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BRIEF COMMUNICATION

Prodigiosin R2, a new prodigiosin from the roseophilin producer *Streptomyces griseoviridis* 2464-S5

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Abstract

Roseophilin (**2**) is a unique prodigiosin-related compound produced by *Streptomyces griseoviridis* 2464-S5, and is characterized by a central furan ring and a bicyclic alkyl chain. During a search for biosynthetic intermediates of **2**, a new metabolite designated prodigiosin R2 (**1**) was isolated from the culture of the roseophilin producer. The molecular formula of **1** was established as $C_{27}H_{35}N_3O$ by high-resolution FAB-MS. The structure of **1** was determined by NMR spectroscopic analyses as a prodigiosin derivative with the same bicyclic alkyl chain as **2**. Prodigiosin R2 (**1**) showed potent cytotoxicity against HeLa human cervical carcinoma cells and HT1080 human fibrosarcoma cells with IC_{50}s of 0.41 and 0.82 μM, respectively.

Introduction

Prodigiosins are cytotoxic antibiotics characterized by three pyrrole rings in the structure [1]. Roseophilin (**2**) and dechlororoseophilin (**3**) are unique prodigiosin-related compounds produced by *Streptomyces griseoviridis* [2, 3]. These metabolites contain a furan ring in place of the central pyrrole ring and a bicyclic alkyl chain (Fig. 1). The roseophilin producer also produces prodigiosin R1 (**4**) [4], which is a typical prodigiosin containing a simple cyclic alkyl chain with the same carbon skeleton as **2**. It was suggested that most of the biosynthesis pathways of **2** and **4** are common. The *rph* gene cluster has been found in *S. griseoviridis* as involved in the biosynthesis of these prodigiosin derivatives [5], although the cluster seems not to contain genes for furan ring formation or chlorination. Then, we searched for biosynthetic intermediates of **2** to add more information for its biosynthesis.

Results and discussion

S. griseoviridis 2464-S5 was cultured at 27 °C for 60 h with reciprocal shaking in a seed medium (glycerol 1%, Bacto Soytone 1%, molasses 1%, pH 7.2). The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glycerol 4%, molasses 1%, soybean meal 1.5%, and $CaCO_3$ 0.4% (pH 6.8). The fermentation was carried out on a rotary shaker at 27 °C for 6 days. The ethyl acetate extract obtained from the mycelial acetone extract was analyzed by HPLC (PEGASIL ODS SP-100, Senshu Scientific, Tokyo, Japan) with 90% methanol-5 mM disodium hydrogen citrate at 530 nm. The chromatogram revealed peaks for **2**, **3**, **4**, and 11-methyldodecylprodiginine (**5**) as shown in Fig. 2. No other distinct peak was observed in various culture conditions. The productivity of each prodigiosin in 1-L cultivation was calculated as 82 mg for **2**, 36 mg for **3**, 22 mg for **4**, and 5 mg for **5**. In the previous study, only 8.2 mg of **3** was isolated from the 2-L culture under the similar condition [3], while this fraction was expected to contain 72 mg of prodigiosin derivatives. Silica gel TLC analysis and silica gel column fractionation identified a new prodigiosin designated prodigiosin R2 (**1**) in the HPLC fraction containing **3**.

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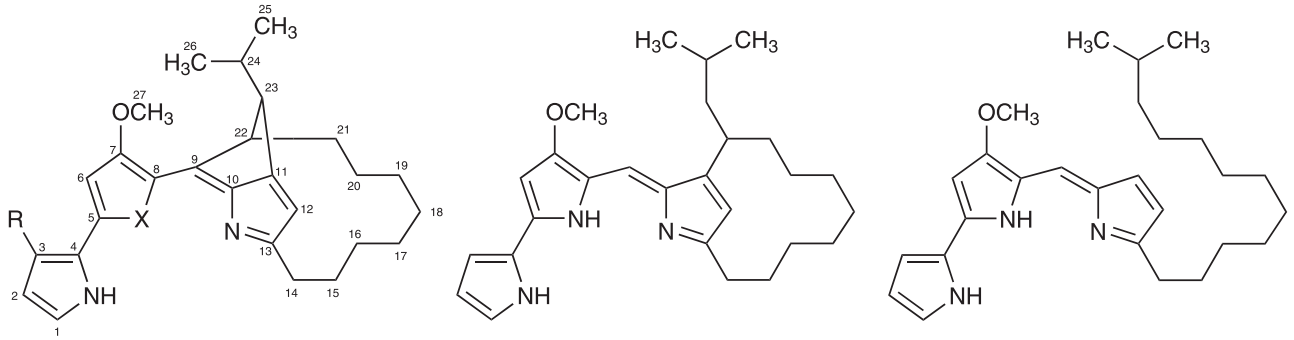

Fig. 1 Structure of prodigiosin derivatives from the roseophilin producer

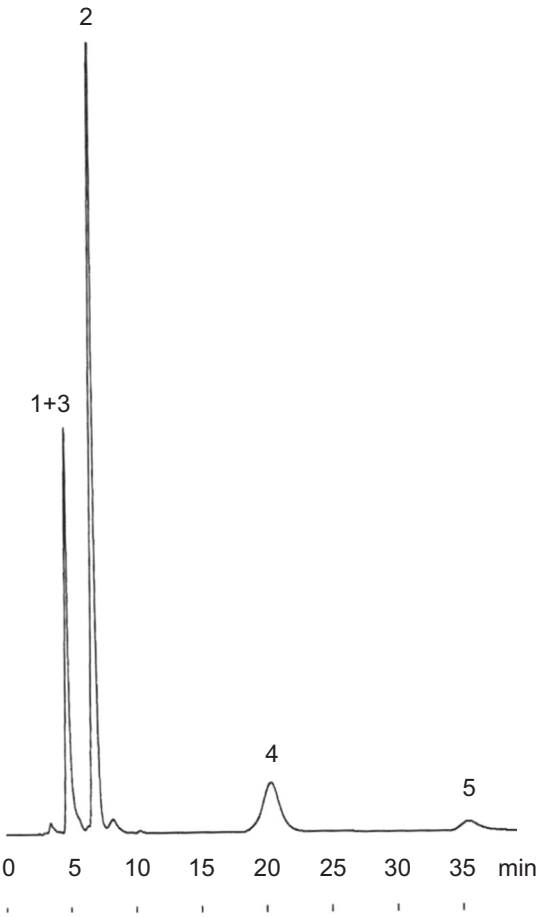

Fig. 2 HPLC analysis of prodigiosin derivatives produced by *S. griseoviridis* 2464-S5

The fermented 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 was partitioned between hexane and 90% methanol. The 90% methanol layer was concentrated and applied to a silica gel column, which was washed with hexane-chloroform (2:1) and eluted with hexane-chloroform (1:1). The eluate contained **1** and **4**, and the two pigment bands (**1**: Rf 0.84, **4**: Rf 0.93) were separated by preparative silica gel TLC with chloroform-methanol-29% ammonia water (200:20:1). The fraction containing **1** was dissolved in ethyl acetate, and the solution was washed with 0.1 M NaOH and water. After evaporation, the residue was dissolved in methanol and acidified by addition of equivalent hydrogen chloride. The material was concentrated to dryness to give a hydrochloride salt of **1** (5.6 mg).

The molecular formula of **1** was established as $C_{27}H_{35}N_3O$ by high-resolution FAB-MS. The ^{13}C and ^{1}H NMR data for **1** are summarized in Table 1. All one-bond ^{1}H–^{13}C connectivities were confirmed by an HMQC experiment [6].

COSY and HMBC [7] analyses established the structure of **1** as shown in Fig. 3. Three aromatic protons (H-1 to H-3) and an exchangeable proton (1-NH) were coupled with each other. Long-range correlations from H-1 to C-2, C-3, and C-4, from H-2 to C-1 and C-4, and from H-3 to C-1 and C-4 indicated the presence of a 2-pyrrolo group. ^{1}H–^{13}C long-range couplings were observed from H-6 to C-5 and C-8, from an exchangeable proton (5-NH) to C-7, and from a methoxy proton (H-27) to C-7. In addition to these correlations, an NOE from H-27 to H-6 and a high-field chemical shift for C-6 (δ 93.9) constructed a 2,5-disubstituted 3-methoxypyrrole moiety. The third pyrrole ring was identified by ^{1}H–^{13}C long-range correlations from H-12 to C-10, C-11, and C-13, and a ^{1}H–^{1}H long-range correlation between H-12 and an exchangeable proton (10-NH). Proton spin networks from H-14 to a methine (H-22) and from a methine (H-23) to two doublet methyls (H-25 and H-26) revealed the presence of bridged alkyl chains, which were connected by ^{1}H–^{13}C long-range couplings from H-23 to C-

Table 1 ^{13}C and ^{1}H NMR data for **1** and **2** in $CDCl_3$

1			2		
No	δ_C	δ_H (*J* in Hz)	No	δ_C	δ_H (*J* in Hz)
1	125.6	7.15 br s	1	126.6	7.24 dd (3.5, 2.5)
2	110.9	6.29 m	2	112.0	6.26 dd (2.5, 2.0)
3	115.2	6.83 d (2.5)	3	119.4	
4	122.1		4	116.7	
5	144.8		5	159.2	
6	93.9	6.15 d (2.5)	6	96.3	6.91 s
7	164.5		7	166.7	
8	116.8		8	132.8	
9	146.9		9	144.7	
10	133.9		10	135.6	
11	156.7		11	160.3	
12	108.6	6.08 s	12	110.8	6.17 s
13	157.6		13	165.7	
14	28.6	2.92 m, 2.88 m	14	28.2	3.54 ddd (13.0, 11.0, 6.5)
					2.82 ddd (13.0, 5.5, 3.5)
15	28.1	2.01 m, 1.31 m	15	27.8	2.06 m, 1.30 m
16	25.3	1.35 m, 1.12 m	16	24.8	1.30 m, 1.16 m
17	28.2	0.96 m, 0.41 m	17	28.2	1.00 m, 0.39 m
18	27.1	0.95 m, 0.78 m	18	26.8	0.90 m, 0.76 m
19	27.7	0.75 m, 0.41 m	19	27.4	0.76 m, 0.36 m
20	24.6	0.98 m	20	24.3	1.00 m, 0.90 m
21	34.7	1.98 m, 1.78 m	21	33.9	1.99 m, 1.79 m
22	56.0	3.96 m	22	55.4	3.81 dd (4.5, 3.0)
23	51.3	2.61 d (8.0)	23	51.5	2.69 d (6.5)
24	33.1	1.78 m	24	32.9	1.79 m
25	21.4	0.99 3 H d (8.0)	25	21.3	0.98 d (6.5)
26	19.5	0.77 3 H d (8.0)	26	19.5	0.76 d (6.5)
27	58.8	4.01 3 H s	27	60.0	4.12 s
1-NH		12.60 br s	1-NH		13.79 br
5-NH		11.99 br s			
10-NH		13.19 br s	10-NH		13.49 br

21 and C-22. Furthermore, H-23 displayed long-range correlations to C-9, C-10, and C-11, and an NOE to H-12, indicating that C-23 was located on C-11 and a five-membered ring was composed of C-9, C-10, C-11, C-22, and C-23. A long-range correlation between H-14 and C-13 connected the alkyl chain to the C-13 position in the third

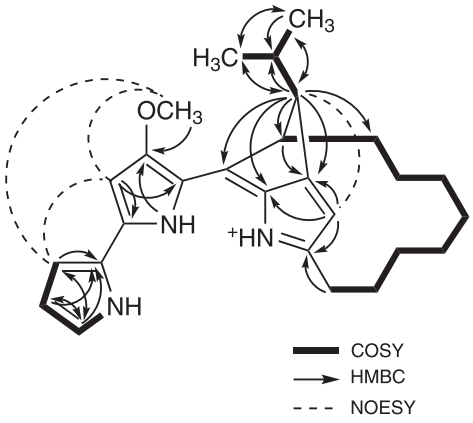

Fig. 3 NMR analysis of prodigiosin R2 (**1**)

pyrrole ring. NOEs from H-3 to H-6 and H-27 confirmed the connection of the first and the central pyrrole rings. The remaining bond was formed between C-8 and C-9 to complete the planar structure of **1**. The ^{13}C chemical shift similarity (±0.8 p.p.m.) in the alkyl region of **1** and **2** (Table 1) established the structure including the relative stereochemistry.

The cytotoxic activities of the prodigiosin derivatives were evaluated using the MTT method against HeLa human cervical carcinoma cells and HT1080 human fibrosarcoma cells. The IC_{50} values against HeLa and HT1080 cells were 0.41 and 0.82 μM for **1**, 4.2 and 5.7 μM for **2**, and 1.7 and 3.3 μM for **4**, respectively. Prodigiosin derivatives isolated from the roseophilin producer exhibited potent cytotoxicity against both the cell lines. Prodigiosin R2 (**1**) was more cytotoxic than the other prodigiosin derivatives.

Prodigiosins are known to possess antimicrobial, antifungal, antitumor, antimalarial, and immunosuppressive activities [8], and are reported to be H^+/Cl^- symporters, which uncouple lysosomal vacuolar-type ATPase [9]. The activity of prodigiosin derivatives as H^+/Cl^- symporters can be evaluated by cytotoxicity. The similar cytotoxic profiles of **2** and **3** have been reported [3]. The higher cytotoxicity of **1** indicated that the bicyclic alkyl chain and the central pyrrole ring increase the activity of prodigiosins. These structure-activity relationships will be helpful for structural optimization.

Prodigiosin R2 (**1**) contains a tripyrrole moiety with a bicyclic alkyl chain. Among known prodigiosin derivatives, such an alkyl chain has been found in only **1**, **2**, and **3**. In the *rph* gene cluster, four genes *rphG*, *rphG2*, *rphG3*, and *rphG4* exhibited similarity to prodigiosin cyclization genes. Among them, *rphG* has been established to cyclize undecylprodiginine [10] and is considered to be involved in the formation of prodigiosin R1 (**4**) from 11-methyldodecylprodiginine (**5**). Since only the roseophilin producer has four genes for prodigiosin cyclization, *rphG2, rphG3*, and *rphG4* might construct a system for cyclization

to form the bicyclic alkyl chain. In this study, no chlorinated derivative of **1** has been found in the culture extract (data not shown). This result suggests that the substrate of chlorination is only **3** or chlorinated **1** is immediately converted into **2**. Further biosynthetic studies are in progress.

Experimental section

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 were referenced relative to $CDCl_3$ (δ_H 7.24 and δ_C 77.0).

Physico-chemical properties of prodigiosin R2 (1)

Red amorphous powder; m.p. 65–68 °C; high-resolution FAB-MS *m/z* 418.2856 ($[M + H]^+$, calcd for $C_{27}H_{36}N_3O$, 418.2858); UV λ_{max} (ε) 271 nm (4000), 293 nm (4700), 342 nm (4100), 513 nm (80300) in MeOH, 293 nm (4800), 342 nm (4400), 513 nm (82800) in 0.01 M HCl–MeOH, 289 nm (6800), 459 nm (29100) in 0.01 M NaOH–MeOH; IR (ATR) ν_{max} 3140, 2930, 1580, 1320 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 bovine 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.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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