, including hydroquinone, arbutin, kojic acid and placenta liquid[10], and these are known to possess tyrosinase inhibitory activity. Therefore, it is of interest to examine the mechanism of the inhibitory effect of **1** and **2** on melanogenesis.

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