Azicemicins A and B, a New Antimicrobial Agent Produced by *Amycolatopsis*

I. Taxonomy, Fermentation, Isolation, Characterization and Biological Activities

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A new structural class of the antibiotic, azicemicins A (**1**) and B (**2**) were isolated from the culture broth of the strain MJ126-NF4, which was closely related to *Amycolatopsis sulphurea*. They were purified by adsorption on Diaion HP-20, silica gel column chromatography and preparative TLC. The molecular formulas of **1** and **2** were determined to be $C_{23}H_{25}O_9N$ and $C_{22}H_{23}O_9N$ by HRFAB-MS, respectively. Azicemicins A and B have moderate growth inhibiting activity against Gram-positive bacteria and mycobacteria.

In the course of our screening for antibiotics, a new type antibiotic, azicemicins A (**1**) and B (**2**) were found in the fermentation broth of *Amycolatopsis* sp. MJ126-NF4 (Fig. 1). A preliminary communication of this work has been reported[1]. In this paper, the details of taxonomy, production, isolation, physico-chemical properties and biological properties of **1** and **2** are described. The structural elucidation study of **1** and **2** will be reported in another paper[2].

Materials and Methods

Azicemicin-producing Strain

Strain MJ126-NF4 was isolated from a soil sample collected in Setagaya-ku, Tokyo, Japan.

Amycolatopsis orientalis[3] IMC A-0161 (ISP 5040^T), *A. orientalis subup. lurida*[3] IMC A-0168 (IFO 14500^T), *A. azurea*[4] IMC A-0138 (JCM 3275^T), *A. fastidiosa*[4] IMC A-0166 (JCM 3276^T), *A. mediterranei*[3] IMC A-0162 (ISP 5501^T), *A. methanolica*[5] IMC A-0167 (IFO 15065^T), *A. rugosa* IMC A-0169 (IFO 14506^T), *A. sulphurea*[3] IMC A-0170 (IFO 13270^T) and "*A. tolypophorus*" IMC A-0171 (IFO 14664) were compared as reference strains.

Morphological Characteristics

Morphological observations were made with a scanning electron microscope (model Hitachi S-570) on cultures grown on yeast extract-malt extract agar (ISP medium No. 2)[6], inorganic salts-starch agar (ISP medium No. 4)[6] and sucrose-nitrate agar[7] at 30°C for 2 to 4 weeks.

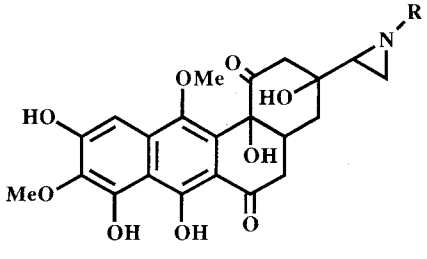
Cultural and Physiological Characteristics

The media and procedures used for cultural and physiological characteristics of the strain MJ126-NF4 were those described by SHIRLING and GOTTLIB[3] and by WAKSMAN[7]. Cultures were incubated at 30°C for 2 to 4 weeks. Color determinations were made by comparing the cultures with color chips from the *Color Harmony Manual* (Container Corporation of America). The temperature range for growth was determined on yeast-starch agar (soluble starch 1.0%, yeast extract 0.2%, agar 2.0%, pH 7.0). Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[8].

Cell Chemistry

The strain MJ126-NF4 were grown in YD medium (yeast extract 1.0% , glucose 1.0%, pH 7.2) at 30°C for 7 days on a rotary shaker. The mycelia were centrifuged and washed with distilled water. The washed and packed mycelia were then freeze-dried as a whole-cell preparation. Cell walls were prepared as described by KAWAMOTO *et al.*[9] except for treating them with pronase AS (Kaken Chemicals Co.). For determination of diaminopimelic acid isomers, cell wall hydrolysates were prepared by the methods of BECKER *et al.*[10] and analyzed by high performance liquid chromatography (HPLC) according

Fig. 1. The structure of azicemicins A (**1**) and B (**2**).



A: R=Me
B: R=H

to the method of TAKAHASHI *et al.*[11]. Cell wall sugars and whole-cell sugars were determined by the methods of LECHEVALIER and LECHEVALIER[12], and MIKAMI and ISHIDA[13], respectively. Phospholipids and mycolic acids were analyzed by the procedures of MINNIKIN *et al.*[14,15]. Menaquinones were extracted and purified by the method of COLLINS *et al.*[16] and were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.*[17], using a CAPCELL PAK C_{18} AG120 column (4.6 by 250 mm, Shiseido) with a solvent of methanol - isopropyl alcohol (2 : 1, v/v) at a flow rate of 1 ml/minute. The fatty acids were analyzed by gas chromatography of whole-cell methanolysates[18]. Fatty acid methyl esters were determined by using a Hewlett-Packard model 5890 gas chromatography with a flame ionization detector and a Hewlett-Packard model 3392A integrator. Column was used a Megabore DB-1 (15 m by 0.53 mm, film 1 mm).

Preparation and Hybridization Analysis of DNA

After incubation in YD medium supplemented with 0.5% glycine at 30°C for 3 to 5 days on a rotary shaker, mycelia were centrifuged and washed with a buffer (25 mM Tris-HCl - 25 mM EDTA - 25 mM NaCl, pH 7.4). Total DNA was extracted from the washed mycelia by the method described[19] and dissolved in 1 × TE (10 mM Tris-HCl - 1 mM EDTA, pH 7.6).

DNA homologies between strains were determined fluorometrically by the method of EZAKI *et al.*[20]. Fluorescence intensity in the wells was measured with a microplate reader MTP-32 (Corona Electric).

Measurement of Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of **1** and **2** were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial test which was incubated at 37°C for 18 hours and a nutrient agar containing 1% glycerol for mycobacteria test which was incubated at 37°C for 42 hours.

Spectroscopic Methods

UV absorption spectra were measured with a Hitachi U-3210 spectrophotometer. IR absorption spectra were obtained with a Hitachi I-5020 FT-IR spectrometer. FAB-MS and HRFAB-MS were obtained on a Jeol JMS-SX102 mass spectrometer. Optical rotations were taken by a Perkin-Elmer 241 porarimeter using a micro-cell (light path 10 cm).

Results and Discussion

Taxonomic Studies

Strain MJ126-NF4 had a branched vegetative hyphae which tended to break down into squarish fragments. The aerial hyphae broke down into chains of squarish fragments in straight to flexuous. The fragments were 0.3 to 0.5 by 0.8 to 1.1 μm in size with smooth surfaces. No sporangia, motile spores or synnemata were observed. The cultural characteristics of strain MJ126-NF4 were summarized in Table 1. The physiological characteristics and carbohydrate utilization showed Table 2.

Cell wall hydrolystates contained *meso*-diaminopimelic acid, arabinose and galactose, and whole-cell hydrolysates were rhamnose, ribose, mannose, arabinose, galactose and glucose. These data indicated that strain MJ126-NF4 has a type IV cell wall and a type A whole-cell sugar pattern. A type P II phospholipid pattern (phosphatidylethanolamine present, phosphatidylcholine and glucosamine-containing phospholipids absent) was found. Mycolic acids were not present. The major menaquinone was MK-9(H_4). This strain were contained major amounts of iso-branched 14-methylpentadecanoic acid (iso-16 : 0), hexadecanoic acid (16 : 0), 10-methyl-branched hexadecanoic acid (10Me-16 : 0) and other minor components.

The properties described above of strain of MJ126-NF4 suggested that the strain belonged to the genus *Amycolatopsis*[3]. As a result, it was found that the strain

Table 1. Cultural characteristics of strain MJ126-NF4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose - nitrate agar	Lt Ivory [2ca]	White	None
Yeast extract - malt extract agar (ISP No. 2)	Cinnamon [3 le]	White	Brownish
Oatmeal agar (ISP No. 3)	Lt Ivory [2ca]	White	None
Inorganic salts - starch agar (ISP No. 4)	Lt Tan [3gc] ~ Clove Brown [3ni]	White	None
Glycerol - asparagine agar (ISP No. 5)	Lt Tan [3gc] ~ Yellow Maple [3ng]	White ~ Pearl [3ba]	Faint, brownish
Tyrosine agar (ISP No.7)	Clove Brown [3ni]	White ~ Oyster White [b]	None ~ Dark Brown
Glucose - asparagine agar	Lt Ivory [2ca] ~ Dusty Olive [1ie]	White	Faint, greenish
Nutrient agar	Lt Tan [3gc]	Thin, white	Brownish
Glycerol - nitrate agar	Lt Tan [3gc] ~ Lt Amber [3ic]	Pearl [3ba]	Yellowish
Starch agar	Colorless ~ Pearl Pink [3ca]	White	None
Calcium malate agar	Citron [1 gc]	Thin, white	Pale Olive

Table 2. Physiological characteristics of strain MJ126-NF4.

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

+: positive utilization, ±: doubtful utilization, −: no utilization

MJ126-NF4 resembled *Amycolatopsis sulphurea* with difference about the fatty acids composition, formation of melanoid pigment, and the utilization of inositol and D-xylose (Table 3). We examined a DNA hybridization test between strain MJ126-NF4 with 9 strains 8 species of genus *Amycolatopsis*. The strains MJ126-NF4 showed the low homology with each strain in genus *Amycolatopsis*. We considered that the strain MJ126-NF4 was different from *A. sulphurea*.

Thus, the strain MJ126-NF4 is tentatively identified as *Amycolatopsis* sp. MJ126-NF4.

This strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-13362.

Fermentation

A slant culture of the azicemicin-producing organism was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, corn steep liquor 0.5%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The inoculated medium was incubated at 30°C for 8 days on a rotary shaker (220 rpm/minute). Two ml of the seed culture was transferred to each 500 ml Erlenmeyer flask containing 110 ml of a producing medium which was composed of glycerol 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, yeast extracts 0.3%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The fermentation was carried out at 27°C for 5 days on a rotary shaker.

Table 3. Comparison of strain MJ126-NF4 and *Amycolatopsis sulphurea*.

	Strain MJ126-NF4	*A. sulphurea* IMC A-0170 (IFO 12370)
Major fatty acids[a]	i-16:0, 16:0, 10Me-16:0	i-16:0, 16:0, 17:0
Aerial mycelium	Straight~flexuous	Straight
Formation of melanoid pigment		
ISP No. 1	Positive	Negative
ISP No. 6	Positive	Negative
ISP No. 7	Doubtful	Doubtful
Utilization of[b]		
Inositol	+	−
D-Xylose	+	−

[a] i-16:0, iso-branched 14-methylpentadecanoic acid; 16:0, hexadecanoic acid; 10Me-16:0, 10-methyl-branched hexadecanoic acid.

[b] +, positive utilization; −, no utilization.

Isolation and Purification

The culture broth was filtrated and the filtrate (4.4 liters) was adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited, 350 ml). The column was washed with water (2.0 liters) and 30% aqueous MeOH (1.5 liters). The antibiotic was eluted with 80% aqueous MeOH (1.5 liters). The active fractions were collected and concentrated under reduced pressure to dryness. The dried residue (1.4 g) was chromatographed on a silica gel (Merck Kieselgel 60, 70 g) using mixtures of $CHCl_3$-MeOH (20:1, 10:1, 7:1, 5:1, 4:1 and 2:1). The crude antibiotic 1 was eluted with the mixture of $CHCl_3$-MeOH (7:1 and 5:1). The fractions which contained a UV-absorbing substance at Rf 0.26 on silica gel TLC (Merck Kieselgel $60F_{254}$, $CHCl_3$-MeOH, 10:1) were collected and concentrated under reduced pressure to give a dark yellow solid (0.202 g). A sample (13 mg) of the crude **1** was purified by silica gel TLC ($CHCl_3$-90% aqueous MeOH, 10:1) providing 9.5 mg of **1** as a yellow powder.

The crude antibiotic **2** (0.205 g) was obtained from the column chromatography eluting by the mixtures of $CHCl_3$-MeOH (4:1 and 2:1). A sample (56 mg) was purified by silica gel TLC ($CHCl_3$-MeOH-H_2O, 4:1:0.1) and **2** (8.1 mg) was provided from giving a UV-absorbing spot at Rf 0.42.

Physico-chemical Properties

Physico-chemical properties of azicemicins A (**1**) and B (**2**) are summarized in Table 4. The antibiotics **1** and **2** are soluble in methanol, chloroform, acetone, slightly soluble in water and insoluble in hexane. The molecular formula $C_{23}H_{25}NO_9$ and $C_{22}H_{23}NO_9$ were determined by HRFAB-MS. The IR spectrum of azicemicins showed the nonchelated carbonyl and chelated carbonyl at 1720

Table 4. Physico-chemical properties of azicemicins A and B.

	Azicemicin A	Azicemicin B
Appearance	Yellow powder	Yellow powder
Nature	Amphoteric	Amphoteric
Molecular formula	$C_{23}H_{25}NO_9$	$C_{22}H_{23}NO_9$
FAB-MS (*m/z*)	$(M+H)^+$ 460, $(M-H)^-$ 458	$(M+H)^+$ 446, $(M-H)^-$ 444
HRFAB-MS (*m/z*)		
Calcd:	459.1529 (as $C_{23}H_{25}NO_9$)	446.1451 (as $C_{22}H_{24}NO_9$)
Found:	459.1524 (M^-)	446.1457$(M+H)^+$
UV λ_{max} (log ε) in MeOH	234 (4.23), 277 (4.46), 322 (3.65), 335 (sh, 3.55), 410 (3.95)	234 (4.30), 278 (4.55), 322 (3.72), 336 (sh, 3.62), 409 (4.06)
in 0.01 N NaOH-MeOH	241 (4.02), 290 (4.44), 414 (4.15)	242 (4.13), 290 (4.58), 414 (4.27)
in 0.01 N HCl-MeOH	234 (4.22), 278 (4.43), 322 (3.62), 335 (sh, 3.51), 409 (3.96)	234 (4.35), 277 (4.56), 323 (3.75), 335 (sh, 3.65), 409 (4.05)
IR ν_{max}^{KBr} cm^{-1}	3430, 2955, 1720, 1630, 1615, 1525, 1395	3410, 2940, 1720, 1620,1520, 1390
$[\alpha]_D^{24}$	−185 (*c* 1.04, MeOH)	−121 (*c* 0.2, MeOH)
Rf	0.26[a]	0.42[c]
Rm (L-alanine: 1)	0.51[b]	0.51[b]

[a] Silica gel TLC (Merck Art. No. 5715) $CHCl_3$: MeOH (10 : 1).
[b] 3500 V, 15 min (Formic acid - Acetic acid - Water 25 : 75 : 900, pH 1.6).
[c] Silica gel TLC (Merck Art. No. 5715) $CHCl_3$: MeOH : H_2O (4 : 1 : 0.1).

Table 5. Antimicrobial activities of azicemicins A and B.

Test organism	MIC (μg/ml)	
	Azicemicin A	Azicemicin B
Staphylococcus aureus FDA209P	>100	100
S. aureus Smith	>100	100
S. aureus MS9610	>100	100
S. aureus No. 5 (MRSA)	>100	100
S. aureus No. 17 (MRSA)	>100	100
Micrococcus luteus FDA 16	50	6.25
M. luteus IFO 3333	12.5	1.56
M. luteus PCI 1001	12.5	1.56
Bacillus anthracis	100	100
B. subtilis NRRL B-558	>100	>100
B. subtilis PCI 219	>100	>100
B. cereus ATCC 10702	>100	>100
Corynebacterium bovis 1810	25	6.25
Escherichia coli NIHJ	50	25
E. coli K-12	>100	100
E. coli K-12 MLI629	>100	>100
E. coli BEM11	100	100
E. coli BE1126	100	100
E. coli BE1186	100	100
Shigella dysenteriae JS11910	100	25
S. flexneri 4b JS11811	50	100
S. sonnei JS11746	>100	100
Salmonella typhi T-63	>100	100
S. enteritidis 1891	100	50
Proteus vulgaris OX19	>100	100
P. mirabilis IFM OM-9	>100	100
Providencia rettgeri GN311	>100	100
P. rettgeri GN466	>100	100
Serratia marcescens	100	50
Pseudomonas aeruginosa A3	>50	>50
P. aeruginosa GN315	>100	>100
Klebsiella pneumoniae PCI602	100	100

and 1630 cm^{-1}, respectively[21]. The UV spectrum showed absorption maxima at 234, 277, 322, 335 and 419 nm. These absorption appeared similar to those of the aureolic acid group such as chromomycins[21] and indicative of a presence of an anthracenone moiety[22] in the molecule of **1** and **2**.

Table 6. Anti-mycobacteria activities of azicemicins A and B.

Test organism	MIC (μg/ml)	
	Azicemicin A	Azicemicin B
M. smegmatis ATCC 607	50	12.5
M. vaccae ATCC 15483	50	6.25
M. smegmatis ATCC 607 rifamycin-resistant	50	25
M. smegmatis ATCC607 paromomycin-resistant		6.25
M. smegmatis ATCC 607 capreomycin-resistant		12.5
M. smegmatis ATCC 607 streptothricin-resistant		25
M. megmatis ATCC 607 streptomycin-resistant		6.25

Antimicrobial Activities and Acute Toxicity

Antimicrobial activities of **1** and **2** are shown in Tables 5 and 6. The antimicrobial activities of **1** and **2** are moderate against Gram-positive bacteria and *Mycobacteria*. **1** did not show any toxic sign in mice at a dose of 150 mg/kg when administered intraperitoneally. **2** did not show any toxic sign in mice at a dose of 75.5 mg/kg when administered intravenously.

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