Nothramicin, a New Anthracycline Antibiotic from *Nocardia* sp. MJ896-43F17

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A new antibiotic designated nothramicin was isolated from the culture broth of *Nocardia* sp. MJ896-43F17 which was closely related to *Nocardia brasiliensis*. It was isolated by the Diaion HP-20 column chromatography, butyl acetate extraction and purified by HPLC. It inhibited the growth of mycobacteria at the concentration of 1.56~25 μg/ml. Nothramicin was revealed to be a new member of anthracycline antibiotics by the various spectroscopies.

During our screening for new antibiotics, we have isolated a new anthracycline antibiotic, nothramicin (Fig. 1) which was produced by *Nocardia* sp. MJ896-43F17. In this paper, taxonomy of the producing strain, isolation, physico-chemical properties, biological activities and structure of nothramicin are reported.

Materials and Methods

Microorganism

The nothramicin producing strain, MJ896-43F17 was isolated from a soil sample collected at Miura city, Kanagawa prefecture, Japan.

Nocardia brasiliensis IMC A-0198 (JCM 3374^T) was used as reference strain.

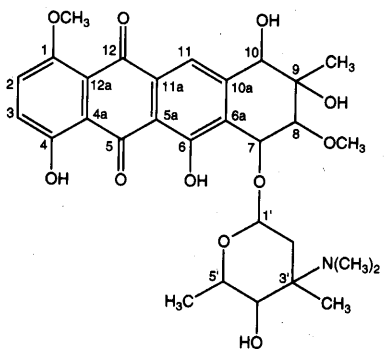
Taxonomic Studies

Morphological observations under a scanning electron microscope (model Hitachi S-570) were made on cultures grown on sucrose-nitrate agar, incubated at 27°C for 2 to 3 weeks. Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[1], WAKSMAN[2], and GORDON *et al.*[3]. The substrate and aerial mass color including soluble pigments were assigned by the Color Harmony Manual, 1958 (Container Corporation of America, Chicago). Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[4]. 2,6-Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[5] and STANECK and ROBERTS[6]. Whole-cell sugars were determined by the method of LECHEVALIER and LECHEVALIER[7], and MIKAMI and ISHIDA[8]. Muramic acid was analyzed by the glycolate test as described by UCHIDA[9]. Phospholipids and mycolic acids were detected by the procedure of MINNIKIN *et al.*[10] and MINNIKIN *et al.*[11], respectively. Menaquinones were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.*[12]. Fatty acids were detected by gas chromatography as described by SUZUKI and KOMAGATA[13].

Production of Nothramicin

A slant culture of the strain MJ896-43F17 was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of seed medium consisting of galactose 2.0%,

Fig. 1. Structure of nothramicin.



dextrin 2.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki) 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicon oil (Shin-Etsu Chemical Industry, KM-70) (adjusted to pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 30°C for 72 hours. This seed culture (550 ml) was transferred into a 30-liter fermentor containing 15 liters of production medium consisting of yeast extract 0.5%, glucose 1.0%, potato starch 2.0%, casamino acid (Difco) 0.5%, $CaCO_3$ 0.4% and silicon oil (Toho kagaku, Pronal 502). Production of nothramicin was carried out at 27°C for 192 hours under aeration (15 liters/minute) and agitation (200 rpm/minute).

Biological Activities

Antimicrobial activity of nothramicin was assayed by cup or paper-disk diffusion methods against *Mycobacterium smegmatis* ATCC 607. The MICs of nothramicin against mycobacteria were determined by the agar dilution method in a nutrient agar containing 1.0% glycerol.

Results and Discussion

Taxonomy of the Nothramicin Producing Strain

The vegetative mycelium of strain MJ896-43F17 was well-branched and fragmented. Aerial hyphae were either straight or irregularly spiral, and fragmented into cylindrical or ellipsoidal elements. They often twined oneself at the tip of one's aerial hyphae, which seemed to be spherical. The mature spore was $0.3 \sim 0.5 \times 0.8 \sim 1.2\,\mu m$ in size with a smooth surface. Spores were not motile. No synnemata, sclerotia or sporangia were observed. The cultural characteristics of the strain MJ896-43F17 on various agar media are shown in Table 1. The color of growth was colorless to pale orange, and the color of aerial mycelia was white to pinkish white on some media tested. Shade of pale pink or pale orange soluble pigments were produced. The physiological properties of the strain MJ896-43F17 were shown in Table 2.

An analysis of cell wall hydrolysate of the strain revealed the presence of *meso*-diaminopimelic acid. The whole-cell hydrolysate of the strain showed the presence of arabinose and galactose as diagnostic sugars (whole-cell sugar pattern A). The phospholipid type was PII, which contained phosphatidylethanolamine, but neither phosphatidylcholine nor unknown glucosamine-containing phospholipids. The acyl type of muramic acid in peptideglycan was glycolyl. The major menaquinone was MK-8 (H_4) and MK-8 (H_2). Mycolic acids were present. Hexadecanoic acid, 10-methyl-octadecanoic acid, hexadecenoic acid, octadecenoic acid were detected as the cellular fatty acids.

On the basis of these characteristics, the strain MJ896-43F17 was belonged to the genus *Nocardia*, and related to *Nocardia brasiliensis*[14] as shown in Table 3. The physiological characteristics of strain MJ896-43F17 was similar to those of *N. brasiliensis* IMC A-0198 (JCM 3374^T) except for the decomposition of tyrosine and the acid production from rhamnose. Therefore, strain

Table 1. Cultural characteristics of strain MJ896-43F17.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless ~ pale yellow [3ca, Pearl Pink]	White	None
Glycerol-asparagine agar (ISP No. 5)	Pale orange [3ea. Lt Melon Yellow]	Pinkish white [5ba, Shell Pink]	Shade of pale orange
Inorganic salts-starch agar (ISP No. 4)	Colorless	Thin, white	None
Tyrosine agar (ISP No. 7)	Pale orange [3ea, Lt Melon Yellow] ~ pale yellowish brown [3ic, Lt Amber]	Pinkish white [5ba, Shell Pink]	Shade of pale orange
Yeast extract-malt extract agar (ISP No. 2)	Dull orange [4ic, Pastel Orange]	Pinkish white [4ca, Flesh Pink]	Pinkish
Oatmeal agar (ISP No. 3)	Colorless ~ pale yellow [3ca, Pearl Pink]	White ~ pinkish white [5ba, Shell Pink]	Shade of pale pink

Observation after incubation at 27°C for 21 days.

Table 2. Physiological characteristics of strain MJ896-43F17.

		Utilization of	
Temperature range for growth (℃)	10 ~ 37	L-Arabinose	(-)
Optimum temperature (℃)	27 ~ 30	D-Xylose	-
Formation of melanoid pigment	-	D-Glucose	+
Hydrolysis of starch	-	D-Fructose	(+)
Reduction of nitrate	+	Rhamnose	+
		Sucrose	-
		Raffinose	-
		Inositol	+
		D-Mannitol	(+)

+: Positive; (+): probably positive; (−): probably negative; −: negative.

Table 3. Physiological characteristics of strain MJ896-43F17 and *Nocardia brasiliensis* IMC A-0198 (JCM 3374^T).

	MJ896-43F17	IMC A-0198		MJ896-43F17	IMC A-0198
Decomposition of:			Survival at 50°C/8h	+	+
Adenine	-	-	Acid production from:		
Casein	+	+	Adonitol	-	-
Hypoxanthine	(+)	+	L-Arabinose	-	-
Tyrosine	-	+	D-Cellobiose	-	-
Urea	+	+	*meso* -Erythritol	-	-
Xanthine	-	-	D-Galactose	+	+
Utilization of:			D-Glucose	+	+
Benzoate	-	-	Glycerol	+	+
Citrate	+	+	Inositol	+	+
Mucate	-	-	Lactose	-	-
Succinate	+	+	Maltose	-	-
DL-Tartrate	-	-	D-Mannitol	+	+
Hydrolysis of:			D-Mannose	+	+
Esculin	+	+	D-Melezitose	-	-
Hippurate	-	-	Melibiose	-	-
Growth in the presence of:			α-Methyl-D-glucoside	-	-
4%NaCl	+	+	Raffinose	-	-
5%NaCl	+	+	Rhamnose	+	-
Growth at:			D-Sorbitol	-	-
37°C	(+)	+	Treharose	+	+
45°C	-	-	D-Xylose	-	-

+: Positive; (+): probably positive; −: negative.

MJ896-43F17 was closely related to *Nocardia brasiliensis*. This strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan, under the accession number FERM P-16068.

Isolation

The culture broth (30 liters) was centrifuged to separate a supernatant and a mycelial cake. The supernatant was applied to a column of Diaion HP-20 (1.5 liters). The column was washed with water (3 liters) and then with

Table 4. Physico-chemical properties of nothramicin.

Appearance	Red powder
mp	140 ~ 145℃
Molecular formula	$C_{30}H_{37}NO_{11}$
FAB-MS (*m/z*)	588 $(M+H)^+$
	587 (M^-)
HRFAB-MS (*m/z*)	
Calcd:	588.2445 (as $C_{30}H_{38}NO_{11}$)
Found:	588.2431 $(M+H)^+$
UV λ_{max} nm (log ε) in	
MeOH	233 (4.71), 257 (4.31), 290 (sh, 3.96), 472 (4.13)
MeOH-NaOH	238 (4.65), 280 (3.98), 323 (3.62), 536 (4.13)
MeOH-HCl	233 (4.73), 257 (4.33), 290 (sh, 3.98), 472 (4.15)
IR ν_{max}(KBr) cm^{-1}	3400, 2930, 1620, 1470, 1410, 1290, 1225, 1180, 1010, 990
TLC (Rf value)[a]	0.21

[a] Silica gel TLC (Merck Art. 1.05715.): $CHCl_3$ - MeOH (3 : 1).

30% aq MeOH (2 liters). The active substance was eluted with 50% aq acetone (6 liters) and concentrated to 2 liters under reduced pressure. The concentrate was adjusted to pH 8.0 with 1 N NaOH, and was extracted twice with BuOAc (2 liters). The extract was evaporated to dryness to give a red oil (437.2 mg). The oily substance was purified by reverse phase HPLC using Capcell Pak UG (Shiseido, 2.0 × 25 cm) with a solvent mixture of CH_3CN - 0.2 N phosphoric acid (20 : 80) running at the flow rate of 10 ml/minute. The active fraction was evaporated under reduced pressure to remove CH_3CN after neutralization with 0.1 N NaOH. The aqueous solution was adjusted to pH 8.0 with 0.1 N NaOH and extracted with an equal volume of $CHCl_3$. The organic layer was concentrated to dryness to give pure nothramicin (105.1 mg).

Physico-chemical Properties and Its Structure

Physico-chemical properties of nothramicin are shown in Table 4. It was soluble in organic solvents such as MeOH, $CHCl_3$ and DMSO, but insoluble in *n*-hexane and water. Nothramicin gave positive color reaction with molybdophosphoric acid-sulfuric acid, 2,4-dinitrophenylhydrazine, $FeCl_3$ and anisaldehyde-sulfuric acid reagents, but negative with ninhydrin and Rydon-Smith reagents.

The UV spectrum of nothramicin showed absorption maxima at 233, 257, 290 and 472 nm in MeOH and visible absorption band in alkaline solution exhibited characteristic bathochromic shifts. The UV data suggested that nothramicin was related to the anthracycline antibiotics[15,16].

The molecular formula for nothramicin was established as $C_{30}H_{37}NO_{11}$ by HRFAB-MS and NMR spectra. Chemical shifts of the ^1H and ^{13}C NMR spectra in nothramicin are shown in Table 5. The ^1H, ^{13}C NMR, DEPT and HMQC spectra of nothramicin revealed the presence of sixteen sp^3 carbons consisting of three methyl, two methoxy, a dimethylamino, a methylene, six methine and two quaternary carbons. In addition, nothramicin contains fourteen sp^2 carbons consisting of three methine and eleven quaternary carbons. The ^1H NMR spectrum indicated the presence of five hydroxyl groups.

The ^1H-^1H COSY spectrum showed the presence of five partial structures. The connectivity among the partial structures was elucidated by the HMBC experiment. Furthermore, to observe very small long range ^{13}C-^1H couplings, D-HMBC[17] experiment was performed. The results of the ^1H-^1H COSY, HMBC and D-HMBC experiments are summarized in Fig. 2.

In the planar structure, the structure of the aglycone in nothramicin was closely similar to that in 10-dihydrosteffimycin[18]. However, the ^1H and ^{13}C NMR spectra of nothramicin showed the presence of a methoxy group at position 1 instead of position 2 reported in the aglycone of 10-dihydrosteffimycin. Moreover, the ^1H and ^{13}C NMR spectra of nothramicin indicated the presence of a sugar moiety because the characteristic signals at δ_H

Table 5. ^{13}C and ^{1}H NMR assignments of nothramicin in $CDCl_3$.

Position	δ_C (ppm)	δ_H (ppm)
1	154.79	
1-OCH_3	57.08	4.02 s
2	124.44	7.44 d (9.4)*
3	126.76	7.33 d (9.4)
4	157.40	
4-OH		12.35 br
4a	115.68	
5	192.64	
5a	114.81	
6	162.35	
6-OH		12.67 br
6a	126.95	
7	72.49	5.06 d (1.8)
8	87.18	3.65 br
8-OCH_3	59.72	3.60 s
9	69.61	
9-CH_3	23.20	1.34 s
9-OH		3.72**
10	75.04	4.33 br
10-OH		3.26 br
10a	147.10	
11	121.58	7.88 br
11a	134.74	
12	180.41	
12a	119.08	
1'	101.36	5.60 br d (5.0)
2'	35.22	1.67 br d (14.0)
		1.82 dd (5.0, 14.0)
3'	56.03	
3'-CH_3	11.95	0.97 s
3'-$N(CH_3)_2$	36.29	2.12 s
4'	70.20	3.26 br
4'-OH		2.40**
5'	64.14	3.99 br q (6.4)
6'	17.87	1.45 d (6.4)

Chemical shifts in ppm from TMS as an internal standard.
* The coupling constants (Hz) are in parentheses.
** These assignments are exchangeable.

Fig. 2. Summary of ^{1}H-^{1}H COSY, HMBC and D-HMBC experiments of nothramicin.

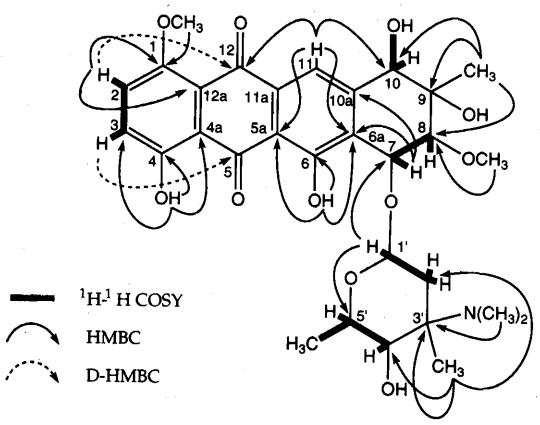


Table 6. The antimycobacterial activity of nothramicin.

Test organism	MIC (μg/ml)
Mycobacterium smegmatis ATCC 607	1.56
M. smegmatis ATCC 607 paromomycin-resistant	6.25
M. smegmatis ATCC 607 capreomycin-resistant	6.25
M. smegmatis ATCC 607 viomycin-resistant	6.25
M. smegmatis ATCC 607 streptothricin-resistant	6.25
M. smegmatis ATCC 607 kanamycin-resistant	6.25
M. smegmatis ATCC 607 streptomycin-resistant	6.25
M. smegmatis ATCC 607 rifampicin-resistant	6.25
M. phlei	6.25
M. vaccae ATCC 15483	12.5
M. fortuitum	25

Nutrient agar + 1% Glycerol, 37°C, 42 hours.

5.60 (1'-H) and δ_C 101.36 (C-1') were assignable to the anomeric signals. The anomeric proton 1'-H was coupled to C-7 (δ_C 72.49) indicating the glycosidic bond between C-1' and C-7. As a result, the structure of nothramicin was proposed as shown in Fig. 1.

Biological Activities

The antimycobacterial activity of nothramicin are shown in Table 6. Nothramicin inhibited the growth of mycobacteria. Its MICs were 1.56~25 μg/ml. But nothramicin did not inhibit the growth of Gram-positive and -negative bacteria, and yeast.

The acute toxicity (LD_{50}, iv) of nothramicin in mice was estimated to be >100 mg/kg.

Experimental

General

NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz

for ^{13}C NMR. Chemical shifts are given in ppm using TMS as an internal standard. UV absorption spectra were measured with a Hitachi U-3210 spectrophotometer. IR absorption spectra were recorded with a HORIBA FT-210 spectrometer. FAB-MS and HRFAB-MS were measured with a JEOL JMS-SX 102 spectrometer. Optical rotations were taken by a Perkin-Elmer 241 polarimeter.

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