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NOTE

Identification of a prodigiosin cyclization gene in the roseophilin producer and production of a new cyclized prodigiosin in a heterologous host

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The prodigiosins are reddish-colored tripyrrole antibiotics biosynthesized by several microorganisms such as *Serratia*,[1] *Pseudomonas*[2] and *Streptomyces*.[3] *Streptomyces griseoviridis* 2464-S5 produces prodigiosin R1[4] and roseophilin,[5] a unique prodigiosin-related compound containing two pyrrole and one furan moieties (Figure 1), and carries the *rph* gene cluster involved in their biosynthesis.[6] Prodigiosin R1 and roseophilin possess an alkyl chain with the same length and differ in the cyclization pattern (Figure 1). Twenty-one of 25 genes in the *rph* cluster are homologous to *red* genes in *Streptomyces coelicolor* A3(2),[7] which produces undecylprodiginine[3] and butyl-*meta*-cycloheptylprodiginine (streptorubin B).[8] In the *rph* cluster, *rphG*, *rphG2*, *rphG3* and *rphG4* showed sufficient homology to *redG*, a gene involved in cyclization of undecylprodiginine to butyl-*meta*-cycloheptylprodiginine.[9] Thus, we attempted to analyze the functions of these *rph* genes using a heterologous expression system in *S. coelicolor*.

S. coelicolor M511, an actinorhodin-nonproducing mutant of *S. coelicolor* A3(2),[10] also produced undecylprodiginine (**3**) and butyl-*meta*-cycloheptylprodiginine (**4**). Disruption of *redG* in *S. coelicolor* M511 resulted in the lack of **4** production. The *redG* disruptant (Δ*redG*) transformed with a *redG*-expressing plasmid restored the productivity of **4**. To analyze the function of *redG* homologous genes, the *redG* disruptant was transformed with plasmids bearing *rph* genes. Two prodigiosins (**1** and **2**) in addition to **3** were detected in the culture of *S. coelicolor* M511 Δ*redG* carrying an *rphG*-expressing plasmid, whereas the strain expressing *rphG2*, *rphG3* and *rphG4* produced only **3** (Figure 2).

Compound **1** was identified as metacycloprodigiosin (Figure 1)[11,12] by its ^{1}H and ^{13}C NMR spectra (Table 1) and by high-resolution FAB-MS (*m/z* 392.2703 $[M+H]^+$, calcd. for $C_{25}H_{34}N_3O$, 392.2702). Compound **2** had the same molecular formula ($C_{25}H_{33}N_3O$) as **1**, as indicated by high-resolution FAB-MS (*m/z* 392.2702 $[M+H]^+$, calcd. for $C_{25}H_{34}N_3O$, 392.2702). In the ^{1}H and ^{13}C NMR spectra, aromatic signals for **2** were almost identical to those for **1** (Table 1), thereby showing that these compounds only differ in the alkyl-chain branching position. The existence of a propyl group in **2** was required by ^{1}H–^{13}C long-range correlations from H_3-24 to C-23 and C-22 in the HMBC spectrum. A COSY experiment revealed a proton spin network from the propyl group to H_2-20 through a methine proton at δ 3.00 (H-21). The cyclization position of the alkyl chain at C-21 was confirmed by HMBC data as shown in Figure 3. The structure of **2** was thus determined as propyl-*meta*-cyclooctylprodiginine (Figure 1). To our knowledge, this prodigiosin has not been reported previously.

Prodigiosins are known to show various biological properties such as antimicrobial, antimalarial, antitumor and immunosuppressive activities.[13] The cytotoxic activities of linear or cyclized prodigiosins were evaluated using HeLa human cervical carcinoma cells and HT1080 human fibrosarcoma cells. The IC_{50} values against HeLa and HT1080 cells were 1.1 and 3.7 μM for **1**, 0.89 and 3.5 μM for **2**, 3.5 and 4.2 μM for **3** and 3.2 and 3.9 μM for **4**, respectively. The results indicate that cyclization of the alkyl chain does not significantly affect the cytotoxicity of prodigiosins.

Known prodigiosin cyclization genes, *redG* and *mcpG*, are involved in cyclization of undecylprodiginine (**3**) to butyl-*meta*-cycloheptylprodiginine (**4**) and metacycloprodigiosin (**1**), respectively.[9] The *rph* cluster contained four *redG* homologous genes, *rphG*, *rphG2*, *rphG3* and *rphG4*. Among them, *rphG* was identified as a prodigiosin cyclization gene based on two cyclized prodigiosins produced by an *rphG*-expressing strain of *S. coelicolor* M511 Δ*redG*. These prodigiosins were determined to be metacycloprodigiosin (**1**) and propyl-*meta*-cyclooctylprodiginine (**2**) by spectroscopic analyses. Metacycloprodigiosin possesses the same 12-membered carbocyclic ring as prodigiosin R1 and propyl-*meta*-cyclooctylprodiginine has an 11-membered carbocyclic ring. RphG is presumed to cyclize isotridecylprodiginine to prodigiosin R1, although no prodigiosin with an 11-membered carbocyclic ring has been isolated from *S. griseoviridis* 2464-S5. Neither *rphG2*, *rphG3* nor *rphG4* was able to cyclize

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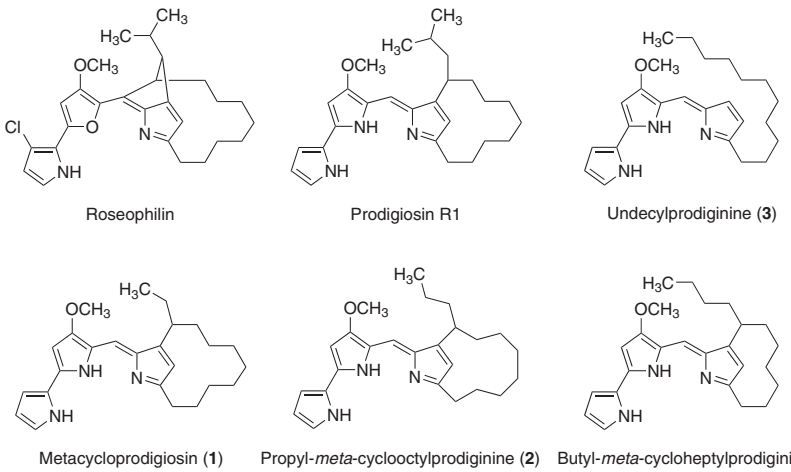

Figure 1 Structures of prodigiosin derivatives.

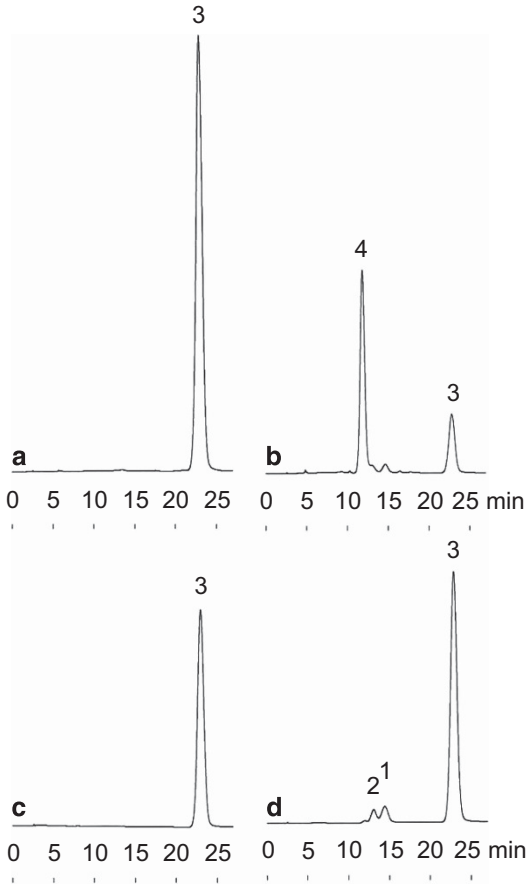

Figure 2 HPLC analysis of the culture extract of *S. coelicolor* M511 Δ*redG* expressing *rph* genes. (**a**) Δ*redG*, (**b**) Δ*redG*+*redG*, (**c**) Δ*redG*+*rphG2*–*rphG4*, (**d**) Δ*redG*+*rphG*. **1**: metacycloprodigiosin, **2**: propyl-*meta*-cyclooctylprodiginine, **3**: undecylprodiginine, **4**: butyl-*meta*-cycloheptylprodiginine.

undecylprodiginine. Since a substrate for the cyclization enzyme is not undecylprodiginine in the roseophilin producer, these *rph* genes might be involved in cyclization of the true substrate to form a roseophilin-type carboskeleton. Further biosynthetic studies are in progress.

EXPERIMENTAL PROCEDURE

General experimental procedures

UV and visible spectra were measured on a UV-1700 spectrometer (Shimadzu, Kyoto, Japan). IR spectra were obtained on a Spectrum 100 FT-IR spectrometer (PerkinElmer, Waltham, MA, USA) in the ATR (attenuated total reflection) mode. Mass spectra were measured on a JMS-SX102A spectrometer (JEOL, Akishima, Tokyo, Japan) in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. NMR spectra were obtained on a JNM-LA400 spectrometer (JEOL) with ^{1}H NMR at 400 MHz and with ^{13}C NMR at 100 MHz. Chemical shifts are given in parts per million relative to $CDCl_3$ (δ_H 7.24 and δ_C 77.0).

Bacterial strains and culture conditions

Actinobacterial strains, *S. griseoviridis* 2464-S5 and *S. coelicolor* M511, were used in this study. Growth condition and medium of *S. griseoviridis* were previously described.[5] *S. coelicolor* and its derivatives were grown in yeast extract-malt extract (YEME) medium for making protoplasts or R5 agar medium for prodigiosin production.[12] *Escherichia coli* XL1-blue MRF′ was used for genetic engineering and grown in Lennox broth (LB) medium supplemented with 100 μg ml^{-1} ampicillin as required. *E. coli* JM110 was used for preparation of non-methylated DNA for transformation of *S. coelicolor* protoplasts.

DNA isolation and manipulation

Actinobacterial genetic procedures and other general procedures were carried out as described by Kieser *et al.*[14] and Sambrook *et al.*,[15] respectively. Isolated genomic DNA was farther purified by QIAGEN Genomic-tip (QIAGEN, Venlo, Netherlands).

Disruption of *redG* in *S. coelicolor* M511

A 1.8-kbp fragment upstream and a 1.6-kbp fragment downstream of *redG* were amplified from *S. coelicolor* genomic DNA using two sets of primers with additional restriction sites, and KOD plus PCR system (Toyobo, Osaka, Japan) and digested with XbaI/BglII and BglII/HindIII, respectively. These fragments were cloned between XbaI and HindIII sites of pGEM-11Z (Promega, Madison, WI, USA). A BglII fragment of the apramycin resistance gene *apr* was cloned into a BglII site between the fragment upstream and downstream of *redG*. The plasmid was digested with XbaI/HindIII and the *apr*-containing fragment was ligated into an XbaI/HindIII site of pWHM3-DIS, a gene disruption plasmid obtained by BclI digestion of pWHM3. The *redG* disruption plasmid was introduced in *S. coelicolor* M511 and apramycin-resistant/thiostrepton-sensitive mutants were selected on R5 plates.

Table 1 ^{13}C and ^{1}H NMR data for metacycloprodigiosin (1) and Propyl-*meta*-cyclooctylprodiginine (2) in $CDCl_3$

*Metacycloprodigiosin (**1**)*			*Propyl-meta-cyclooctylprodiginine (**2**)*		
No.	δ_C	δ_H *(J in Hz)*	*No.*	δ_C	δ_H *(J in Hz)*
1	126.8	7.20 td (2.5, 1.0)	1	126.7	7.20 td (2.5, 1.0)
2	111.6	6.33 dt (4.0, 2.5)	2	111.6	6.32 m
3	116.8	6.90 ddd (4.0, 2.5, 1.0)	3	116.6	6.89 ddd (4.0, 2.5, 1.0)
4	122.3		4	122.3	
5	147.4		5	147.2	
6	92.7	6.08 d (2.0)	6	92.7	6.08 d (2.0)
7	165.7		7	165.5	
8	120.6		8	120.3	
9	113.3	7.04 s	9	112.7	7.07 s
10	125.9		10	124.7	
11	150.3		11	149	
12	112.4	6.25 d (1.5)	12	115.7	6.31 m
13	154.2		13	153.3	
14	28.9	3.19 ddd (14.5, 8.5, 4.0)	14	29	3.28 dt (13.0, 4.0)
					2.53 td (13.0, 4.5)
		2.74 ddd (14.5, 8.5, 4.0)			
15–21	34.4	0.81–1.99 14H m	15	28.6	1.98 m, 1.36 m
	27.3		16–19	29.7	0.86–1.58 8H m
	26.8		27.9		
	26.6		27		
	25.5		24.9		
	24.5		20	36.7	1.83 m, 1.13 m
	22.8		21	36.5	3.00 m
22	39.6	2.53 m	22	34.7	1.70 m, 1.58 m
23	29.9	1.72 m, 1.59 m	23	21.1	1.35 m
24	12.6	0.86 3H t (7.5)	24	14.3	0.93 3H t (7.5)
25	58.7	4.01 3H s	25	58.6	4.01 3H s
1-NH		12.56 br	1-NH		12.58 br
5-NH		12.75 br	5-NH		12.68 br
10-NH		12.62 br	10-NH		12.62 br

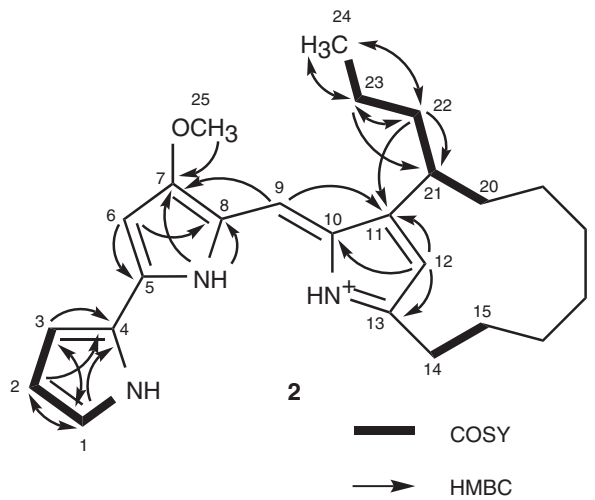

Figure 3 NMR analysis of propyl-*meta*-cyclooctylprodiginine (**2**).

Construction of expression plasmids for prodigiosin cyclization genes

A promoter for the *rphM* gene in *S. griseoviridis* was used for expression of *redG*, *rphG*, *rphG2*, *rphG3* and *rphG4* in *S. coelicolor*. A cosmid containing *rphM* was digested with NheI and PstI and an *rphM–rphG4* region was cloned between XbaI and PstI sites of pWHM3. An 875 bp fragment containing the *rphM* promoter was amplified by KOD plus PCR system (Toyobo) using the *rphM–rphG4*-containing plasmid as template with M13 primer (5′-GTTTTCCCAGTCACGAC-3′) and a primer containing an XbaI site (5′- CATTCTAGAGTCCCTCCGCGTGTCACC-3′). The fragment was cloned between EcoRI and XbaI sites of pWHM3 to construct the expression plasmid pWHMexM. Prodigiosin cyclization genes were amplified by KOD plus or Tks Gflex (Takara Bio, Kusatsu, Japan) using *S. griseoviridis* genomic DNA as template with primers containing XbaI or HindIII site (*rphG*: 5′-GCTCTA GAATGATCCCGAATCAGTGGTACGCC-3′ and 5′-ATCAAGCTTTCATG CGGAGGCCGCGATGT-3′, *rphG2–rphG4*: 5′-GCTTCTAGACGCAGTACGG AAAGGCAGCTGAAC-3′ and 5′-GGGAATTCAGCATGGACACAATGAAA GCTTGGT-3′) and cloned between XbaI and HindIII sites of pWHMexM.

HPLC analysis of prodigiosins

Fermented mycelium on R5 plates with 10 μg ml^{-1} thiostrepton was extracted with 2 volumes of acetone. The extract was concentrated and partitioned between ethyl acetate and water. The organic layer was evaporated and dissolved in methanol. The methanol solutions were analyzed by HPLC using a Senshu-Pak PEGASIL ODS SP100 column (Senshu Scientific, Tokyo, Japan) with 90% methanol containing 5 mM disodium hydrogen citrate. Absorption peaks for prodigiosins were detected at 530 nm.

Isolation of cyclized prodigiosins

The *rphG*-expressing strain was cultured on R5 plates (20 ml × 300 plates) at 27 °C for 7 days. The culture was extracted with acetone and the aqueous concentrate was extracted with ethyl acetate. The extract was partitioned between hexane and 90% methanol. The 90% methanol layer was evaporated and applied to a silica gel column, which was washed with hexane-chloroform (2:1) and eluted with chloroform. The eluate was subjected to preparative silica gel TLC developed with chloroform–methanol–29% ammonia water (200:20:1). Two fractions (**1**: Rf 0.86, **2**: Rf 0.84) thus obtained were separately purified by HPLC using a Senshu-Pak PEGASIL ODS SP100 column with 90% methanol containing 5 mM disodium hydrogen citrate. The prodigiosin fractions were evaporated and the aqueous concentrates were extracted with ethyl acetate. Each extract was acidified by addition of equivalent hydrogen chloride and concentrated to dryness to give a hydrochloride salt of prodigiosin (**1**: 9.9 mg, **2**: 3.6 mg).

Propyl-*meta*-cyclooctylprodiginine (**2**): red amorphous powder; m.p. 73 °C; high-resolution FAB-MS *m/z* 392.2702 ($[M+H]^+$, calcd. for $C_{25}H_{34}N_3O$, 392.2702); UV λ_{max} (ε) 535 nm (62 700) in MeOH, 535 nm (82 700) in 0.01 M HCl–MeOH, 473 nm (32 700) in 0.01 M NaOH–MeOH; IR (ATR) ν_{max} 3150, 3100, 1600, 1260 cm^{-1}.

Cell culture and cell viability assay

HeLa and HT1080 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose. The cells were plated and incubated for 48 h with various concentrations of samples. After the cells were treated with 0.5 mg ml^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C, the relative cell number was measured as absorbance at 540 nm. IC_{50} values were calculated by linear interpolation between the two drug concentrations above and below the 50% inhibition line.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)