BIOSYNTHESIS OF THE PRADIMICIN FAMILY OF ANTIBIOTICS

I. GENERATION AND SELECTION OF PRADIMICIN-NONPRODUCING MUTANTS

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(Received for publication October 8, 1992)

Germinated spores of *Actinomadura verrucosospora* subsp. *neohibisca* E-40, a high pradimicins producer, were mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and/or UV treatment. Thirty-seven mutants which did not produce pradimicin were selected to test for cosynthesis ability, and classified into nine classes. On the basis of their cosynthesis ability and bioconversion results, we concluded that strain JN-213 (class III) was a true converter and that strains JN-219 (class IV), JN-47 (class V) and JNU-46 (class VI) were secretors accumulating biosynthetic intermediates of pradimicin, and that strains JN-59 (class VII), JN-58 (class VIII) and JN-207 (class IX) were producers of shunt metabolites of pradimicin biosynthesis. TLC and HPLC analyses of the fermentation broths of individual strains showed that 8 new compounds were produced along with pradinone I, pradimicinone I, 11-*O*-demethylpradimicinone II and 7-*O*-methylpradimicinone II.

In the study of microbial products, it is widely accepted that the generation and selection of a variety of mutant strains serve to characterize different steps involved in the biosynthetic pathway of antibiotics. This type of study might also lead to a discovery of biosynthetic intermediates which may be useful in elucidating the biosynthetic pathway of antibiotics[1]. In this context, such a study has not been reported for the dihydrobenzo[*a*]naphthacenequinone antibiotics pradimicin and benanomicin[2,3]. Biosynthetic studies of both antibiotics have established that their aglycones are of polyketide origin, apparently derived from a dodecaketide and an amino acid[4,5]. Consequently, we generated mutants of *Actinomadura verrucosospora* subsp. *neohibisca* E-40, blocked in pradimicin biosynthesis and selected biosynthetic intermediates- or shunt metabolite-producing mutants by a cosynthesis test, resulting in the characterization of 11 aglycones of pradimicin containing 7 new compounds and a new pradimicin A analog.

In this paper, we describe the generation of non-pradimicin-producing mutants and their accumulated metabolites. The structure determination of their metabolites and the biosynthetic subunit assembly of both pradimicin and benanomicin will be reported in the following papers[6,7].

Materials and Methods

Bacterial Strains

A. verrucosospora subsp. *neohibisca* E-40, which overproduces pradimicins A and C, was derived from the parental strain R103-3[8] by *N*-methyl-*N'*-nitro-*N*-nitrosoguanigine (NTG) treatment. *Candida albicans* A9540 was used for the bioassay of pradimicin.

Media

Yeast starch (YS) agar medium composed of yeast extract 0.2%, soluble starch 1% and agar 1.8%

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was used for the propagation of strain E-40 and its mutants. GBS agar medium consisting of glucose 1%, soluble starch 0.2%, Bacto soytone 1%, yeast extract 0.2%, malt extract 0.5% and agar 2% was used for cosynthesis experiments between pairs of mutants. Yeast nitrogen base (Difco) supplemented with glucose 1% and agar 1.5% was used for the bioassay of pradimicin. Seed medium (V-15) was prepared with glucose 1%, peptone 0.2%, NZ case (Humko Scheffield Chemical) 0.2%, meat extract 1.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.054% and $CaCl_2 \cdot 2H_2O$ 0.05% (pH was adjusted to 7.2 before autoclaving). Production medium (FR-18) was prepared with glucose 3%, Pharmamedia (Traders Protein, The Procter & Gamble Oilseed Products Company) 3% and $CaCO_3$ 0.3% (pH was adjusted to 8 before autoclaving).

Isolation of Mutants

The spores of strain E-40 grown on a YS agar medium for 20 days at 28°C were suspended in 10 ml of a solution containing Tween 80 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and NaCl 0.5%, sonicated, and then filtrated through cotton wool. The spores were centrifuged at 3,000 rpm for 10 minutes and resuspended in an aliquot of 20% of glycerol solution. The spores (5×10^8) were inoculated into a 500-ml flask containing 50 ml of the V-15 medium and shaken at 38°C and 100 rpm for 18.5 hours. The germinated spores were harvested, resuspended in 10 ml of the V-15 medium, treated with Polytron, sonicated, and then incubated at 38°C and 138 rpm. After 2.5 hours, the germinated spores thus obtained were spun down and resuspended in 20 ml of 0.01 M Tris-HCl buffer (pH 8.5). This suspension was diluted 2-fold with the same buffer containing 6 mg/ml of NTG and kept standing at 37°C for 30 minutes or exposed to UV light (10 W., distance: 30 cm) for 2 minutes. The mutagen-treated spores were spread onto the YS agar plates and incubated at 30°C for 7 days. Colonies which produced unusual diffusible pigments were selected and cultivated in the FR-18 medium to confirm a lack of pradimicin production.

Cosynthesis

Two mutants were streaked in a straight line, about 3~5 mm apart from each other on a GSB agar plate and incubated at 28°C for 1~2 weeks. The appearance of a diffusible red pigment on the side of only one of the mutants was considered a sign of pradimicin production. The agar layer which produced the diffusible red pigment was taken from the plate and then placed on the surface of an agar plate embedded with *C. albicans*. The plate was incubated overnight at 30°C, and the appearance of an inhibition zone associated with the diffusible red pigment was considered a sign of the production of pradimicin. A mutant which produced a diffusible red pigment was designated as a converter, whereas the other one was a secretor.

Bioconversion of Metabolites

The mutants JN-219, JN-47, JNU-46, JN-59 and JN-207 were fermented in 100 ml of the FR-18 medium for the preparation of their metabolites. After incubation at 28°C for 7 days on a rotary shaker (200 rpm), each of the mutants metabolic complexes were extracted by Diaion HP-20 adsorption, eluted with 60% aqueous acetone, evaporated to dryness and then dissolved in 10 ml of water. This solution (1 ml) was added to a 50-ml flask containing 5 ml of a 48 hour-old culture of each mutant, strains JN-51, JN-236, JN-213, JN-219, JN-47, JNU-46 or JN-59, in the FR-18 medium and incubated at 28°C for an additional 72 hours on a rotary shaker (200 rpm). Bioconverted products were analysed by TLC, HPLC and anti-*Candida* activity.

Analysis and Determination of Metabolites or Bioconverted Products

Samples of the broth (0.5 ml) were centrifuged at 10,000 rpm for 10 minutes, diluted with 50% DMSO if necessary, and filtered (Gelman Science Japan, Ltd., Ekicrodisc 13 CR, pore size: 0.45 μm). Metabolites or bioconverted products in the filtrate were determined by HPLC (Hitachi L-6000 high pressure system) on a YMC-ODS A-301-5 column (Yamamura Chemical Lab., 10 cm × 4.6 mm i.d.) at 35°C and by TLC on silica gel thin layer plates (Kieselgel $60F_{254}$ 0.25 mm; mfd, E. Merk). The mobile phase used for HPLC was a stepwise gradient of 0.01 M phosphate (pH 3.5) and acetonitrile; 23% of acetonitrile for the first 15 minutes, a linear gradient from 23 to 40% of acetonitrile for the next 12 minutes, and 40% of acetonitrile for the final 10 minutes. The flow-rate was 1.0 ml/minute. Metabolites were detected by UV absorption at 290 nm or 460 nm. TLC plate was developed with *n*-butanol - acetic acid - water (2 : 1 : 1).

Isolation of Metabolites Produced by Strains JNU-46 and JN-59

A 5% inoculum of strain JN-59 or JNU-46 growing in the V-15 medium was transferred to 100 ml of the FR-18 medium in a 500-ml flask and incubated at 28°C for 10 days on a rotary shaker (200 rpm). The cultured broth of strain JNU-46 was centrifuged at 3,000 rpm for 15 minutes. The supernatant (500 ml) was passed through a Diaion HP-20 column (300 ml), washed with 0.001 N HCl (1 liter), and the metabolite was eluted with 60% aqueous acetone (300 ml). The solvent was removed *in vacuo* and the aqueous layer (500 ml) was extracted with the same volume of ethyl acetate at pH 2. The solvent was re-extracted with water (300 ml) at pH 8 and then lyophilized to give the powder (616 mg). A part (50 mg) of the sample was dissolved in water (20 ml) at pH 7.2 and chromatographed on a column of YMC GEL ODS A-60 (500 ml) using water as eluent. The fraction (2.3 liters) containing pradimicinone I (PMN I) was desalted using a column of Diaion HP-20 and then lyophilized to give pure PMN I (43 mg). By a similar procedure, 7-methoxypradimicinone II (7M-PMN II, 198 mg), pradimicinone II (PMN II, 15 mg) and 11-*O*-demethylpradimicinone II (11dM-PMN II, 43 mg) were isolated from the fermented broth (500 ml) of strain JN-59. The three compounds, PMN I, 7M-PMN II and 11dM-PMN II were finally identified as pradimicinone (AG-2)[2,9], pradimicin N[9] and pradimicin P[10], respectively, by the direct comparison with these authentic samples. The structure of PMN II will be reported in the following paper[6].

Results

Generation of Mutants

Approximately 10,000 colonies were tested for the production of unusual diffusible pigments aimed at a quick visual selection of pradimicin-nonproducing mutants, as putative mutants were expected to produce diffusible pigments which differed from those characteristic of pradimicin (red.). Four hundred colonies were found to produce colorless or non-red pigments which differed from those of the parental strain. These colonies were fermented in the FR-18 medium, and a lack of the pradimicin production was confirmed by TLC and anti-*Candida* activity. As the result, 37 stable mutants blocked in pradimicin biosynthesis were selected as pradimicin-nonproducing strains (blocked mutants). They were subjected to the cosynthesis test.

Characterization of Mutants

As an example of the experimental result shown in Fig. 1, pradimcin cosynthesis is demonstrated when strains JN-213 and 219 are paired. A diffusible red pigment appears on the side of strain JN-213 only. An inhibition zone against *C. albicans* is also detected on the side of strain JN-213, which correspond to the diffusible red pigment (Fig. 2). The agar containing this diffusible red pigment was cut from the plate. The red pigment was eluted from the agar piece by frozen and thaw method[10] and identified as

Fig. 1. Cosynthesis between a pair of strains JN-213 and JN-219.

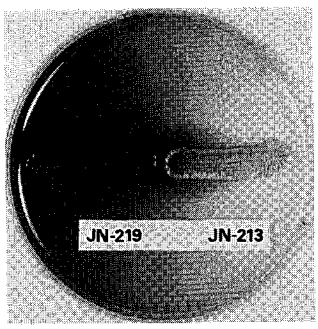


Fig. 2. Bioassay of cosynthesized product.

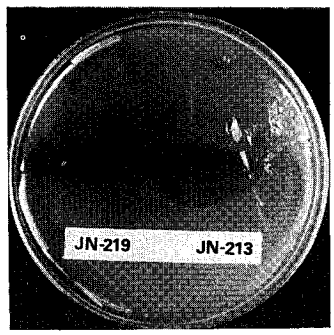


Table 1. Cosynthesis between pairs of mutants.

Converter	Secretor								
Class (color)[a]	I	II	III	IV	V	VI	VII	VIII	IX
I (Colorless)		+	+	+	+	+	+	+	+
II (Colorless)			+	+	+	+	+	+	+
III (Colorless)				+	+	+	−	−	−
IV (Purple)					−	−	−	−	−
V (Colorless to pale pink)						−	−	−	−
VI (Pink)							−	−	−
VII (Reddish purple)								−	−
VIII (Colorless)									−
IX (Pink)									

[a] Color : Color of diffusible pigment in glucose soytone agar.
+ : Cosynthesis was observed on the site of the secretor strain.
Class I : JN-51, JN-279
II : JN-236, JN-302
III : JN-213, JN-56, JN-214, JN-216, JN-217, JN-218, JN-227, JN-277, JN-280, JN-282, JN-283, JN-284, JN-287, JN-307, JN-314, JN-346, JN-349, JN-351
IV : JN-219, JN-204
V : JN-47
VI : JNU-46, JN-48, JN-49, JN-54, JN-106, JN-276
VII : JN-59, JN-43, JN-212
VIII : JN-58
IX : JN-207, JN-320

pradimicin A by TLC and HPLC analyses. In brief, strain JN-219 produced an intermediate(s) which was (were) converted into pradimicin A by strain JN-213, indicating that strains JN-213 and JN-219 are blocked at different biosynthetic steps, the former being blocked at an earlier step than the latter in the biosynthetic pathway. Thus, strain JN-213 is a "converter" and strain JN-219 is a "secretor". Thirty-seven blocked mutants were similarly analyzed and classified into nine classes as summarized in Table 1. A colorless class I mutant always act as a converter, and the other mutants of classes II and III act either as a converter or a secretor. Mutants of classes IV, V and VI act as secretors in their behavior against strains JN-51 (class I), JN-236 (class II) and JN-213 (class III).

Table 2. Bioconversion of metabolite complex produced by a secretor.

Converter	Metabolite complex produced by a secretor of					
Class (strain)	IV	V	VI	VII	VIII	IX
I (JN-51)	−	−	−	−	−	−
II (JN-236)	−	−	−	−	−	−
III (JN-213)	+	+	+	−	−	−
IV (JN-219)		−	−	−	−	−
V (JN-47)			−	−	−	−
VI (JNU-46)				−	−	−
VII (JN-59)					−	−
VIII (JN-58)						−
IX (JN-207)						

One-ml aliquot of metabolite complex produced by a secretor was added to 5 ml of 48 hour-growing cultures of a converter strain and incubated for additional 3 days. Products were analyzed by TLC and HPLC.
+: Production of pradimicin A was observed.

The remaining mutants of classes VII, VIII and IX show the same cosynthetic pattern, but we classify these mutants into separate classes because they produce different colors of diffusible pigments. To confirm this result, bioconversion by a converter strain using the 6 metabolite complexes from the mutants of classes IV, V, VI, VII, VIII and IX was carried out. The metabolites of strains JN-219, JN-47 and JNU-46 are bioconverted to pradimicin A by the strain JN-213 only (Table 2).

It is reasonable think that different classes of mutants might accumulate different types of biosynthetic

Table 3. Metabolites accumulated by 6 secretors.

Class	Secretor	Metabolites accumulated	TLC		HPLC[b] minute
			Rf[a]	Color	
IV	JN-219	11dM-7M-PN II	0.76	Purple	6.10
		11dM-PN II	0.75	Purple	7.55
		11dM-PN I	0.78	Red	18.11
		PN I	0.80	Red	30.03
V	JN-47	11dM-7M-PMN II	0.75	Purple	4.41
		11dM-PMN II	0.74	Purple	5.79
		11dM-PMN I	0.76	Red	11.77
VI	JNU-46	PMN I	0.75	Red	27.40
VII	JN-59	11dM-PMN II	0.74	Purple	5.79
		7M-PMN II	0.71	Red	20.04
		PMN II	0.72	Purple	21.48
VIII	JN-58	7OH-PRM A	0.36	Purple	3.11
		11dM-PMN II	0.74	Purple	5.79
		PMN II	0.72	Purple	21.48
IX	JN-207	11dM-PMN II	0.74	Purple	5.79
		11dM-6dO-PN I	0.82	Orange	31.57

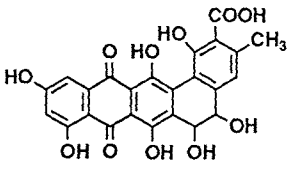
[a] Thin-layer chromatography, E. Merck silica gel $60F_{254}$, *n*-butanol-acetic acid-water (2:1:1).
[b] HPLC; Hitachi L-6000 high pressure system. Column; YMC ODS A-301-5 column (Yamamura Chemical Lab. Ltd., 10 cm × 4.6 mm i.d.) at 35°C. The mobile phase used was a stepwise gradient of 0.01 M phosphate (pH 3.5) and acetonitrile: 23% acetonitrile for the first 15 minutes, increasing concentrations of acetonitrile from 23 to 40% of acetonitrile for the next 12 minutes, and 40% of acetonitrile for the final 10 minutes. The flow-rate was 1.0 ml/minute. Metabolites were detected by UV absorption at 290 nm.

intermediate(s). Consequently, it is assumed that strain JN-213 (class III) is a true converter, and strains JN-219 (class IV), JN-47 (class V) and JNU-46 (class VI) which act as secretors only, would accumulate biosynthetic intermediates of pradimicin, whereas strains JN-59 (class VII), JN-58 (class VIII) and JN-207 (class IX) might produce shunt metabolites of pradimicin.

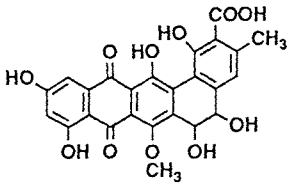
Metabolites Produced by Mutants

Mutants of classes IV, V, VI, VII, VIII and IX were expected to produce biosynthetic intermediates or shunt metabolites of pradimicin. Thus, their metabolites in cultured broths were analyzed by TLC and HPLC, and the results are summarized as follows: (1) Class IV mutant strains JN-219 and JN-204 produced 4 aglycone analogs of pradimicin (named 11-*O*-demethylpradinones I and II: 11dM-PN I and II, 11-*O*-demethyl-7-methoxypradinone II: 11dM-7M-PN II and pradinone I: PN I), (2) class V mutant strain JN-47 accumulated 3 aglycone analogs (11-*O*-demethylpradimicinones I and II: 11dM-PMN I and II, 11-*O*-demethyl-7-methoxypradimicinone II: 11dM-7M-PMN II), (3) class VI mutant strains JNU-46, JN-48, JN-49, JN-54, JN-106 and JN-276 produced an aglycone of pradimicin (pradimicinone I: PMN I), (4) class VII mutants JN-59, JN-43 and JN-212 produced 3 aglycone analogs (7-methoxypradimicinone II: 7M-PMN II, pradimicinone II: PMN II and 11dM-PMN II), (5) class VIII mutant JN-58 produced a new analog of pradimicin A (7-hydroxypradimicin A: 7OH-PRM A) and two aglycone analogs (11dM-PMN II and PMN II) and (6) class IX mutants JN-207 and JN-320 produced 2 aglycone analogs (11-*O*-demethyl-6-deoxypradinone I: 11dM-6dO-PN I and 11dM-PMN II). Among these aglycones, 11dM-PMN II, 7M-PMN II and PMN I were isolated from the culture broths of strains JN-59 and JNU-46 and identified as pradimicin P[9], pradimicin N[9] and pradimicinone (AG-2)[2,9], respectively. The

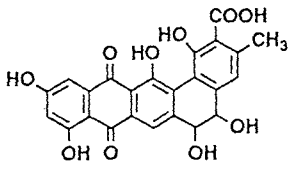
Fig. 3. Structures of metabolites isolated from the mutants.



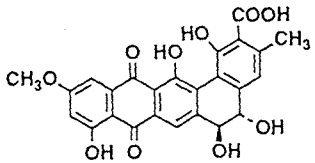
11-*O*-Demethylpradinone II
(11dM-PN II)



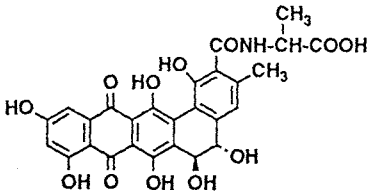
11-*O*-Demethyl-
7-methoxypradinone II
(11dM-7M-PN II)



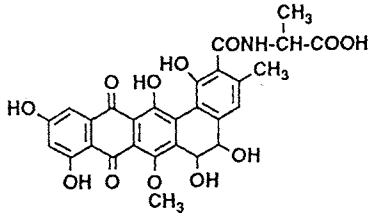
11-*O*-Demethylpradinone I
(11dM-PN I)



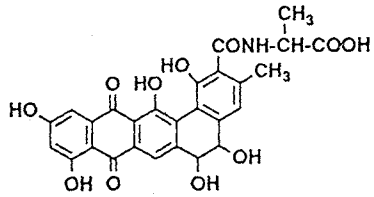
Pradinone I
(PN I)



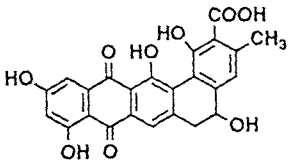
11-*O*-Demethylpradimicinone II
(11dM-PMN II)



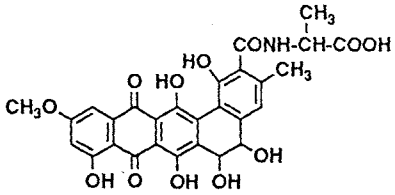
11-*O*-Demethyl-
7-methoxypradimicinone II
(11dM-7M-PMN II)



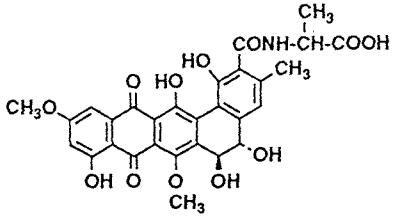
11-*O*-Demethylpradimicinone I
(11dM-PMN I)



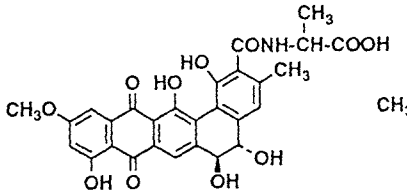
11-*O*-Demethyl-6-deoxy-
pradinone I
(11dM-6dO-PN I)



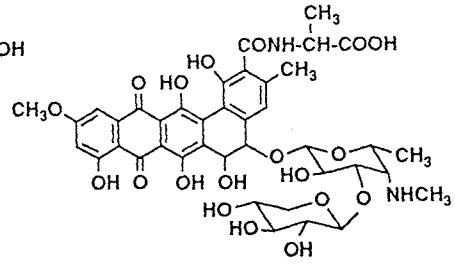
Pradimicinone II
(PMN II)



7-Methoxypradimicinone II
(7M-PMN II)



Pradimicinone I
(PMN I)



7-Hydroxypradimicin A
(7OH-PRM A)

remaining metabolites are new compounds[6]. Their Rf values, color on silica gel TLC, and retention times in HPLC are shown in Table 3.

Discussion

In general, the methods for obtaining antibiotic-blocked mutants in the antibiotic-producing actinomycetes are more inconvenient than those for selecting auxotrophs. For this purpose, time-consuming screens such as bacterial overlays or the transfer of colonies on agar plugs to assay plates seeded with a test bacterium have been used. Therefore, we first wanted to design the simplest screen for isolating mutants blocked in pradimicin biosynthesis. Since the characteristic color of a pradimicin producer is red due to the presence of pradimicin, mutants producing pradimicin intermediates would produce either unusual diffusible pigments which differed from the those characteristic of pradimicin, or no pigment at all, and they would be easily identified against a background of predominantly red pigmented colonies. In this context, about 10,000 progenies were collected from *A. verrucosospora* subsp. *neohibisca* E-40 by NTG and/or UV treatment. Expectedly, 37 strains among them are easily obtained as non-pradimicin-producing mutants, and the phenotype of these mutants appears to be stable.

Since DELIĆ[11] first demonstrated the application of "cosynthesis by an agar plate method" for selecting the mutants that produced antibiotic intermediates, the biosynthetic pathways of several antibiotics have been elucidated using this technique[9,12~14]. The present study performed with the cosynthesis test between pairs of 37 mutants and the bioconversion test using metabolite complexes produced by secretors show that strain JN-213 is a true converter, and that strains JN-219, JN-47 and JNU-46 accumulate biosynthetic intermediates of pradimicin and that strains JN-59, JN-58 and JN-207 produce shunt metabolites. Moreover, nine new metabolites and three known compounds (11dM-PMN II, 7M-PMN II and PMN I) were detected in the broths of the above 6 strains. Their structures are shown in Fig. 3.

It is also noted that 3 metabolites of strain JN-59 are identified as PMN II, 11dM-PMN II and 7M-PMN II, respectively, among which 7M-PMN II has also been isolated from the benanomicin-producing strain as a minor component[15].

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