NISAMYCIN, A NEW MANUMYCIN GROUP ANTIBIOTIC FROM *Streptomyces* sp. K106

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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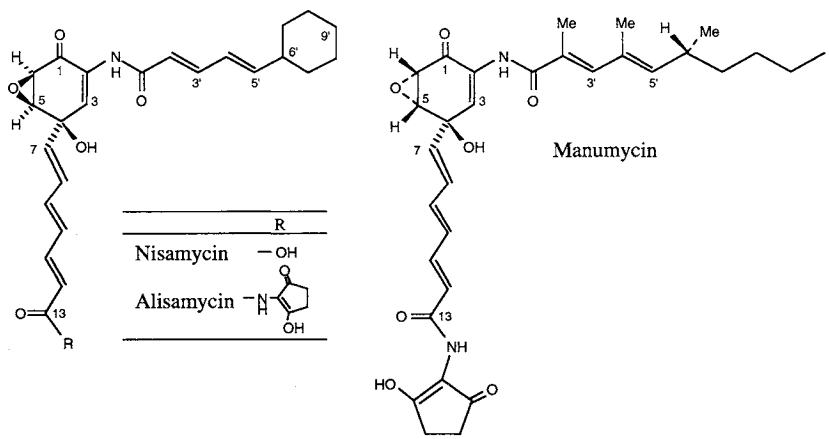
Nisamycin, a new manumycin group antibiotic, was isolated from the culture broth of *Streptomyces* sp. K106. This strain was designated to genus *Streptomyces* by the taxonomic features. Nisamycin was purified by ethyl acetate extraction, silica gel column chromatography, preparative silica gel TLC, and Sephadex LH-20 column chromatography as a pale yellow powder. Nisamycin is active against Gram-positive bacteria and fungi, and exhibits a cytotoxic activity.

In the course of a screening program for new antibiotics, we found a new manumycin group antibiotic named nisamycin in the culture broth of *Streptomyces* sp. K106. This strain also produced a known manumycin group antibiotic, alisamycin. In the previous studies on the manumycin group antibiotics, manumycin[1], asukamycin[2], U-56,407[3], U-62162[4], colabomycin A[5], alisamycin[6], and manumycins B, C, D, E, F and G[7,8] were discovered. A preliminary communication of this work has been reported[9]. This paper deal with the taxonomy and the fermentation of the producing microorganism, the isolation, the physico-chemical and the biological properties of nisamycin (Fig. 1). The structural elucidation and some structure-activity relationships of nisamycin are described in an accompanying paper[10].

Fig. 1. Structure of nisamycin, alisamycin and manumycin.



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Materials and Methods

Taxonomic Studies

Streptomyces sp. K106 was isolated from a soil sample collected in Sakai city, Osaka prefecture. The taxonomic studies were carried out according to the International Streptomyces Project (ISP)[11]. Micro-morphological observations were made with a light microscope and a scanning electron microscope from the cultures grown at 27°C for 14 days on yeast extract-malt extract agar and inorganic salts-starch agar. The type of diaminopimelic acid in the cell wall was determined by the method of BECKER *et al.*[12]. Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[13].

Fermentation Studies

A well grown agar slant of the strain K106 was inoculated into a 500-ml flask containing 100 ml of the fermentation medium consisting of glycerol 1%, NZ Amine type A 0.25%, yeast extract 0.1%, and meat extract 0.1% (pH 7.0 before sterilization). The flask was incubated at 28°C for 2 days on a reciprocal shaker. A fifteen-ml portion of this culture was transferred to a 5-liter Erlenmeyer flask containing 1.4-liters of the same medium as above. The fermentation was carried out at 28°C for 2 days on a reciprocal shaker.

Antimicrobial Activity

Antimicrobial activity was examined by the agar-dilution method on nutrient agar for Gram-positive and Gram-negative bacteria and on Sabouraud agar for fungi and yeast. The inoculum was adjusted to 5×10^5 cfu/ml for bacteria and 1×10^6 cfu/ml for fungi and yeast. Minimum inhibitory concentration (MIC) is expressed as μg/ml after 18 hours at 37°C for bacteria and 48 hours incubation at 28°C for fungi and yeast. The plate of *Micrococcus roseus* was incubated for 48 hours.

Cytotoxic Assay

Mouse leukemia P388 cells, mouse melanoma B16 cells, and human T cell lymphocyte Molt-4 cells were used for *in vitro* cytotoxic assay. All cell lines except B16 cells were cultivated in RPMI1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) at 37°C in a humidified atmosphere of 5% CO_2. B16 cells were cultivated in DULBECCO's modified EAGLE's medium (Nikken Bio-medical Laboratory, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum in the same condition. 2×10^4 cells of each cell line were inoculated into each well of 96-well microtiter plates containing 200 μl of the medium. Nisamycin was dissolved in DMSO. The serially diluted nisamycin solution was added to each well of the plates. The final concentration of DMSO in the cultures was adjusted to 1% (v/v). The cells were incubated at 37°C for 3~4 days. After incubation, the cells were treated with trypan blue. Living cells on hematocytometer were counted using the light microscope .

Results and Discussion

Taxonomy

Morphological and Chemical Properties

The substrate mycelia of strain K106 were well-branched and not fragmented. The spore chains were the *Spirals* type and each chain had more than 50 spores per chain. The spores were spherical ($0.6 \sim 1.0 \times 0.6 \sim 0.9$ μm) and their surface was spiny (Fig. 2). Sclerotia, sporangia, and zoospores were not observed. Whole-cell hydrolysates of strain K106 contained LL-diaminopimelic acid but no *meso*-diaminopimelic acid. Accordingly, the cell wall of

Fig. 2. Scanning electron micrograph of spore chains of strain K106.

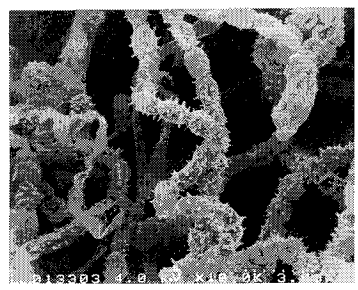

Table 1. Cultural characteristics of strain K106.

Medium		Cultural characteristics	Medium		Cultural characteristics
Yeast extract-malt extract agar (ISP 2)	G:	Good, cottony	Tyrosine agar (ISP 7)	G:	Good, powdery
	AM:	Abundant, brownish gray		AM:	Abundant, pale brownish gray
	R:	Brown		R:	Blacknish brown
	SP:	None		SP:	Dark brown
Oatmeal agar (ISP 3)	G:	Good, cottony	Sucrose-nitrate agar	G:	Poor
	AM:	Abundant, brownish gray		AM:	Scant, white
	R:	Brown		R:	Colorless
	SP:	None		SP:	None
Inorganic salts-starch agar (ISP 4)	G:	Good, powdery	Nutrient agar	G:	Moderate-good
	AM:	Abundant, pale brownish gray		AM:	None
	R:	Brown		R:	Colorless
	SP:	None		SP:	None
Glycerol-asparagine agar (ISP 5)	G:	Good, cottony	Glucose-asparagine agar	G:	Good, powdery
	AM:	Abundant, pale brownish gray		AM:	Abundant, brownish gray
	R:	Light brown		R:	Brown
	SP:	None		SP:	None

Abbreviations: G, growth; AM, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2. Physiological properties of strain K106.

Temperature:		Nitrate reduction	Negative
Growth range	20~40°C	Milk peptonization	Positive
Optimum growth	28°C	Milk coagulation	Negative
Melanoid pigment production	Positive	NaCl tolerance	>6%
Starch hydrolysis	Positive	H_2S production	Positive
Gelatin liquefaction	Positive		

the strain was classified as type I.

Table 3. Utilization of carbon sources by strain K106.

Response	Carbon source
Positive	D-Glucose, D-fructose, D-galactose, lactose, glycerol, inositol, D-mannitol, D-mannose, L-arabinose, raffinose, L-rhamnose, D-xylose, salicin, sucrose, melibiose
Negative	Melezitose

Cultural Characteristics

The cultural characteristics of strain K106 on various media are summarized in Table 1. Mature aerial mycelia corresponded to the gray color series. The reverse side of the colony was light to dark brown. Dark brownish soluble pigment was observed in tyrosine agar.

Physiological Characteristics

The physiological characteristics and the utilization of carbohydrate are shown in Tables 2 and 3, respectively. Starch hydrolysis, gelatin liquefaction, milk peptonization and H_2S production were positive, but milk coagulation and nitrate reduction were negative. Melanoid pigment was produced in peptone-yeast-iron agar and tyrosine agar. NaCl tolerance of strain K106 was less than 6% on yeast extract-malt extract agar. This strain grew within a temperature range of 20°C to 40°C, with the optimum at about

28°C. This strain used D-glucose, D-fructose, D-galactose, lactose, glycerol, inositol, D-mannitol, D-mannose, L-arabinose, raffinose, L-rhamnose, D-xylose, salicin, sucrose, and melibiose as sole carbon sources. Of all carbon sources tested, only melezitose was not utilized.

Taxonomic Position of Strain K106

On the basis of the morphological, cultural, physiological and chemotaxonomic characteristics, strain K106 belongs to the genus *Streptomyces*. Among the published description of various *Streptomyces* species, strain K106 was found to resemble to *Streptomyces gannmycicus*[14]. As there are some differences in the utilization of carbon sources between strain K106 and *Streptomyces gannmycicus*, strain K106 was designated *Streptomyces* sp. K106. *Streptomyces* sp. Y-88,31582 was known to produce alisamycin[6]. *Streptomyces* sp. K106 also produce alisamycin. From the taxonomic features, the two strains belong to different species. The strain K106 has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan under the accession number FERM P-12701.

Fermentation

Fig. 3 shows a typical time course for the production of nisamycin in a 500-ml flask. The growth of the strain increased during 72 hours incubation. The production of nisamycin started at 21 hours and reached a maximum at 48 hours after incubation.

Isolation

The purification scheme is shown in Fig. 4. During the isolation process, antimicrobial activity was monitored by the agar diffusion paper-disc method using *Bacillus subtilis* IFO 12210 as a test organism. The cultured broth of strain K106 was filtered with the aid of diatomaceous earth. The filtrate (16.8 liters) was adjusted to pH 3.0 with 4N HCl and extracted with 10 liters of EtOAc. The extraction was carried out twice, and the EtOAc extracts were combined. This organic layer was evaporated *in vacuo* to give the EtOAc solution (2 liters). After dehydration with

Fig. 3. Time course of antibiotic production of strain K106.

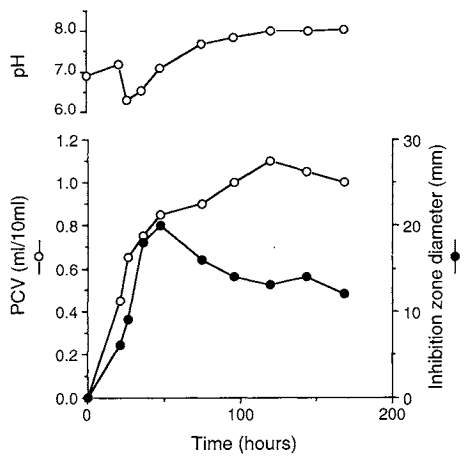

Fig. 4. Isolation of Nisamycin.

Culture filtrate (16.8 liters)
↓ extracted with EtOAc at pH 3.0
Organic layer (20liters)
↓ concd *in vacuo*
Crude product (2.7g)
↓ silica gel column
eluted stepwise with Benzene-Acetone
Active fraction (Benzene:Acetone=8:2)
↓ silica gel column
eluted stepwise with $CHCl_3$-MeOH
Active fraction ($CHCl_3$:MeOH=95:5)
↓ preparative TLC ($CHCl_3$:MeOH=95:5)
eluted with $CHCl_3$-MeOH (9:1)
Semi-pure nisamycin
↓ Sephadex LH-20 column
eluted with MeOH
Nisamycin (120mg)

Table 4. Physico-chemical properties of nisamycin.

Appearance	Pale yellow powder
Molecular formula	$C_{24}H_{27}NO_6$
HRFAB-MS (*m/z*)	
Calcd:	426.1917
Found:	426.1918 $(M+H)^+$
MP (°C)	122~124 (dec)
IR ν_{max} (KBr) cm^{-1}	3346, 1694, 1668, 1615, 1521, 1005
UV λ_{max}^{MeOH} nm (ε)	278 (32,100), 305 (22,600)
$[\alpha]_D^{25}$ (*c* 0.35, EtOH)	−143°
CD $\lambda_{max}^{CHCl_3}$ nm ($\Delta\varepsilon$)	261 (+15.24), 315 (−14.78)

Table 5. Antimicrobial spectra of nisamycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus IFO 3060	0.19
Bacillus subtilis IFO 12210	0.39
B. cereus IFO 3514	0.19
Arthrobacter globiformis IFO 12140	0.39
Micrococcus roseus IFO 3768	0.39
Escherichia coli K-12 IFO 3301	50
Pseudomonas aeruginosa IFO 3923	>100
Serratia marcescens IFO 12648	>100
Saccharomyces cerevisiae	>100
Candida albicans	>100
C. tropicalis IFO 0589	100
Aspergillus niger van Tieghem IFO 4416	100
Fusarium oxysporum IFO 5880	50

anhydrous Na_2SO_4, the EtOAc solution was further concentrated *in vacuo* to an oily residue (2.7 g). The residue was applied to a silica gel column (400 ml) and then eluted stepwise with benzene-acetone solvent system. The active fraction was eluted with benzene-acetone (8:2) and concentrated *in vacuo* to dryness. The residue was subjected again to a silica gel column (200 ml). The column was developed stepwise with $CHCl_3$-MeOH solvent system. Two active fractions were eluted with $CHCl_3$-MeOH (97:3) and (95:5), respectively. Alisamycin, a known manumycin group antibiotic, was obtained from the active fraction (97:3) with crystallization in $CHCl_3$-MeOH solution. The other active fraction (95:5) was concentrated *in vacuo* to a crude powder. The crude powder was then purified with preparative thin-layer chromatography with development of $CHCl_3$-MeOH (95:5). Active band (Rf=0.32) was scrapped off and eluted with $CHCl_3$-MeOH (9:1). The elute was concentrated *in vacuo* to a yellow powder. The powder was subjected to Sephadex LH-20 column (200 ml) eluting MeOH to afford pale yellow powder of nisamycin (120 mg).

Physico-chemical Properties

The physico-chemical properties of nisamycin are summarized in Table 4. Nisamycin was soluble in MeOH, EtOH, ethyl acetate, pyridine, DMSO, and $CHCl_3$, but insoluble in *n*-hexane and water. The molecular formula of nisamycin was established to be $C_{24}H_{27}NO_6$ by HRFAB-MS. Nisamycin has carboxylic group in the structure and lacks 2-amino-3-hydroxycyclopent-2-enone moiety characteristic of the manumycin family (Fig. 1). The structure of nisamycin is closely related to that of alisamycin including stereochemistry[15]. The details of the structure elucidation of nisamycin will be reported in an accompanying paper[10].

Biological Properties

Antimicrobial activity of nisamycin are shown in Table 5. Nisamycin mainly inhibits the growth of Gram-positive bacteria within a concentration of 0.19~0.39 μg/ml and is slightly active against fungi. Nisamycin was about 6-fold more active than alisamycin on Gram-positive bacteria in our experiment (data not shown). It displays no significant effects on Gram-negative bacteria. In addition, nisamycin showed a weak cytotoxicity in the proliferation assay against mouse leukemia P388 cells (IC_{50} 4.8 μg/ml), mouse melanoma B16 cells (IC_{50} 2.5 μg/ml), and human T cell lymphocyte Molt-4 cells (IC_{50} 6.5 μg/ml).

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