NEIHUMICIN, A NEW CYTOTOXIC ANTIBIOTIC FROM *MICROMONOSPORA NEIHUENSIS*

I. THE PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

RONG-YANG WU, LI-MING YANG†, TOSHIO YOKOI† and KUO-HSIUNG LEE†

Institute of Botany, Academia Sinica,
Taipei, Taiwan, Republic of China
†Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina,
Chapel Hill, North Carolina 27514, U.S.A.

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A new cytotoxic and antifungal antibiotic, neihumicin, was isolated from the culture broth of a soil isolate identified as *Micromonospora neihuensis* Wu, sp. nov. Neihumicin shows *in vitro* cytotoxicity against KB tissue culture cells (ED_{50} 0.94 μg/ml) as well as antifungal activity against *Saccharomyces cerevisiae* ATCC 9763.

In the course of our screening program for novel antitumor and antifungal antibiotics, an antibiotic-producing culture designated as NH3-1 was found to show significant ($ED_{50} \leqq 4.0$ μg/ml) cytotoxicity against *in vitro* KB tissue culture cells. Bioassay-directed fractionation of the active mycelial extract led to the isolation of a new cytotoxic antibiotic, neihumicin. This paper describes the producing strain, the fermentation conditions, the isolation and the biological properties of neihumicin. Studies on the characterization, total synthesis and structure-activity relationships of neihumicin and related compounds will be reported in the subsequent papers[1,2].

Materials and Methods

Isolation and Purification of NH3-1

Strain NH3-1 was isolated from a soil sample collected in Nei-Hu, near Taipei, Taiwan. A 2-g sample of soil was added to 10 ml of phosphate buffer (pH 7.0), shaken for 10 minutes and diluted 10-, 100- and 1,000-fold in pH 7.0 phosphate buffer. The dilutions were plated on TYG agar consisting of Tryptone 5 g, yeast extract 3 g, glucose 10 g, K_2HPO_4 1 g, KH_2PO_4 1 g and agar-agar 20 g in 1,000 ml of distilled water. Plates were incubated at 28°C for 14 days the red-brownish colonies (1~2 mm in size) were isolated. The taxonomic studies on the microbial isolate were conducted according to ARAI[3], KRASIL'NIKOV[4], WAKSMAN[5], SYKES and SKINNER[6], ALEXANDER[7], and BUCHANAN and GIBBONS[8].

Cultural Characteristics

Spores of NH3-1 were collected from 21-day old cultures grown on TYG agar and suspended in pH 7.0 phosphate buffer saline solution. One drop of the suspension was used to inoculate the various media according to WAKSMAN[5], LUEDEMANN[9] and BECKER *et al.*[10]. Cultural characteristics of strain NH3-1 were based on observations made after 7, 14 and 21 days incubation at 28°C on various agar media (Table 1).

Scanning Electron Microscopic Observations

Strain NH3-1 was cultivated on TYG agar plates at 28°C for 3 weeks. Each week colonies were cut out from agar plates and fixed in 2.5% of glutaraldehyde buffer solution at pH 7.4 for 2 hours.

After dehydration through graded ethanol series, drying with a critical point apparatus, and coating with gold-palladium, the colonies and spores were examined in Hitachi S-520 and Zeiss DSM 950 scanning electron microscopes.

Utilization of Carbon Sources

Carbon source utilization was investigated by the methods of WAKSMAN[5], PRIDHAM and GOTTLIEB[11], and LECHEVALIER and LECHEVALIER[12] using the twenty-two compounds listed in Table 3. Growth and carbohydrate utilization was measured 21 days after incubation at 28°C.

Physiological Studies

Methods and media used for these studies were prepared according to the procedures of WAKSMAN[5], LUEDEMANN[9], LUEDEMANN and BRODSKY[13], and NEYRA *et al.*[14]. Mature spores and mycelia on TYG agar were used for inoculation. All cultures were incubated at 28°C for 21 days, except for gelatin liquefaction which was incubated at 15°C for 21 days.

Cell-wall Composition

Determination of the cell-wall composition including the analyses of amino acid and sugar samples prepared from the cells was based upon methods of BOONE and PINE[15], PINE and BOONE[16], KAWAMOTO *et al.*[17], and LECHEVALIER and LECHEVALIER[12,18].

Fermentation

Spores of *Micromonospora* NH3-1 were inoculated into 125-ml Erlenmeyer flasks containing 50 ml of a seed medium composed of Tryptone 0.25 g, yeast extract 0.15 g, glucose 0.5 g, K_2HPO_4 0.05 g and KH_2PO_4 0.05 g (pH 7.0). Flasks were incubated on a rotary shaker at 200 rpm at 28°C for 4 to 5 days, after which 25 ml of the vegetative seed was transferred into a 2-liter fermenter containing 1 liter of the fermentation medium (glucose 10 g, molasses 10 g, peptone 6 g and $CaCO_3$ 4 g). The pH of the media was adjusted to 7.0 prior to sterilization. The fermentation was carried out at 28°C on a rotary shaker at 200 rpm. The total antibacterial activity reached a maximum after 7 day's fermentation.

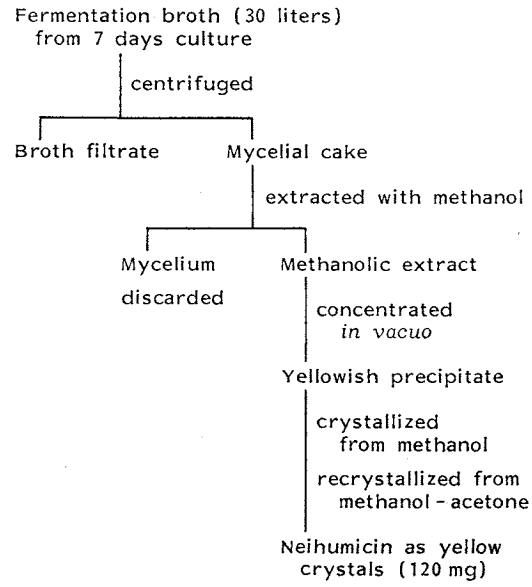
The activity was determined by the following procedure. The culture broth (10 ml) was centrifuged at $1,200 \times g$ for 15 minutes. Methanol (5 ml) was added to the packed cells, mixed vigorously and centrifuged at $1,200 \times g$ for 10 minutes. Antibacterial activity of the extract was measured by paper-disc method using *Bacillus subtilis* PCI-219, grown on Nutrient agar, as the test organism. Zones of inhibition were recorded after 48 hours of incubation at 28°C. Approximately 95% of the antibacterial activity was found in the mycelium.

Isolation and Purification

The mycelial cake was collected by continuous centrifugation, washed with water and extracted approximately 10 times with methanol (4 liters). The methanolic extracts were combined and concentrated in vacuum to a 400-ml solution. This solution was allowed to stand at room temperature overnight to give a yellowish precipitate. Crystallization from methanol gave greenish needles (300 mg). Further recrystallization from methanol - acetone furnished neihumicin as yellow crystals (120 mg).

The isolation and purification of neihumicin from strain NH3-1 is shown in Fig. 1.

Fig. 1. Isolation and purification of neihumicin from strain NH3-1.



Antimicrobial Spectrum and Cytotoxic Activity

Methods and media used for the antimicrobial assay were according to the procedures of U.S.A. Code of Federal Regulations for foods and drugs[19] using the paper-disc agar diffusion method. Because of the poor water solubility of neihumicin, it was dissolved into dimethylformamide and then diluted with 2-fold 0.1 M phosphate buffer. The final concentration of dimethylformamide was 1.0% at pH 6.5. A portion (50 μl) of the test solution was applied on paper disc (0.8 cm in diameter), which was then placed on agar plates seeded with appropriate organisms. The common bacteria, human pathogenic fungi and plant pathogenic fungi were incubated at 37°C for 24 hours, 37°C for 48 hours and 28°C for 72 hours respectively. The lowest concentration of neihumicin that inhibited the formation of colonies completely was determined.

In vitro cytotoxicity assay was carried out according to standard National Cancer Institute guidelines[20,21]. The KB cells were maintained on Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 5% fetal calf serum and 100 μg/ml kanamycin. The cells (5×10^4 cell/ml) were plated 24 hours before the addition of compound to be tested. Test compounds were added on day 1. Results were expressed as the dose that inhibits growth to 50% control growth three days after drug addition. All counting of KB cells was done on a hemacytometer. The ED_{50} values were estimated from a semi-log plot of the drug concentration (μg/ml) vs. the percent of viable cells. The ED_{50} values less than or equal to 4.0 μg/ml are considered to be of significant cytotoxicity.

Results

Culture Characteristics

The culture characteristics of the strain NH3-1 grown on various media are shown in Table 1. The growth was slower than that of *Streptomyces* in general. Strain NH3-1 grew better on organic media than synthetic media. On organic media, vegetative mycelia raised from the agar surface and developed into the medium. The size of colonies ranged from 2 to 3 mm with yellow to brown color. No soluble pigment was produced in most of media, except for TYG agar. A faint diffusible reddish pigment appeared on TYG agar 1 week after cultivation.

Morphological Characteristics

This organism formed no true aerial mycelium. It had well-developed and branched straight vegetative mycelia. Scanning electron micrographs indicated that spores are borne singly at the ends of the sporophores branching from vegetative hypha (Fig. 2) or form at tips of short sporophore (Fig. 3) appearing in monopodially arranged clusters (Fig. 4). The spore was either spherical (0.8~1.2 μm) or elongate (0.6~1.2×0.8~2.5 μm). Its surface appeared smooth or with only minor ir-

Table 1. Growth characteristics of NH3-1 on various media[a].

Medium	Growth characteristics	Diffusible pigment
Oatmeal	Moderate, white, 1~2 mm, smooth	Absent
Yeast-maltose	Moderate, brown, 2~3 mm, wrinkled rough	Absent
CZAPEK's	Moderate, ivory, 2~3 mm, smooth	Absent
Potato-peptone-glycerol	Moderate, brown, 2~3 mm, smooth	Absent
Glycerol-tyrosine	Moderate, ivory, 3 mm, smooth	Absent
Peptone	Poor, ivory, 1~2 mm, smooth	Absent
Peptone-yeast	Moderate, ivory, 2 mm, smooth	Absent
Glycerol-asparagine	No growth	
Nutrient	Moderate, brown, 3 mm, wrinkled rough	Absent
Tryptone-yeast-glucose	Moderate, brown, 3 mm, wrinkled rough	Pink
SABOURAUD's	No growth	
Minimal medium	Moderate, ivory, 1~2 mm, wrinkled rough	Absent

[a] Plates were incubated at 28°C for 3 weeks.

Fig. 2. Scanning electron micrograph of strain NH3-1 cultured on TYG agar plates for 2 weeks at 28°C.

Bar indicates 2 μm.

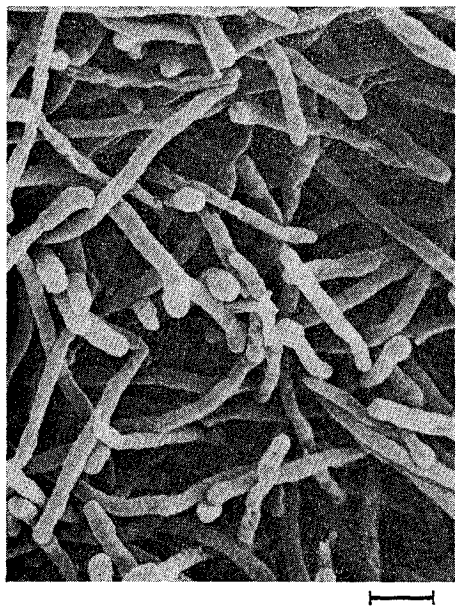
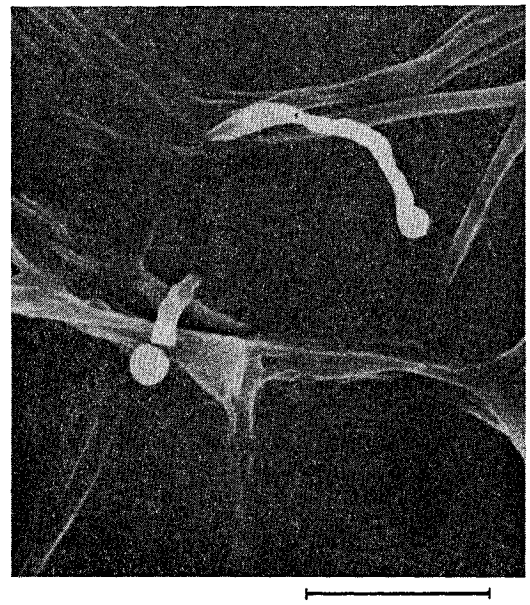


Fig. 3. Scanning electron micrograph of strain NH3-1 cultured on TYG agar plates for 3 weeks at 28°C.

A single spore formed at the tip of sporophore.

Bar indicates 5 μm.



regularities. The spore morphology of strain NH3-1 is shown in Table 2.

Utilization of Carbon Source

As shown in Table 3, D-galactose, D-maltose, D-sorbitol, lactose, D-xylose, D-mannose, D-glucose, L-glucose, L-arabinose, α-melibiose and starch were well utilized. L-Rhamnose and cellulose were poorly utilized. Raffinose, *i*-inositol, sucrose, inulin, D-mannitol, D-cellobiose, dulcitol, glycerol and salicin were not utilized at all.

Physiological Properties

Physiological properties of the strain NH3-1 are summarized in Table 4. Hydrolysis of starch, coagulation and peptonization of milk as well as reduction of nitrate are positive. However, tyrosinase reaction, liquefaction of gelatin, production of H_2S, production of melanin pigment and liquefaction of serum are negative. The temperature range for growth is rather narrow. Good growth occurred at 25 to 28°C. No growth was observed at 10 and 40°C or higher.

Fig. 4. Scanning electron micrograph of strain NH3-1 cultured on TYG agar plates for 1 week at 28°C.

Spores and sporophore found in monopodially arranged clusters.

Bar indicates 4.3 μm.

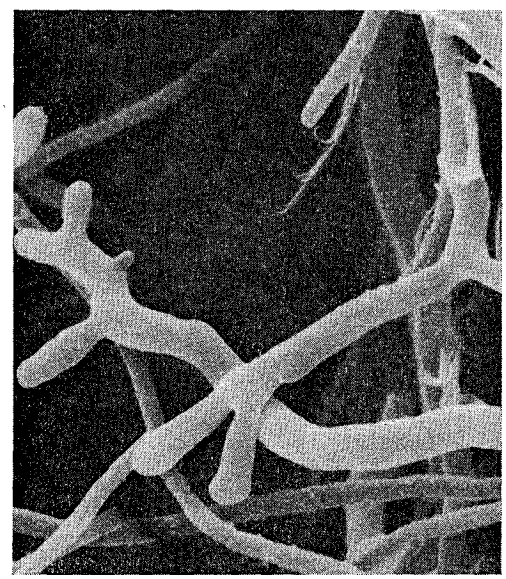


Table 2. Spore morphology of strain NH3-1.

Spore surface:	Smooth
Spore shape:	Spherical or elongate
Spore chain:	Single spore on short sporophore
Spore size:	Spherical spore: 0.8~1.2 μm in diameter
	Elongate spore: 0.6~1.2 by 0.8~2.5 μm
Sporophore:	Develop from substrate mycelium,
	0.8~10 μm in width, 2~10 μm in length

Table 3. Utilization of various carbon sources by strain NH3-1.

Carbon source	Response	Carbon source	Response
D-Galactose	+	Cellulose	±
D-Maltose	+	Raffinose	−
D-Sorbitol	+	*i*-Inositol	−
Lactose	+	Sucrose	−
D-Xylose	+	Inulin	−
D-Mannose	+	D-Mannitol	−
D-Glucose	+	D-Cellobiose	−
L-Glucose	+	Dulcitol	−
L-Arabinose	+	Glycerol	−
α-Melibiose	+	Salicin	−
Starch	+	No carbon source	−
L-Rhamnose	±		

+: Carbohydrate well utilized, ±: carbohydrate poorly utilized, −: carbohydrate not utilized at all.

Table 4. Physiological characteristics of strain NH3-1.

Reaction	Medium	Response
Gelatin liquefaction	Gelatin medium	−
Starch hydrolysis	Starch agar	+
Milk coagulation	Litmus milk	+
Milk peptonization	Litmus milk	Weak
Nitrate reduction	Nitrate broth	+
Tyrosinase reaction	Tyrosine agar	−
H_2S production	Peptone iron agar	−
Serum liquefaction	LÖFFLER's medium	−
NaCl tolerance	TYG slant with NaCl	4%
Growth temperature	TYG slant	20~35°C
Melanin formation	PY slant	−
Growth in potato plug	Potato plug	Good
Growth on carrot plug	Carrot plug	Good
Growth on cellulose	0.5% cellulose agar	Poor

Sodium chloride tolerance of strain NH3-1 is 4%. It does not grow in 5% NaCl. The strain grows well on potato and carrot plugs without adding calcium carbonate.

Cell-wall Compositions

An analysis of the whole cell hydrolysate by paper chromatography demonstrated that the cell-wall contained *meso*-diaminopimelic acid. The whole cell hydrolysate contains glucose, galactose, maltose, mannose, xylose and lactose. This evidence coupled with the morphological and colonial

characteristics described above indicated that the strain NH3-1 is a member of the genus *Micromonospora*.

Antimicrobial Spectrum and Cytotoxic Activities

The antimicrobial spectrum of neihumicin is shown in Table 5. This antibiotic shows moderate activity against the growth of *Saccharomyces cerevisiae* and *Penicillium italicum* Wehmer in acidic condition at pH 6.5, but is devoid of any significant activity against other microorganisms. Neihumycin showed potent cytotoxicity (ED_{50} 0.94 μg/ml) against KB cells.

Table 5. Antimicrobial activities of neihumicin.

Test organism	Inhibition (μg/disc)[a]
Escherichia coli ATCC 10536	—
Staphylococcus aureus ATCC 6538	—
Bacillus subtilis ATCC 6633	—
Candida albicans ATCC 10231	—
Saccharomyces cerevisiae ATCC 9763	30
Penicillium italicum Wehmer	30~60
Gibberella fujikuroi	—

[a] Inhibition was determined by paper disc method. Approximately 50 μl of test solution was placed on a thick paper disc (0.8 cm diameter).
—: No inhibition.

Discussion

According to BUCHANAN and GIBBONS[8] strain NH3-1 resembles *Micromonospora narashinoensis*. However, some of the cultural and physiological characteristics of strain NH3-1 differed from those of *M. narashinoensis*. Based upon ARAI[3], WAKSMAN[5], SYKES and SKINNER[6], and BUCHANAN and GIBBONS[8], the colony of *M. narashinoensis* showed the following properties: When it grew on yeast extract - glucose agar, it yielded abundant folded growth of deep orange colored spore layer with no production of soluble pigment; on Nutrient agar, it was minute, orange to tan colored with spore developed at the periphery of the colony as a moist, drab, and dark brown to black layer; on tyrosine agar, it was minute, orange in color with spore developed as a brown layer with production of purplish diffusible pigment. It showed no growth in cellulose solution. It liquefied gelatin. It did not reduce nitrate. On the other hand, the strain NH3-1 grew well on Nutrient and tyrosine agar. Its colonies on Nutrient and tyrosine agar were moderate, about 2 to 3 mm in size, convened with indented edges, and ivory to brown color. It produced no soluble pigments in most of the media except in TYG agar. Strain NH3-1 did not liquefy gelatin but could reduce nitrate to nitrite. Thus, strain NH3-1 is clearly distinguished from *M. narashinoensis* in its growth characters, color of vegetative growth, production on a soluble pigment, gelatin liquefaction and nitrate reduction patterns. Moreover, it produces different antibiotics. The *M. narashinoensis* strain No. 76-N_3-5 produces antibiotic nocardorubin (rufinosporin[22]), which is active against the growth of Gram-positive bacteria. Strain NH3-1 produces new piperazine-2,5-dione antibiotic, which is active against the growth of fungi and KB tumor cells. From these results, strain NH3-1 is considered to be a new species of *Micromonospora* and the name *Micromonospora neihuensis* Wu, sp. nov. is proposed.

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