BIOSYNTHESIS OF THE PRADIMICIN FAMILY OF ANTIBIOTICS

II. FERMENTATION, ISOLATION AND STRUCTURE DETERMINATION OF METABOLITES ASSOCIATED WITH THE PRADIMICINS BIOSYNTHESIS

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Ten metabolites produced by 4 mutants derived from *Actinomadura verrucosospora* subsp. *neohibisca* E-40, a high pradimicins producer, were isolated and their structures were determined. Strain JN-219 produced 3 novel analogs of the pradimicin A aglycone, *i.e.* 11-*O*-demethyl-7-methoxypradinone II and 11-*O*-demethylpradinones I and II together with a known aglycone analog, pradinone I, while the metabolites from strain JN-47 were determined to be 2 new aglycone analogs, 11-*O*-demethylpradimicinone I and 11-*O*-demethyl-7-methoxypradimicinone II and a known aglycone analog, 11-*O*-demethylpradimicinone II (11dM-PMN II). Products of strain JN-207 were identified as 11-*O*-demethyl-6-deoxypradinone I and 11dM-PMN II. Interestingly, a new pradimicin analog, 7-hydroxypradimicin A was isolated from strain JN-58 together with a new aglycone analog, pradimicinone II and 11dM-PMN II. None of these metabolites showed antifungal activity.

In order to elucidate the subunit assembly of pradimicin biosynthesis in *Actinomadura verrucosospora* subsp. *neohibisca* E-40, we have generated 37 mutants blocked in the pradimicins production and selected 6 strains of classes IV~VI and VII~IX as biosynthetic intermediates of and shunt metabolites of pradimicin-producing mutants, respectively[1]. This paper presents fermentation, isolation and structural studies of 8 new metabolites produced by blocked mutant strains JN-219 (class IV), JN-47 (class V), JN-58 (class VIII) and JN-207 (class IX). Metabolites accumulated by strains JNU-46 (class VI) and JN-59 (class VII) were previously described[1]. Structures of 8 novel metabolites and known related compounds are shown in Fig. 1.

Materials and Methods

Strain

Strains JN-47, JN-58, JN-207 and JN-219 used in this study are blocked mutants derived from *A. verrucosospora* subsp. *neohibisca* E-40, a pradimicin A high-producing strain[1]. They were propagated on yeast-starch agar composed of yeast extract (Difco Laboratories Inc.) 0.2%, soluble starch 1% and agar 1.8% under incubation at 28°C for 2 weeks. For preparation of the vegetative inoculum and the production of metabolites, the FR-18 medium was used, refer[1].

Fermentation

Mature spores of each slant culture were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the FR-18 medium and cultivated at 32°C for 6 days on a rotary shaker. Then, the resulting vegetative inoculum was transferred to 100 ml of the FR-18 medium prepared in 500-ml Erlenmeyer flasks and fermented at 28°C for 10 days on a rotary shaker. Products were analyzed by TLC and HPLC[1].

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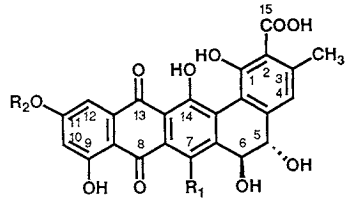
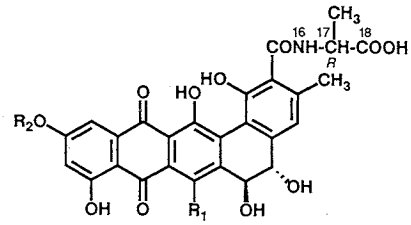
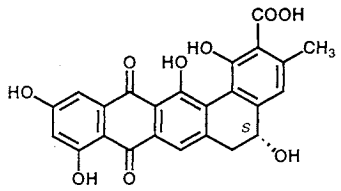
Fig. 1. Structures of new compounds **1**~**8** and related compounds **9**~**12**.



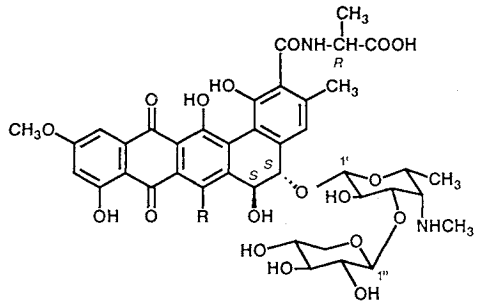
		R_1	R_2
11dM-PN II	**1**	OH	H
11dM-7M-PN II	**2**	OCH_3	H
11dM-PN I	**3**	H	H
PN I	**9**	H	CH_3



		R_1	R_2
11dM-PMN I	**4**	H	H
11dM-7M-PMN II	**5**	OCH_3	H
PMN II	**6**	OH	CH_3
PMN I	**10**	H	CH_3
11dM-PMN II	**11**	OH	H



11dM-6dO-PN I **7**



		R
7OH-PRM A	**8**	OH
PRM A	**12**	H

PN I (**9**), PMN I (**10**) and 11dM-PMN II (**11**) were reported as dealanylpradimicinone (AG-3)[2,3], pradimicinone (AG-2)[2,3] and pradimicin P[3], respectively.

General

UV and IR spectra were recorded on a Shimadzu UV-260 spectrophotometer and an Analect JIR-fx6160 spectrophotometer, respectively. FAB-MS spectra were measured on a JEOL JMS-AX505H with 5 keV beam of energetic xenon atoms and 3 kV as an acceleration voltage using *meta*-nitrobenzyl alcohol as a matrix. MP's were determined with a Yanaco MP-3S micro melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX 400 spectrometer with DMSO-d_6 as an internal reference. The CD spectra were measured in methanol on a JASCO J-600 spectrometer.

Results

Fermentation

The strain JN-219 produced 4 analogs of pradimicin A aglycone, named 11-*O*-demethylpradinones I and II (11dM-PN I, **3** and 11dM-PN II, **1**), 11-*O*-demethyl-7-methoxypradinone II (11dM-7M-PN II, **2**) and pradinone I (PN I, **9**), the strain JN-47 produced 3 aglycone analogs, designated 11-*O*-demethyl-

pradimicinones I and II (11dM-PMN I, **4** and known 11dM-PMN II, **11**) and 11-*O*-demethyl-7-methoxypradimicinone II (11dM-7M-PMN II, **5**), the strain JN-207 produced 11-*O*-demethyl-6-deoxy-pradinone I (11dM-6dO-PN I, **7**) and 11dM-PMN II (**11**), and the strain JN-58 produced two aglycones, named pradimicinone II (PMN II, **6**) and 11dM-PMN II (**11**) and a pradimicin A analog, 7-hydroxypradimicin A (7OH-PRM A, **8**). Among 10 metabolites described above, compounds **1**~**7** are novel analogs of the pradimicin A aglycone (pradimicinone I, **10**) and compound **8** is a new analog of pradimicin A (**12**). Fifty 500-ml Erlenmeyer flasks each containing 100 ml of the FR-18 medium were used to produce 4~5 liters of individual broths for isolation of these metabolites. The progress of fermentations was monitored by checking the visible adsorption[3] and HPLC analysis[1]. The metabolites production in these 4 strains reached maximum at day 10 fermentation, and their titers are summarized in Table 1.

Table 1. Production of metabolites by strains JN-219, JN-47, JN-58 and JN-207.

Metabolite	Titer (μg/ml)			
	JN-219	JN-47	JN-58	JN-207
11dM-PN II	2,270			
11dM-7M-PN II	70			
11dM-PN I	358			
11dM-6dO-PN I				802
PN I	68			
11dM-PMN I		466		
11dM-PMN II		1,024	109	225
11dM-7M-PMN II		235		
PMN II			1,153	
7OH-PRM A			165	

Isolation

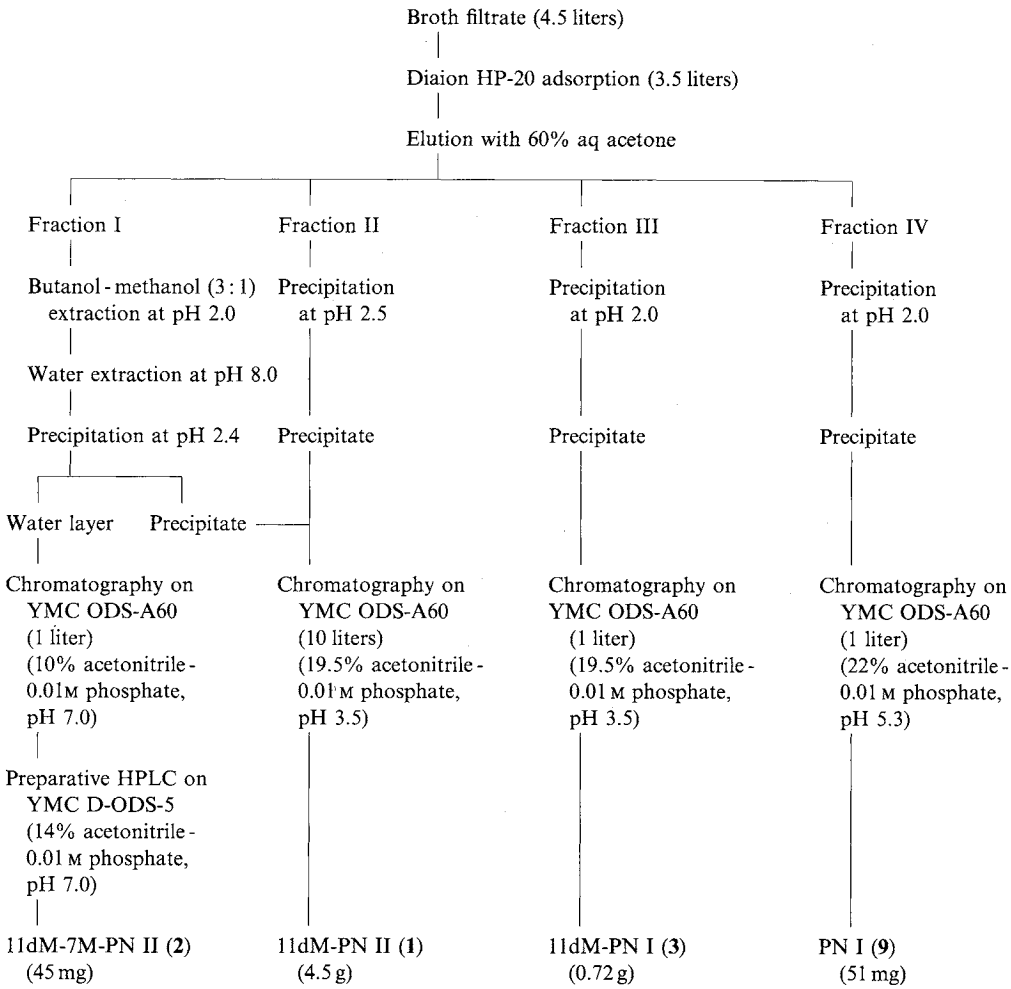
Metabolites accumulated by 4 mutants, strains JN-219, JN-47, JN-58 and JN-207, were easily isolated from the cultured broths with Diaion HP-20 and purified by solvent extraction and/or acidic precipitation, followed by a reverse phase silica gel column chromatography (YMC ODS-A60, Yamamura Chemical Lab. Ltd.). Fig. 2 summarized a typical example for the isolation of compounds **1**, **2**, **3** and **9** produced by the strain JN-219. The fermentation broth was centrifuged at 5,000 rpm for 10 minutes. The products in the supernatant (4.5 liters) were adsorbed on 3.5 liters of Diaion HP-20, washed with water (5 liters) and eluted with 60% aq acetone. The eluted fractions (10 ml each) were monitored by silica gel TLC as described previously[1], and the 4 fractions containing each desired products were pooled and concentrated *in vacuo*. Fraction I (400 ml) was extracted with a mixture of butanol (300 ml) and methanol (100 ml) at pH 2. The solvent layer was re-extracted with alkaline water (100 ml) at pH 8. This water layer was acidified to pH 2.4 with 6 N HCl and centrifuged to remove compound **1**. The supernatant thus obtained was neutralized, concentrated to dryness and then applied to a YMC ODS-A60 column chromatography, followed by preparative HPLC to yield compound **2** (45 mg). The acid precipitates of both fractions I and II were combined and purified by an ODS column chromatography to give compound **1** (4.5 g). The acid precipitates of fractions III and IV were rechromatographed on an ODS column to afford compound **3** (720 mg) and compound **9** (51 mg), respectively.

By a similar procedure, compounds **4** (592 mg), **5** (302 mg) and **11** (1.29 g) were isolated from the broth (4.5 liters) of strain JN-47. Compounds **6** (750 mg), **8** (227 mg) and **11** (151 mg) were obtained from the strain JN-58 (4 liters). Compounds **11** (1.53 g) and **7** (324 mg) were isolated from the strain JN-207 (4.5 liters). Among them, compounds **9** and **11** were identified as dealanylpradimicinone (AG-3)[2,3] and pradimicin P[3], respectively, based on the direct comparison.

Physico-chemical Properties

The physico-chemical properties of compounds **1**~**8** are summarized in Table 2. They are orange-red,

Fig. 2. Isolation procedure of **1**, **2**, **3** and **9** produced by strain JN-219.



amorphous powders, which are readily soluble in dimethyl sulfoxide and *N*,*N*-dimethylformamide and slightly soluble in methanol, ethanol, and alkaline water, but insoluble in other organic solvents and acidic water. The molecular formulae of these compounds were established as shown in Table 2 by positive- or negative-ion HRFAB-MS. The UV and visible adsorption spectra of these compounds under acidic and alkaline conditions are consistent with the chromophore of the pradimicin family of antibiotics, indicating that they have a polyhydroxy-5,6-dihydrobenzo[*a*]naphthacenequinone skeleton. This is supported by the IR absorption band at around $1599 \sim 1618\,cm^{-1}$ due to the stretching of the quinonecarbonyl hydrogen-bonded with the *peri*-hydroxyl[4]. The presence of an amido carbonyl group at around $1660\,cm^{-1}$ in the IR spectra of **4**, **5**, **6** and **8**, and the detection of a characteristic fragment ion $(M-88)^+$ due to the cleavage of alanine in the FAB-MS indicate that these compounds have an alanine moiety bonded to the chromophore. Alanine in **4**, **5**, **6** and **8** was determined to have the *R*-configuration based on the HPLC mobility of their hydrolysis products by a chiral column method as previously reported[2].

Structure

The structures of the compounds **1**~**8** were determined by a combination of two dimensional ^1H-^1H

Table 2. Physico-chemical properties of **1**~**8**.

	11dM-PN II (**1**)	11dM-7M-PN II (**2**)	11dM-PN I (**3**)	11dM-PMN I (**4**)
MP (°C, dec.)	191~215	224~230	220~230	208~220
Molecular formula	$C_{24}H_{16}O_{11}$	$C_{25}H_{18}O_{11}$	$C_{24}H_{16}O_{10}$	$C_{27}H_{21}NO_{11}$
HRFAB-MS obs.	481.0770	493.0771*	463.0663*	536.1184
m/z $(M+H)^+$ calc.	481.0771	493.0771*	463.0665*	536.1193
UV λ_{max} nm (ε)				
in H_2O-MeOH (1:9)	239 (32,000), 285 (24,000), 521 (17,600)	241 (26,300), 285 (15,700), 489 (9,800)	239 (24,500), 285 (16,000), 473 (7,600)	232 (31,800), 293 (28,000), 461 (10,400)
in 0.1 N HCl-MeOH (1:9)	239 (29,600), 287 (23,000), 520 (16,700)	240 (25,800), 287 (17,400), 480 (10,400)	238 (25,100), 290 (18,400), 462 (8,400)	234 (30,700), 302 (29,000), 461 (10,400)
in 0.1 N NaOH-MeOH (1:9)	245 (24,300), 304 (21,800), 339 (8,300), 559 (19,700)	243 (21,700), 306 (15,600), 518 (13,200)	248 (20,900), 305 (15,400), 506 (11,500)	248 (33,000), 307 (21,900), 320 (21,900), 501 (16,900)
IR ν_{max} (KBr) cm^{-1}	3467, 1716, 1603	3565, 1715, 1613	3397, 1734, 1716, 1611	3290, 1728, 1664, 1607

	11dM-7M-PMN II (**5**)	PMN II (**6**)	11dM-6dO-PN I (**7**)	7OH-PRM A (**8**)
MP (°C, dec.)	210	180~185	248~260	205~218
Molecular formula	$C_{28}H_{23}NO_{12}$	$C_{28}H_{23}NO_{12}$	$C_{24}H_{16}O_9$	$C_{40}H_{44}N_2O_{19}$
HRFAB-MS obs.	565.1208*	556.1336	449.0886	857.2634
m/z($M+H)^+$ calc.	565.1220*	566.1298	449.0873	857.2617
UV λ_{max} nm (ε)				
in H_2O-MeOH (1:9)	238 (26,000), 289 (20,600), 480 (10,600)	235 (30,800), 282 (25,800), 514 (16,400)	236 (27,500), 300 (21,200), 465 (11,700)	235 (32,400), 280 (26,200), 520 (16,300)
in 0.1 N HCl-MeOH (1:9)	238 (25,000), 289 (19,600), 477 (10,600)	237 (30,600), 285 (26,000), 510 (17,100)	238 (29,300), 300 (24,100), 460 (12,200)	237 (36,000), 286 (28,500), 512 (20,300)
in 0.1 N NaOH-MeOH (1:9)	236 (28,200), 306 (18,700), 330 (14,800), 513 (15,400)	226 (32,400), 302 (13,600), 330 (8,800), 563 (18,500)	241 (26,700), 298 (14,900), 330 (23,100), 514 (10,200)	228 (41,000), 320 (11,600), 567 (22,400)
IR ν_{max} (KBr) cm^{-1}	3382, 1734, 1664, 1617	3374, 1725, 1660, 1599	3467, 1701, 1618	3397, 1720, 1666, 1601

* The data by negative ion HRFAB-MS: $(M-H)^-$ for compounds **2** and **3**, $(M)^-$ for compound **5**.

COSY, NOESY and ^{13}C-^1H COSY NMR experiments and comparison with the data of published pradimicin compounds[2,3]. The ^1H and ^{13}C NMR data of these metabolites are summarized in Tables 3, 4 and 5.

Structure of 11dM-PN II (1)

An excellent structural homology between **1** and known 11dM-PMN II (**11**)[3] is immediately apparent from a comparison of UV, MS and NMR spectroscopic data. In the FAB-MS spectra of **1**, the strong fragment ions m/z 463 $(M-OH)^+$ and 445 $(463-H_2O)^+$ together with the pseudomolecular ion m/z 481 $(M+1)^+$ are observed. The same fragment ions are also observed in case of **11**, which suggests **1** is a dealanine derivative of **11**. The NOE correlation peaks are displayed between the singlet signal (4-H, 6.84 ppm) in the aromatic region and methine proton 5-H and 3-methyl protons in the NOESY spectrum

Table 3. ^{1}H NMR data for pradinones and pradimicinones (400 MHz, in DMSO-d_6).

Position	11dM-PN II (1)	11dM-7M-PN II (2)	11dM-PN I (3)	11dM-PMN I (4)
3-CH_3	2.34 (s)	2.45 (s)	2.55 (s)	2.33 (s)
4-H	6.84 (s)	6.70 (s)	6.95 (s)	7.06 (s)
5-H	4.53 (d, J=3.0)	4.43 (d, J=3.0)	4.20 (d, J=10.7)	4.22 (d, J=10.7)
6-H	5.10 (d, J=3.0)	5.01 (d, J=3.0)	4.29 (d, J=10.7)	4.23 (d, J=10.7)
7-H	—	—	8.05 (s)	8.08 (s)
7-OMe	—	3.86 (s)	—	—
10-H	6.67 (d, J=2.1)	6.60 (d, J=2.1)	6.59 (d, J=2.1)	6.65 (d, J=2.6)
11-OMe	—	—	—	—
12-H	7.29 (d, J=2.1)	7.12 (d, J=2.1)	7.15 (d, J=2.1)	7.22 (d, J=2.6)
16-NH	—	—	—	8.56 (d, J=7.3)
17-H	—	—	—	4.40 (qui, J=7.3)
17-Me	—	—	—	1.33 (d, J=7.3)

Position	11dM-7M-PMN II (5)	PMN II (6)	11dM-6dO-PN I (7)
3-CH_3	2.31 (s)	2.29 (s)	2.57 (s)
4-H	6.76 (s)	6.68 (s)	6.97 (s)
5-H	4.43 (d, J=3.2)	4.43 (d, J=3.0)	4.57 (dd, J=4.7, 9.8)
6-H	5.02 (d, J=3.2)	5.04 (d, J=3.0)	2.75 (dd, J=9.8, 15.8), 3.11 (dd, J=4.7, 15.8)
7-H	—	—	8.80 (s)
7-OMe	3.86 (s)	—	—
10-H	6.64 (d, J=2.4)	6.88 (d, J=2.4)	6.61 (d, J=2.1)
11-OMe	—	3.95 (s)	—
12-H	7.18 (d, J=2.4)	7.30 (d, J=2.4)	7.22 (d, J=2.1)
16-NH	8.64 (d, J=7.5)	8.65 (d, J=6.8)	—
17-H	4.39 (qui, J=7.5)	4.39 (dq, J=6.8, 7.3)	—
17-Me	1.36 (d, J=7.5)	1.35 (d, J=7.3)	—

Chemical shifts are expressed by δ (ppm), multiplicity and coupling constants (Hz) are in parentheses.

of **1**. The structure of **1** was further confirmed by the ^{13}C-^{1}H COSY and long range COSY experiments. As the ^{13}C-^{1}H long range correlation map shown in Fig. 3, all ^{13}C chemical shifts of **1** are unambiguously assigned. A particularly close correspondence between **1** and **11** is seen in their chemical shifts through C-4 to C-14b skeleton. This indicates that **1** and **11** share the identical benzo[*a*]naphthacenequinone core. From these facts, the structure of **1** is established as dealanyl-11-*O*-demethylpradimicinone II and named 11-*O*-demethylpradinone II (11dM-PN II).

Absolute stereochemistry at C-5 and C-6 is elucidated as follows. For the quaternary carbon assignment, without decoupling (QUATN) for **1**, each of the doublet signals for C-7 and C-14a reveal heteronuclear coupling constants $^3J_{C,H\text{-}6}$=5.0 Hz, while the C-14b is split into a doublet of doublets having $^3J_{C,H}$=4.4 Hz (C-14b–5-H) and 6.2 Hz (C-14b–4-H). Meanwhile, a pair of coupled signals at 4.53 and 5.10 ppm reveals the presence of a CH(OH)CH(OH) moiety located in the 5,6-position in **1**. The observed coupling constants of 3.0 Hz suggest the dihedral angle between 5-H and 6-H to be approximately 56°[5]. Both results indicate the dihydrodiol is in a *trans*-diaxial orientation. This result is also supported by the CD spectrum of **1** [$\lambda^{MeOH}_{extreme}$ nm ($\Delta\varepsilon$) 207 (−10.4), 243 (11.6), 290 (−5.7)], which shows the same sign as that of **11** [$\lambda^{MeOH}_{extreme}$ nm ($\Delta\varepsilon$) 211 (−11.4), 245 (26.5), 285 (−6.5)] and the opposite sign to those of known compounds **9** and **10** having (5*S*,6*S*)-diequatorial diol orientation ($J_{5,6}$=~10 Hz)[2]. From these facts, **1** is found to have a (5*S*,6*S*)-diaxial diol unit.

Table 4. ^{13}C NMR data for pradinones and pradimicinones (100 MHz, in DMSO-d_6).

Position	11dM-PN II (1)	11dM-7M-PN II (2)	11dM-PN I (3)	PMN II (6)	11-dM-6dO-PN I (7)
C-1	155.8 (s)	155.9 (s)	157.9 (s)	154.7 (s)	160.5 (s)
C-2	120.0 (s)	120.1 (s)	115.3 (s)	127.3 (s)	112.9 (s)
C-3	141.9 (s)	141.6 (s)	143.3 (s)	137.0 (s)	142.8 (s)
3-Me	20.7 (q)	20.7 (q)	21.9 (q)	18.9 (q)	23.3 (q)
C-4	123.6 (d)	123.6 (d)	118.6 (d)	122.5 (d)	119.3 (d)
C-4a	139.3 (s)	143.5 (s)	140.4 (s)	139.8 (s)	139.4 (s)
C-5	70.8 (d)	70.9 (d)	72.1 (d)	71.1 (d)	69.6 (d)
C-6	62.9 (d)	63.8 (d)	71.5 (d)	62.9 (d)	29.0 (t)
C-6a	137.6 (s)	138.9 (s)	148.2 (s)	137.3 (s)	146.6 (s)
C-7	155.1 (s)	153.1 (s)	117.3 (d)	155.4 (s)	119.3 (d)
7-OMe	—	62.3 (q)	—	—	—
C-7a	110.8 (s)	121.7 (s)	131.5 (s)	113.2 (s)	129.4 (s)
C-8	187.7 (s)	185.0 (s)	185.3 (s)	187.7 (s)	189.3 (s)
C-8a	109.2 (s)	110.1 (s)	109.2 (s)	110.1 (s)	109.0 (s)
C-9	165.4 (s)	164.5 (s)	164.4 (s)	163.7 (s)	165.4 (s)
C-10	108.1 (d)	107.2 (d)	107.3 (d)	105.4 (d)	107.5 (d)
C-11	164.3 (s)	164.4 (s)	164.4 (s)	165.4 (s)	164.3 (s)
11-OMe	—	—	—	56.0 (q)	—
C-12	108.3 (d)	108.5 (d)	108.0 (d)	106.2 (d)	108.6 (d)
C-12a	134.9 (s)	134.0 (s)	135.5 (s)	135.6 (s)	135.2 (s)
C-13	186.0 (s)	187.7 (s)	180.5 (s)	181.8 (s)	181.1 (s)
C-13a	112.2 (s)	115.2 (s)	116.5 (s)	111.3 (s)	113.3 (s)
C-14	154.8 (s)	155.6 (s)	158.3 (s)	154.7 (s)	158.2 (s)
C-14a	132.3 (s)	130.3 (s)	133.2 (s)	134.7 (s)	130.1 (s)
C-14b	115.6 (s)	115.9 (s)	116.5 (s)	117.0 (s)	116.5 (s)
C-15	170.9 (s)	170.9 (s)	169.9 (s)	167.5 (s)	173.2 (s)
C-17	—	—	—	47.5 (d)	—
17-Me	—	—	—	17.0 (q)	—
C-18	—	—	—	173.7 (s)	—

Chemical shifts are expressed by δ (ppm), multiplicity is in parentheses.

Structures of 11dM-7M-PN II (2), 11dM-7M-PMN II (5) and PMN II (6)

The protonated molecular ions of **2** (*m/z* 495) and **5** (*m/z* 566) in the FAB-MS spectra are 14 mass unit higher than those of **1** and **11**, respectively. When the ^1H NMR spectra of **2** and **5** are compared to those of **1** and **11**, most protons are assigned quite similar positions, except for an additional methoxy signal, found in both **2** and **5** at 3.86 ppm. Moreover, their NOESY spectra exhibit a NOE correlation peak between the methoxy and methine proton 6-H. Thus, the compounds **2** and **5** are the 7-methoxy analogs of **1** and **11** and called 11-*O*-demethyl-7-methoxypradinone II (11dM-7M-PN II) and 11-*O*-7-methoxypradimicinone II (11dM-7M-PMN II), respectively.

Compound **6** is closely similar to **11** in UV and IR spectra. The MS of **6** displays 14 mass units more than **11** and one additional methoxy group (3.95 ppm) is observed in ^1H NMR, which shows a NOE to two aromatic protons 10-H and 12-H. Hence, the structure of **6** is assigned as 11-methoxy analog of **11** and named pradimicinone II (PMN II). The NMR data ($J_{5,6} = \sim 3.0$ Hz) as shown in Table 3 indicate that compounds **2**, **5** and **6** have 5,6-*trans*-diaxial diol.

Structures of 11dM-PN I (3) and 11dM-PMN I (4)

The FAB-MS spectra of compounds **3** and **4** show protonated ions at *m/z* 465 and 536, respectively, together with the common dehydrated fragment ions at *m/z* 447 and 429, suggesting that they correspond

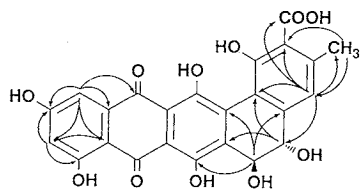
Table 5. ^{1}H and ^{13}C NMR data in DMSO-d_6 for **8**.

Position	δ_C	δ_H	Position	δ_C	δ_H
1	158.9 (s)		14	162.6 (s)	
2	127.6 (s)		14a	136.7 (s)	
3	137.2 (s)		14b	119.4 (s)	
3-Me	19.7 (q)	2.26 (s)	15	168.2 (s)	
4	122.7 (d)	6.59 (s)	16-NH	—	8.67 (d, J=7.3)
4a	132.8 (s)		17	47.8 (d)	4.40 (quint, J=7.3)
5	80.2 (d)	4.50 (d, J=3.0)	17-Me	17.4 (q)	1.35 (d, J=7.3)
6	60.8 (d)	5.19 (d, J=3.0)	18	174.3 (s)	
6a	140.8 (s)		1′	103.1 (d)	4.57 (d, J=7.6)
7	153.9 (s)		2′	69.3 (d)	3.12 (dd, J=7.6, 9.7)
7a	112.1 (s)		3′	80.4 (d)	3.79 (dd, J=3.9, 9.7)
8	188.2 (s)		4′	63.1 (d)	3.23 (bd, J=3.9)
8a	110.3 (s)		4′-NMe	36.2 (q)	2.50 (s)
9	163.5 (s)		5′	67.2 (d)	3.82 (q, J=6.6)
10	104.3 (d)	6.72 (d, J=2.4)	6′	16.2 (q)	1.20 (d, J=6.6)
11	165.8 (s)		1″	105.2 (d)	4.36 (d, J=7.7)
11-OMe	56.0 (q)	3.92 (s)	2″	73.5 (d)	3.12 (dd, J=7.7, 8.8)
12	105.5 (d)	7.19 (d, J=2.4)	3″	75.8 (d)	3.11 (dd, J=8.8, 9.2)
12a	138.5 (s)		4″	69.1 (d)	3.22~3.29 (m)
13	178.2 (s)		5″	65.9 (t)	3.08 (t, J=11.2),
13a	114.6 (s)				3.71 (dd, J=5.4, 11.2)

Multiplicity and coupling constants (Hz) are in parentheses.

to compounds lacking one hydroxy group (16 mass units) from **1** and **11**, respectively. The ^{1}H NMR spectra of **3** and **4** reveal new singlet aromatic protons at 8.05 and 8.08 ppm in addition to those of **1** and **11**, respectively. These new signals indicate each NOE to benzylic proton 6-H, suggesting that the structures of **3** and **4** are 7-deoxy analogs of **1** and **11**, respectively. The values of vicinal coupling constants between 5-H and 6-H ($^{3}J_{5,6}$=10.7 Hz) in both ^{1}H NMR spectra of **3** and **4**, however, are larger than 3.0~3.4 Hz in **1** and **11**. Moreover, the magnitudes of $^{3}J_{C,H}$ between C-14a and 6-H and between C-14b and 5-H in compound **3** are very small (0.5 Hz) compared with those of compound **1**. Therefore, the conformational relation at C-5 and C-6 of both **3** and **4** is assigned as a *trans*-diequatorial diol. In fact, the CD spectra of **3** displays $\lambda_{extreme}^{MeOH}$ nm ($\Delta\varepsilon$) 209 (20.3), 230 (−28.9), 270 (2.0), the same sign as for the known compounds (5*S*,6*S*)-**9** and **10**[2,3]. From these facts, the structures of **3** and **4** are determined to be 11-*O*-demethylpradinone I (11dM-PN I) and 11-*O*-demethylpradimicinone I (11dM-PMN I), respectively.

Fig. 3. The summary of ^{13}C-^{1}H correlation map of **1**.



Structure of 11dM-6dO-PN I (**7**)

The FAB-MS spectra of compound **7** reveal a protonated ion at *m/z* 449 with the characteristic fragment ions at *m/z* 431 and 413, which show 16 mass units lower than the corresponding ions of **3**. If the ^{1}H NMR spectrum of **7** is compared with compound **3**, three proton signals, a methine at 4.57 ppm and methylene protons at 2.75 and 3.11 ppm (corresponding to a methine at 69.6 ppm and methylene carbon at 29.0 ppm, respectively, by the ^{13}C-^{1}H COSY experiment) appear as each a doublet of doublets (AMX spin system) whereas these are two methine signals for 5-H and 6-H in **3**. NOEs in **7** are observed between the methine proton (4.57 ppm) and 4-H, and one of the methylene protons (3.11 ppm) and 7-H.

This indicates that a methine proton is assignable to 5-H and methylene protons at C-6. The vicinal coupling constant values between the methine at C-5 position and methylene protons at C-6, are 4.7 Hz and 9.8 Hz, indicating that the hydroxy group at C-5 of **7** is in the equatorial orientation. From these results, the structure of **7** is determined to be 6-deoxy analog of **3** and named 11-*O*-demethyl-6-deoxypradinone I (11dM-6dO-PN I).

Structure of 7OH-PRM A (**8**)

In the FAB-MS spectra of **8**, the pseudomolecular ions at *m/z* 857 $(M+H)^+$ and 879 $(M+Na)^+$ appear together with the characteristic fragment ions at *m/z* 768 resulting from a loss of alanine moiety, *m/z* 725 $(M-xylose)^+$, 548 (carbonium ion of **6**), *m/z* 477, 459 and 443 due to the aglycone of **6** and oxonium ions of sugar parts at *m/z* 292 (disaccharide) and 160 (*N*-methylthomosamine). These data suggest that **8** is composed of the aglycone **6** and xylosyl-*N*-methylthomosamine, the same sugar moiety as in pradimicin A (**12**). The structure is further confirmed by comparing the acid hydrolysis products and NMR data of **8** and **12**. Upon acid hydrolysis, **8** afforded aglycone **6**, xylose and ninhydrin positive substances, and these degradation products except for **6** are identical with the hydrolysates of **12** on TLC. The NMR data for compound **8** assigned by double quantum filter COSY, relayed COSY, heteronuclear COSY and COLOC experiments, are summarized in Table 5. Except for 5-H, 6-H ($J=3.0$ Hz) and disappearance of H-7, these data for **8** closely correspond to those of **12**, particularly in the sugar moiety. In the rotating frame Nuclear Overhauser Effect (ROESY) and NOESY spectra of **8**, NOE correlation peaks display between 1′-H–3′-H, 1′-H–5′-H, 4′-H–4′-NMe and 4-H′–6′-H in the thomosamine part and among 1″-H–3″-H–axial 5″-H (3.08 ppm) in the xylose part. Moreover, NOE's peaks between H-5 and the anomeric proton of *N*-methylthomosamine (1′-H) and between 3′-H and the anomeric proton of xylopyranose (1″-H) are observed in their spectra of **8**. The magnitudes of coupling constants of two anomeric protons are 7.6 Hz for 1′-H and 7.7 Hz for 1″-H, indicating that the sugar moiety has a β-pyranoside linkage of xylose to 3′-OH and β-linkage of C-1′ to 5-OH.

From the results mentioned above, the structure of **8** is composed of an aglycone **6** attached to the same disaccharide as in PRM A (**12**), *i.e.*, 7-hydroxypradimicin A (7OH-PRM A). However, the geometry between C-5 and C-6 of **8** is found to be *trans*- diaxial diol based on the coupling constants ($^3J_{H\text{-}5,H\text{-}6}=3.0$ Hz, $^3J_{C\text{-}14a,H\text{-}6}=5.9$ Hz, $^3J_{C\text{-}14b,H\text{-}5}=4.4$ Hz). Furthermore, the CD curve of **8** [$\lambda^{MeOH}_{extreme}$ nm ($\Delta\varepsilon$) 212 (−21.6), 238 (34.5), 282 (−6.0)] shows opposite sign to that of **12**. Therefore, the stereochemistry at C-5 and C-6 of **8** are established to be (5*S*,6*S*) with different conformation from that of the pradimicin A (**12**).

Discussion

The results of this study demonstrate that the 4 strains derived from *A. verrucosospora* subsp. *neohibisca* E-40 are mutants with modified biosynthesis of pradimicins and producing 5,6-dihydrobenzo[*a*]naphthacenquinone chromophores with different substitution patterns. It is noted that: compounds **1**, **6** and **11** possess a hydroxy group at C-7 position; compounds **2** and **5** possess a methoxyl group at C-7 position; and compounds **3**, **4** and **9** lack a hydroxyl group at C-7 position. It is important to note that these compounds produced by 4 mutants have all (5*S*,6*S*) configuration, and compounds **1**, **2**, **5**, **6**, **11** and 7-hydroxypradimicin A (**8**) have different conformation at C-5- and C-6-positions from that observed for compounds **3**, **4**, **7** and **9** as well as **10** and pradimicin A (**12**).

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