PRADIMICINS L AND FL: NEW PRADIMICIN CONGENERS FROM *Actinomadura verrucosospora* subsp. *neohibisca*

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Pradimicin L, a new congener of pradimicin A having the D-glucosyl-D-thomosamine moiety at the C-5 position, was isolated from *Actinomadura verrucosospora* subsp. *neohibisca* subsp. nov. The structure of pradimicin L was deduced to be *N*-[[(5*S*,6*S*)-5-*O*-[4,6-dideoxy-4-(methylamino)-3-*O*-(β-D-glucopyranosyl)-β-D-galactopyranosyl]-5,6,8,13-tetrahydro-1,5,6,9,14-pentahydroxy-11-methoxy-3-methyl-8,13-dioxobenzo[*a*]naphthacene-2-yl]carbonyl]-D-alanine by MS and NMR spectrometry and degradation studies. Pradimicin FL which has the D-serine moiety instead of D-alanine was produced by directed biosynthesis in D-serine-supplemented medium. Pradimicins L and FL have a broad spectrum of *in vitro* antifungal activity. Pradimicin L was equiactive to pradimicin A and pradimicin FL was more active than pradimicin L.

Pradimicins A, B, C[1~4], D, E[5], FA-1 and FA-2[6] were previously reported to exhibit broad activity against pathogenic fungi and yeasts. They share 5,6-dihydrobenzo[*a*]naphthacenequinone as the common skeleton and can be distinguished from each other in the amino acid moiety (D-alanine[1~4], glycine[5] and D-serine[6]) and in the 4′-amino group of D-thomosamine in the disaccharide moiety. Benanomicin A, which also has a 5,6-dihydrobenzo[*a*]naphthacenequinone skeleton, was reported to have D-alanine as the amino acid moiety and D-xylosyl-D-fucose as the C-5 disaccharide moiety[7,8]. In the continued search for new antifungal compounds, a new strain of *Actinomadura* numbered R103-3 was originally discovered which produced pradimicin L. Based on taxonomic studies described in this paper, *Actinomadura verrucosospora* subsp. *neohibisca* subsp. nov. is proposed here for strain R103-3. Compared with known pradimicin analogs, pradimicin L was found to differ by a distal D-glucose in the C-5 disaccharide moiety. Like pradimicins FA-1, FA-2[6] and BMS-181184[9], pradimicin FL was produced by directed biosynthesis in D-serine-supplemented medium so that the D-alanine moiety of pradimicin L could be replaced by D-serine. This paper deals with the taxonomy of strain R103-3, structure elucidation of pradimicins L and FL and their antifungal evaluation.

Materials and Methods

Taxonomy

Strain R103-3 was isolated from a soil sample collected in Puerto Viejo Costa, Peru. The cultural and physiological characteristics were examined by the methods of SHIRLING and GOTTLIEB[10] and GORDON *et al.*[11]. The whole cell hydrolyzates, phospholipids, menaquinone and cellular fatty acid compositions were analyzed by the methods of LECHEVALIER[12], LECHEVALIER *et al.*[13], COLLINS *et al.*[14] and SUZUKI and KOMAGATA[15], respectively.

Isolation of a High Producer Strain

According to the method previously reported[5,6], strain R103-3 was treated with *N*-methyl-*N*′-nitro-

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N-nitroguanidine (5 mg/ml) and a high producer of pradimicin L, strain A10019, was selected. Briefly each clony grown on Bn-2 agar medium [soluble starch (Nichiden Kagaku) 0.5%, glucose 0.5%, fish meat extract (Mikuni Kagaku Sangyo) 0.1%, yeast extract (Oriental Yeast) 0.1%, NZ-case (Humko Sheffield Chemical) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar (Junsei Chemical) 1.6%, pH 7.0] was inoculated to production medium A consisting of soluble starch 1.0%, glucose 1.0%, L-glutamic acid 0.1%, L-methionine 0.05%, L-arginine 0.05%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.6%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.05%, $CaCO_3$ 0.3% and salts solution ($FeSO_4 \cdot 7H_2O$ 0.1g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 0.1 g in 1 liter of water) 1.0% (v/v), pH 7.0 before autoclaving (10 ml/50 ml-Erlenmeyer flask) and incubated at 28°C for 10 days at 200 rpm on a rotary shaker. Fermentation broth was adjusted to pH 2.0 with 6 N HCl and mixed with an equal volume of BuOH. The BuOH layer was separated by centrifugation at 10,000 rpm for 10 minutes at room temperature and 10 μl of the BuOH extract was spotted on a silica gel TLC plate (Kieselgel $60F_{254}$, 0.25 mm thick, E. Merck) for identification of pradimicin L. Rf values of pradimicins with a solvent system of MeOAc-*n*-PrOH-28% NH_4OH (3:7:4): 0.47 for A, 0.52 for B, 0.44 for C, 0.15 for L, (0.20 for FL).

Quantitation of Pradimicin Congeners

For quantitation of pradimicin congeners, the fermentation broth was centrifuged as described above. The supernatant was diluted with 0.02 N NaOH - MeOH (1 : 1), and the identity of the red color characteristic of pradimicins was measured spectrophotometrically at 500 nm. The total production of pradimicins is expressed in μg/ml as the equivalent amount of pradimicin A (zwitterionic form, $E^{1\%}_{1cm}$ at 500 nm = 180 at pH $\geqq$ 11).

Quantitative component analysis was carried out by reverse-phase HPLC. After centrifugation, the broth supernate was adjusted to pH 2.0 with 0.1 N HCl and recentrifuged. The supernate was adjusted to pH 5.0 with 0.1 N NaOH and recentrifuged. The resulting sediment was dissolved in water at pH 3.5, diluted with CH_3CN-0.15% KH_2PO_4 (pH 3.5) (1 : 1) and passed through a Sep-Pak filter (Millipore-Waters). The filtrate was mixed with DMSO (1 : 1) and filtered again through a Millipore filter HVA (0.45 μm). HPLC (Waters M600, YMC A301-3, 4.6 mm i.d. × 100 mm, 3 μm, ODS, YMC Co., Ltd.) using a solvent system of CH_3CN-0.15% KH_2PO_4 (pH 3.5) (1 : 3). Flow rate: 0.8 ml/minute, detection at 254 nm, retention times in minutes: 4.64 for FA-2, 5.10 for FA-1, 6.95 for E, 7.75 for D, 8.48 for L, 8.99 for C, 10.07 for A and 11.40 for B, (4.34 for FL).

Fermentation of Pradimicins L and FL

Strain A10019 was grown and maintained on a slant of Bn-2 agar medium. Spores of the microorganism was inoculated in a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of soluble starch 1%, glycerol 1%, yeast extract 1%, peptone (Daigo Eiyo) 0.5%, NaCl 0.3% and $CaCO_3$ 0.2%, pH 7.0 before autoclaving. The seed culture was fermented at 32°C for 7 days on a rotary shaker at 200 rpm. For production of pradimicin L, a 5-ml aliquot of the seed culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium A. For production of pradimicin FL, 0.25% D-serine was supplemented to medium A before autoclaving. The fermentation was carried out at 28°C for 11 days.

Isolation

The fermentation broth (10 liters) of strain A10019 was centrifuged and the supernate was adjusted to pH 4.1 with 6 N HCl and kept at 5°C for 2 hours. The dark red precipitates deposited were collected by filtration. The precipitates were dissolved in 900 ml of water (adjusted to pH 9.1 with 6 N NaOH), and the solution was again filtered for removal of insoluble impurities. The filtrate was adjusted to pH 2.0 and applied onto a column of Diaion HP-20 (800 ml). The resin was washed with 3 liters of water and then eluted with 2 liters of acetone-H_2O (pH 2.5) (3 : 2). Acetone in the eluate was evaporated *in vacuo* and the remaining red-colored solution was washed 3 times with 400 ml each of ethyl acetate. The resulting water layer was concentrated and lyophilized to yield a dark red solid of pradimicin complex hydrochloride (4.5 g). The solid was dissolved in 450 ml of CH_3CN-0.15% KH_2PO_4 (pH 3.5) (22 : 78) and subjected to reverse-phase column chromatography of ODS-A60 (10 liters, YMC Co., Ltd.) which had been preequilibrated with the same solvent mixture. Elution was carried out with the above solvent mixture, and the eluate was collected in 1 liter fractions. Each fraction was analyzed by HPLC (Waters M600;

column: YMC A-301-3, 4.6 mm i.d. × 100 mm, 3 μm, ODS; mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (25:75); flow rate: 0.8 ml/minute; detection: UV absorption at 254 nm). Pradimicin L-containing fractions were pooled and concentrated *in vacuo* for removal of CH_3CN. The concentrate was applied onto a column of Diaion HP-20 (0.3 liter) and eluted with 200 ml of acetone-H_2O (pH 2.5) (60:40) to give a red powder of semi-pure pradimicin L hydrochloride (563 mg). The red powder (50 mg) was dissolved in CH_3CN-KH_2PO_4 (pH 3.5) (24:76) and chromatographed on an ODS (RP-18, 2.2 liters, E. Merck) column using the same solvent mixture. Fractions containing pradimicin L were combined and concentrated *in vacuo* for removal of CH_3CN. The concentrate was again passed through a column of Diaion HP-20 (0.2 liter). The column was washed with 600 ml of water and eluted with acetone-H_2O (pH 3.0) (60:40). The eluate (40 ml) was concentrated and lyophilized to yield 24 mg of pradimicin L hydrochloride.

Pradimicin FL hydrochloride (35 mg) was also isolated from the fermentation broth (20 liters) of strain A10019, which was cultivated in the D-serine-supplemented medium A.

In vitro Antifungal Activity

The MICs of the antifungal agents were determined by an agar dilution method on yeast morphology agar adjusted to pH 7.0 with 1/15 M phosphate buffer[4]. A 5-μl aliquot of fungal suspension containing 10^6 cells/ml, except for *Trichophyton mentagrophytes* No. 4329 which contained 10^7 cells/ml, was inoculated onto the surface of the antibiotic-containing agar plates. After incubation at 28°C for 40 hours, the lowest concentration of antibiotic causing complete inhibition of fungal growth was determined.

Vigorous Acid Hydrolysis of Pradimicins L and FL

Pradimicin L (100 mg) was dissolved in 10 ml of 6 N HCl and refluxed at 115°C for 15 hours. After cooling, the precipitates were collected by filtration, washed with water and dried to afford pradimicinone I (40 mg): MP >200°C (dec.); FAB-MS (m-NBA), *m/z* 550 $(M+H)^+$; molecular formula, $C_{28}H_{23}NO_{11}$; UV (0.02 N NaOH-MeOH, 1:1) λ_{max} 240 nm (ε 36,000), 314 (13,900), 504 (14,100). The filtrate was passed through a column of Diaion HP-20 (20 ml). Ninhydrin-positive fractions were pooled and concentrated to give a white solid (0.8 mg) which was identical with an authentic sample of alanine by silica gel TLC and amino acid analysis. The stereochemistry of the alanine was determined to be the *R*-configuration using the chiral HPLC method (column: Excel pak SIL-C18 5B, 4.6 mm i.d. × 150 mm, 5 μm, Yokogawa Electronic Co., mobile phase: MeOH-22 mM sodium phosphate buffer (pH 7.0) containing 1% THF (20:80), flow rate: 1.0 ml/minute, detection: UV absorption at 344 nm, Rt 12.0 minutes, L-alanine 14.3 minutes).

Pradimicin FL (50 mg) was similarly treated, resulting in pradimicinone Is (29 mg): MP >200°C (dec.); HRFAB-MS (m-NBA), $(M+H)^+$: obsd *m/z* 566.1299, calcd 566.1298; molecular formula, $C_{28}H_{23}NO_{12}$; UV (0.02 N NaOH-MeOH, 1:1) λ_{max} 242 nm (ε 28,300), 319 (14,000), 497 (11,500); IR ν_{max} (KBr) 3369, 3250, 2946, 1716, 1604, 1446, 1378, 1339, 1296, 1256 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.34 (3H, s), 3.73 (2H, dq, J=10.3 and 5.1 Hz), 3.94 (3H, s), 4.22 (1H, d, J=10.7 Hz), 4.28 (1H, d, J=10.7 Hz), 4.46 (1H, t, J=5.1 Hz), 6.90 (1H, d, J=2.4 Hz), 7.06 (1H, s), 7.26 (1H, d, J=2.4 Hz), 8.03 (1H, s) and D-serine (1.8 mg), Rt 9.09 minutes, L-serine 9.72 minutes.

Acid Methanolysis of Pradimicin L

Pradimicin L hydrochloride (108 mg) was dissolved in 36 ml of 1.5 N HCl-MeOH and refluxed at 80°C for 20 hours. The reaction mixture was concentrated to dryness *in vacuo*. The residue was dissolved in water (15 ml) and applied onto a column of Diaion HP-20 (200 ml). The HP-20 column was eluted first with 300 ml of water for recovery of methyl D-glucopyranoside and then with 360 ml of acetone-H_2O (pH 3.0) (60:40) for recovery of pradimicins B and L methyl esters. Fractions which were positive to anthrone-H_2SO_4 reagent, were further purified by Sephadex LH-20 column chromatography (80 ml) using MeOH-H_2O (1:1, 20 ml) to give a white powder (16.3 mg). The powder was identified as methyl D-glucopyranoside with $[\alpha]_D^{24}$ +116° (*c* 0.5, H_2O, a ratio of α to β was estimated to be 7:3 by ^1H NMR spectrometry). The aqueous acetone eluate (360 ml) containing pradimicins B and L methyl esters was evaporated to give a red powder (80 mg). The powder was dissolved in CH_3CN-0.15% KH_2PO_4 (pH 3.5) (24:76) and applied onto a reverse-phase silica gel column (YMC ODS-A60, 350/250 mesh, 200 ml), which was eluted with the same solvent. Each fraction was analyzed by HPLC (column: YMC A301-3, 3 μm,

4.6 mm i.d. × 100 mm, mobile phase: CH_3CN - 0.15% KH_2PO_4 (pH 3.5) (30 : 70), flow rate: 1.0 ml/minute, detection: UV absorption at 254 nm). Fractions containing pradimicin L methyl ester (Rt: 13.55 minutes, 1.2 liters) were concentrated. The concentrate was adsorped on a column of Diaion HP-20 (200 ml), washed with water (1 liter) and then eluted with acetone - H_2O (pH 3.0) (60 : 40). The eluate (100 ml) was concentrated and lyophilized to yield pradimicin L methyl ester hydrochloride (27 mg): MP >190°C (dec.); FAB-MS (m-NBA), *m/z* 885 $(M+H)^+$; molecular formula, $C_{42}H_{46}N_2O_{19}$; UV (0.02 N NaOH - MeOH, 1 : 1) λ_{max} 319 nm (ε 11,500), 498 (10,800); IR ν_{max} (KBr) 3370, 2930, 1730, 1620, 1450, 1390~1380, 1295, 1160, 1060 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.67 (3H, s); TLC, Rf 0.39, MeOAc - *n*-PrOH - 28% NH_4OH (45 : 105 : 60). By the same way, pradimicin B methyl ester hydrochloride[1,2] (Rt: 10.33 minutes, 15 mg) was obtained.

Mild Acid Hydrolysis of Pradimicin FL

Pradimicin FL hydrochloride (70 mg) was dissolved in 18 ml of 1 N HCl and refluxed at 70°C for 9.5 hours. The solution was neutralized with 6 N NaOH and applied onto a column of Diaion HP-20 (25 ml). The resin was washed with water (100 ml) and eluted with acetone - H_2O (pH 3.0) (60 : 40). The eluate (50 ml) was concentrated and lyophilized to give a crude deglucosylpradimicin FL solid (40 mg). The solid was dissolved in 10 ml of CH_3CN - 0.15% KH_2PO_4 (pH 3.5) (25 : 75) and subjected to reverse-phase silica gel (RP-18, 2.2 liters) column chromatography using the same solvent system. Fractions containing deglucosylpradimicin FL were combined (5.1 liters) and concentrated *in vacuo*. The concentrate was applied onto a column of Diaion HP-20 (60 ml). The column was washed with water and eluted with acetone - H_2O (pH 3.0) (4 : 1). The eluate (50 ml) was evaporated to yield pure deglucosylpradimicin FL hydrochloride (33 mg): MP >195°C (dec.); FAB-MS (m-NBA), *m/z* 725 $(M+H)^+$; molecular formula, $C_{35}H_{36}N_2O_{15}$; UV (0.02 N NaOH - MeOH, 1 : 1) λ_{max} 300 nm (ε 24,500), 458 (10,200); IR ν_{max} (KBr) 3400, 1720, 1620~1610, 1390, 1340, 1295, 1260, 1130, 1065 cm^{-1}.

General

Spectral data were recorded with the following instruments: IR, JASCO IR-810 spectrometer; UV-vis, JASCO UVIDEC-610 spectrometer; ^1H and ^{13}C NMR, JEOL JMN-GX 400 spectrometer; FAB-MS and HRFAB-MS, JEOL JMS-AX 505H spectrometer.

Results and Discussion

Taxonomy

Strain R103-3 formed well-branched, non-fragmenting substrate mycelia and short aereal mycelia on agar media. Loop or spiral short spore chains (5~12 spores per chain) were formed on the aerial hyphae. The spores were oval (0.8 × 1.2~1.5 μm) with warty surface (Figs. 1 and 2). Flagellate spores,

Fig. 1. Photomicrograph of spore chains of strain R103-3 on ISP medium No. 4 grown at 28°C for 3 weeks (× 600).

Fig. 2. Scanning electron micrograph of spore chains with warty spore surface of strain R103-3 on ISP medium No. 4 grown at 28°C for 3 weeks.

Bar represents 2.0 μm.

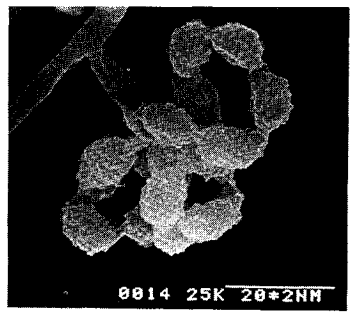


Table 1. Cultural characteristics of strain R103-3.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Moderate	None	Very deep red (14)	Very deep purplish red (257)
Tryptone-yeast extract broth (ISP No. 1)	Poor, not turbid	None	Deep red (13)	Moderate red (15)
Yeast extract-malt extract agar (ISP No. 2)	Good	None	Very deep red (14)	Very dark red (17)
Oatmeal agar (ISP No. 3)	Moderate	Moderate; pale pink (7)	Moderate pink (5)	Grayish pink (8) to light grayish red (18)
Inorganic salts-starch agar (ISP No. 4)	Moderate	Poor; white	Moderate pink (5)	Light grayish red (18)
Glycerol-asparagine agar (ISP No. 5)	Poor	Poor; white	Colorless	None
Peptone-yeast extract-iron agar (ISP No. 6)	Good	Scant; white	Grayish pink (8) to deep red (13)	Very deep red (14)
Tyrosine agar (ISP No. 7)	Moderate	Poor; white	Moderate red (15)	Light yellowish pink (28)
Glucose-asparagine agar	Poor	None	Colorless	Light pink (4)
Nutrient agar	Moderate	Poor; white	Dark pink (6)	Dark red (16)
BENNETT's agar	Good	None	Blackish red (21)	Blackish red (21)

Observation after incubation at 28°C for 3 weeks. Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of strain R103-3.

Decomposition of:		Acid production from[a]:	
Adenine	−	Adonitol	−
Casein	+	D-Arabinose	−
Hippuric acid	+	L-Arabinose	+
Hypoxanthine	−	Cellobiose	+
Tyrosine	+	Dulcitol	−
Xanthine	−	Erythritol	−
Utilization of:		D-Fructose	+
Benzoate	−	D-Galactose	−
Citrate	−	D-Glucose	+
Mucate	−	Glycerol	−
Succinate	+	Inositol	−
Tartrate	−	Lactose	−
Production of:		D-Mannitol	+
Amylase	−	D-Mannose	−
Esculinase	+	D-Melezitose	−
Gelatinase	+	Melibiose	−
Nitrate reductase	+	Methyl-α-glucoside	−
Tyrosinase	−	Raffinose	−
Urease	−	L-Rhamnose	+
Growth in/at:		D-Ribose	+
Lysozyme, 0.001% (w/v)	−	Salicine	+
NaCl, 3%	+	Soluble starch	+
5%	+	D-Sorbitol	−
8%	−	L-Sorbose	−
10°C	−	Sucrose	+
20°C	+	Trehalose	−
45°C	−	D-Xylose	+

[a] Basal medium: Pridham-Gottlieb medium (ISP 9), $CuSO_4 \cdot 7H_2O$ omitted.

sporangium-like vesicles and sclerotia were not observed. Well sporulating aerial mycelia were formed on ISP media Nos. 3, 4, 5 and 7. Reddish diffusible pigments including pradimicins were produced in CZAPECK's agar and organic media such as ISP medium No. 2. Cultural and physiological characteristics are shown in Tables 1 and 2, respectively.

The whole cell hydrolyzate contained *meso*-diaminopimelic acid, glucose and madurose. The phospholipids consisted of phosphatidylglycerol and phosphatidylinositol without nitrogenous phospholipids. Therefore, strain R103-3 belonged to the wall type III, sugar pattern B and phospholipid type P-I. The major menaquinones consisted of MK-9 (H_6), MK-9 (H_4) and MK-9 (H_8) (53%, 24% and 13%, respectively) together with minor components such as MK-9 (H_2) and MK-9 (H_{10}). The major cellular fatty acid compositions contained *n*-16:0, 18:1, 10Me 18:0 and *n*-18:0 (39%, 27%, 15% and 6%, respectively).

Based on the morphological and chemotaxonomical results, strain R103-3 is placed in the genus *Actinomadura* Lechevalier and Lechevalier 1970[16]. Among hitherto published species of *Actinomadura*, strain R103-3 seemed similar to *Actinomadura madurae* (Vincent 1894) Lechevalier and Lechevalier 1970, *Actinomadura verrucosospora* Nonomura and Ohara 1971 and *Actinomadura cremea* Preobrazhenskaya, Lavrova, Ukholina and Nechaeva 1975[17,18], while it was fairly different from *Actinomadura hibisca*, a pradimicin producer (Table 3). As shown in Table 4, strain R103-3 seemed to be more closely related to *A. verrucosospora* than the others, although the former was differentiated from *A. verrucosospora* in

Table 3. Differential characteristics of strain R103-3 from *Actinomadura hibisca* P157-2.

	Strain R103-3	Strain P157-2
Morphology:		
Spore-chain	Short, hook	Long, straight
Spore sulface	Warty	Smooth
Cultural and physiological characteristics:		
Tyrosine agar:		
Brownish pigment	Not formed	Formed
Glucose-asparagine agar:		
Growth	Poor	Abundant
Reddish pigment	Scant	Abundant
Utilization of:		
L-Arabinose	+	−
D-Mannitol	+	−
L-Rhamnose	+	−
D-Xylose	+	−

Table 4. Differential characteristics of *Actinomadura madurae* ATCC 19425^T, *Actinomadura verrucosospora* ATCC 27299^T and *Actinomadura cremea* INA 292^T from R103-3.

	Strain R103-3	*A. madurae* ATCC 19425^T	*A. verrucosospora* ATCC 27299^T	*A. cremea* INA 292^T
Color of aerial mycelium	White to pink	White	White to pink or blue	White to pink or yellow
Brown substrate mycelium	−	+	−	+
Production of:				
Nitrate reductase	+	+	−	+
Urease	−	−	−	+
Acid production from:				
Adonitol	−	+	+	+
Cellobiose	+	+	+	−
Glycerol	−	+	+	+
Lactose	−	+	−	−
D-Mannose	−	+	−	−
Starch	+	+	+	−
Trehalose	−	+	+	−
Decomposition of:				
Hypoxanthine	−	+	+	−

terms of the production of nitrate reductase, the absence of acid production from adonitol, glycerol and trehalose and the lack of decomposition of hypoxanthine. In addition, no strain of *A. verrucosospora* was reported to produce a diffusible red pigment generated by benzo[*a*]naphthacenequinone or related chromophores. Thus, strain R103-3 was identified as a new subspecies of *A. verrucosospora* and is proposed to name *Actinomadura verrucosospora* subsp. *neohibisca* subsp. nov. Tomita (Origin: neo'hi-biska, L, n.; Gr *hibiskos* rose mallow, a plant with reddish flower referring to a new red pigmented organism). Type strain, strain R103-3, is a single isolate and has been deposited with the American Type Culture Collection under the accession number ATCC 53930.

Fermentation and Purification

Total production and the component ratio of pradimicins including L and FL by strain A10019 in the presence and absence of D-serine are shown in Table 5. Pradimicin FL was not produced in 0.25% L-serine-supplemented medium A (data not shown). Pradimicin L (or FL) was purified by HP-20 adsorption and reverse-phase silica gel column chromatography.

Physico-chemical Properties and Structural Elucidation

The physico-chemical properties of pradimicins L and FL hydrochlorides are shown in Table 6. The UV and visible spectra of these antibiotics under acidic and alkaline conditions, along with the IR spectra were quite similar to those of pradimicin[1,2], indicating that they share a common chromophore of

Table 5. Production of pradimicin congeners by strain A10019 in the presence and absence of D-serine.

D-Serine (%)	Total production (μg/ml)	Ratio of pradimicins (%)								
		A	B	C	D	E	L	FA-1	FA-2	FL
0	310	51	1	6	3	—	27	—	—	—
0.25	330	17	11	5	1	—	1	41	7	6

Quantitative component analysis was carried out by reverse-phase HPLC as described in Materials and Methods.

Table 6. Physico-chemical properties of pradimicins L and FL hydrochlorides.

	Pradimicin L	Pradimicin FL
Nature	Dark red amorphous powder	Orange amorphous powder
Solubility (covers both antibiotics)		
soluble in	DMSO, DMFA, acidic or alkaline water	
slightly soluble in	Water, MeOH, EtOH, *n*-BuOH	
insoluble in	Other common organic solvents	
MP (°C, dec.)	>200	>200
$[\alpha]_D^{27}$ (*c* 0.1, 0.1 N HCl)	+415°	Not determined
Molecular formula	$C_{41}H_{46}N_2O_{19}$	$C_{41}H_{46}N_2O_{20}$
FAB-MS (*m/z*)	871 $(M+H)^+$, 870 (M^-)	887 $(M+H)^+$, 886 (M^-)
HRFAB-MS (*m/z*)	Obsd: 871.2769 $(M+H)^+$ Calcd for $C_{41}H_{46}N_2O_{19}$: 871.2773	
UV λ_{max} nm (ε)		
in 0.02 N HCl-MeOH (1:1)	234 (33,400), 298 (29,900), 460 (12,000)	234 (31,900), 299 (27,800), 459 (10,600)
in 0.02 N NaOH-MeOH (1:1)	241 (32,700), 319 (14,500), 498 (13,900)	242 (34,500), 319 (15,200), 497 (13,600)
IR (KBr) cm^{-1}	3380, 2900, 1620~1600, 1385, 1295, 1260, 1160, 1060	3400, 2940, 1720, 1630~1610, 1390, 1335, 1295, 1260, 1160, 1080~1060

Table 7. ^{1}H NMR spectra of pradimicins L, FL and deglucosylpradimicin FL hydrochlorides (400 MHz, DMSO-d_6).

Position	Pradimicin L	Pradimicin FL	Deglucosylpradimicin FL
3-CH_3	2.31 (s)	2.33 (s)	2.33 (s)
4-H	6.98 (s)	7.02 (s)	6.98 (s)
5-H	4.54 (d, J=9.4)	4.56~4.58 (m)	4.49 (d, J=9.4)
6-H	4.56 (br d, J=9.4)		4.55 (br d, J=9.4)
7-H	7.85 (s)	7.89 (s)	7.84 (s)
10-H	6.83 (d, J=2.1)	6.86 (d, J=2.1)	6.81 (d, J=2.1)
11-OCH_3	3.93 (s)	3.94 (s)	3.93 (s)
12-H	7.21 (d, J=2.1)	7.23 (d, J=2.1)	7.19 (d, J=2.1)
16-NH	8.70 (br s)	8.46 (br s)	8.54 (br s)
17-H	4.40 (qui, J=7.8)	4.44~4.50 (m)	4.46 (dt, J=4.7, 7.3)
17'-CH_3	1.34 (d, J=7.3)	—	—
17'-CH_2	—	3.70~3.80 (m)	3.72~3.78 (m)
1'-H	4.80 (d, J=7.7)	4.81 (d, J=7.7)	4.71 (d, J=7.7)
2'-H	3.51 (m)	3.50 (m)	3.41 (m)
3'-H	3.96 (m)	3.92~3.97 (m)	3.80~3.83 (m)
4'-H	3.53 (m)	3.54 (d, J=3.4)	3.24 (br s)
4'-NCH_3	2.71 (s)	2.71 (s)	2.68 (br s)
5'-H	3.90 (br q, J=6.8)	3.92 (br q, J=6.8)	3.89 (q, J=6.6)
6'-CH_3	1.28 (d, J=6.8)	1.28 (d, J=6.8)	1.28 (d, J=6.6)
1''-H	4.46 (d, J=7.7)	4.46 (d, J=7.3)	
2''-H	3.15 (t, J=7.7)	3.13 (dd, J=7.3, 7.7)	
3''-H	3.18~3.24 (m)	3.20 (dd, J=7.7, 9.0)	
4''-H	3.06 (m)	3.05 (t, J=9.0)	
5''-Hax	3.18~3.24 (m)	3.18~3.24 (m)	
5''-Heq	—	—	
6''-Hax	3.44 (dd, J=6.0, 12.0)	3.44 (dd, J=6.4, 12.0)	
6''-Heq	3.74 (dd, J=3.8, 12.0)	3.73 (dd, J=2.6, 12.0)	

ppm (multiplicity, J=Hz).

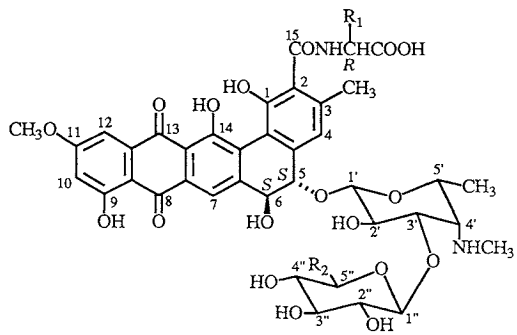
5,6-dihydrobenzo[*a*]naphthacenequinone. The molecular formula of pradimicin L was established as $C_{41}H_{46}N_2O_{19}$ by HRFAB-MS [$(M+H)^+$ m/z 871.2769]. Pradimicin L differed by 30 mass units higher than pradimicin A. In the ^{1}H NMR spectrum of pradimicin L (Table 7), a signal at δ 3.75 corresponding to 5''-Heq (J=5.4 and 11.1 Hz) in pradimicin A[2] is missing, while new signals are observed at δ 3.44 (1H, dd, J=6.0 and 12.0 Hz) and δ 3.74 (1H, dd, J=3.8 and 12.0 Hz), assignable to 6''-H of a hexose by ^{1}H-^{1}H COSY. Methanolysis (1.5 N HCl - MeOH, 80°C, 20 hours) of pradimicin L yielded methyl D-glucoside and pradimicin B methyl ester (AG-1)[12], suggesting that pradimicin L has D-glucose in place of D-xylose in pradimicin A. Vigorous acid hydrolysis (6 N HCl, 115°C, 15 hours) of pradimicin L gave alanine which was detected by silica gel TLC and amino acid analysis. Alanine was determined to have the *R*-configuration by a chiral column method[19]. Pradimicinone I (formerly called AG-2)[1,2] was also isolated from the hydrolyzate, which supported pradimicin L having the 5*S*, 6*S* configurations. The structure of the disaccharide moiety in pradimicin L was studied by ^{1}H and ^{13}C NMR spectrometry. The anomeric proton of 1''-H in the D-glucoside moiety showed the NOE with 3'-H of thomosamine in the NOESY experiment, and the β-pyranoside configuration was determined by a coupling constant of J=7.7 Hz. Moreover, the 3'-C signal at δ 79.7 of pradimicin L is deshielded as compared with that of pradimicin B (δ 71.0)[2]. The D-glucose moiety in pradimicin L was, therefore, elucidated to be attached on the 3'-*O*-position of thomosamine, which was consistent with the sugar linkage in known pradimicin antibiotics. These results indicate the structure of pradimicin L to be 3'-*O*-(β-D-glucopyranosyl) pradimicin B.

Table 8. ^{13}C NMR spectra of pradimicin L and deglucosylpradimicin FL hydrochlorides (100 MHz, DMSO-d_6).

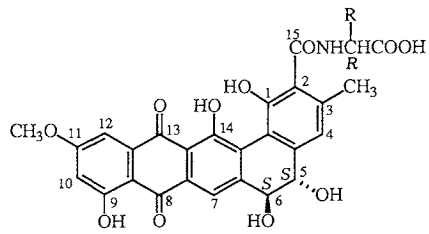
Position	Pradimicin L	Deglucosylpradimicin FL
1	151.5 (s)	151.8 (s)
2	127.3 (s)	126.3 (s)
3	137.2 (s)	137.1 (s)
3-CH_3	19.0 (q)	19.5 (q)
4	118.5 (d)	118.2 (d)
4a	137.5 (s)	137.8 (s)
5	80.8 (d)	81.3 (d)
6	71.3 (d)	71.4 (d)
6a	147.4 (s)	146.1 (s)
7	114.1 (d)	114.1 (d)
7a	131.2 (s)	131.3 (s)
8	184.9 (s)	184.5 (s)
8a	110.0 (s)	110.0 (s)
9	164.5 (s)	164.2 (s)
10	106.6 (d)	105.7 (d)
11	165.9 (s)	165.8 (s)
11-OCH_3	56.2 (q)	56.0 (q)
12	107.3 (d)	106.8 (d)
12a	134.4 (s)	135.5 (s)
13	186.9 (s)	185.6 (s)
13a	117.2 (s)	116.8 (s)
14	157.2 (s)	153.8 (s)
14a	126.1 (s)	128.4 (s)
14b	115.7 (s)	115.7 (s)
15	166.7 (s)	167.4 (s)
17	47.5 (d)	54.8 (d)
17′	16.8 (q)	61.2 (t)
18	173.6 (s)	171.5 (s)
1′	103.8 (d)	104.5 (d)
2′	69.5 (d)	70.9 (d)
3′	79.7 (d)	70.8 (d)
4′	62.9 (d)	63.8 (d)
4′-NCH_3	35.8 (q)	36.3 (q)
5′	67.2 (d)	67.4 (d)
6′	16.0 (q)	16.0 (q)
1″	104.3 (d)	
2″	73.5 (d)	
3″	75.9 (d)	
4″	69.9 (d)	
5″	76.9 (d)	
6″	60.8 (t)	

ppm (multiplicity).

Fig. 3. Structures of pradimicins A, FA-1, L and FL and their aglycones pradimicinones I and Is.



	R_1	R_2
Pradimicin A	CH_3	H
Pradimicin FA-1	CH_2OH	H
Pradimicin L	CH_3	CH_2OH
Pradimicin FL	CH_2OH	CH_2OH



	R
Pradimicinone I	CH_3
Pradimicinone Is	CH_2OH

Pradimicin FL is closely similar to pradimicin L in the UV and IR spectra (Table 6). The molecular formula of pradimicin FL was estimated to be $C_{41}H_{46}N_2O_{20}$ by FAB-MS spectrometry. Pradimicin FL differs by 16 mass units from pradimicin L. In the ^1H NMR spectrum of pradimicin FL (Table 7), the 17′-CH_3 signal at δ 1.34 (3H, d, J=7.3 Hz) in pradimicin L or δ 1.35 in pradimicin A is missing, while new signals around δ 3.70~3.80 (m) assignable to 17′-CH_2OH are observed. Proton signals at C-6″ in pradimicin FL are exactly identical with those of pradimicin L, which indicates that pradimicin FL also possesses the D-glucose moiety like pradimicin L.

Mild acid hydrolysis (1 N HCl, 70°C, 9.5 hours) of pradimicin FL yielded deglucosylpradimicin FL, whose structure was established by comparing with an authentic sample prepared from pradimicin FA-1 (Tables 7 and 8). Vigorous acid hydrolysis of pradimicin FL yielded pradimicinone Is along with a small

Table 9. *In vitro* antifungal spectra of pradimicins L, FL and A[a].

Test organism	MIC (μg/ml)		
	Pradimicin L	Pradimicin FL	Pradimicin A
Saccharomyces cerevisiae ATCC 9763	6.3	3.1	6.3
Candida albicans A9540	25.0	6.3	12.5
C. albicans ATCC 32354	6.3	6.3	6.3
C. albicans 83-2-14	100.0	6.3	12.5
C. albicans ATCC 38247	3.1	3.1	6.3
C. tropicalis 85-8	>100.0	6.3	12.5
C. tropicalis IAM 10241	100.0	6.3	>100.0
Cryptococcus neoformans D49	1.6	3.1	1.6
C. neoformans IAM 4514	0.8	1.6	1.6
Aspergillus fumigatus IAM 2034	3.1	3.1	1.6
Trichophyton mentagrophytes[b] No. 4329	3.1	3.1	3.1

[a] Yeast morphology agar adjusted to pH 7.0 with 1/15 M phosphate buffer. Determined after incubation for 40 hours at 28°C (*Trichophyton mentagrophytes*: 60 hours, 28°C).
[b] Inoculum size 10^6 cells/ml (*Trichophyton mentagrophytes*: 10^7 cells/ml).

quantity of D-serine, which was confirmed to have the *R*-configuration as the amino acid side chain. Consequently, pradimicin FL was concluded to have D-serine in place of D-alanine of pradimicin L.

The structures of pradimicins L and FL and their degradation products, pradimicinones I and Is, were determined as illustrated in Fig. 3.

In Vitro Antifungal Activity

MICs for pradimicins L, FL and A against fungi and yeasts are shown in Table 9. Pradimicins L and FL show a broad-spectrum of antifungal activity with MICs ranging from 1.6 to 6.3 μg/ml. Pradimicin FL appears to be more active than pradimicins L and A against *Saccharomyces cerevisiae*, *Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes*. The antifungal activities of pradimicins L and FL indicate a probability that the distal sugar in pradimicin A can be replaced with other sugars without significant loss of anitifungal activity. In view of urgency in the development of fungicidal and less toxic antifungal agents for life-threatening fungal infections, screening of microbial products in the pradimicin family should be continued.

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