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Takanawaenes, Novel Antifungal Antibiotics Produced by *Streptomyces* sp. K99-5278

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Three new pentaene macrolides having a 28-membered ring, designated takanawaenes A, B and C, were isolated from the fermentation broth of *Streptomyces* sp. K99-5278 by solvent extraction, silica-gel column chromatography and HPLC. Takanawaenes showed antifungal activity against *Aspergillus niger*, *Mucor racemosus*, *Candida albicans* and *Saccharomyces cerevisiae*.

During our screening for novel bioactive compounds of microbial origin, we isolated four structurally related pentaene macrolides as antifungal antibiotics from the culture broth of *Streptomyces* sp. K99-5278. From the structure elucidation as described in the accompanying paper[1], all these compounds were 28-membered pentaene macrolides. Three were new and designated takanawaenes A, B and C, but the other was identified as compound AB023a, which was previously isolated as an antifungal antibiotic produced by an actinomycete[2] (Fig. 1). In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of takanawaenes A, B and C are described.

Materials and Methods

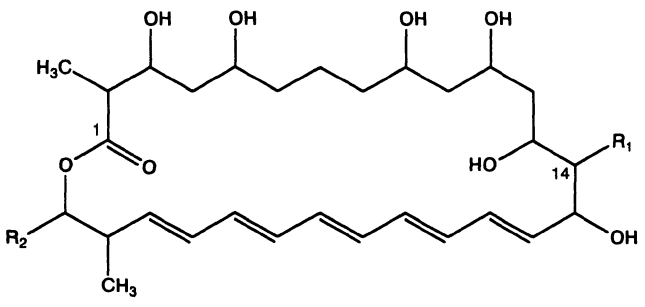
General Experimental Procedures

Strain K99-5278 was isolated from a soil sample collected at Minato-ku, Tokyo, Japan, and was used for production of takanawaenes A, B and C and AB023a. For determination of the amounts of takanawaenes in the culture broths, the samples, dissolved in acetonitrile, were analyzed on an HP1100 system (Huwllet Packerd Inc.) under the following conditions: column, Symmetry (2.1×150 mm, Waters Inc.); flow rate, 0.2 ml/minute; mobile phase, a 20-minute linear gradient from 30% CH_3CN/0.05% H_3PO_3 to 70% CH_3CN/0.05% H_3PO_3; detection, UV at 320 nm.

Taxonomic Studies

The International *Streptomyces* Project (ISP) media recommended by SHIRLING and GOTTLIEB[3] and media recommended by WAKSMAN[4] were used to investigate the

Fig. 1. Structures of takanawaenes A, B, C and AB023a.



Takanawaene A :	$R_1 = H$	$R_2 = CH_3$
Takanawaene B :	$R_1 = CH_3$	$R_2 = CH_2CH_3$
Takanawaene C :	$R_1 = CH_3$	$R_2 = CH(CH_3)_2$
AB023a :	$R_1 = CH_3$	$R_2 = CH_3$

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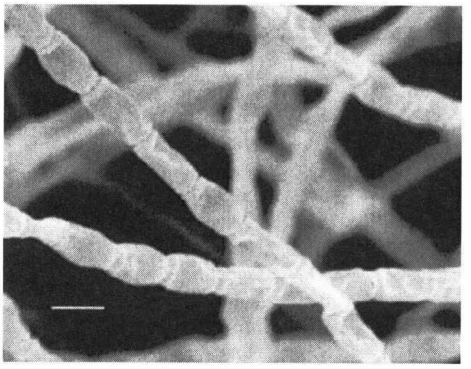
cultural and physiological characteristics. Cultures were routinely observed after the incubation for two weeks at 27°C. Color names and hue numbers were determined according to the Color Harmony Manual[5]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon at 27°C[6]. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL). The isomer of diaminopimelic acid (DAP) was determined by the method of BECKER *et al.*[7]. Menaquinones were extracted and purified by the method of COLLINS *et al.*[8], then were analyzed by HPLC (Gulliver System, JASCO) equipped with a Capcell Pak C18 column (4.6×250 mm, Shiseido)[9].

Antimicrobial Activity

Paper disk method: Antimicrobial activity against 15 species of microorganisms was measured. The microorganisms were as follows; *Bacillus subtilis* PCI 219, *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Escherichia coli* NIHJJ-2 IFO 12734, *Pseudomonas aeruginosa* P-3, *Xanthomonas campestris* pv. *oryzae*, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC6275, *Mucor racemosus* IFO4581, *Candida albicans* and *Saccharomyces cerevisiae*. Media for microorganisms were as follows: GAM agar (Nissui Seiyaku Co.) for *B. fragilis*; Bacto PPLO agar (Difco) supplemented with 15% horse serum, 0.1% glucose, 0.25% phenol red (5 mg/ml) and 1.5% agar for *A. laidlawii*; Taiyo agar (Shimizu Syokuhin Kaisya Ltd.) for the other bacteria; a medium composed of 1.0% yeast extract, and 0.8% agar for fungi and yeasts. A paper disk (i.d. 6 mm, ADVANTEC) containing 10 μg of a sample was placed on an agar plate. Bacteria except *X. oryzae* were incubated at 37°C for 24 hours. Yeasts and *X. oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

Liquid culture method: The sample dissolved in the DMSO was added to each well of a 96-well microplate (Corning) to make the final concentration at 0 to 500 μg/ml, and the solvent was dried up. The fungus (*A. niger*, *M. racemosus*, *C. albicans* or *S. cerevisiae*) was suspended in the medium containing glucose 1.0% and yeast extract 0.5% (pH 6.0) at a concentration of 2.0×10^5~3.0×10^5 spores/ml, and the suspension (200 μl) was inoculated into each well. The growth was measured at OD_{550} with a microplate reader (model ELX808, BIO-TEK Instruments) after a 24-hour incubation at 27°C for *M. racemosus*, *C. albicans* and *S. cerevisiae* or after a 48-hour incubation at 27°C for *A. niger*.

Fig. 2. Scanning electron micrograph of spore chains of strain K99-5278 grown on oatmeal agar at 27°C for 14 days.



Bar represents 1 μm.

Results

Taxonomy of the Producing Strain K99-5278

The vegetative mycelia grew abundantly on yeast extract-malt extract agar, oatmeal agar and other agar media, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract-malt extract and oatmeal agar. The spore chains were straight and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.2×0.8 μm in size and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia and flagellate spores were not observed.

The isomer of DAP in whole-cell hydrolysates of strain K99-5278 was determined to be LL-form. Major menaquinones were MK-9(H_8) and MK-9(H_6).

The cultural characteristics, the physiological properties and the utilization of carbon sources are shown in Tables 1, 2 and 3. The color of vegetative mycelia showed yellow to brown and the aerial mass color showed white to gray. Melanoid pigment was not produced, but yellowish pigment was produced.

Based on the taxonomic properties described above, strain K99-5278 is considered to belong to the genus *Streptomyces*[10]. The strain was deposited in National Institute of Advanced Industrial Science and Technology,

Table 1. Cultural characteristics of strain K99-5278.

Medium		Cultural characteristics	Medium		Cultural characteristics
Yeast extract-malt extract agar [a]	G:	Good, chestnut brown (4ni)	Tyrosine agar [a]	G:	Poor, ivory (2db)
	R:	Light spice brown (4lg)		R:	Ivory (2db)
	AM:	Abundant, sand (3cb) to no name (5cb)		AM:	Poor, white (a)
	SP:	None		SP:	None
Oatmeal agar [a]	G:	Good, camel (3ie)	Sucrose-nitrate agar [b]	G:	Moderate, alabaster tint (13ba)
	R:	Fawn (4ig)		R:	Pearl (3ba)
	AM:	Abundant, rosewood (5ge)		AM:	Moderate, white (a)
	SP:	None		SP:	None
Inorganic salts-starch agar [a]	G:	Good, light ivory (2ca)	Glucose-nitrate agar [b]	G:	Poor, pearl (3ba)
	R:	Light wheat (2ea)		R:	Pearl (3ba)
	AM:	Moderate, white (a)		AM:	None
	SP:	None		SP:	None
Glycerol-asparagine agar [a]	G:	Good, biscuit (2ec)	Glycerol-calcium malate agar [b]	G:	Good, bisque (3ec) to camel (3ie)
	R:	Biscuit (2ec)		R:	Bisque (3ec) to camel (3ie)
	AM:	Moderate, orchid tint (10ba)		AM:	Scant
	SP:	Yellow		SP:	None
Glucose-asparagine agar	G:	Moderate, biscuit (2ec)	Glucose-peptone agar [b]	G:	Moderate, camel (3ie)
	R:	Biscuit (2ec)		R:	Light amber (3ic)
	AM:	None		AM:	Moderate, white (a)
	SP:	None		SP:	Yellow
Peptone-yeast extract-iron agar [a]	G:	Poor, light mustard tan (2ie)	Nutrient agar [b]	G:	Moderate, bisque (3ec)
	R:	Mustard (2le)		R:	Light wheat (2ea)
	AM:	None		AM:	None
	SP:	None		SP:	Yellow

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

Table 2. Physiological properties of strain K99-5278.

Melanin formation	
Tyrosine agar	—
Peptone-yeast extract-iron agar	—
Tryptone-yeast extract broth	—
Gelatin medium	—
Reduction of nitrate	+
Liquefaction of gelatin (21~23°C)	—
Hydrolysis of starch	+
Coagulation of milk (27°C)	—
Peptonization of milk (27°C)	+
Decomposition of cellulose	—
Temperature range for growth	19~35°C

+, Positive : —, Negative

Table 3. Utilization of carbon sources by strain K99-5278.

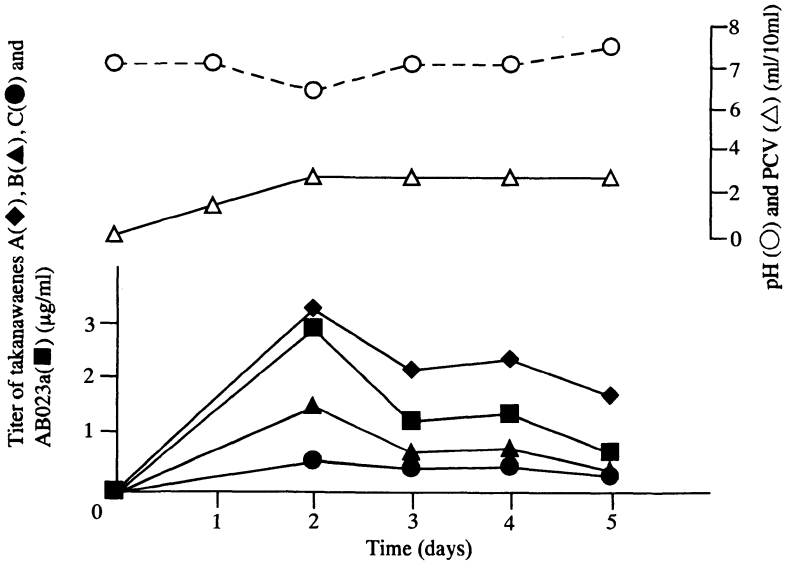
Utilized:	D-Glucose, Melibiose
Weakly utilized:	L-Arabinose, Raffinose
Not utilized:	D-Fructose, *myo*-Inositol, D-Mannitol, L-Rhamnose, Sucrose, D-Xylose

Japan, as *Streptomyces* sp. K99-5278 with the accession number FERM BP-8219.

Fermentation

A slant culture of the strain K99-5278 grown on Seino agar (starch 1.0%, N-Z amine 0.3%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.0%, pH 7.0) was used to inoculate a 50-ml test tube containing 10 ml of the seed medium (glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0). The tube was shaken on a reciprocal shaker for 4 days at 27°C. One ml of the seed culture was incubated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (glycerol 1.5%, Defatted Wheat Germ

Fig. 3. A typical course of production of takanawaenes A, B, C and AB023a by *Streptomyces* sp. K99-5278.



The amounts of takanawaenes A (◆), B (▲), C (●) and AB023a (■) in culture broths were determined by HPLC as described in Materials and Methods.

PCV, packed cell volume (ml) from 10 ml of the whole culture broth after centrifugation at 3000 rpm.

(Nisshin Pharma Inc.) 1.0%, $CaCO_3$ 0.3%, pH 7.0). The fermentation was carried out at 27°C for 5 days. A typical time course of the fermentation is shown in Fig. 3. Takanawaenes and AB023a were detected in the culture broth on day 1 after inoculation. Their concentrations reached maximal levels (0.5~3 μg/ml) on day 2, then gradually decreased until day 5.

Isolation

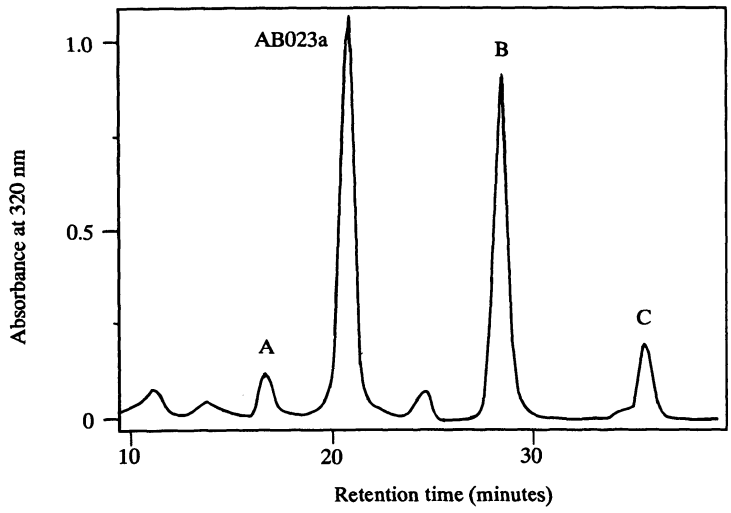
To the 5-day old culture broth (15 liters) was added 15 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with ethyl acetate. The ethyl acetate extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield 2.4 g of brown material. The material was dissolved in $CHCl_3$, applied on a silica gel column (100 g, 2×16 cm, 70~230 mesh, Merck), and eluted stepwise with 100% $CHCl_3$ and 100:1, 50:1, 10:1, 5:1 and 1:1 (v/v) of $CHCl_3$-CH_3OH solvents (500 ml each). Antifungal activity against *A. niger* was observed in the 5:1 fraction, which was concentrated to give a yellow powder (401 mg). The powder was subjected to a second column (30 g, 1×18 cm, 230~400 mesh, Merck). The material was eluted with the $CHCl_3$-CH_3OH (5:1, v/v) solvent and each 50 ml of the elution was successively collected. The 19th to 27th fractions containing takanawaenes and AB023a were concentrated to give a yellow powder (173 mg). The compounds were finally purified by HPLC (column, Senshu Pak PEGASIL ODS 20×250 mm; solvent, 50% CH_3CN; detection, UV at 320 nm; flow rate, 8.0 ml/minute). Under these conditions, takanawaene A, AB023a, takanawaene B and takanawaene C were eluted as peaks with retention times of 17.6, 21.2, 28.0 and 36.9 minutes, respectively (Fig. 4). They were concentrated to yield pure takanawaene A (12.8 mg), takanawaene B (22.4 mg), takanawaene C (8.6 mg) and AB023a (46.2 mg) as yellow powders.

Biological Properties

Antifungal Activities

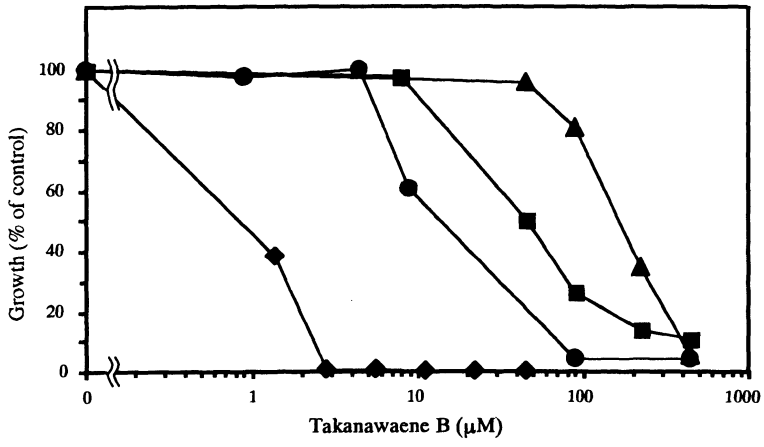
Takanawaenes A, B and C and AB023a showed antifungal activity against *A. niger* (diameter of inhibition zone: 18, 16, 15 and 17 mm, respectively), *M. racemosus* (9, 9, 8 and 9 mm), *C. albicans* (9, 7, 8 and 9 mm) and *S. cerevisiae* (12, 12, 9 and 12 mm) at a concentration 10 μg/6 mm disk. No activity of takanawaenes and AB023a

Fig. 4. A chromatographic profile of purification of takanawaenes A, B, C and AB023a by preparative HPLC.



Column, Senshu Pak PEGASIL ODS (20×250 mm); solvent, 50% CH_3CN; detection, UV at 320 nm; flow rate, 8.0 ml/minute; sample, 10 μg of active materials dissolved in 100 μl of MeOH.

Fig. 5. Antifugal activity of takanawaene B.



C. albicans (▲), *M. racemosus* (■), *A. niger* (◆) and *S. cerevisiae* (●) were grown in the liquid medium in the presence of takanawaene B. The grown was plotted as percent of control (without takanawaene B).

was observed against the other microorganisms: *B. subtilis*, *S. aureus*, *M. luteus*, *M. smegmatis*, *E. coli*, *E. coli*, *P. aeruginosa*, *X. oryzae*, *B. fragilis*, *A. laidlawii* and *P. oryzae*. These data suggested that these compounds show the most potent antifungal activity against *A. niger* among the microorganisms tested.

The antifungal activity of takanawaene B was confirmed in the liquid culture method. The growth of *A. niger*, *M. racemosus*, *C. albicans* and *S. cerevisiae* was inhibited by the compound in a dose-dependent fashion with IC_{50} values of 0.94, 44.3, 177.0 and 20.9 μM, respectively (Fig. 5). Takanawaenes A and C also inhibited the growth of

S. cerevisiae with IC_{50} values of 21.8 and 28.7 μM, respectively. The results are comparable to those by the paper disk method.

Thus, this series of compounds showed antifungal and anti-yeast activities, especially potent anti-*A. niger* activity among the microorganisms.

Discussion

Twenty eight-membered pentaene macrolides have been reported, but the number is not so many[11]. They are classified into three groups. The well-defined methylpentaenes, where one end of the pentaene moiety is substituted with a methyl group, include filipins[12,13], chainin[14], and fungichromin[15]. Especially filipin III is known for its antifungal activity by interacting with sterols of plasma membranes[16] to form membrane inclusion, and is also indicated to inhibit ergosterol biosynthesis[17]. Takanawaenes, AB023s[2] and polyene I[18], all produced by *Streptomyces* sp. are simple pentaenes with no substituent at the end of the pentaene moiety. Takanawaenes and AB023s[2] showed antifungal and anti-yeast activity, and polyene I was reported as an inhibitor of NADPH oxidase[18]. Strevertenes[19], produced by *Streptoverticillum* sp., are also 28-membered pentaenes with a carboxylic acid at C-14 (Fig. 1). They were reported to show antifungal and anti-yeast activity, and inhibitory activities against ergosterol biosynthesis.

Thus, these 28-membered pentaene macrolides seem to show similar biological activities, but further structure-activity study remains to be done.

Acknowledgments

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