A Prodigiosin from the Roseophilin Producer *Streptomyces griseoviridis*

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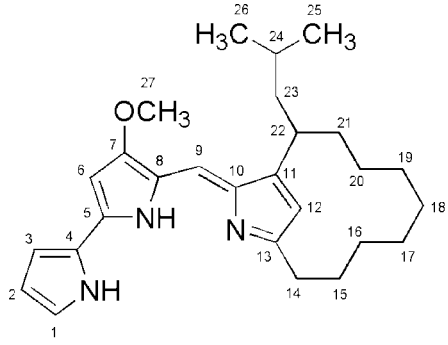
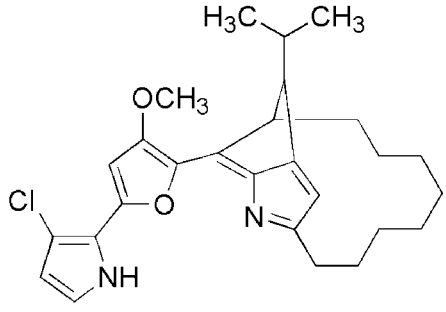
Roseophilin is a unique metabolite containing two pyrrole and one furan ring and is structurally related to tripyrrole antibiotics, prodigiosins. Each homologous gene in the roseophilin producer *Streptomyces griseoviridis* was amplified by PCR using primers designed from the prodigiosin-biosynthesis genes *redH*, *redM*, and *redW*. A search for prodigiosins produced by *S. griseoviridis* resulted in the isolation of a new prodigiosin designated prodigiosin R1 (**1**). The molecular formula of **1** was established as $C_{27}H_{37}N_3O$ by high-resolution FABMS. The structure of **1** was elucidated by NMR spectroscopic analysis including COSY, HMQC, HMBC, and NOESY. Prodigiosin R1 (**1**) is a new member of the prodigiosin family possessing a cyclic alkyl side chain.

Roseophilin is a reddish-purple pigment isolated from the culture broth of *Streptomyces griseoviridis* 2464-S5.[1] The compound has a unique structure, containing two pyrrole and one furan ring. Tripyrrole antibiotics, prodigiosins, show a similar structural feature and are produced by *Streptomyces* or *Serratia*.[2–6] Roseophilin is considered to be biosynthesized partially with the same pathway as prodigiosins.[7–9] We attempted to amplify genes homologous to *redH*, *redM*, and *redW* involved in the biosynthesis of prodigiosin from the roseophilin producer. Nucleotide sequencing of the PCR products identified each homologous gene in *S. griseoviridis* genomic DNA. This result suggested that prodigiosins are also produced by the roseophilin producer. Color-guided fractionation of the culture extract afforded prodigiosin R1 (**1**), a new prodigiosin with a cyclic alkyl side chain. We report herein the isolation and structure elucidation of **1**.



Prodigiosin R1 (**1**)



Roseophilin

Amplification of Prodigiosin-Biosynthesis Gene Fragments from *S. griseoviridis*. A pair of primers were designed from the amino acid sequences of biosynthetic genes *redH*/*pigC*, *redM*/*pigI*, and *redW*/*pigA* in the three prodigiosin producers *Streptomyces coelicolor* A3(2), *Serratia marcescens* ATCC274, and *Serratia* sp. ATCC39006.[7–9] PCR amplification of *S. griseoviridis* genomic DNA gave each candidate fragment. Nucleotide sequencing and homology search showed that the fragments shared amino acid identities of 61%, 76%, and 71% with RedH, RedM, and RedW, respectively.

Table 1. NMR Data for **1** in $CDCl_3$

position	δ_C	δ_H multiplicity (*J* in Hz)
1	126.8	7.20 m
2	111.6	6.32 dt (8.4, 2.7)
3	116.8	6.90 m
4	122.3	
5	147.5	
6	92.7	6.08 d (2.0)
7	165.7	
8	120.6	
9	113.2	7.06 s
10	125.7	
11	150.5	
12	112.4	6.24 d (2.0)
13	154.2	
14	29.0	3.18 m, 2.72 m
15	26.8	1.75 2H m
16	27.4	1.48 m, 1.18 m
17	25.4	0.83 m, 0.18 m
18	24.5	1.18 m, 1.02 m
19	22.4	1.33 2H m
20	26.6	1.02 m, 0.86 m
21	35.1	1.67 m, 1.35 m
22	35.5	2.72 m
23	46.4	1.58 m, 1.39 m
24	26.0	1.46 m
25	23.7	0.84 3H d (6.4)
26	22.1	0.88 3H d (6.4)
27	58.4	4.00 3H s
1-NH		12.61 br
5-NH		12.75 br
10-NH		12.53 br

Production and Isolation of a New Prodigiosin. *Streptomyces griseoviridis* 2464-S5 was cultivated at 27 °C for 7 days on a rotary shaker in 500 mL Erlenmeyer flasks containing 100 mL of a medium consisting of glycerol 4.0%, soybean meal 1.5%, molasses 1%, and calcium carbonate 0.4% (pH 6.8). The fermentation broth (2.0 L) was centrifuged, and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract contained several red pigments and was chromatographed on a silica gel column with chloroform. The eluted pigment was subjected to reversed-phase

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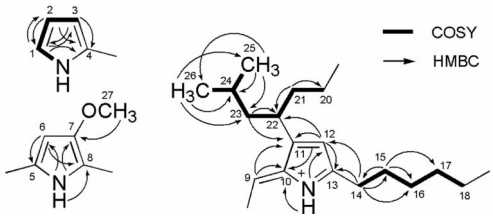

Figure 1. Partial structures of **1** derived from COSY and HMBC.

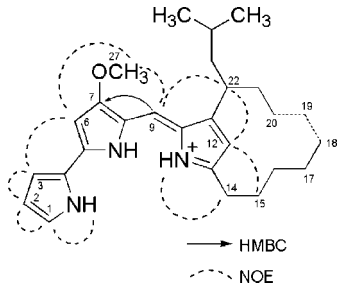

Figure 2. Connection of the partial structures of **1**.

HPLC (YMC Pack D-ODS-7) using 85% methanol with 0.2% trifluoroacetic acid. A major pigment fraction was further purified by reversed-phase HPLC (Waters XBridge C_{18}) using 90% methanol with 0.2% triethylamine. A single-peak fraction was concentrated and dissolved in ethyl acetate. The solution was washed with 0.1 M HCl and water and concentrated to dryness to give a red powder of **1** (8.4 mg).

Structure Elucidation of Prodigiosin R1. The molecular formula of **1** was established as $C_{27}H_{37}N_3O$ by high-resolution FABMS (*m*/*z* 420.3014 MH^+, Δ −0.1 mmu error). ^1H and ^{13}C NMR data for **1** are summarized in Table 1. All one-bond $^1H-^{13}C$ connectivities were confirmed by HMQC.[10]

COSY and HMBC experiments identified three pyrrole moieties, as shown in Figure 1. Three aromatic protons (1-H−3-H) coupled with each other exhibited correlations to an exchangeable proton (1-NH) and two or three of four aromatic carbons (C1−C4), indicating the presence of a pyrrolyl group. Long-range couplings were observed from 6-H to C-5 and C-8, from 5-NH to C-6, C-7, and C-8, and from a methoxy proton (27-H_3) to C-7. These correlations and a high-field chemical shift for C-6 (δ 92.7) showed that a 2,5-disubstituted 3-methoxypyrrole moiety consisted of C-5 to C-8 and C-27. The existence of a 2-alkenyl-3,5-dialkylpyrrole moiety was required by $^1H-^{13}C$ long-range correlations from 12-H to C-10, C-11, C-13, and C-22, from 10-NH to C-10 and C-12, from 9-H to C-10 and C-11, from 14-H_2 to C-12 and C-13, and from 23-H_2 to C-11. Proton spin networks from 14-H_2 to 18-H_2 and from 20-H_2 to 26-H_3 indicated the start and end of an alkyl chain, which was supported by HMBC data (Figure 1). NOEs between 1-NH, 1-H, 2-H, and 3-H confirmed a 2-pyrrolyl group and their assignments. NOEs between 3-H and 6-H, between 6-H and 7-OMe, and between 7-OMe and 9-H together with a long-range coupling between 9-H and C-7 established the connection of the three heterocyclic rings as shown in Figure 2. The remaining methylene (C-19) joined C-18 and C-20 to construct a cyclic alkyl chain (Figure 2). The structure of prodigiosin R1 (**1**) thus obtained consists of the same carbon skeleton as roseophilin except for the number and position of carbon−carbon bridges. Structurally related prodigiosins possessing a cyclic alkyl side chain have been found in microbial metabolites including cycloprodigiosin,[11] metacycloprodigiosin,[12] cyclononylprodiginine,[13] methylcyclodecylprodiginine,[14] and butyl-*meta*-cycloheptylprodiginine,[15] which showed antibacterial, antifungal, and antimalarial activities. Among them, prodigiosin R1 (**1**) is closely related to metacycloprodigiosin, which contains an ethyl group in place of the isobutyl group of **1**, and only roseophilin has a bicyclic alkyl ring and a chlorinated pyrrole moiety. Studies on the biosynthetic relationship between prodigiosin R1 and roseophilin are in progress.

Experimental Section

General Experimental Procedures. UV and IR spectra were measured on Shimadzu UV-1700 and JASCO FT/IR-410 spectrometers. NMR spectra were obtained on a JEOL JNM-LA400 spectrometer with ^1H NMR at 400 MHz and with ^{13}C NMR at 100 MHz. Mass spectra were obtained on a JEOL JMS-SX102A spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard.

DNA Isolation and Manipulation. *Escherichia coli* XL1-blue MRF′ was used as a host for the manipulation of plasmid DNA and grown in LB medium. Plasmids from *E. coli* were prepared using a QIAprep spin miniprep kit (QIAGEN). All restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Nippon Gene. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a Gene Pulser II electroporation system (Bio-Rad). Other general procedures were performed as described by Sambrook et al.[16]

Amplification and Cloning of Gene Fragments. To perform the cloning of roseophilin-biosynthetic genes, degenerate primers were prepared on the basis of the conserved region of *redH*/*pigC*, *redM*/*pigI*, and *redW*/*pigA* in *Streptomyces coelicolor* A3(2), *Serratia marcescens* ATCC 274, and *Serratia* sp. ATCC39006. Each gene fragment was amplified by using *S. griseoviridis* genomic DNA and specific primers for a *redH*-like gene (5′-ARGGNYAYGTSTAYCT-SAAYAT-3′ and 5′-NGGNGTCCASGASGCRTCSGTYTA-3′), a *redM*-like gene (5′-ACNGGSACSCCSAARGGSGTSCA-3′ and 5′-RCANACRTTSGTYTCSGTSGGSCC-3′), and a *redW*-like gene (5′-TTYATHACSAAYGCSCCSRTSGC-3′ and 5′-YTTYTGDATNT-CRTTSGTSCC-3′). The reaction mixture consisted of 1 × GC buffer I, 0.4 mM dNTP mixture, 2 μM each primer, 0.5 ng/μL genomic DNA, and 0.05 U/μL Takara *LA Taq* (*Taq* DNA polymerase). After heating at 95 °C for 4 min, the mixture was followed by 29 cycles of three steps: 95 °C 1 min, 50 °C 30 s, 72 °C each time (1 min: 1 kbp). After purification, the PCR products were linked with pGEM-T Easy Vector (Promega) by using T4 DNA ligase. *E. coli* was transformed with the plasmid DNA by electroporation.

Sequence Analysis. PCR products were selected and used for sequencing analysis. Sequencing was carried out with an automatic DNA sequencer (3100 genetic analyzer, Applied Biosystems). Nucleotide sequences reported here have been deposited in the GenBank, DDBJ, and EMBL databases under accession numbers AB372878 (*redH*-like gene), AB372879 (*redM*-like gene), and AB372880 (*redW*-like gene).

Prodigiosin R1 (1): red powder; mp 80−85 °C; UV λ_{max} (ϵ) 472 nm (13 000) in MeOH, 533 nm (34 600) in 0.01 M HCl−MeOH, 470 nm (15 100) in 0.01 M NaOH−MeOH; IR (KBr) ν_{max} 3450, 2900, 1600, 1260 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; HRFABMS *m*/*z* 420.3014 (MH^+, calcd for $C_{27}H_{38}N_3O$, 420.3015).

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Supporting Information Available: The ^1H NMR, ^{13}C NMR, HMQC, HMBC, COSY, NOESY, IR, and mass spectra of **1**, and the proposed pathway of prodigiosin biosynthesis are available free of charge via the Internet at http://pubs.acs.org.

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