VOL. 53 NO. 10, OCT. 2000 THE JOURNAL OF ANTIBIOTICS pp. 1096 — 1101

Tubelactomicin A, a Novel 16-Membered Lactone Antibiotic, from *Nocardia* sp.

I. Taxonomy, Production, Isolation and Biological Properties†

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(Received for publication June 27, 2000)

A novel 16-membered lactone antibiotic named tubelactomicin A was isolated from the culture broth of an actinomycete strain. The producing organism, designated MK703-102F1, was identified as a member of *Nocardia*. Tubelactomicin A was isolated from the culture broth by Diaion HP20 absorption, ethyl acetate extraction, silica gel and Sephadex LH-20 column chromatographies and centrifugal liquid-liquid partition chromatography (CPC). Tubelactomicin A showed strong activity against acid-fast bacteria including the drug-resistant strains.

In recent years, the prevalence of HIV has caused the increase of tuberculosis patient and the morbidity of tuberculosis with the drug resistant strains has increased in the world[1]. However, the number of antitubercular drugs is less than that of other antibacterial drugs. In addition, the drugs for the atypical mycobacterial disease are far less. Therefore, new effective drugs are needed for treatment of *Mycobacterium tuberculosis* and the atypical mycobacterial disease[1].

In the course of screening for new anti-microbial substances from microorganisms, we found that an actinomycete strain which was isolated from a soil collected at Suwa-shi, Nagano prefecture, Japan, produced a novel 16-membered lactone named tubelactomicin A (**1**, Fig. 1), which showed strong and specific activity against some acid-fast bacteria.

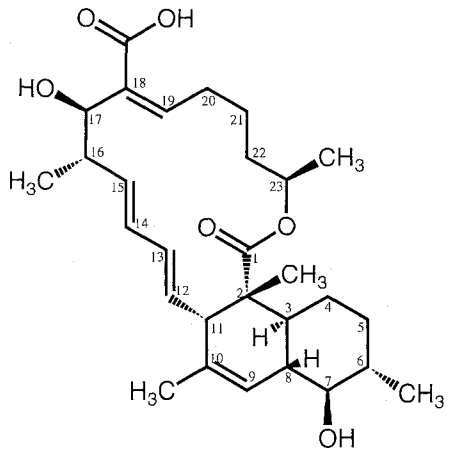
In this paper, we describe the identification of the producing organism together with the isolation, fermentation and biological activities of **1**. Physico-chemical properties and structure elucidation of the compound will be described in the accompanying paper[2].

Materials and Methods

Taxonomy

Tubelactomicin producing organism, strain MK703-102F1, was isolated from a soil sample collected at Suwa-shi, Nagano prefecture, Japan. Morphological, cultural and physiological properties of the strain MK703-102F1 were

Fig. 1. Absolute structure of tubelactomicin A (**1**).



† This article is dedicated to Sir EDWARD P. ABRAHAM, a pioneer in the field of antibiotics.

examined according to the methods described by SHIRLING and GOTTLIEB[3] and WAKSMAN[4]. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Model S-570, Hitachi) after strain MK703-102F1 was incubated on sucrose-nitrate agar and glucose-asparagine agar at 27°C for 6 to 10 days. Chemical composition of cell wall was analyzed using thin layer chromatography (TLC) according to the method of STANECK and ROBERTS[5]. Whole-cell sugars were determined by the method of LECHEVALIER and LECHEVALIER[6]. The acyl type of cell wall was analyzed according to the method of UCHIDA[7]. Menaquinone was analyzed with the method of TAMAOKA *et al.*[8] Phospholipids and mycolic acids were analyzed by the procedures of MINNIKIN *et al.*[9,10] The fatty acids were analyzed by gas chromatography of whole-cell methanolysates[11].

Fermentation

A slant culture of the tubelactomicin-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, glycerol 1.0%, Soytone Peptone (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 3 days. The seed culture (3.3 ml) of the strain were transferred into a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium consisting of starch 2.0%, glucose 1.0%, casamino acid 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4% in deionized water. The fermentation was carried out on a rotary shaker (180 rpm) at 27°C for 6 days.

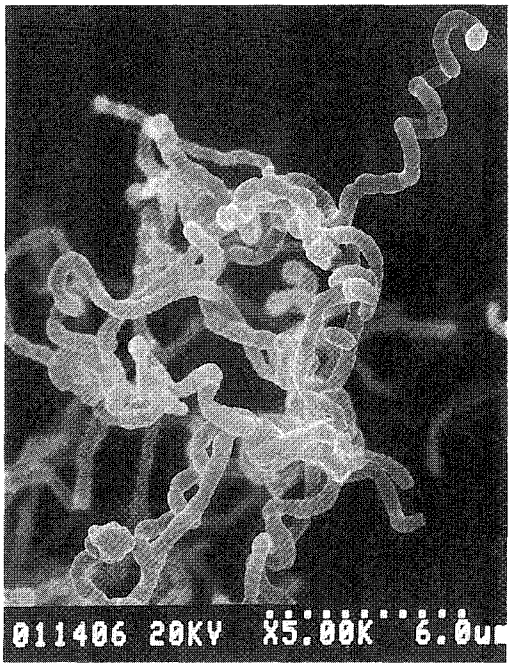
Analytical Procedure

Content of **1** in the fermentation broth and its purification steps were monitored with reversed phase HPLC and silica gel TLC. HPLC was performed with a CAPCELL PAK C_{18} column (4.6×150 mm, Shiseido Co. Ltd., Japan; mobile phase, acetonitrile : H_2O : formic acid=55 : 45 : 1; flow rate, 1.5 ml/minute; column temperature, 60°C; detection, UV at 238 nm). It was eluted at 3.8 minutes. TLC was performed with Kieselgel 60 F_{254} (Art. No. 5715, Merck) developed with $CHCl_3$-MeOH-AcOH=10 : 1 : 0.1. Spot of the antibiotic on a TLC was detected by molybdophosphoric acid-sulfuric acid and UV quenching (254 nm). Rf value of tubelactomicin A was 0.28.

Biological Activity

The minimum inhibitory concentrations (MIC) of tubelactomicin A were examined by serial agar dilution method using Nutrient agar containing 1% glycerol for acid-fast bacteria and Mueller-Hinton agar (Difco) for other bacteria and yeast. The MIC was observed after an incubation for 42 hours at 37°C against acid-fast bacteria, and incubation for 18 hours at 37°C against other bacteria and yeast, respectively.

Fig. 2. Scanning electron micrograph of *Nocardia* sp. MK703-102F1 grown on sucrose-nitrate agar for 6 days.



Results

Taxonomic Features of Strain MK703-102F1

Strain MK703-102F1 produced well-branched vegetative mycelia. This strain formed aerial hyphae which was straight, flexous or spirals. Both vegetative and aerial hyphae formed nocardioform fragmentation. The spore was cylindrical and ellipsoidal with smooth surface and 0.4~0.6×0.8~1.8 μm in size (Fig. 2). No synnemata, sclerotia, sporangia nor motile spores was observed.

The cultural characteristics of strain MK703-102F1 on various agar media are shown in Table 1. The aerial mycelia were white to brownish white. The vegetative mycelia were colorless to pale yellow to pale brown. The soluble pigments were not produced or shade of vinaceous. Physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature ranges for

Table 1. Cultural characteristics of strain MK703-102F1.

Medium	Growth	Aerial mycelium	Soluble pigmemt
Sucrose-nitrate agar	Colorless	Thin, white	None
Yeast extract-malt extract agar (ISP No.2)	Pale yellow [2 ec, Sand] ~ pale brown [3 ie, Camel]	Brownish white [2 ba, Pearl]	None
Oatmeal agar (ISP No.3)	Colorless	Thin, white	None
Inorganic salts-starch agar (ISP No.4)	Colorless	Thin, white	None
Glycerol-asparagine agar (ISP No.5)	Pale yellow [2 ec, Sand] ~ pale brown [3 ie, Camel]	Brownish white [2 ba, Pearl]	Shade of pale reddish purple
Tyrosine agar (ISP No.7)	Pale yellow [2 ec, Sand] ~ pale brown [3 ie, Camel]	Brownish white [2 ba, Pearl]	None

Observation after incubation at 27°C for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.[13]

growth of the strain were 20°C to 37°C. The optimal temperature for growth of strain MK703-102F1 was 30°C.

Whole-cell hydrolysates of strain MK703-102F1 contained *meso*-diaminopimelic acid, galactose and arabinose. The strain has type PII phospholipid (phosphatidyl ethanolamine +, phosphatidyl choline −, unknown glucosamine containing phospholipid −) and MK-8(H_4) as the major components of menaquinone. *N*-acyl type of muramic acid in cell wall was glycolyl type. Mycolic acids were present. Cellular fatty acids consisted 16 : 0 as major components and 18 : 1, 10-methyl-18 : 0, 16 : 1 and 18 : 0 as minor components.

These taxonomic properties suggested that strain MK703-102F1 belonged to the genus *Nocardia*[12,13].

Therefore, the strain was identified as *Nocardia* sp. and designated *Nocardia* sp. MK703-102F1. Detailed taxonomic study of strain MK703-102F1 is now progress. Strain MK703-102F1 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba, Japan, under the accession No. FERM P-16580.

Table 2. Physiological characteristics of strain MK703-102F1.

Temperature range for growth (°C)	20 ~ 37
Optimum temperature (°C)	30
Formation of melanoid pigment	
ISP No.1	Negative
ISP No.6	Negative
ISP No.7	Negative
Hydrolysis of starch	Negative
Reduction of nitrate	Positive
Utilization of *	
L-Arabinose	+
D-Xylose	-
D-Glucose	+
D-Fructose	+
Sucrose	-
Inositol	-
Rhamnose	-
Raffinose	-
D-Mannitol	+

* +: utilization, −: no utilization.

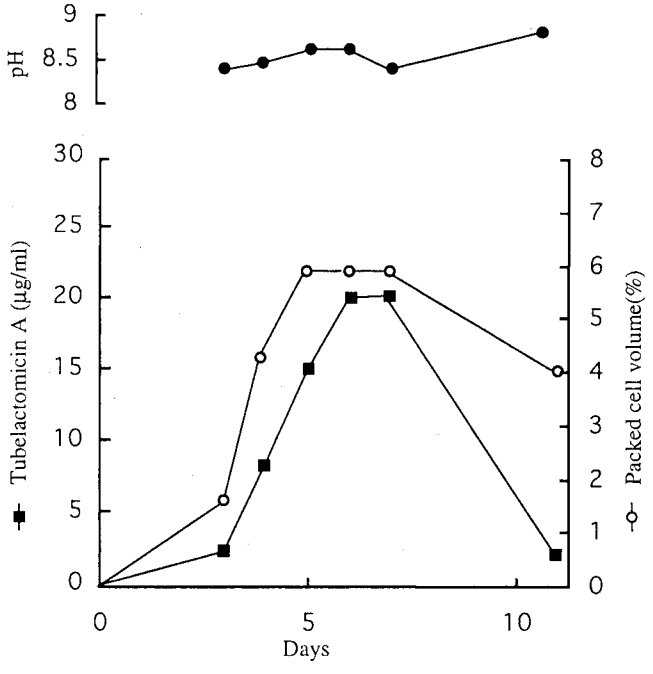
Fermentation and Isolation

A typical time course of **1** production in a 500-ml baffled Erlenmeyer flask is shown in Fig. 3. The production of **1** began at 3-days and maximum (*ca.* 20 mg/liter) was observed at 6-days after incubation.

The fermentation broth (5 liters) was separated to the mycelial cake and supernatant by centrifugation. The supernatant was adjusted at pH 2.0 with 6 M HCl and applied to a Diaion HP20 (Mitsubishi Chemical Co.) column (56×200 mm). The column was washed with

deionized water (1500 ml) and 50% aqueous MeOH (1500 ml). The active principle was eluted with 50% aqueous acetone (1000 ml). The aqueous acetone was concentrated *in vacuo* to 400 ml and adjusted to pH 9.0 with 1 M NaOH. The solution was extracted with EtOAc (800 ml). The EtOAc solution was washed with H_2O and concentrated under reduced pressure to yield a brown oil (236 mg). The concentrate was chromatographed by using silica gel column ($CHCl_3$, $CHCl_3$: MeOH = 10 : 1 and $CHCl_3$: MeOH : formic acid = 10 : 1 : 0.1). The active fractions were collected and concentrated *in vacuo* to give a pale brown oil (81 mg). The oil was chromatographed on a column of Sephadex LH-20 (Amersham Pharmacia Biotech, 28×200 mm) developing with MeOH. The active fractions were collected and concentrated *in vacuo* to give a crude tubelactomicin A (**1**, 54 mg). The crude antibiotic was further purified by CPC (EtOAc : 10 mM K_2HPO_4 = 1 : 1, decending method). The pure antibiotic, it was kept in stationary phase, was collected, concentrated *in vacuo* and obtained as colorless powder of **1** (37.9 mg, Fig. 4). Structure of **1** is shown in Fig. 1. The studies on the structure determination of this antibiotic will be reported in the accompanying paper[2].

Fig. 3. Production of tubelactomicin A.



Biological Activity

The antimicrobial activities of **1** is shown in Table 3. Tubelactomicin A (**1**) showed strong and specific anti-microbial activities against rapid growers *Mycobacterium* including drug-resistant strains except for a strain of viomycin resistant (Table 3). Its MIC's was 0.1 μg/ml. Moreover, its resistant strain of *Mycobacterium smegmatis* did not have tolerance against anti-tuberculotic antibiotics

Fig. 4. Isolation procedure of tubelactomicin A.

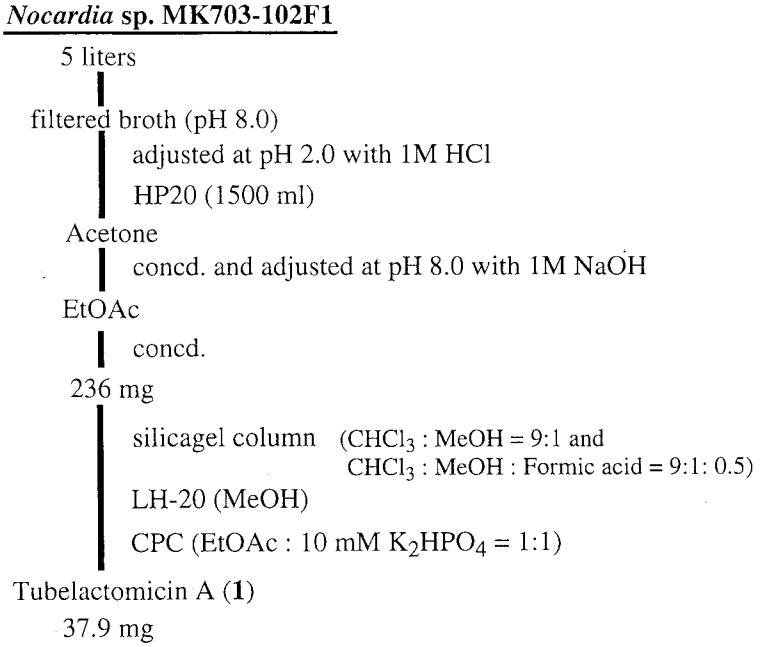


Table 3. Antimicrobial activities of tubelactomicin A.

Test organisms	MIC(μg/ml)
Staphylococcus aureus FDA 209P[a]	50
Micrococcus luteus IFO 3333[a]	100
Bacillus subtilis NRRL B-558[a]	>100
Escherichia coli NIHJ[a]	>100
Pseudomonas aeruginosa A3[a]	>100
Candida albicans 3147[b]	>100
Mycobacterium smegmatis ATCC 607[c]	0.10
M. smegmatis ATCC 607 PM-R[c]	0.10
M. smegmatis ATCC 607 VM-R[c]	50
M. smegmatis ATCC 607 KM-R[c]	0.10
M. smegmatis ATCC 607 SM-R[c]	0.10
M. smegmatis ATCC 607 RFP-R[c]	0.10
M. phlei [c]	0.20
M. vaccae [c]	0.10
M. fortuitum [c]	0.78

a: Mueller Hinton agar 37°C 18 hours. b: Nutrient agar +1% glucose 27°C 18 hours. c: Nutrient agar +1% glycerol, 37°C, 42 hours. PM-R: paromomycin resistant. VM-R: viomycin resistant. KM-R: kanamycin resistant. SM-R: streptomycin resistant. REP-R: rifampicin resistant.

Table 4. Cross-resistance test on tubelactomicin A.

Chemicals (100 μg/ml)	Inhibition zone (mm)	
	M. smegmatis ATCC607	*M. smegmatis* ATCC607 tubelactomicin R*
Tubelactomicin A	59.0	0
Viomycin	24.5	20.0
Streptomycin	40.0	34.5
Kanamycin	39.5	36.0
Paromomycin	40.0	34.5
Rifampicin	(17.0)	(17.0)
INH	33.0	29.5

Cylinder agar plate method, Nutrient agar + 1% glycerol, 37°C, 42 hours.
( ): partial inhibition.
*: tubelactomicin A resistant.

including viomycin (Table 4). Compound **1** did not show acute toxicity in mice at a dose of 100 mg/kg when administered intravascural. However, the activity of **1** against pathogenic *Mycobacterium* such as *M. tuberculosis*, *M. avium* and *M. intracellulare* is very weak (data not shown).

Discussion

Our screening program for new antibiotics gave a new antiacid-fast bacterial compound designated tubelactomicin A (**1**). Compound **1** showed strong antimicrobial activities against rapid growers *Mycobacterium* including drug-resistant strains. Moreover, the resistant strain of **1** did not show cross resistance with other anti-tuberculotic antibiotics. It was suggested that **1** has new mechanism of growth inhibition against acid-fast bacteria. Structural study revealed that **1** is a 16-membered cyclic lactone antibiotic. Avermectin[15] and milbemycin[16] having 16-membered lactone, were isolated from a strain of *Streptomyces* as anthelmetic and insecticidal antibiotics. Chlorothricin[17] was having 14-membered lactone and decaline ring was isolated from a strain of *Streptomyces* as antitumour antibiotics. Although their structures are related to that of **1**, they are 16-membered lactone or decaline ring contained compounds. However, **1** did not exhibit anthelmetic, insecticidal and antitumour activity in spite of a member of these macrocyclic lactone. For that reason, mode of action of **1** is interesting as a lead for the development of novel type of antitubercular drugs. Mode of action of **1** and the structural relationships are under investigation.

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