BIOSYNTHESIS OF THE PRADIMICIN FAMILY OF ANTIBIOTICS

III. BIOSYNTHETIC PATHWAY OF BOTH PRADIMICINS AND BENANOMICINS

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The biosynthetic pathway of the pradimicin-benanomicin family of antibiotics was investigated by using sinefungin and blocked mutants derived from *Actinomadura verrucosospora* subsp. *neohibisca* E-40 (a high pradimicin producer) or *Actinomadura* sp. AB1236 (a benanomicin producer). Addition of sinefungin to strain E-40, pradimicin A aglycone-producing mutant or strain AB1236 inhibited the formation of 11-*O*-demethyl-7-methoxypradinone II (11dM-7M-PN II), resulting in the accumulation of 11-*O*-demethylpradimicinone II and pradimicinone II. By feeding pradimicin A aglycone and its analogs to mutants blocked early in pradimicin or benanomicin biosynthesis, the following results were obtained: 11-*O*-demethylpradinone II, 11dM-7M-PN II, 11-*O*-demethylpradinone I, 11-*O*-demethylpradimicinone I and pradimicinone I were converted to pradimicin A or benanomicin A; the remaining 6 aglycone analogs were not incorporated into the antibiotics. Pradimicin B, dexylosylpradimicin C and dexylosylbenanomicin A were converted to pradimicin A, pradimicin C and benanomicin A, respectively. A biosynthetic pathway for the antibiotics is proposed.

The pradimicins and benanomicins are new antifungal antibiotics of dihydrobenzo[*a*]naphthacenequinone family produced by strains belonging to the single genus *Actinomadura*[1~5]. Both antibiotics have a dihydrobenzo[*a*]naphthacenequinone chromophore with a methoxyl group at C-11. Biosynthetic studies of benanomicins revealed that this methoxyl group was introduced from a methionine-derived methyl group[6]. Sinefungin, an analog of *S*-adenosyl methionine (SAM) is a potent and specific inhibitor of *O*-methyltransferase and other methyltransferase[7~11]. Consequently, addition of sinefungin to the above 2 strains and 6 mutants which accumulate analogs of pradimicin A aglycone is worth examining for understanding of these antibiotics biosynthesis. In the mean time, we have reported[12,13] that some of 11-*O*-demethylpradinone II (11dM-PN II), 11-*O*-demethyl-7-methoxypradinone II (11dM-7M-PN II), 11-*O*-demethylpradinone I (11dM-PN I), pradinone I (PN I), 11-*O*-demethylpradimicinone I (11dM-PMN I) and pradimicinone I (PMN I) accumulated by 3 mutants of classes IV, V and VI would be true biosynthetic intermediates of pradimicins, whereas 11-*O*-demethyl-6-deoxypradinone I (11dM-6dO-PN I), 11-*O*-demethylpradimicinone II (11dM-PMN II), pradimicinone II (PMN II) and 7-methoxypradimicinone II (7M-PMN II) might be shunt metabolites associated with the pradimicins biosynthesis. To prove this, we investigated the bioconversion of these compounds using growing cultures of both pradimicin- and benanomicin-nonproducing mutants (blocked mutants). From the above information, we propose the subunit assembly in the biosynthetic pathway for both pradimicins and benanomicins as summarized in Fig. 1.

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Materials and Methods

Bacterial Strains

Actinomadura verrucosospora subsp. *neohibisca* E-40 and its blocked mutants used in this report have been described in preceding papers[12,14]. *Actinomadura* sp. AB1236 which produces benanomicins has been described[15].

Media

TP medium consisting of glucose 3%, defatted soybean meal (Esusan mi-to, Ajinomoto Co., Ltd.) 3% and $CaCO_3$ 0.3% was used for bioconversion experiments. Seed medium (BS) was prepared with soluble starch 1% and defatted soybean meal 3%. Production medium (BP) was prepared with glucose 2%, Pharmamedia (Traders Protein) 1% and KH_2PO_4 0.1%. The BS and BP media were used for the production of benanomicins.

Isolation of Converter Strain from Strain AB1236

Mutagenesis of the strain AB1236 was carried out as previously described[12]. Colonies which produce no diffusible pigment were fermented in the BP medium to confirm a lack of the production of benanomicins. Benanomicin-nonproducing mutants thus obtained were inoculated as a patch on a GBS agar medium[12] supplemented with 11dM-PN II (250 γ/ml) and incubated at 30°C for 7 days. Mutant strain B-6 which formed red pigment around the colony was selected as a converter strain.

Fermentation

A 5% inoculum of strain E-40 or its mutants grown in V-15 medium was transferred to 100 ml of FR-18 medium supplemented with different concentration of sinefungin (Calbiochem Corporation) in a 500-ml flask and was incubated at 28°C for 7 days. 5% seed of strain AB1236 grown in the BS medium was transferred to 100 ml BP medium supplemented with sinefungin in a 500-ml flask and incubated at 28°C for 7 days. Metabolites in the broth supernatant were determined by TLC and HPLC[12] and quantitated by absorbance at 460 nm[12].

Bioconversion

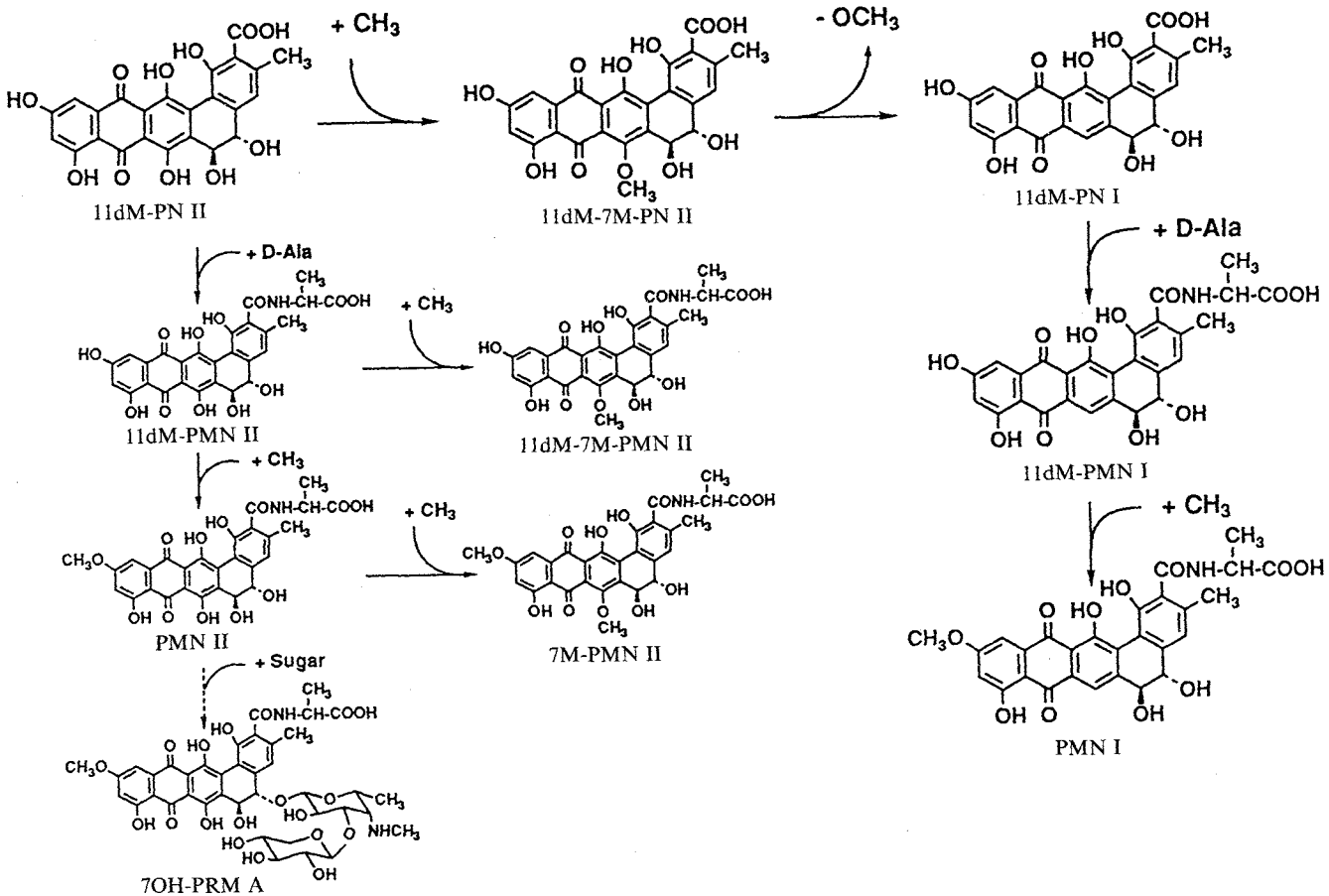
5% inoculum of strain JN-213 (or strain B-6) grown in V-15 medium (or BS medium) was transferred to 100 ml of TP medium (or BP medium) in a 500-ml flask and incubated at 28°C for 48 hours. Substrate was added to 4 ml of a 48-hour growing culture prepared and incubated at 28°C and 200 rpm on a shaker. Products in the supernatant were analysed by TLC and HPLC[12].

Results

Effect of Sinefungin on the Production of Pradimicins and Benanomicins

Normally, *A. verrucosospora* subsp. *neohibisca* E-40 and *A.* sp. AB1236 produce pradimicins A and C (PRM A and C) and benanomicins (BNM A and B; BNM B is identical to PRM C), respectively, as major components together with several minor components. As both antibiotics have a methoxyl group at C-11 on the aglycone moiety, the effect of sinefungin was investigated on the production of both antibiotics by strains E-40 and AB1236. As shown in Table 1, addition of sinefungin (0.0228 mM) inhibits the formation of pradimicins and causes the accumulation of two aglycone analogs, 11dM-PMN II and PMN II. 11dM-PMN II and PMN II were isolated and identified by direct comparison. In the case of benanomicins (Table 2), sinefungin (0.114 mM) results in a concomitant accumulation of 11dM-PMN II and PMN II together with two new components which are thought to be the 7-hydroxyl analogs of BNM A and PRM C (7OH-BNM A and 7OH-PRM C). We reported earlier that 10 analogs of pradimicin A aglycone including three 7-methoxyl compounds (11dM-7M-PN II, 11dM-7M-PMN II and 7M-PMN

Fig. 1. *Biosynthetic pathway of pradimicin A and benanomicin A.*



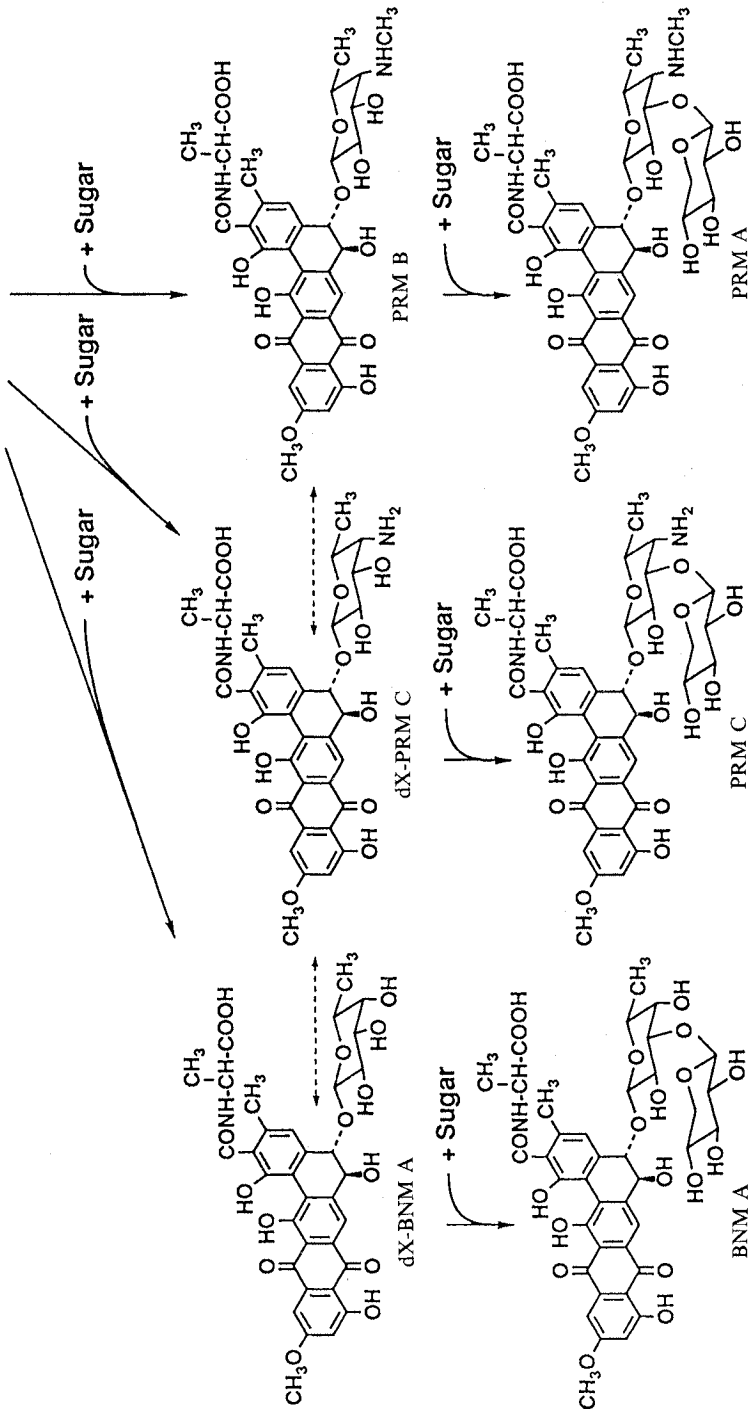

+ Sugar
+ Sugar
+ Sugar
+ Sugar
+ Sugar
+ Sugar
dX-BNM A
dX-PRM C
PRM B
BNM A
PRM C
PRM A

Table 1. Effect of sinefungin on the production of pradimicins by strain E-40.

Sinefungin (mM)	Metabolite (μg/ml)				
	11dM-PMN II	PMN II	PMN I	PRM C	PRM A
0	74	23	53	89	1,690
0.00228	79	257	0	42	297
0.0057	124	267	0	37	235
0.0114	166	384	0	31	166
0.0228	273	279	0	17	31

Medium (FR-18): Glucose 3%, Pharmamedia 3% and $CaCO_3$ 0.3%.
Fermentation: 28°C, 7 days.

Table 2. Effect of sinefungin on the production of benanomicins by strain AB1236.

Sinefungin (mM)	Metabolite (μg/ml)					
	11dM-PMN II	PMN II	7OH-PRM C	7OH-BNM A	PRM C	BNM A
0	0	0	0	0	394	918
0.0228	0	0	0	0	506	108
0.057	0	0	0	58	387	88
0.114	6	389	223	594	92	64
0.228	117	410	183	567	0	0

Medium (BP): Glucose 2%, Pharmamedia 1% and KH_2PO_4 0.1%.
Fermentation: 28°C, 7 days.

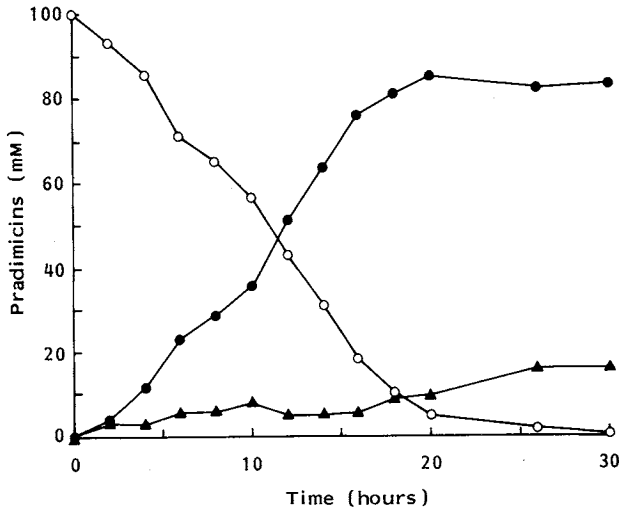
Table 3. Effect of sinefungin on the production of aglycones by 4 mutant strains.

Strain	Sine-fungin (mM)	Metabolites (μg/ml)								
		11dM-PN II	11dM-7M-PN II	11dM-PN I	PN I	11dM-PMN I	PMN I	11dM-PMN II	11dM-7M-PMN II	PMN II	7M-PMN II
JN-219	0	2,090	60	163	80						
	0.0057	3,389	0	0	0						
	0.0228	3,434	0	0	0						
JN-47	0	16				363		917	249		
	0.0057	10				95		2,051	35		
	0.0228	28				45		2,001	0		
JNU-46	0						1,800	21		0	
	0.0057						276	174		324	
	0.0228						91	300		431	
JN-59	0	0						232		173	1,106
	0.0057	0						339		411	46
	0.0228	0						369		400	0

Medium (FR-18): Glucose 3%, Pharmamedia 3% and $CaCO_3$ 0.3%.
Fermentation: 28°C, 7 days.

II) were produced by 6 mutant strains[12,13]. Therefore, we also examined the effect of sinefungin on the formation of these compounds by strains JN-219, JN-47, JNU-46, JN-58, JN-59 and JN-207. Expectedly, sinefungin (0.00228 mM) completely blocks the formation of 7-methoxyl compounds (11dM-7M-PN II, 11dM-7M-PMN II and 7M-PMN II), 11-methoxyl compounds (PN I and PMN I except for PMN II), 11dM-PN I and 11dM-PMN I and causes the accumulation of 11dM-PMN II and PMN II (Table 3). The formation of 11dM-6dO-PN I and 7OH-PRM A produced by strains JN-58 and JN-207 was not

Fig. 2. Bioconversion of 11dM-PN II to PRM by blocked mutant JN-213.
11dM-PN II (○), PRM A (●), PRM B (▲).



affected by sinefungin (data not shown). It also appeared that sinefungin specifically inhibits the conversion of 11dM-PN II, 11dM-PMN II and PMN II to 11dM-7M-PN II, 11dM-7M-PMN II and 7M-PMN II, respectively, catalyzed by 7-*O*-methyltransferase, resulting in the accumulation of 11dM-PMN II and PMN II as shunt products. The information with sinefungin indicates that the inhibition of 11dM-7M-PN II formation causes a coupled disappearance of 11dM-PN I, PN I, 11dM-PMN I and PMN I and PN I thus 11dM-PMN I and PMN I may be biosynthesized from 11dM-PN II *via* 11dM-7M-PN II and 11dM-PN I. This would suggest that *O*-methylation at the C-7 position followed by demethoxylation is required for the biosynthetic process from 11dM-PN II to 11dM-PN I.

Bioconversion by Pradimicin- and Benanomicin-nonproducing Mutants

From the results with sinefungin and cosynthesis, it appeared that one or more of the accumulated compound *i.e.* 11dM-PN II, 11dM-7M-PN II, 11dM-PN I, PN I, 11dM-PMN I and PMN I would be true biosynthetic intermediates of pradimicin biosynthesis. Subsequently, they were fed to converter strains. Preliminary experiments showed that these aglycone analogs, except for PN I, were slowly incorporated into pradimicins, though the yields of bioconverted products were quite variable depending on the culture age of the mutants used. For example, when exogenously added 11dM-PN II was incubated with the strain JN-213, PRM A and B appeared within 4 hours and disappearance of the substrate occurred after 24 hours (Fig. 2). In contrast, attempts to convert 11dM-PMN II, 11dM-7M-PMN II, PMN II, 7M-PMN II and 11dM-6dO-PN I to PRM A failed (data not shown). Concerning the biosynthesis of PN I and 11dM-6dO-PN I, PN I may be biosynthesized from 11dM-PN I as a shunt metabolite and the biosynthetic pathway to 11dM-6dO-PN I remains to be resolved.

Bioconversion of 11dM-PN II, 11dM-7M-PN II, 11dM-PN I, 11dM-PMN I and PMN I

Table 4 presents typical results which demonstrate the bioconversion of possible biosynthetic intermediates by the converter strain JN-213. After 17 hours, 11dM-PN II is converted to PRM B (11.2%)

Table 4. Bioconversion of 11dM-PN II, 11dM-7M-PN II, 11dM-PN I, 11dM-PMN I and PMN I by growing cultures of blocked mutant JN-213.

Added substrate	Substrates and products (mol%)									RS	BP
	11dM-PN II	11dM-7M-PN II	11dM-PN I	11dM-PMN I	PMN I	dX-PRM C	PRM C	PRM B	PRM A		
11dM-PN II	17.5	—	t	t	t	—	—	11.2	71.2	17.5	99.9
11dM-7M-PN II	—	61.0	t	6.4	t	—	—	7.3	25.1	61.0	99.5
11dM-PN I	—	—	10.0	14.2	—	—	—	14.4	61.4	10.0	100.0
11dM-PMN I	—	—	—	90.2	—	—	—	t	9.7	90.2	95.9
PMN I	—	—	—	9.2	83.3	—	—	3.4	3.7	83.3	97.6

—: Not detected.
t: Traces.
RS: Recovery of substrate.
BP: Yield of bioconverted products (mol%: Total bioconverted products/consumed substrate $\times 100$).
Culture medium (TP): Glucose 3%, defatted soybean meal 3% and $CaCO_3$ 0.3%.
Method: Each substrate (0.5~0.8 μmol) was added to 4 ml of 48-hour growing cultures of a converter strain. After incubation for additional 17 hours at 28°C, products were determined by HPLC and TLC.

Table 5. Effect of sinefungin on bioconversion of 11dM-PN II, 11dM-7M-PN II and 11dM-PN I by strain JN-213.

Substrate	Sinefungin (mM)	Time (hours)	Substrates and products (mol%)							RS*	BP*
			11dM-PN II	11dM-7M-PN II	11dM-PN I	11dM-PMN I	PRM A	11dM-PMN II	PMN II		
11dM-PN II	—	0	100	—	—	—	—	—	—	100	—
	—	17	2.8	—	—	—	97.0	—	—	2.8	99.8
	0.0005	17	11.5	—	—	—	71.7	18.2	—	11.5	101.6
	0.0009	17	39.4	—	—	—	22.8	34.4	—	39.4	94.4
	0.0018	17	41.3	—	—	—	—	38.4	16.6	41.3	93.7
	0.0036	17	55.6	—	—	—	—	21.7	24.0	55.6	102.9
11dM-7M-PN II	—	0	—	100	—	—	—	—	—	100	—
	—	17	—	56.0	—	—	44.0	—	—	56.0	100
	0.0018	17	—	72.1	—	—	28.0	—	—	72.1	100
11dM-PN I	—	0	—	—	100	—	—	—	—	100	—
	—	17	—	—	0	38.4	58.9	—	—	0	97.3
	0.0018	17	—	—	26.8	36.7	36.3	—	—	26.8	99.7

and PRM A (71.2%) as well as trace amounts of 11dM-PN I, 11dM-PMN I and PMN I. 11dM-7M-PN II is similarly converted to 11dM-PMN I (6.4%), PRM B (7.3%) and PRM A (25.1%). 11dM-PN I is also converted to 11dM-PMN I (14.2%) and PRM B (14.4%) and PRM A (61.4%). 11dM-PMN I is converted to PRM A (9.7%), and PMN I is converted to PRM B (3.4%), PRM A (3.7%) and 11dM-PMN I (9.2%), among which the accumulation of 11dM-PMN I may be caused by degradation.

These results suggest that pradimicins are biosynthesized from 11dM-PN II *via* 11dM-7M-PM II, 11dM-PN I, 11dM-PMN I and PMN I.

To clarify this, the effect of sinefungin on the bioconversion of 11dM-PN II, 11dM-7M-PN II and 11dM-PN I by strain JN-213 was also investigated. As shown in Table 5, during the conversion of exogenously added 11dM-PN II to PRM A, sinefungin (0.0018 mM) completely inhibits the formation of PRM A and the accumulation of 11dM-PMN II (38.4%) and PMN II (16.6%) appears within 17 hours

Table 6. Bioconversion of 11dM-PN II, 11dM-7M-PN II, 11dM-PN I, 11dM-PMN I and PMN I by growing cultures of blocked mutant B-6.

Added substrate	Substrates and products (mol%)									RS	BP
	11dM-PN II	11dM-7M-PN II	11dM-PN I	11dM-PMN I	PMN I	dX-PRM C	dX-BNM A	PRM C	BNM A		
11dM-PN II	8.0	—	—	—	—	—	—	91.8		8.0	99.8
11dM-7M-PN II	—	0	—	—	—	—	2.1	90.0	7.8	0	99.9
11dM-PN I	—	—	0	—	—	—	2.4	90.5	5.0	0	97.9
11dM-PMN I	—	—	—	0	—	—	2.2	91.0	4.6	0	97.8
PMN I	—	—	—	—	0	—	7.7	44.2	48.0	0	99.9

cf. Footnote of Table 4, except below.

Culture medium (BP): Glucose 2%, Pharmamedia 1% and KH_2PO_4 0.1%.

Method: The substrate (0.5~0.8 μmol) was added to 4 ml of 48-hour growing cultures of strain B-6. After incubation for additional 72 hours at 28°C, products were determined by HPLC and TLC.

after addition of the substrate. Interestingly, in the presence of sinefungin (0.0018 mM), 11dM-7M-PN II is converted into PRM A (28%), and 11dM-PN I is incorporated into PRM A (36.3%) *via* 11dM-PMN I (36.7%). PMN I was also incorporated into PRM A (data not shown).

Since the aglycone of benanomicins is identical with PMN I[4], it is assumed that benanomicins would also be biosynthesized from 11dM-PN II *via* 11dM-7M-PN II, 11dM-PN I, 11dM-PMN I and PMN I. These 5 compounds were examined to determine whether or not they were incorporated into benanomicins by strain B-6. Strain B-6 is a mutant blocked in benanomicin production and acts as a converter strain (see Materials and Methods). As shown in Table 6, the antibiotics produced are benanomicins. The result is quite identical to those observed on the formation of pradimicins. From the information obtained above, a biosynthetic sequence, namely 11dM-PN II→11dM-7M-PN II→11dM-PN I→11dM-PMN I→PMN I→PRM A or BNM A, was determined. Since 11dM-PMN II, 11dM-7M-PMN II, PMN II and 7M-PMN II failed to be converted to PRM A or BNM A and 11dM-PN II is converted to 11dM-PMN II and PMN II in the presence of sinefungin, a shunt metabolic pathway is proposed as follows:

11dM-PN II→11dM-PMN II→PMN II→7OH-PRM A or 7OH-BNM A

↓ ↓

11dM-7M-PMN II 7M-PMN II

Bioconversion of the Aglycone Glycosides

Tables 7 and 8 present results on the bioconversion of dexylosyl analogs of both pradimicins and benanomicins by strains JN-213 and B-6, respectively. Strain JN-213 converts dexylosylpradimicin C (dX-PRM C) into PRM C (3.3%) and PRM A (28.9%), PRM B into PRM A (2.3%) and PRM C into PRM B (26.1%) and PRM A (27.5%). In case of strain B-6, PRM B is converted to BNM A (46.7%), whereas dX-PRM C and dexylosylbenanomicin A (dX-BNM A) are incorporated into PRM C (19.5% and 15.3%) and BNM A (40.4% and 51.7%), respectively (Table 8). These results indicate that PRM B, dX-PRM C and dX-BNM A are precursors for PRM A, PRM C and BNM A, respectively. In conclusion, the biosynthetic pathway of pradimicin A and benanomicin A is as described in Fig. 1.

Table 9 summarizes the possible blocked site of each mutant used and the metabolites accumulated. Mutants JN-213 and B-6 are unable to convert acetate into 11dM-PN II. Mutants JN-219, JN-47 and

Table 7. Bioconversion of PRM B and dX-PRM C by growing cultures of blocked mutant JN-213.

Added substrate	Substrates and products (mol%)				RS	BP
	dX-PRM C	PRM B	PRM C	PRM A		
dX-PRM C	63.6	—	3.3	28.9	63.6	95.5
PRM B	—	84.1	—	2.3	84.1	98.7
PRM C	—	26.1	46.4	27.5	46.4	100
PRM A	—	14.4	—	85.6	85.6	100

cf. Footnote of Table 4.

Table 8. Bioconversion of PRM B, dX-PRM C and dX-BNM A by growing cultures of blocked mutant B-6.

Added substrate	Substrates and products (mol%)						RS	BP
	PRM B	dX-PRM C	dX-BNM A	PRM C	PRM A	BNM A		
PRM B	43.4	2.0	8.6	—	—	46.7	43.4	101.2
dX-PRM C	—	30.1	7.8	19.5	—	40.4	30.1	96.9
dX-BNM A	—	—	33.0	15.3	—	51.7	33.0	100
PRM C	—	—	—	100	—	—	100	0
PRM A	—	—	—	—	100	—	100	0
BNM A	—	—	—	—	—	100	100	0

cf. Footnote of Table 6.

Table 9. Characteristics of mutant strains (JN series) of *A. verrucosospora* subsp. *neohibisca* E-40 and a mutant strain (B-6) of *A.* sp. AB1236.

Mutant	Possible site blocked	Metabolite
JN-213	Between acetate and 11dM-PN II	—
B-6	Between acetate and 11dM-PN II	—
JN-219	D-Alanylation at C-15 position on the aglycone	11dM-PN II, 11dM-7M-PN II, 11dM-PN I and PN I
JN-47	11-*O*-Methylation	11dM-PMN I, 11dM-PMN II and 11dM-7M-PMN II
JNU-46	5-*O*-Glycosidation	PMN I
JN-58	7-*O*-Methylation	11dM-PMN II, PMN II and 7OH-PRM A
JN-59	Between 11dM-7M-PN II and 11dM-PN I as well as 5-*O*-glycosidation	11dM-PMN II, 7M-PMN II and PMN II

JNU-46 are blocked on the pathway involving D-alanylation at the C-15 position on the aglycone, 11-*O*-methylation and 5-*O*-glycosidation, respectively. The blocked site of mutant JN-58 must be on the pathway which is associated with 7-*O*-methylation. The blocked site of mutant JN-59 seems to be located on the steps for 5-*O*-glycosidation and/or demethoxylation at the C-7 position.

Discussion

Pradimicins and benanomicins have a methoxyl group at C-11 on the aglycone moiety, which has been shown to be derived from the methyl of methionine[6]. The present findings with sinefungin suggest that *A. verrucosospora* subsp. *neohibisca* possesses two distinct methyltransferases, which catalyze methylation of the aglycone moiety at C-7 or C-11. 7-*O*-Methylation would occur prior to 11-*O*-methylation followed by 5-*O*-glycosidation in the biosynthesis of the antibiotics. These methoxyl groups arise *via* an

SAM-dependent methyltransferase. It is important to note than the *O*-methylation at C-7 position is crucial in the early steps of the biosynthetic pathway, since blocking the *O*-methylation resulted in the formation of shunt products.

Antibiotic-blocked mutants have provided essential information that has led to understanding of antibiotic biosynthesis. Thus, it is of interest to point out that in Step 1 (formation of 11dM-PN II), 11dM-PN II isolated from mutant strain JN-219 appears to be biosynthesized from a dodecaketide precursor[6,16] and is considered to be the initial intermediate. In Steps 2 and 3 (formation of 11dM-7M-PN II and 11dM-PN I), the pathway, represented by strain JN-219, is *O*-methylation at C-7 position followed by demethoxylation. 11dM-7M-PN II undergoes an unusual demethoxylation process leading to the formation of 11dM-PN I. The precise mechanism by which the methoxyl is lost and the conformation at C-5/C-6 is changed[13] remains to be resolved. In Step 4 (formation of 11dM-PMN I), the reaction, represented by strain JN-47, is D-alanylation at the C-15 position of 11dM-PN I. The D-alanine may be biosynthesized from L-alanine by alanine racemase[16]. In Step 5 (formation of PMN I), the reaction, represented by strain JNU-46, is 11-*O*-methylation of 11dM-PMN I. This methoxyl group may also be derived from the methyl of methionine. In Steps 6 and 7 (formation of dexylosyl analogs and pradimicins or benanomicins), these steps are 5-*O*-glycosidation of PMN I followed by C-3′-glycosidation of dexylosyl analogs of the antibiotics. The precise pathway of dexylosyl analog formation cannot be determined until the blocked mutants corresponding to each of them are isolated.

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