NOVEL ANTIBIOTICS, AMYTHIAMICINS

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, AND ANTIMICROBIAL ACTIVITY

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Novel antibacterial antibiotics, amythiamicins A, B, C and D, have been isolated from the fermentation broth of *Amycolatopsis* sp. MI481-42F4. In this paper, the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of amythiamicins are reported. Amythiamicins inhibit the growth of Gram-positive bacteria including multi-drug resistant strains.

In the course of our screening for antibacterial substances from various microorganisms, new antibiotics that we named amythiamicins were obtained from the fermentation broth of a strain of *Amycolatopsis* sp. MI481-42F4. The producing microorganism, strain MI481-42F4, was isolated from a soil sample collected in Nerima-ku, Tokyo, Japan. 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-12739.

In this paper we report the taxonomy, fermentation, isolation, physico-chemical properties and biological activity of amythiamicins A, B, C and D. The structural studies of amythiamicins A, B, C and D will be described in the following papers[1,2].

Materials and Methods

Morphological Characteristics

Morphological observations were made with a light microscope on cultures grown on yeast extract - malt extract agar (ISP med. 2)[3], sucrose - nitrate agar[4] and glycerol - nitrate agar[4] at 30°C for 2 to 4 weeks. Spore morphology was studied with a scanning electron microscope (model Hitachi S-570).

Cultural and Physiological Characteristics

The media and procedures used for cultural and physiological characteristics of strain MI481-42F4 were those described by SHIRLING and GOTTLIEB[3], by WAKSMAN[4], and by GORDON *et al.*[5]. 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 glucose - asparagine agar[4]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[6].

Cell Chemistry

The strain MI481-42F4 was grown in YD medium (yeast extract 1.0%, glucose 1.0%, pH 7.2) at 30°C for 5 days on a rotary shaker. The mycelia were centrifuged and was washed with distilled water. The washed and packed mycelia were then freeze-dried as a whole-cell preparation. The whole cell

hydrolysates were analyzed for diaminopimelic acid isomers and sugars according to the methods of BECKER *et al.*[7] and LECHEVALIER and LECHEVALIER[8], which were modified by STANECK and ROBERTS[9] for separation on thin layer plates. Phospholipids and mycolic acids were analyzed by the procedures of MINNIKIN *et al.*[10,11]. Menaquinones were extracted with chloroform-methanol (2:1) from the freeze-dried cells, purified by thin layer chromatography[12], and analyzed by electron impact mass spectrometry. The fatty acids were analyzed by gas chromatography of whole-cell methanolysates[13]. Fatty acid methyl esters were determined with a Hewlett-Packard model 5890 gas chromatograph, 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 μm, J & W Scienctific).

Preparation and Hybridization Analysis of DNA

After incubation in YD medium supplemented with 0.2% glycine at 30°C for 3 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[14] and dissolved in TE (10 mM Tris-HCl - 1 mM EDTA, pH 7.6).

Southern hybridization was carried out as described previously[15] by using 250 ng of *Sph* I-digested total DNAs from organisms tested. Following prehybridization at 70°C for 6 hours, hybridization was performed at 70°C for 45 hours in the presence of 5~10 ng/ml of the following probe. To prepare the probe, total DNA from the strain MI481-42F4 was fragmented by sonication to about 4 Kb or smaller and labelled with α-^{32}P-dCTP by nick translation. The specific activity of the probe was 0.5×10^8 cpm/μg. After hybridization the filter was washed 3 times with 200 ml of 2 × SSC (0.15 M NaCl - 15 mM sodium citrate, pH 7.0) at 80°C for 1 hour each. Autoradiography was carried out by exposing X-ray film (Fuji RX) to the filter at −80°C and the film was scanned on GC 300 Scanning Densitometer (Hoefer Scientific Instruments).

Detection of Amythiamicins

Amythiamicins were assayed by the paper disc diffusion method, based on their *in vitro* antibacterial activity against *Staphylococcus aureus* Smith grown on agar of Polypepton (Nihon Seiyaku) 1%, meat extract 1% and NaCl 0.2% (pH 7.0).

Amythiamicin A concentrations were measured by reversed phase HPLC (Capcell Pak C_{18}, Shiseido) with a solvent of CH_3CN - water (47:53) at the UV absorption of 254 nm.

Measurement of Antimicrobial Activity

The minimum inhibitory concentration (MIC) of amythiamicins 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% glucose for antifungal test which was incubated at 27°C for 42 hours.

Results and Discussion

Taxonomic Studies

Strian MI481-42F4 had a branched vegetative hyphae which was a slight tendency to zig-zag-shaped, and which tended to break down into squarish subunits. The aerial hyphae produced cylindrical conidia in straight to flexuous chains. The spores were 0.6 to 0.7 by 0.7 to 1.2 μM in size with smooth surfaces. No sporangia, motile spores or synnemata were observed.

The cultural characteristics of strain MI481-42F4 were summarized in Table 1.

The physiological characteristics and carbohydrate utilization of strain MI481-42F4 showed in Table 2.

The whole-cell hydrolysate contained *meso*-2,6-diaminopimelic acid, arabinose and galactose, which was typical of IV cell walls and a type A whole-cell sugar pattern. A type PII phospholipid pattern (phosphatidylethanolamine present, phosphatidylcholine and glucosamine-containing phospholipids absent) was found. Mycolic acids were absent. The predominant menaquinones were MK-9(H_4) and MK-9

Table 1. Cultural characteristics of strain MI481-42F4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Canary yellow (1 ea)	Thin, white	None
Yeast extract-malt extract agar (ISP No. 2)	Mustard gold (2 ne)	White	None
Oatmeal agar (ISP No. 3)	Lt antique gold ($1\frac{1}{2}$ ic)	Thin, white	Faint, yellowish
Inorganic salts-starch agar (ISP No. 4)	Lt antique gold ($1\frac{1}{2}$ ic)~Bamboo (2 gc)	Thin, white	None
Glycerol-asparagine agar (ISP No. 5)	Butter yellow ($1\frac{1}{2}$ ga)	Cream ($1\frac{1}{2}$ ca)	Faint, yellowish
Tyrosine agar (ISP No. 7)	Gold ($1\frac{1}{2}$ lc)	Lt ivory (2 ca)	Faint, yellowish
Glucose-asparagine agar	Sunlight yellow ($1\frac{1}{2}$ ia)	Pale yellow (1 ca)	Faint, yellowish
Nutrient agar	Bamboo (2 gc)	Thin, white	None
Glycerol-nitrate agar	Lt lemon yellow (1 ga)	Thin, white	None
Starch agar	Lemon yellow (1 la)	Scant, white	None
Calcium malate agar	Lt lemon yellow (1 ga)	Scant, white	None

Observation after incubation at 30°C for 21 days. Color name and numbers from Color Harmony Manual, Container Corporation of America.

Table 2. Physiological characteristics of strain MI481-42F4.

Temperature range for growth (°C)	20~30	*meso*-Erythritol	Positive
Optimum temperature (°C)	27~30	D-Glucose	Positive
Formation of melanoid pigment	Negative	Raffinose	Positive
Liquefaction of gelatin	Positive	Utilization of	
Coagulation of milk (30°C)	Negative	L-Arabinose	+
Peptonization of milk (30°C)	Positive	D-Xylose	+
Hydrolysis of starch	Positive	D-Glucose	+
Reduction of nitrate	Positive	D-Fructose	(+)
Decomposition of		Rhamnose	(+)
Hypoxanthine	Positive	Sucrose	−
Xanthine	Positive	Raffinose	(+)
Growth on 5% NaCl	Negative	Inositol	+
Acid from		D-Mannitol	+
Cellobiose	Positive		

+, Positive utilization; (+), probably positive utilization; −, no utilization.

Table 3. Comparison of strain MI481-42F4, *Amycolatopsis orientalis* and *A. mediterranei*.

	Strain MI481-42F4	*A. orientalis* IMC A-0161 (ISP 5040)	*A. mediterranei* IMC A-0162 (ISP-5501)
Fatty acids[a]	i-16[b], i-15, ai-15, ai-17	i-15[b], i-16, 16:0, 17:0, ai-17	i-16[b], ai-17, i-15, 16:0
Aerial mass color	White	White	−~White~Lt melon yellow (3 ea)
Color of growth	Lt lemon yellow (1 ga)~Lt antique gold ($1\frac{1}{2}$ ic)	Lt wheat (2 ea)~Lt melon yellow (3 ea)	Melon yellow (3 ga) Amber (3 lc)
Decomposition of			
Xanthine	Positive	Positive	Negative
Acid from			
meso-Erythritol	Positive	Positive	Negative
Raffinose	Positive	Negative	Positive
Utilization of[c]			
Sucrose	−	−	+
Raffinose	+	−	(−)

[a] i-15, iso-branched 13-methyltetradecanoic acid; ai-15, anteiso-branched 12-methyltetradecanoic acid; 16:0, saturated hexadecanoic acid.

[b] Under line, main compornent.

[c] +, Positive utilization; (−), probably no utilization; −, no utilization.

(H_2). This strain contained major amounts of *iso*-branched 14-methyl-pentadecanoic acid (*i*-16), *iso*-branched 13-methyltetradecanoic acid (*i*-15), *anteiso*-branched 12-methyltetradecanoic acid (*ai*-15) and *anteiso*-branched 14-methylhexadecanoic acid (*ai*-17).

Based on these characteristics, strain MI481-42F4 was placed in the genus *Amycolatopsis*[16,17] Among the genus *Amycolatopsis*, *Amycolatopsis mediterranei* and *Amycolatopsis orientalis* were similar to strain MI481-42F4. As shown in Table 3, strain MI481-42F4 was different from *A. orientalis* in the fatty acids composition, the acid production from raffinose, and the utilization of raffinose. And it was also different from *A. mediterranei* in the fatty acids composition, the decomposition of xanthine, the acid production from *meso*-erythritol, and the utilization of raffinose and sucrose. We examined DNA hybridization between strain MI481-42F4 and *A. orientalis* and *A. mediterranei*. Strain MI481-42F4 showed low homology with *A. orientalis* and *A mediterranei*, 32.8% and 20.8%, respectively. Therefore, strain MI481-42F4 was designated *Amycolatopsis* sp. MI481-42F4.

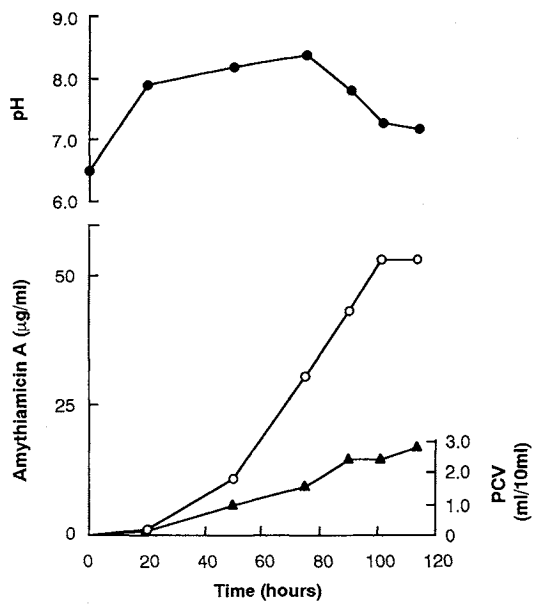
Fermentation

The seed medium consisted of glycerol 2.0%, dextrin 2.0%, Bacto soytone (Difco) 1.0%, dried yeast extract-S (Nihon Seiyaku) 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, one drop of silicone oil (Shin-Etsu Kagaku, KM-70) and pH of the medium was not adjusted. Growth of strain MI481-42F4 from an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of the medium and cultured at 27°C for 5 days on a rotary shaker (180 rpm/minute). The seed culture (200 ml) was transferred into a 30-liter fermentor containing 12 liters of a medium consisting of glycerol 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, dried yeast extract-S (Nippon Seiyaku) 0.3%, $CaCO_3$ 0.2% and silicon oil (Toho Kagaku, Pronal 502) 0.01%. Fermentation was carried out at 27°C for 110 hours with aeration (12 liters/minute) and agitation (200 rpm/minute).

The time course of the production of amythiamicin A is shown in Fig. 1. After 100 hours cultivation, amythiamicin A accumulated in mycelium up to 50 μg/ml. Amythiamicins in the culture broth were much less than 1/10 in the mycelial cake.

Fig. 1. Time course of amythiamicin A production by *Amycolatopsis* sp. MI481-42F4.

○ Amythiamicin A (μg/ml), ● pH, ▲ packed cell volume (ml/10 ml).



Isolation and Purification

The fermentation broth was centrifuged to separate the supernatant and the mycelial cake. The mycelial cake was extracted with 30 liters of MeOH. The MeOH extract was concentrated to remove MeOH under reduced pressure and the residue was extracted with 1-BuOH. The 1-BuOH extract was concentrated to dryness under reduced pressure, and the residue was dissolved in a small amount of $CHCl_3$. The $CHCl_3$ solution was put onto a column of silica gel (500 ml). The column was washed with 1 liter of $CHCl_3$ and then eluted with 2 liters of $CHCl_3$-MeOH (10:1). The active fractions were combined and condensed to dryness under reduced

Table 4. Physico-chemical properties of amythiamicins A, B, C and D.

	A	B
Molecular formula	$C_{50}H_{51}N_{15}O_8S_6$	$C_{50}H_{53}N_{15}O_9S_6$
HRFAB-MS ($(M+H)^+$, m/z)		
Calcd:	1182.2447	1200.2553
Found:	1182.2463	1200.2549
UV λ_{max}^{MeOH} nm ($\log\varepsilon$)	203 (4.92), 221 (4.89), 250 (sh, 4.73), 310 (4.55), 345 (sh, 4.13)	204 (4.78), 220 (4.75), 250 (sh, 4.52), 308 (4.37), 345 (sh, 4.13)
$\lambda_{max}^{MeOH-HCl}$	203 (4.93), 223 (4.90), 250 (sh, 4.72), 303 (4.62), 345 (sh, 4.07)	204 (4.77), 222 (4.75), 250 (sh, 4.52), 308 (4.38), 345 (sh, 3.97)
$\lambda_{max}^{MeOH-NaOH}$	204 (5.46), 250 (sh, 4.73), 309 (4.55), 345 (sh, 4.13)	202 (5.44), 250 (sh, 4.52), 308 (4.37), 345 (sh, 3.97)
Optical rotation	$[\alpha]_D^{28}$ +133° (c 0.745, DMSO)	$[\alpha]_D^{23}$ +155° (c 0.25, MeOH)
IR ν_{max}^{KBr} cm^{-1}	3300, 2980, 1660, 1540, 1500, 1250, 1100, 990, 760	3395, 2965, 1655, 1540, 1500, 1245, 1070, 755

	C	D
Molecular formula	$C_{50}H_{50}N_{14}O_9S_6$	$C_{43}H_{42}N_{12}O_7S_6$
HRFAB-MS ($(M+H)^+$ m/z)		
Calcd:	1183.2288	1031.1702
Found:	1183.2285	1031.1688
UV λ_{max}^{MeOH} nm ($\log\varepsilon$)	203 (4.80), 221 (4.76), 250 (sh, 4.52), 306 (4.40), 345 (sh, 3.97)	204 (4.72), 224 (4.79), 250 (sh, 4.56), 307 (4.49), 345 (sh, 4.04)
$\lambda_{max}^{MeOH-HCl}$	203 (4.78), 221 (4.76), 250 (sh, 4.53), 306 (4.41), 345 (sh, 3.97)	205 (4.72), 224 (4.80), 250 (sh, 4.57), 307 (4.50), 345 (sh, 4.04)
$\lambda_{max}^{MeOH-NaOH}$	203 (5.44), 250 (sh, 4.53), 306 (4.39), 345 (sh, 3.97)	204 (4.78), 224 (4.79), 250 (sh, 4.57), 308 (4.49), 345 (sh, 4.04)
Optical rotation	$[\alpha]_D^{24}$ +112° (c 0.25, MeOH)	$[\alpha]_D^{22}$ +179° (c 0.5, MeOH)
IR ν_{max}^{KBr} cm^{-1}	3385, 3115, 2965, 1735, 1665, 1540, 1495, 1210, 755	3385, 3115, 2960, 1725, 1665, 1535, 1495, 1220, 1025, 755

pressure to give 5 g of the residue.

The residue was dissolved in MeOH. HPLC analysis of the solution suggested that at least 4 active related compounds were contained in the residue.

The solution was applied to a reverse phase HPLC column (Capcell Pak C_{18} 30×250 mm, flow rate 20 ml/minute) and eluted with a gradient of CH_3CN-water (40:60~48:52 linear gradient in 17 minutes and 48:52~80:20 linear gradient in 15 minutes). The fractions containing amythiamicins A, B, C and D were collected separately and concentrated under reduced pressure to give residues of crude amythiamicins A, B, C and D, respectively. The crude amythiamicins were each dissolved in MeOH and further purified by applying on a column of Capcell Pak C_{18} (20×250 mm) and eluted with CH_3CN-water (47:53) in the case of A, B and C, and CH_3CN-water (70:30) in the case of D to give pure amythiamicins (A: 550 mg, B: 9 mg, C: 20 mg, and D: 38 mg), respectively.

Physico-chemical Properties

The physico-chemical properties of amythiamicins A, B, C and D are summarized in Table 4. Amythiamicins are soluble in MeOH and DMSO, but is insoluble in *n*-hexane and water. The UV spectra of amythiamicins show the absorption maxima near 204, 222, 250 (sh), 308 and 345 nm (sh) in MeOH. The IR spectra of those indicated the presence of peptide bonds (1660 and 1500 cm^{-1}). The molecular formulae of amythiamicins A, B, C and D were determined to be $C_{50}H_{51}N_{15}O_8S_6$, $C_{50}H_{53}N_{15}O_9S_6$, $C_{50}H_{50}N_{14}O_9S_6$ and $C_{43}H_{42}N_{12}O_7S_6$, respectively, by HRFAB-MS. In particular, from the UV spectra

Table 5. Antimicrobial activities of amythiamicins.

Microorganisms	MIC (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA209P	0.1	<0.78	3.12	0.2
S. aureus Smith	0.2	<0.78	6.25	0.2
S. aureus MS9610	0.2	1.56	>100	0.78
S. aureus No. 5 (MRSA)	0.2	1.56	100	0.39
S. aureus No. 17 (MRSA)	0.2	1.56	>100	0.39
Micrococcus luteus FDA16	0.39	1.56	3.12	0.78
M. luteus IFO3333	0.78	1.56	1.56	0.78
M. luteus PCI1001	0.2	<0.78	0.78	0.39
Bacillus anthracis	0.1	<0.78	1.56	0.2
B. subtilis NRRL B-558	0.2	>100	>100	3.12
B. subtilis PCI219	0.2	3.12	>100	0.78
B. cereus ATCC 10702	0.1	<0.78	1.56	0.2
Corynebacterium bovis 1810	0.78	100	>50	1.56
Escherichia coli NIHJ	>100	>100	>100	>100
E. coli K-12	>100	>100	>100	>100
E. coli K-12 ML1629	>100	>100	>100	>100
E. coli BEM11	>100	>100	>100	>100
E. coli BE1121	>100	>100	>100	>100
E. coli BE1186	>50	>50	>100	>50
Shigella dysenteriae JS11910	>50	>50	>50	1.56
S. flexneri 4b JS11811	>50	>50	>50	>50
S. sonnei JS11746	>100	>100	>100	>100
Salmonella typhi T-63	>50	>50	>50	>50
S. enteritidis 1891	0.39	>50	>50	1.56
Proteus vulgaris OX19	>100	>50	>50	>100
P. mirabilis IFM OM-9	>100	>50	>50	>100
Providencia rettgeri GN311	100	>50	>50	>100
P. rettgeri GN466	>50	>50	>50	>50
Serratia marcescens	>50	>50	>50	>50
Pseudomonas aeruginosa A3	>50	>50	>50	>50
P. aeruginosa GN315	>50	>50	>50	>50
Klebsiella pneumoniae PCI602	>50	>50	>50	>50
Mycobacterium smegmatis ATCC 607	50	100	>50	3.12
Aeromonas punctata IAM1646	>50	ND	ND	>50
A. salmonecida ATCC 14174	25	ND	ND	>50
Aeromonas sp. (KT-444)	>50	ND	ND	>50
Vibrio anguillarum NCMB6	>50	ND	ND	>100
Pseudomonas fluorescens	>50	ND	ND	>50
P. lachrymans	50	ND	ND	>50
Erwinia aroideae	>50	ND	ND	>50
Candida tropicalis F-1	>50	ND	ND	>50
C. pseudotropicalis F-2	>50	ND	ND	>50
C. albicans 3147	>50	>50	ND	>50
Candida Yu-1200	>100	ND	ND	>100
C. krusei F-5	>100	ND	ND	>100
Saccharomyces cerevisiae F-7	>50	ND	ND	>50
Cryptococcus neoformans F-10	>50	ND	ND	>50
Cochliobolus miyabeanus	>25	ND	ND	50
Pyricularia oryzae	>50	ND	ND	>50
Pellicularia sasakii	>25	ND	ND	>25
Xanthomonas citri	25	ND	ND	>50
X. oryzae	6.25	ND	ND	>50
Trichophyton asteroides 429	>50	ND	ND	>50
T. mentagrophytes F-15 (833)	>50	ND	ND	>50
Aspergillus nigar F-16	>50	ND	ND	>50
A. fumigatus F-181	>50	ND	ND	>50

MRSA: Methicillin-resistant *S. aureus*.

and molecular formulae the amythiamicins were clearly different from other known antibiotics.

Antimicrobial Activity and Acute Toxicity

The antimicrobial activities of amythiamicins are shown in Table 5. Amythiamicins A, B, C and D inhibit the growth of Gram-positive bacteria including multi-drug resistant strains such as *Staphylococcus aureus* MS9610 and Methicillin-resistant *S. aureus*, but are not active against most Gram-negative bacteria and fungi.

Amythiamicins A, B, C and D showed no signs of toxicity when administered once to mice intraperitoneally at the dose of 100 mg/kg.

Details of the biological properties of amythiamicins, including its *in vitro* and *in vivo* antibacterial activity, will be described elsewhere.

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