PRADIMICINS A, B AND C†: NEW ANTIFUNGAL ANTIBIOTICS

I. TAXONOMY, PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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New antifungal antibiotics, pradimicins A, B and C were isolated from the culture broth of actinomycete strains proposed as *Actinomadura hibisca*. They are orange to red pigments containing a benzo[*a*]naphthacenequinone chromophore substituted with a D-alanine, an aminosugar and a D-xylose (pradimicins A and C).

In a systematic search for microbial metabolites effective against fungi and yeasts, actinomycete strains No. P157-2 and Q278-4 isolated from soil samples collected in the Fiji Islands and India, respectively, were found to produce a complex of novel antibiotics designated pradimicin[1~3]. The producing strains were classified as an undescribed species of the genus *Actinomadura* and named *Actinomadura hibisca* sp. nov. after taxonomical studies. The active principle was precipitated from the broth filtrate at pH 5.0 and purified by solvent partition and column chromatography to yield a major component pradimicin A and two minor components pradimicins B and C. Pradimicins A, B and C showed moderate *in vitro* activity against a wide variety of fungi and yeasts, some Gram-positive bacteria, and viruses. Most interestingly, they exhibited marked *in vivo* therapeutic activity against systemic fungal infections caused by *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* strains in mice. Through chemical degradation and spectral analyses, the structures of pradimicins have been determined to be a benzo[*a*]naphthacenequinone carrying D-alanine and sugars[4~6]. In this paper, we report the taxonomy of the producing strains and the production, isolation and physico-chemical properties of pradimicins. The biological activities of the antibiotics are reported in a companion paper[7].

Taxonomy of the Producing Strain

The producing organisms, strain P157-2 and strain Q278-4 were isolated from soil samples collected in the Fiji Islands and Andhra Pradesh State, India, respectively.

Morphological Characteristics

Both strains form white aerial mycelium and non-fragmentary substrate mycelium. Long straight spore-chains (10 to 50 spores per chain) are formed on all parts of the aerial mycelium. The spores are oblong in shape, 0.4 to 0.6 by 0.7 to 1.5 μm in size, non-flagellate, and have smooth surface (Fig. 1). A fused coil of spore chain is occasionally formed intercalary or at the tip of long chains, which is observed by photomicroscopy as a small globose body (1.5 to 4 μm in diameter) (Fig. 2).

† Pradimicin A was originally called as BU-3608 A or BMY-28567, pradimicin B as BU-3608 B or BMY-28634 and pradimicin C as BU-3608 C or BMY-28747.

Fig. 1. Scanning electron micrograph of spore chains of strain P157-2 on inorganic salts-starch agar (ISP medium No. 4) incubated at 28°C for 14 days.

Bar represents 3 μm.

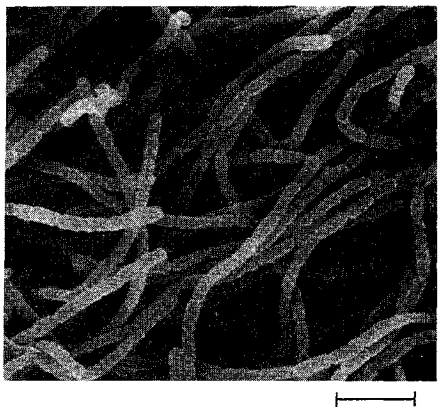
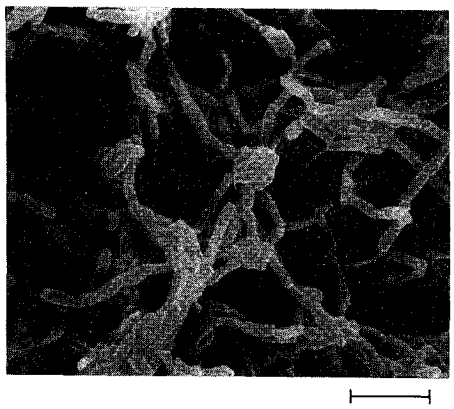


Fig. 2. Scanning electron micrograph of spore chains with intercalary globose body, of strain P157-2 on yeast extract-malt extract agar (ISP medium No. 2) incubated at 28°C for 14 days.

Bar represents 3 μm.

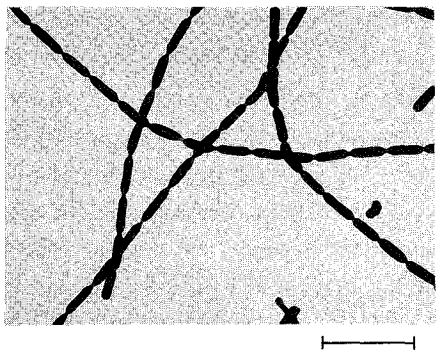


Cultural and Physiological Characteristics

White aerial mycelium is formed on some media such as yeast extract-malt extract agar (ISP medium No. 2) (Fig. 3). A reddish pigment, which is recognized as pradimicins, is produced abundantly in ISP media No. 2 and 6, but slightly in ISP media No. 3, 4 and 5, and a brown melanoid pigment only in ISP medium No. 7. The growth temperature range is 18 to 40°C for strain P157-2 and 16 to 43°C for strain Q278-4. The NaCl tolerance for growth is seen at 3% (w/v) but not at 6% (w/v). The cultural characteristics of strains P157-2 and Q278-4 are shown in Table 1, and the physiological characteristics examined by the methods of GORDON *et al.*[8] are given in Table 2.

Fig. 3. Transmission electron micrograph of spore chains of strain Q278-4 on yeast extract-malt extract agar (ISP medium No. 2) at 28°C for 14 days.

Bar represents 5 μm.



Chemotaxonomical Studies

The whole-cell hydrolysate contains *meso*-diaminopimelic acid, glucose and a small amount of madurose, hence the cell wall is type III and the whole cell-sugar is Pattern B. The phospholipids contain phosphatidylglycerol and phosphatidylinositol but lack nitrogenous phospholipids, hence belong to type P-I. The major menaquinone is MK-9 (H_6). Glycolate test is negative. Mycolate is absent.

Taxonomic Position

Based on the major characteristics of strains P157-2 and Q278-4, both strains were placed in the genus *Actinomadura*. Many strains of *Actinomadura* produce reddish pigments such as those identified as prodigiosins or anthracyclines, while these two strains produce three new red pigments, pradimicins A, B and C having benzo[*a*]naphthacenequinone as aglycone.

Table 1. Cultural characteristics of strains P157-2 and Q278-4.

Medium	Strain P157-2	Strain Q278-4
Sucrose-nitrate agar (CZAPEK-Dox agar)	G: Scant	G: Poor
	A: None	A: None
	S: Pinkish white (9)	S: Colorless to pale purplish pink (252)
	D: Pale yellowish pink (31)	D: Pale purplish pink (252)
Yeast extract-malt extract agar (ISP No. 2)	G: Moderate	G: Good
	A: Moderate; white	A: Moderate; white
	S: Very deep red (14)	S: Deep red (13)
	D: Very dark red (17)	D: Very dark red (17)
Glycerol-asparagine agar (ISP No. 5)	G: Poor	G: Poor
	A: Scant; white	A: None
	S: Colorless to light pink (4)	S: Light grayish red (18)
	D: Moderate yellowish pink (29)	D: Light yellowish pink (28)
Glucose-asparagine agar	G: Abundunt	G: Moderate
	A: None	A: None
	S: Very deep red (14)	S: Dark red (16), later dark grayish purple (229)
	D: Very dark red (17)	D: Grayish purplish pink (253)
Tyrosine agar (ISP No. 7)	G: Poor	G: Poor
	A: Very scant; white	A: Poor; white, later light gray (264)
	S: Light brown (57)	S: Dark reddish brown (44)
	D: Deep brown (59)	D: Dark grayish reddish brown (47)

Observations after incubation at 28°C for 3 weeks.
Abbreviations: G, growth; A, aerial mycelium; S, substrate mycelium; D, diffusible pigment.
Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of *Actinomadura hibisca* and *Actinomadura pelletieri*.

	A.h.	*A.p.*		*A.h.*	*A.p.*
Decomposition of:			Mannose	−	−
Adenine	+	−	Melezitose	−	−
Casein	+	+	Melibiose	−	−
Hypoxanthine	+	+	Methyl α-glucoside	−	−
Tyrosine	+	+	Raffinose	−	−
Urea	−	−	L-Rhamnose	−	−
Xanthine	−	−	Sorbitol	−	−
Resistance to:			Trehalose	+	+
Lysozyme	+	−	D-Xylose	−	−
Hydrolysis of:			Utilization of:		
Aesculin	+	−	Benzoate	−	−
Hippuric acid	−	−	Citrate	−	−
Starch	−	−	Mucate	−	−
Acid from:			Succinate	v	−
Adonitol	−	−	Tartrate	−	−
L-Arabinose	−	−	Production of:		
Cellobiose	+	−	Gelatinase	+	+
Erythritol	−	−	Nitrate reductase	+	+
Glucose	+	+	Melanoid	+	v
Glycerol	−	−	Prodigiosin	−	+
Inositol	−	−	Collagenase	−	+
Lactose	−	−	Tyrosinase	−	−
Mannitol	−	−			

Abbreviations: *A.h.*, *Actinomadura hibisca*; *A.p.*, *A. pelletieri*.
+: 85 to 100% of strains positive. −: 0 to 14% of strains positive. v: 15 to 84% strains positive.
The data of *A. pelletieri* are cited from the literature of GORDON *et al.*[8].

The chemotaxonomy[9] and numerical taxonomy[10,11] of *Actinomadura* as well as the descriptions of many new species of the genus such as *Actinomadura atramentaria*[12] indicate that the two strains have some similarity to *Actinomadura pelletieri*. However, they are differentiated from *A. pelletieri* in the ability to form long spore-chains, the decomposition of adenine, the resistance to lysozyme, the hydrolysis of aesculin, the acid formation from cellobiose, and the production of pradimicins instead of prodigiosin of *A. pelletieri* (Table 2). Thus, strains P157-2 and Q278-4 are considered to be a new species of *Actinomadura* and are proposed to name *Actinomadura hibisca* sp. nov. TOMITA (Origin: hi-bisika, L.n.; Gr *hibiskos* rose mallow, a plant with reddish flower reffering to the red diffusible pigments. The type strain is No. P157-2 (ATCC 53557).

Antibiotic Production

A slant culture of *A. hibisca* strain P157-2 (ATCC 53557) or strain Q278-4 (ATCC 53646) was prepared using modified BENNETT's agar medium consisting of soluble starch 0.5%, glucose 0.5%, fish meal extract 0.1%, yeast extract 0.1%, NZ-case (Sheffield) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6%, and incubated at 28°C for 7 days. A portion of the microbial growth from the slant culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of glucose 1%, soluble starch 2%, NZ-amine A (Sheffield) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.1%, adjusted to pH 7.2 before sterilization. The seed culture was incubated at 28°C for 4 days on a rotary shaker (200 rpm); 5 ml of the culture was transferred to a 500-ml Erlenmeyer flask which contained 100 ml of fermentation medium consisting of glucose 3%, soybean meal 3%, Pharmamedia 0.5%, yeast extract 0.1% and $CaCO_3$ 0.3%. The fermentation was carried out at 28°C for 5 to 6 days on a rotary shaker.

Antibiotic production in the cultured broth was determined by the conventional broth dilution method using *C. albicans* A9540 as the indicator organism in Sabouraud dextrose medium. The visible absorption at 500 nm in 0.02 N NaOH-MeOH (1:1) solution was used in parallel with the microbial assay. The

Table 3. Physico-chemical properties of pradimicins A, B and C.

	Pradimicin A	Pradimicin B	Pradimicin C
Nature	Red amorphous powder	Red amorphous powder	Red amorphous powder
MP (°C, dec)	193~195	195~198	220~225
$[\alpha]_D^{26}$ (*c* 0.1, 0.1 N HCl)	+685°	+440°	+619°
UV and visible $\lambda_{max}^{50\% \text{ MeOH}}$ nm (ε)	231 (28,300), 284 (22,700) 482 (9,600)	234 (27,900), 278 (23,100), 492 (8,800)	230 (31,400), 285 (23,400), 481 (9,900)
SI-MS *m/z*: Glycerol matrix	843 $(M+3H)^+$	711 $(M+3H)^+$	
mNBA matrix	841 $(M+H)^+$	709 $(M+H)^+$	827 $(M+H)^+$
Molecular formula	$C_{40}H_{44}N_2O_{18}$	$C_{35}H_{36}N_2O_{14}$	$C_{39}H_{42}N_2O_{18}$
TLC[a] Rf	0.36	0.48	0.32
HPLC[b] Rt (minutes)	16.7	21.8	14.0

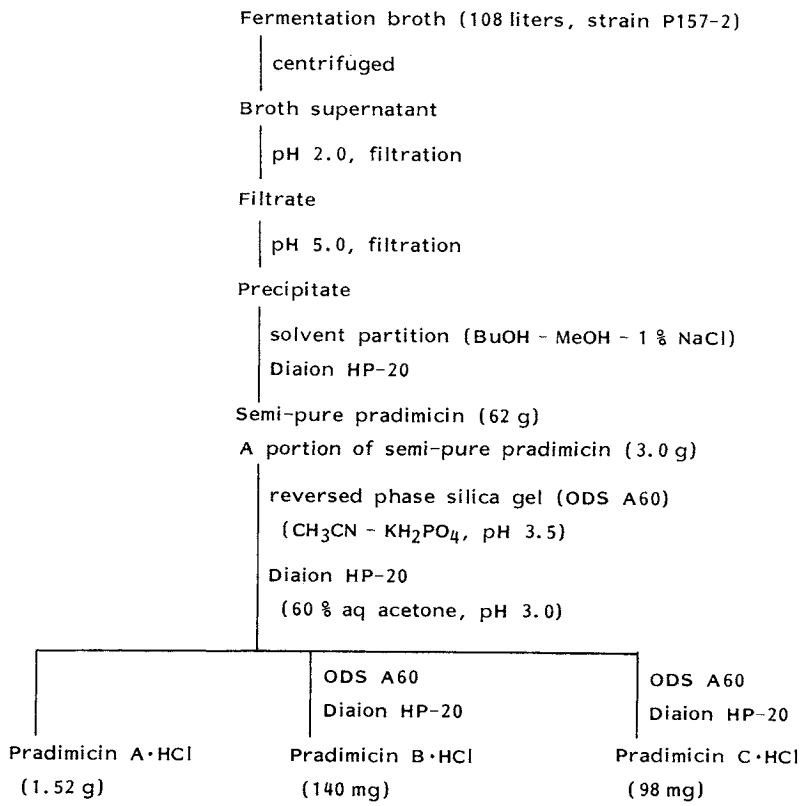
[a] Silica gel 60 (Merck, 5715), MeOAc-1-PrOH-28% NH_4OH (45:105:60).

[b] Column: Microsorb Short One C_{18} (4.6 mm i.d. × 100 mm, Rainin Instrument), mobile phase: CH_3CN-0.15% KH_2PO_4 adjusted to pH 3.5 with H_3PO_4 (7:17), flow rate: 1.2 ml/minute, detection: UV absorption at 254 nm.

Table 4. Production ratio of pradimicins A, B and C.

Producing strain	Potency (μg/ml)	Production ratio (%)		
		Pradimicin A	Pradimicin B	Pradimicin C
Actinomadura hibisca P157-2	820	86.5	6.6	6.9.
A. hibisca Q278-4	780	56.6	6.9	36.5

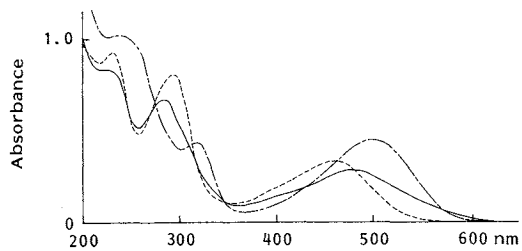
Fig. 4. Isolation and purification of pradimicin components.



antibiotic potency reached a maximum of 650 μg/ml on the 5th day. The fermentation was also carried out in a 200-liter tank fermenter. Three liters of the seed culture were used to inoculate 120 liters of production medium consisting of glucose 3%, Protein S (Ajinomoto Co.) 3% and $CaCO_3$ 0.3%. The tank fermenter was operated at 28°C with agitation at 250 rpm and an aeration rate of 120 liters/minute. The pH of the cultured broth gradually rose with the progress of fermentation and reached 7.9 after 96 hours. The antibiotic potency was 820 μg/ml.

Fig. 5. UV and visible spectrum of pradimicin A (25 μg/ml).

—— in 50% MeOH, ------ in 0.01 N HCl-50% MeOH, —·— in 0.01 N NaOH-50% MeOH.



Strain P157-2 produced pradimicin A as a major component and a small amount of pradimicins B and C in the broth; strain Q278-4 produced pradimicins A and C as major components with a small amount of pradimicin B.

The pradimicin components in the broth were analyzed by HPLC. The HPLC mobility of each component is shown in Table 3. An example of production ratio of pradimicins A, B and C in the tank fermentation using strains P157-2 and Q278-4 is shown in Table 4.

Fig. 6. IR spectrum of pradimicin A (KBr).

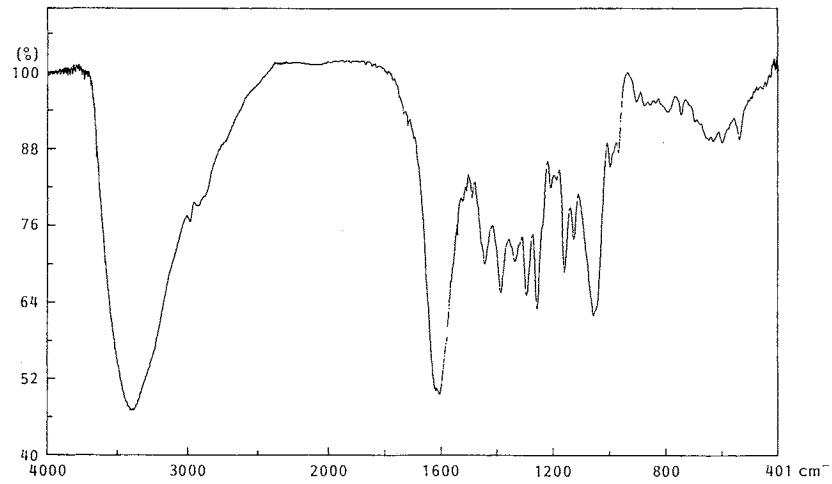
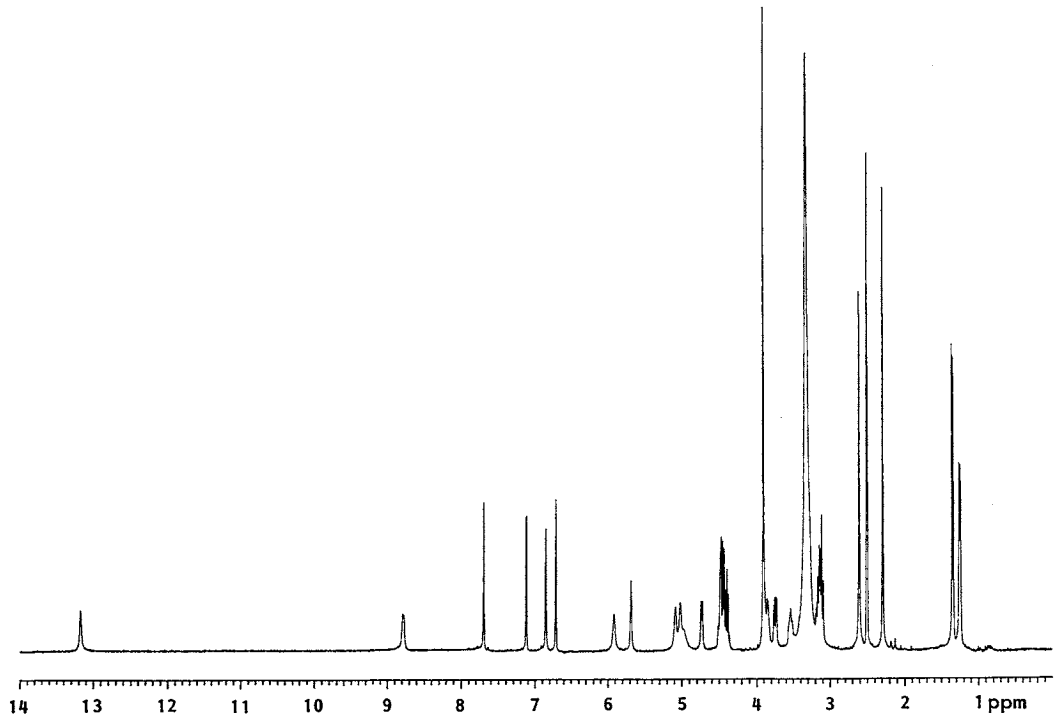


Fig. 7. ^{1}H NMR spectrum of pradimicin A (400 MHz, in DMSO-d_6).

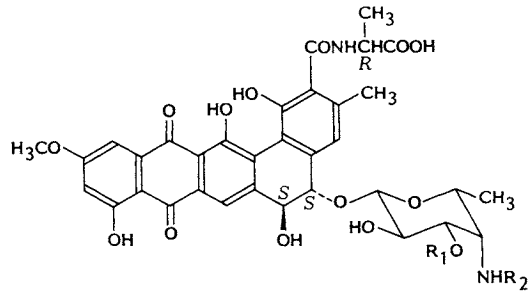


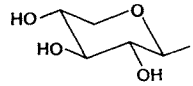
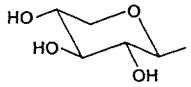
Isolation and Purification

The isolation procedure for the pradimicin components is summarized in Fig. 4 and the details are described in the previous paper[5].

Crystallization of the purified pradimicin A·HCl from MeOAc-1-PrOH-0.1 N NaOH gave fine needles of the monosodium salt of the antibiotic. When the salt was dissolved in water and adjusted to pH 5.0 by 0.1 N HCl, a precipitate of the zwitterionic form of pradimicin A deposited. Aqueous solution of pradimicin B·HCl and pradimicin C·HCl were similarly treated with 0.1 N NaOH to obtain the

Fig. 8. Structures of pradimicins A, B and C.



	R_1	R_2
Pradimicin A		CH_3
Pradimicin B	H	CH_3
Pradimicin C		H

zwitterionic forms.

Physico-chemical Properties

Zwitterionic pradimicins A, B and C exhibited the physico-chemical properties as summarized in Table 3. They are soluble in *N*,*N*-dimethylformamide, dimethyl sulfoxide and acidic and alkaline water, slightly soluble in water, methanol, ethanol and 1-butanol but insoluble in common organic solvents. The UV and visible spectrum and IR spectrum of pradimicin A are shown in Figs. 5 and 6, respectively. The CD spectrum of pradimicin A ($\lambda_{extreme}^{0.01\,N\,HCl}$ nm ($\Delta\varepsilon$) 213 (+1.9), 244 (−17.7), 289 (+15.3), 335 (−6.1), 515 (+6.6)) corresponded well with that of its aglycone reported before[4,5]. Fig. 7 shows the ^1H NMR spectrum of pradimicin A. As has been reported in a separate paper[4,5], the structures of pradimicins A, B and C were determined to have a benzo[*a*]naphthacenequinone nucleus substituted with D-alanine and sugars (Fig. 8).

Discussion

Novel antifungal antibiotics pradimicins A, B and C were discovered as the metabolites of a new species of the genus *Actinomadura*, later named *Actinomadura hibisca* sp. nov. Pradimicins A, B and C are red amorphous powders having characteristic UV absorption. The structural studies have revealed that they share the same unique aglycone, a dihydro benzo[*a*]naphthacenequinone nucleus substituted with D-alanine, and differ from each other in the type and number of sugar substituents (Fig. 8). As discussed in the following paper, pradimicins A, B and C demonstrated moderate *in vitro* activity against various clinically important fungi and yeasts and impressive *in vivo* activity against those fungal infections.

Recently, a complex of closely related antibiotics, benanomicin, was reported[13~15]. Benanomicin B is considered to be identical with pradimicin C from the reported data.

Acknowledgments

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