PRADIMICIN S, A NEW PRADIMICIN ANALOG

I. TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITIES

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A directed search for antibiotics active in a syncytium formation inhibition assay using a co-culture of HeLa-T4 cells expressing CD4 antigen and BSC-1 cells expressing gp-120 led to the isolation of pradimicin S, a new member of the pradimicin family. The producing strain (AA0851) was characterized as a new species of *Actinomadura* for which the name *Actinomadura spinosa* sp. nov. is proposed. Production of pradimicin S by strain AA0851 was significantly improved by addition of ferrous sulfate (0.1~0.4%) to the production medium. Pradimicin S showed potent activity against human immunodeficiency virus (HIV) LAV_{BRU} strain in a CPE assay, and moderate activity against influenza virus type A. The antibiotic was active against a wide variety of fungi and yeasts *in vitro* and demonstrated *in vivo* efficacy against a systemic *Candida albicans* infection in mice.

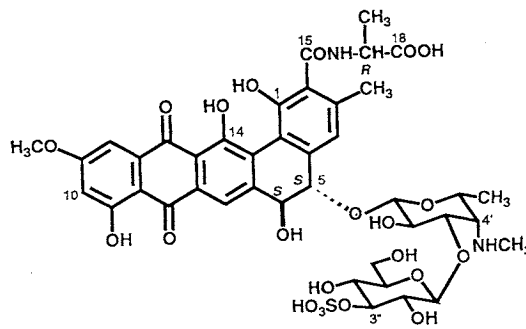
In an effort to screen for antiviral antibiotics among the products of rare actinomycetes, we found strain AA0851 producing an antibiotic active in an assay designed to detect inhibitors of syncytium formation[1]. The antibiotic, pradimicin S (Fig. 1), was also discovered using this assay and recovered from the fermentation broth of strain AA0851 by a series of chromatography and recrystallization procedures. In this report, we describe the taxonomy and fermentation of the pradimicin S-producing organism and the biological activities of pradimicin S. Full details concerning the structure of pradimicin S will be described in the accompanying paper[2].

Materials and Methods

Taxonomic Methods

Taxonomic studies on strain AA0851 were generally carried out by the methods adopted by the International Streptomyces Project (ISP) using media recommended by SHIRLING and GOTTLIEB[3], WAKSMAN[4] and ARAI[5]. The color name and color code were determined by direct comparison of the cultures with color chips from the Manual of Color Names (Japan Color Enterprise Co., Ltd., 1987). Morphological observations were made by light microscopy and transmission electron microscopy. Whole-cell compositions were analyzed by the procedures recommended by BECKER *et al.*[6,7] and STANECK and ROBERTS[8]. Phospholipids and mycolic acids were analyzed by the methods of LECHEVALIER *et al.*[9,10] and MINNIKIN *et al.*[11], respectively. Menaquinones were analyzed by the procedure of COLLINS *et al.*[12]. DNAs were isolated by the procedure of FURUMAI *et al.*[13] and subjected to HPLC for GC content analysis[14,15]. A DNA-DNA hybridization experiment was carried out by the method of MIYADOH *et al.*[16].

Fig. 1. Structure of pradimicin S.



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Fermentation

Strain AA0851 was propagated on 1/2 strength inorganic salts - starch agar[3] (1/2 ISP-4 medium, Difco Laboratories) supplemented with 0.1% yeast extract. After incubation at 30°C for 3 weeks, a portion of this agar slant culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of Trypticase Soy Broth (BBL, Becton Dickinson and Co., Cockeysville, MD). Tap water was used in media preparation. The inoculated flask was incubated for 7 days at 32°C on a rotary shaker (200 rpm). The resulting vegetative mycelia were washed, resuspended in a half volume of 20% glycerol, and stored at −80°C. A portion (1 ml) of this frozen culture was inoculated into 100 ml of the seed medium (Trypticase Soy Broth) in a 500-ml Erlenmeyer flask, and the inoculated flasks were incubated for 4 days at 32°C on a rotary shaker (200 rpm). The seed culture (5 ml) was transferred into each of three hundred 500-ml Erlenmeyer flasks containing 100 ml of production medium. Production medium was composed of sucrose 3.0%, glucose 1.0%, Pharmamedia (Traders Protein) 3.0%, $FeSO_4 \cdot 7H_2O$ 0.1% and $CaCO_3$ 0.3% (pH adjusted to 7.5 with 1 N NaOH and then autoclaved at 121°C for 30 minutes). The production fermentation was carried out at 28°C with rotary agitation at 200 rpm.

Antibiotic Assay

Production of pradimicin S was monitored by optical density at 500 nm in 0.02 N NaOH - MeOH (1 : 1) solution and by HPLC (column: Cosmosil $5C_{18}$-AR, 4.6 mm i.d. × 150 mm, 5 μm, Nacalai Tesque Inc., mobile phase: CH_3CN - 0.01 M phosphate buffer (27 : 73, pH 3.5), flow rate: 1.0 ml/minute, detection: UV absorption at 254 nm, retention time: 12.07 minutes for pradimicin S).

Discovery Screen

The syncytium formation inhibition assay was performed as previously described[1]. Briefly, HeLa-T4 cells expressing CD4 antigen[17] and BSC-1 cells expressing gp-120[18] were incubated in the presence of 2-fold dilutions of antibiotic, and the number of syncytia formed was compared with the number of those formed in the absence of antibiotic. Dextran sulfate was used as a positive control, giving an ID_{50} value of 19 μg/ml.

Antiviral Assays

The activity against human immunodeficiency virus (HIV) was determined by the XTT assay using CEM-SS cells infected with HIV (LAV_{BRU} strain obtained from Luc Montagnier, Institute Pasteur, Paris, France) as described by WEISLOW *et al.*[19]. The CEM-SS cell line used in this assay was obtained from Dr. OWEN S. WEISLOW (National Cancer Institute). Pradimicin A and 2,3'-dideoxyinosine (ddI) were used as positive controls. The activity against herpes simplex virus (HSV) was determined by the cytopathic effect (CPE) reduction assay using Vero cells infected with HSV type 1 (KOS strain)[20]. The activity against influenza virus A was determined by the CPE assay using Madin Darby canine kidney (MDCK) cells infected with Victoria strain[20]. Acyclovir and ribavirin were used as the reference compounds for anti-HSV and anti-influenza virus activities, respectively.

Antifungal Assay

MICs were determined by the conventional agar dilution method on yeast morphology agar containing 1/15 M phosphate buffer, pH 7.0. The *in vivo* efficacy was evaluated against *Candida albicans* A9540 systemic infection in male ICR mice (20~24 g body weight) as previously described[21]. Groups of 5 mice at each dose level were infected intravenously (iv) with *C. albicans* A9540 (10 LD_{50}) and test compounds were given iv once immediately after the fungal challenge. The 50% protective dose (PD_{50}) was calculated from the survival rate recorded 20 days after infection. Amphotericin B and ketoconazole were used as reference compounds.

Antibacterial Assay

The antibacterial activity was determined by the 2-fold serial agar dilution method using Mueller-Hinton agar (pH 7.2) after incubation at 32°C for 18 hours with an inoculum size of 10^6 cfu/ml.

Results and Discussion

Taxonomy

Strain AA0851 was isolated from a soil sample obtained in Manna Village, India in December, 1985. At temperatures between 21°C and 45°C, strain AA0851 showed better growth on agar media containing organic nitrogen sources than on those containing inorganic nitrogen sources. Colonies grew slowly (1 to 2 weeks) and were convex with an irregular margin. Vegetative mycelia did not fragment on agar or in liquid media. Mature aerial mycelia were generally powdery and tinted white to light gray, and were rarely white velvety to cottony on 1/2 ISP-4 medium. The aerial mycelia sometimes became light gray after 1~2 months at 28°C. Straight chains of more than 10 spores were occasionally observed at the tip of sporulating aerial mycelia. The spores were oval to elliptical in shape with a spiny surface and measured 1.0~1.2 to 1.1~1.3 μm (Fig. 2). They were not motile. The color of vegetative mycelia ranged from pale pink to dark red. Pale yellowish pink, grayish pink or dark red diffusible pigments were produced in various agar media. The color of both vegetative mycelia and diffusible pigments acted like a pH indicator and changed to light reddish yellow or dark yellowish brown on addition of 0.1 N HCl. The macroscopic properties of strain AA0851 on various agar media are summarized in

Fig. 2. Transmission electron micrograph of strain AA0851 grown on 1/2 ISP-4 medium at 28°C for 6 weeks, showing spiny spores.

Bar represents 1.0 μm.

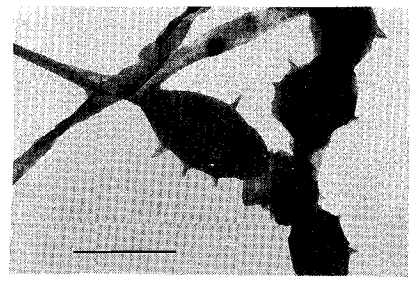

Table 1. Cultural characteristics of strain AA0851.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (Waksman med. No. 1)	Purplish pink (8), good	Purplish pink (8)	None	Colorless~pale yellowish pink (6)
Glycerol-nitrate agar	Pale reddish yellow (130), good	Pale reddish yellow (130)	None	Purplish pink (16)
Glucose-asparagine agar (Waksman med. No. 2)	Light brown (90), good	Light brown (90)	None	Pale yellowish pink (7)
Yeast ext.-malt ext. agar (ISP med. No. 2)	Dark red (57), good	Dark red (57)	Pinkish white (391), scant	Dark red (57)
Oatmeal agar (ISP med. No. 3)	Pale pink (5), good	Pale pink (5)	White (389), scant	Pale pink (5)
Inorganic salts-starch agar (ISP med. No. 4)	Grayish pink (31), moderate	Grayish pink (31)	Pinkish white (391), light gray (398), powdery	Deep pink (22)
Glycerol-asparagine agar (ISP med. No. 5)	Pale reddish yellow (125), scant	Pale reddish yellow (125)	White (388), scant	Pink (12)
Tyrosine agar (ISP med. No. 7)	Pale reddish yellow (125), scant	Pale reddish yellow (125)	White (388), scant	None
Nutrient agar (Waksman med. No. 14)	Grayish pink (30), moderate	Grayish pink (30)	White (389), powdery scant	Grayish pink (30)
Yeast-starch agar	Dark red (57), good	Dark red (57)	Grayish white (390), powdery good	Dark red (57)

Table 1. The physiological characteristics and the utilization profile of carbon sources are shown in Tables 2 and 3, respectively.

Upon hydrolysis, the whole cells of strain AA0851 yielded *meso*-diaminopimelic acid, madurose, ribose, mannose, glucose and galactose. Mycolic acids were not detected. By phospholipids analysis, the cell membrane had a type P1 phospholipid pattern containing phosphatidylinositol mannoside, phosphatidylinositol and diphosphatidylglycerol. The menaquinone and cellular fatty acid compositions of the strain tested are summarized in Tables 4 and 5, respectively.

On the basis of the above-mentioned characteristics, strain AA0851 was determined to belong to the

Table 2. Physiological characteristics of strain AA0851.

Test	Results
Starch hydrolysis (ISP med. No. 4)	Negative
Nitrate reduction (Nitrate broth, Difco)	Positive
10% skimmed milk (10% Skimmed milk, Difco)	
Coagulation	Positive
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a strip of paper as the sole carbon source)	Negative, good growth
Gelatin liquefaction	
On plain gelatin	Doubtful
On glucose peptone gelatin	Positive
Melanin formation	
On ISP med. No. 7	Negative
Temperature range for growth (°C)	21~45
Optimum temperature (°C) (On yeast starch agar)	30~40
pH range for growth	6~9
Optimum pH (On Trypticase Soy Broth, BBL)	8

Table 3. Utilization of carbon sources by strain AA0851.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	−
Mannitol	−
D-Fructose	+
L-Rhamnose	++
Sucrose	+
Raffinose	++

−; Negative, +; weakly positive, ++; strongly positive. (ISP med. No. 9, 28°C for 2 weeks).

Table 4. Menaquinone compositions of strain AA0851 and *Actinomadura echinospora* JCM 3148^T.

Strain	Composition (%)				
	MK-9				
	H_2	H_4	H_6	H_8	H_{10}
AA0851	1	6	53	35	5
JCM 3148^T		10	70	10	10

Table 5. Cellular fatty acid compositions of strain AA0851 and *Actinomadura echinospora* JCM 3148^T.

Strain	Fatty acid composition (%)							
	Normal acids							
	14:0	16:0	16:1^9	18:0	18:1^9	15:0	17:0	17:1^9
AA0851	1	13	2	5	5	7	22	1
JCM 3148^T		6	2	3	11	1	5	3

Strain	Fatty acid composition (%)								
	Iso acids					Anteiso acids	10-Methyl acids		
	16:0	16:1	18:0	18:1	17:0	17:0	16:0	17:0	18:0
AA0851	10		3		1	11	1	10	5
JCM 3148^T	18	2	11	3				5	20

Table 6. Comparison of strain AA0851 with *Actinomadura echinospora* JCM 3148^T.

Characteristic	Strain AA0851	Strain JCM 3148^T
No. of spores per chain	>10	2
Spore surface	Spiny	Spiny
Color of aerial mass	White (389)~light gray (398)	White (389)~pale yellowish pink (6)
Color of diffusible pigment	Dark red (57)	None
Liquefaction of gelatin	+	±
Coagulation of milk	+	−
Utilization of:		
D-mannitol	−	+
L-rhamnose	++	−
raffinose	++	−
Vitamin B requirement	−	+
Diagnostic amino acid	*meso*-DAP	*meso*-DAP
Diagnostic sugar	Madurose, glucose, galactose, ribose and mannose	Madurose, glucose, galactose and ribose
Phospholipid	PI, PG and PIM	PI and PG
G+C content (%)	73.1	67.2

Table 7. Antibiotic susceptibilities of strain AA0851 and *Actinomadura echinospora* JCM 3148^T.

Antibiotics (conc./disc)	Inhibition zone (mm)	
	Strain AA0851	Strain JCM 3148^T
Ampicillin (20 μg)	−	14
Clavulanic acid (15 μg)/ ticarcillin (30 μg)	−	>20
Cephalexin (30 μg)	−	13
Fosfomycin (50 μg)	−	−
Tetracycline (5 μg)	>20	>20
Chloramphenicol (5 μg)	>20	11
Erythromycin (0.5 μg)	18	14
Josamycin (2 μg)	>20	±
Lincomycin (15 μg)	−	13
Kanamycin (10 μg)	10	+
Gentamicin (10 μg)	−	−
Tobramycin (10 μg)	+	+
Nalidixic acid (15 μg)	−	±
Norfloxacin	13	16
Colistin (50 U)	>20	>20
Polymixin B (300 U)	+	+

Table 8. DNA-DNA homology for strain AA0851 and *Actinomadura echinospora* JCM 3148^T.

Strain	% hybridization with ^{32}P-labeled DNA from:	
	AA0851	Strain JCM 3148^T
AA0851	100	11
JCM 3148^T	9	100

genus *Actinomadura* Lechevalier and Lechevalier 1970[22]. The possibility of the strain being *Actinomadura echinospora* (Nonomura and Ohara 1971[23]) Kroppenstedt, Stackebrandt and Goodfellow[24] owing to the similarity in the spore surface ornamentation was eliminated by direct comparison with *A. echinospora* JCM 3148^T (type strain). Differences in the properties are: Compositions of menaquinones and fatty acids (Tables 4 and 5), spore chain morphology, cultural characteristics, utilization of carbon sources (Table 6), antibiotic susceptibility (especially against β-lactams, Table 7), and DNA-DNA hybridization (Table 8). Since there are no descriptions on reference species producing white to light gray aerial mycelia with short chains of more than 10 spiny spores and reddish purple diffusible pigments, strain AA0851 was determined to be a new species of *Actinomadura* and the name *Actinomadura spinosa* Saitoh, Yamamoto and Furumai sp. nov. is proposed. *Actinomadura spinosa* (spi·no' sa. L. adj. *spinosa*, spiny, referring to the spiny spore surface). Type strain, strain AA0851, is a single isolate and has been deposited with the American Type Culture Collection under the accession number ATCC55138.

Fermentation

Strain AA0851 produced pradimicin S as the major product along with two minor products, pardimicins B[21] and L[25]. Production of pradimicin S was influenced by the concentration(s) of some minerals present in the medium. In particular, the effect of sulfur compounds on pradimicin S production was significant because pradimicin S possesses a sulfated sugar moiety[2]. Of several sulfur compounds tested in medium 164, ferrous sulfate enhanced the production yield of pradimicin S, other inorganic sulfates and sulfur containing amino acids were not effective (Table 9). The medium composition study revealed that ferrous sulfate at concentrations of 0.1~0.4% gave the highest titer of pradimicin S when the strain was fermented in medium FR20-1 containing sucrose, glucose and Pharmamedia (Table 10). Based on these results, strain AA0851 was fermented at 28°C for 14 days in production medium. The production of pradimicin S started on day 4 and reached a maximum of 1,242 μg/ml on day 12. Pradimicins B and L were produced in quantities of 693 and 136 μg/ml, respectively.

Biological Properties

Inhibitory Effect on Syncytium Formation

Co-culture of HeLa-T4 cells expressing CD4 antigen and BSC-1 cells expressing gp-120 produced multi-nucleated giant cells (syncytium) in 3 to 5 hours[1]. Pradimicin S at a concentration of 12.5 μg/ml inhibited the syncytium formation almost completely with an ID_{50} value of 5.0 μg/ml. Pradimicin A was more effective in this assay, giving total inhibition at 1.6 μg/ml with an ID_{50} value of 1.1 μg/ml.

Antiviral Activities

Table 11 compares the antiviral activities of pradimicins S and A. Pradimicin S showed potent activity against HIV in the CPE assay with an ID_{50} of 3 μg/ml and moderate activity against influenza virus A (Victoria strain). The reason for the increased selectivity observed with pradimicin S as compared to

Table 9. Effect of sulfur compounds on the pradimicin S production.

Sulfur compound (0.1%)	Titer (μg/ml) of pradimicin S
$MgSO_4 \cdot H_2O$	203
$CaSO_4$	131
$FeSO_4 \cdot 7H_2O$	405
$Fe_2(SO_4)_3$	258
Na_2SO_4	202
$Na_2S_2O_3$	54
Na_2SO_3	154
$(NH_4)_2SO_4$	206
L-Methionine	42
L-Cysteine	21
None	254

Medium 164: Soluble starch 2.0%, glucose 1.0%, Pharmamedia 1.0%, brewer's yeast extract (Kirin Brewery Co., Ltd.) 1.3%, NZ-amine (Humko Sheffield Chemical) 0.3%, $CaCO_3$ 0.1% and Allophane (Shinagawa Brick) 0.5% in tap water, pH adjusted to 7.0 with 1 N NaOH and then autoclaved at 121°C for 30 minutes.

Fermentation conditions: 28°C for 14 days on a rotary shaker (200 rpm).

Table 10. Effect of ferrous sulfate on the pradimicin S production.

Medium	Concentration (%) of $FeSO_4 \cdot 7H_2O$	Titer (μg/ml)
164	0	240
	0.1	369
	0.2	352
	0.3	302
	0.4	257
FR20-1	0	640
	0.1	1,047
	0.2	1,139
	0.3	1,152
	0.4	1,119

Medium FR20-1: Sucrose 3%, glucose 1%, Pharmamedia 3% and $CaCO_3$ 0.3% in tap water, pH adjusted to 7.5 with 1 N NaOH and then autoclaved at 121°C for 30 minutes.

Fermentation conditions: 28°C for 14 days on a rotary shaker (200 rpm).

Table 11. Antiviral activities of pradimicins S and A.

	HIV-CEM-SS cell		Influenza virus-MDCK cell		HSV-Vero cell	
	ID_{50} (μg/ml)	TD_{50}	ID_{50} (μg/ml)	TD_{50}	ID_{50} (μg/ml)	TD_{50}
Pradimicin S	3.0	500	22	>100	>100	>100
Pradimicin A	5.9	15	6.8	>100	>100	>100
Acyclovir					0.09	>100
Ribavirin			9.5	>100		
2,3'-Dideoxyinosine (ddI)	60	>500				

ID_{50}: Concentration to reduce CPE by 50% as compared to drug-free controls.
TD_{50}: Concentration to inhibit the growth of host cells by 50%.

Table 12. *In vitro* antifungal activities of pradimicin S, amphotericin B and ketoconazole.

Test organism	MIC (μg/ml)		
	Pradimicin S	Amphotericin B	Ketoconazole
Saccharomyces cerevisiae ATCC 9763	3.1	0.2	100
Candida albicans A9540	25	0.4	25
C. albicans ATCC 32354	6.3	0.2	50
C. albicans ATCC 38247	6.3	50	6.3
C. albicans 83-2-14	25	0.4	25
C. tropicalis 85-8	50	0.4	100
C. tropicalis IFO 10241	50	0.4	50
Cryptococcus neoformans D49	1.6	0.4	6.3
C. neoformans IAM 4514	1.6	0.4	6.3
Aspergillus fumigatus IAM 2034	3.1	0.4	3.1
Trychophyton mentagrophytes 4329	6.3	0.4	0.8

Medium: Yeast morphology agar + 1/15 M phosphate buffer (pH 7.0).
Inoculum size: 10^4 cells/5-μl spot (10^5 cells/5 μl spot for *T. mentagrophytes* 4329).
Incubation conditions: 28°C, 40 hours (60 hours for *T. mentagrophytes* 4329).

pradimicin A is unknown.

Antifungal Activities

Table 12 summarizes the MICs of pradimicin S, amphotericin B and ketoconazole. Pradimicin S is a broad-spectrum antifungal agent active against *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 32354 and 38247, *Cryptococcus neoformans* D49 and IAM 4514, *Aspergillus fumigatus* IAM 2034 and *Trichophyton mentagrophytes* 4329. Against a systemic *C. albicans* A9540 infection in mice, pradimicin S at single doses of 50 and 25 mg/kg was effective in reducing the mortality of the infected mice with a PD_{50} value of 20 mg/kg (Table 13).

Table 13. *In vivo* activities of pradimicin S, amphotericin B and ketoconazole against a systemic *Candida albicans* A9540 infection in mice.

Compound	PD_{50} (mg/kg)
Pradimicin S	20
Amphotericin B	0.31
Ketoconazole	>50

Groups of 5 mice at each dose level were infected with 10 LD_{50} of *C. albicans* A9540 and compounds given iv once immediately after infection. PD_{50}s were calculated from the survival rate 20 days after infection.

Other Biological Activities

Pradimicin S at 100 μg/ml did not show any effect on the growth of *Staphylococcus aureus* 209P JC-1,

Bacillus subtilis ATCC 6633 or *Escherichia coli* NIHJ JC-2.

No toxic effects were observed when groups of 3 ICR male mice (22~25 g) were treated iv with pradimicin S at doses of up to 150 mg/kg of body weight.

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