Sponge-Derived *Streptomyces* Producing Isoprenoids via the Mevalonate Pathway

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In the course of our screening program for isoprenoids of marine actinobacterial origin, 523 actinobacterial strains were isolated from marine samples. Actinobacteria usually use the 2-*C*-methyl-D-erythritol 4-phosphate pathway for the production of primary metabolites, but some have been reported to use the mevalonate (MVA) pathway for the production of isoprenoids as secondary metabolites. 3-Hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) is a key enzyme and plays an important role in the MVA pathway. Therefore, we screened strains possessing the HMGR gene from the 523 strains mentioned above and also investigated isoprenoid compounds from cultures of strains possessing HMGR genes. As a result, *Streptomyces* sp. SpC080624SC-11 isolated from a marine sponge, *Cinachyra* sp., was shown to possess the HMGR gene and produce novel isoprenoids, JBIR-46 (**1**), -47 (**2**), and -48 (**3**). On the basis of extensive NMR and MS analyses, the structures of **1**−**3** were determined to be phenazine derivatives harboring dimethylallyl moieties. Furthermore, the isoprene units of **2** and **3** were confirmed to be synthesized via the MVA pathway in a feeding experiment using [1-^{13}C]acetate.

Isoprenoids are the largest family of compounds found in nature, comprising more than 30 000 known examples. They contain industrially useful compounds such as flavor molecules, antibiotics, and plant hormones.[1−3] They are essentially composed of units of isopentenyl diphosphate (IPP), which can be synthesized via two independent pathways: the mevalonate (MVA) pathway and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. To synthesize the primary metabolites, all actinobacteria, including *Streptomycetes,* utilize the MEP pathway for the formation of IPP.[4] Some actinobacteria that use the MEP pathway have been reported to also use the MVA pathway for the production of isoprenoids as secondary metabolites. These strains include *Kitasatosporia griseola* (terpentecin producer),[5] *Actinoplanes* sp. A40644 (BE-40644 producer),[6] *Streptomyces* sp. CL190 (naphterpin producer),[7] *Streptomyces* sp. KO-3988 (furaquinocin A and oxaloterpin producer),[8,9] *Chainia rubra* (napyradiomycin A producer),[10] *Streptomyces cinnamonensis* (furanonaphthoquinone I and endophenazine A producer),[11] and *Micromonospora* sp. (diazepinomicin producer).[12] Since these isoprenoids have interesting biological properties, including antitumor, antibacterial, and antioxidative properties, novel isoprenoids produced by actinobacteria are expected to be promising candidates for the discovery of new drugs. In this study, we screened isoprenoids from actinobacteria possessing a key enzyme gene in the MVA pathway, the 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) gene.

A number of reports have been published on the isolation of actinobacteria from marine organisms. Screening bioactive substances from these marine-derived actinobacteria has yielded several new bioactive metabolites. One typical example is the novel compound salinosporamide A, which was produced by members of the genus *Salinispora*.[13] Although more than 70% of the earth's surface is covered by water, the diversity of marine organisms has not yet been fully explored. Marine sponges are of special interest, as they are filter feeders and assimilate bacteria during the process of filtration. Marine sponges and the seawater itself may have a number of undiscovered actinobacteria, as evidenced by denaturing gradient gel electrophoresis (DGGE) and the 16S rRNA clone library.[14] Therefore, these uncultured marine actinobacteria could present new bioactive metabolites.

Recently, our group has been engaged in the isolation of microorganisms from marine sources, including fungi and actinobacteria. Some of the isolated microorganisms have been found to produce novel compounds, namely, sesquiterpenes JBIR-27 and -28,[15] aspochracin derivative JBIR-15,[16] glycosyl benzenediols JBIR-37 and -38,[17] and teleocidins JBIR-31.[18] Thus, we investigated the production of isoprenoids, which are relatively rare secondary metabolites in actinobacteria, in a culture of marine-derived actinobacteria and certified the existence of the HMGR gene as the marker of the MVA pathway. As a result, the novel isoprenoids JBIR-46 (**1**), -47 (**2**), and -48 (**3**) were isolated from the cultures of *Streptomyces* possessing the HMGR gene. Furthermore, it was demonstrated that isoprene units of **2** and **3** are biosynthesized via the MVA pathway in a feeding experiment with [1-^{13}C]acetate. This is the first report with regard to isoprenoid compounds produced via the MVA pathway in marine sponge-derived *Streptomyces*.

Results and Discussion

We isolated a total of 523 strains of actinobacteria from 21 samples of marine origin.[19] To determine whether these strains use the MVA pathway, we investigated the presence of the HMGR gene, which codes a key enzyme in the pathway, using polymerase chain reactions (PCRs) and sequencing. Of the 523 strains, the HMGR genes were amplified in only six. Furthermore, the presence of the isopentenyl diphosphate isomerase gene, which is an essential gene in the MVA pathway, was also recognized in the six strains. Therefore, these strains certainly use the MVA pathway. The six strains possessing the HMGR gene were examined for the production of isoprenoids using LC-MS and NMR analyses. Interestingly, the strain SpC080624SC-11 isolated from a marine sponge, *Cinachyra* sp., produced novel isoprenoids.

We analyzed the sequences of 16S rRNA and HMGR genes from the strain SpC080624SC-11. A Basic Local Alignment Search Tool (BLAST) search of the 16S rRNA gene showed 99% similarity to that of *Streptomyces setonensis* (data not shown); hence, SpC080624SC-11 belongs to the *Streptomyces* genus. The amino acid sequence of the HMGR gene from SpC080624SC-11 was highly similar (78%) to that of the HMGR gene in the furaquinocin biosynthesis gene cluster of *Streptomyces*

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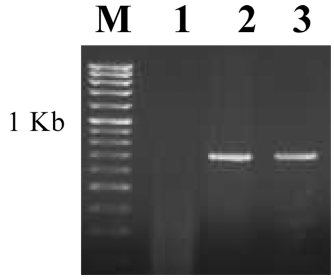

Figure 1. Expression of the HMGR gene. The lane 1 sample is the total RNA without reverse transcriptase reaction as the PCR template. The lane 2 sample is the RT products as the PCR template. Lane 3 is a positive control (DNA as the PCR template). M is the size marker.

sp. KO-3988.[8] Furthermore, we confirmed the expression of the HMGR gene in SpC080624SC-11 using RT (reverse transcriptase)-PCR, since the strain produced isoprenoid compounds. As a result, the HMGR gene was expressed in SpC080624SC-11 at 48 h of culture in a production medium (Figure 1). These results suggest that **1**−**3** can be synthesized via the MVA pathway.

The producing strain SpC080624SC-11 was cultured in a salt-containing media. The isolation of **1**−**3** was carried out by column chromatography. The structures of **1**−**3** were elucidated as follows. The physicochemical properties of **1**−**3** are summarized in Table 1. The molecular formulas of **1**, **2**, and **3** were determined by HRESIMS to be $C_{17}H_{16}N_2O_3$ (found 297.1244 $[M + H]^+$, calculated 297.1239), $C_{22}H_{24}N_2O_2$ (found 349.1924 $[M + H]^+$, calculated 349.1916), and $C_{22}H_{24}N_2O_3$ (found 365.1869 $[M + H]^+$, calculated 365.1865), respectively.

The structures of **1**−**3** were mainly determined by NMR analyses, and the ^{13}C and ^1H NMR data for **1**−**3** are presented in Table 2. Since **1**−**3** produced characteristic resonances in the aromatic regions of the ^1H and ^{13}C NMR spectra, they are considered to comprise the same chromophore. Among these compounds, **3** was used for structure determination because it was the major compound. The structure of **3** was elucidated in a series of NMR analyses such as DQF-COSY and CT-HMBC[20] (Figure 2B). An *ortho*-coupling between H-2 (δ_H 6.99) and H-3 (δ_H 7.52) was observed in the DQF-COSY spectrum of **3**. $^1H-^{13}C$ long-range couplings from H-2 to C-4 (δ_C 129.2) and C-10a (δ_C 126.7) and from H-3 to C-1 (δ_C 150.6) and C-4a (δ_C 142.0) determined the assignments of a 1,2,3,4-tetrasubstituted benzene ring. By taking into consideration the low-field-shifted chemical shift of C-1, an oxygen atom should be substituted at C-1. The singlet methyl protons H-4′ (δ_H 1.76) and H-5′ (δ_H 1.81) were $^1H-^{13}C$ long-range coupled and commonly coupled to an olefinic quaternary carbon C-3′ (δ_C 133.2) and an olefinic methine carbon C-2′ (δ_C 122.3), which in turn was ^1H spin-coupled to methylene protons H-1′ (δ_H 3.85). These results indicated a dimethylallyl moiety. The $^1H-^{13}C$ long-range correlations in the HMBC spectrum from the methylene proton H-1′ to aromatic carbons C-3 (δ_C 131.2), C-4, and C-4a and from H-3 to the methylene carbon C-1′ (δ_C 29.7) determined the substitution position of the dimethylallyl moiety as being at C-4 in the benzene ring. These results indicated a *p*-isoprenylphenol moiety. In the same manner, the same partial structure, a *p*-isoprenylphenol moiety, was confirmed by DQF-COSY and HMBC analyses, as shown in Figure 2B. By taking into consideration the molecular formula, we deduced that these benzene ring moieties formed a phenazine chromophore. The presence of a phenazine chromophore was also supported by UV absorptions (λ_{max} 287 and 510 nm).[21] The remaining oxygen atom should be assigned to an *N*-oxide group, and the connectivity of these *p*-isoprenylphenol moieties is established as shown in Figure 2B from the asymmetric NMR signals. A downfield shift of a hydrogen-bonded phenolic hydroxy proton 1-OH (δ_H 13.61), which in turn was $^1H-^{13}C$ long-range coupled to C-1, C-2 (δ_C 112.5), and C-10a, compared with the chemical shift of 6-OH (δ_H 8.21), which in turn was $^1H-^{13}C$ long-range coupled to C-5a (δ_C 137.1), C-6 (δ_C 150.3), and C-7 (δ_C 109.8), also supported there being an *N*-oxide group at N-10.[22] This structure was further confirmed by an NOE correlation between 6-OH and 1′-H. Therefore, the structure of **3** was determined as 1,6-dihydroxy-4,9-bis(3-methylbut-2-enyl)phenazine 10-oxide (Figure 2A).

The structures of **1** and **2** were determined in the same manner. The molecular formula of **1** suggested the disappearance of an isoprenyl unit in comparison with that of **3**. The analyses of DQF-COSY and HMBC spectra of **1** revealed the presence of 2,3,4-trisubstituted phenol and 2,3-disubstituted phenol ring moieties (Figure 2C). $^1H-^{13}C$ long-range couplings from methylene protons H-1′ (δ_H 3.88) to aromatic carbons C-3 (δ_C 131.3), C-4 (δ_C 129.7), and C-4a (δ_C 142.5) and from an aromatic proton H-3 (δ_H 7.55) to the methylene carbon C-1′ (δ_C 29.4) and C-4a confirmed that this dimethylallyl unit was substituted at C-4. A highly downfield shifted hydrogen-bonded phenolic hydroxy proton, 1-OH (δ_H 13.45), which in turn was $^1H-^{13}C$ long-range coupled to C-1 (δ_C 150.3), C-2 (δ_C 112.7), and C-10a (δ_C 125.8), suggested the presence of an *N*-oxide group at N-10, as observed for **3**. Thus, the structure of **1** was determined to be 4,9-dihydroxy-1-(3-methylbut-2-enyl)phenazine 10-oxide, as shown in Figure 2A.

By taking into consideration the number of carbon signals and molecular formula, **2** should be a symmetric compound. The analyses of DQF-COSY and HMBC spectra revealed the presence of a *p*-isoprenylphenol moiety (Figure 2D). To distinguish the positions of the dimethylallyl moieties (4,6- or 4,9-) on the phenazine-diol skeleton, an NOE experiment was conducted for **2**. An NOE correlation between 1-OH (δ_H 8.08) and 1″-H (δ_H 3.96) was observed. Therefore, the structure of **2** was confirmed as 4,9-bis(3-methylbut-2-enyl)phenazine-1,6-diol (Figure 2A).

To establish that **1**−**3** are synthesized via the MVA pathway, we conducted a feeding experiment for **1**−**3** with [1-^{13}C]acetate, which should be incorporated at C-1′ (C-1″) and C-3′ (C-3″) of IPP via the MVA pathway (Figure 3A). The ^{13}C NMR spectrum of **1** was not observed because only a low amount (approximately 0.1 mg) of **1** was isolated from the culture for the feeding experiment (data not shown). The ^{13}C NMR spectra of nonlabeled and ^{13}C-labeled **2** (1.2 mg of each in 250 μL of $CDCl_3$) were measured under identical conditions (150 MHz, 25 °C, 15 000 scans, acquisition time of 0.45 s, relaxation delay of 3.0 s, pulse width of 45°). The intact incorporation of [1-^{13}C]acetate into the dimethylallyl

Table 1. Physicochemical Properties

	1	**2**	**3**
appearance	red, amorphous solid	orange, amorphous solid	red, amorphous solid
$[\alpha]^{25}_D$	−49.5 (*c* 0.10, MeOH)	not applicable	−46.9 (*c* 0.10, MeOH)
molecular formula	$C_{17}H_{16}N_2O_3$	$C_{22}H_{24}N_2O_2$	$C_{22}H_{24}N_2O_3$
HR-ESIMS			
found $[M + H]^+$	297.1244	349.1924	365.1869
calcd	297.1239	349.1916	365.1865
UV λ_{max} (MeOH) nm (ϵ)	283 (118,100)	278 (108,200)	287 (111,000)
	507 (5060)	373 (3370)	510 (4650)
		460 (3460)	
IR ν_{max} (KBr) cm^{-1}	3410, 1211, 794	3390, 1234, 829	3401, 1253, 794

Table 2. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Data

	1		**2**		**3**	
no.	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]
1	150.3		149.9		150.6	
2	112.7	7.04 (d, 7.9)	108.9	7.18 (d, 7.6)	112.5	6.99 (d, 7.9)
3	131.3	7.55 (d, 7.9)	129.7	7.56 (d, 7.6)	131.2	7.52 (d, 7.9)
4	129.7		130.2		129.2	
4a	142.5		139.6		142.0	
5a	135.7		134.6		137.1	
6	152.4		149.9		150.3	
7	110.5	7.25 (d, 7.6)	108.9	7.18 (d, 7.6)	109.8	7.12 (d, 7.9)
8	132.2	7.68 (dd, 9.0, 7.6)	129.7	7.56 (d, 7.6)	132.6	7.40 (d, 7.9)
9	108.4	8.03 (d, 9.0)	130.2		125.0	
9a	132.6		139.6		132.1	
10a	125.8		134.6		126.7	
1′	29.4	3.88 (d, 7.1)	29.1	3.96 (d, 7.0)	29.7	3.85 (d, 7.1)
2′	122.1	5.40 (t, 7.1)	122.6	5.45 (t, 7.0)	122.3	5.39 (t, 7.1)
3′	133.4		133.0		133.2	
4′	25.8	1.77 (s)	25.8	1.76 (s)	25.8	1.76 (s)
5′	17.9	1.81 (s)	17.9	1.83 (s)	17.9	1.81 (s)
1″			29.1	3.96 (d, 7.0)	34.7	4.14 (d, 7.1)
2″			122.6	5.45 (t, 7.0)	123.6	5.43 (t, 7.1)
3″			133.0		132.8	
4″			25.8	1.76 (s)	25.9	1.75 (s)
5″			17.9	1.83 (s)	18.1	1.77 (s)
1-OH		13.45 (br s)		8.08 (s)		13.61 (s)
6-OH				8.08 (s)		8.21 (s)

[a] Integral, multiplicity, and coupling constants in Hz are given in parentheses.

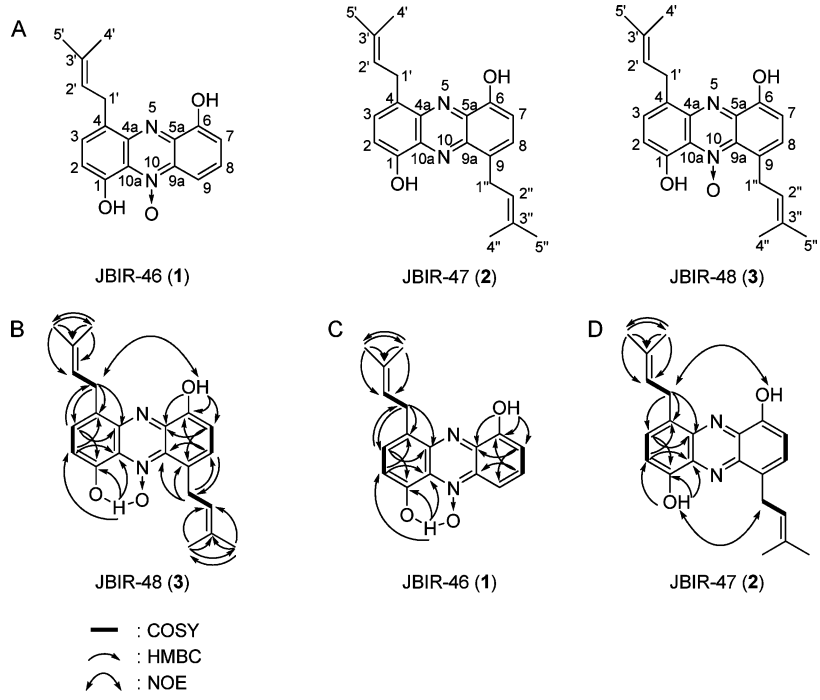

Figure 2. (A) Structures of **1**−**3**. (B) Key correlations observed in DQF-COSY, HMBC, and NOE spectra of **3**: $^{1}H-^{1}H$ (bold lines), $^{1}H-^{13}C$ (single-headed arrows), and NOE (double-headed arrows). (C) Key correlations observed in DQF-COY and HMBC spectra of **1**. (D) Key correlations observed in DQF-COSY, HMBC, and NOE spectra of **2**.

moiety of **2** was indicated by a ^{13}C abundance of 2.2% for C-1′ and C-1″ and 2.7% for C-3′ and C-3″, while no signal enhancement was observed for other carbon resonances of the dimethylallyl moiety (Figure 3). Similarly, [1-^{13}C]acetate incorporations at C-1′ (1.8%), C-3′ (2.6%), C-1″ (2.4%), and C-3″ (2.4%) were observed in **3**. No resonance increase was observed for phenazine moieties in both **2** and **3**. The labeling pattern derived from [1-^{13}C]acetate indicated that **2** and **3** are clearly biosynthesized via the MVA pathway. Detailed study of the biosynthesis pathway requires further genetic experiments.

Experimental Section

General Experimental Procedures. Optical rotation was operated on a Horiba SEPA-300 polarimeter. HRESIMS data were recorded on a Waters LCT-Premier XE mass spectrometer. UV spectra were measured on a Beckman Coluter DU730 UV/vis spectrophotometer. FT-IR spectra were obtained using a Horiba FT-720 spectrophotometer. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR spectra were recorded on a Varian NMR System 600 NB CL. Samples were measured in $CDCl_3$, and the solvent peak was used for spectra calibration (δ_H 7.25 and δ_C 77.0 ppm). Normal-phase medium-pressure liquid chromatography

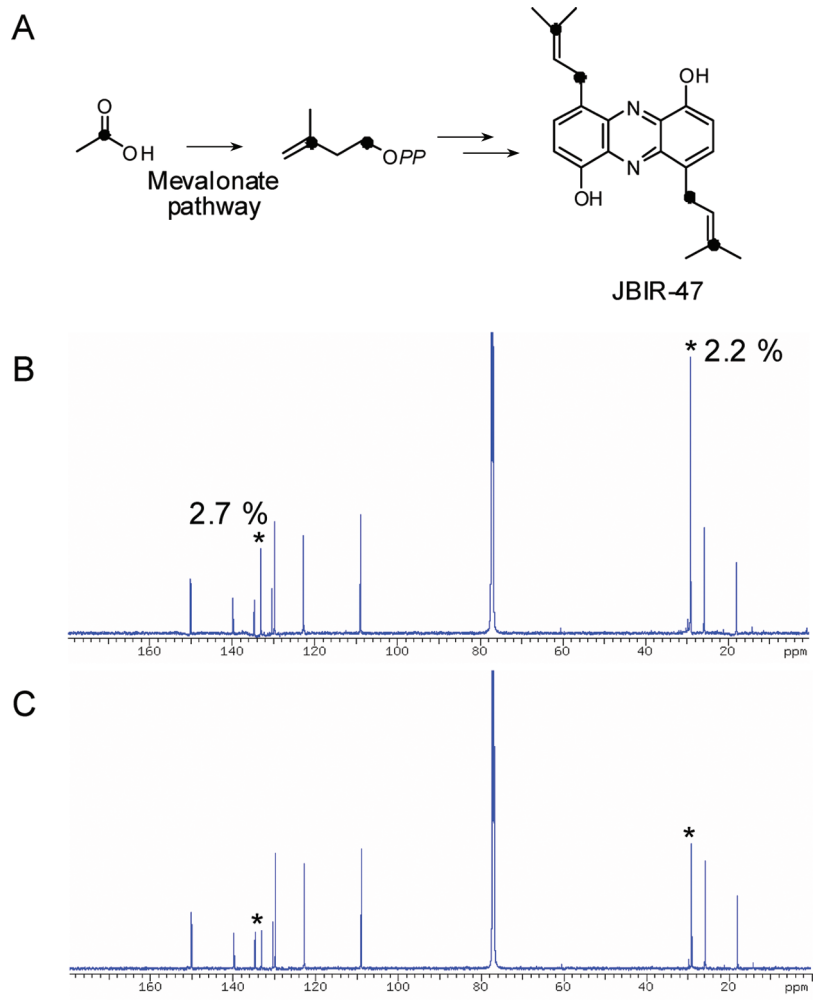

Figure 3. (A) Isoprene units in **2** synthesized via the MVA pathway. (B) ^{13}C NMR spectrum obtained after feeding of [1-^{13}C]acetate with the enrichments (%) indicated. (C) ^{13}C NMR spectrum of the unlabeled standard.

(MPLC) was performed on a Purif-Pack SI-60 column (Moritex, Tokyo, Japan). Analytical reversed-phase HPLC was carried out using an L-column2 ODS column (4.6 i.d. × 150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) with a 2996 photodiode array detector (Waters) and a 3100 mass detector (Waters). Preparative reversed-phase HPLC was carried out using an L-column2 ODS column (20 i.d. × 150 mm) with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. Reagents and solvents were of the highest grade available.

Isolation of *Streptomyces* sp. SpC080624SC-11. A marine sponge, *Cinachyra* sp., was collected from the sea shore at Nagura Bay, Ishigaki, Okinawa Prefecture, Japan. A 20 g wet weight of sponge was ground using a mortar and pestle. These pieces were suspended in 30 mL of sterile seawater, and the suspension was spread on starch casein nitrate agar plates[23] containing 10.0 g starch, 0.3 g casein, 2.0 g KNO_3, 2.0 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 16.0 g agar. The isolated strain was maintained on ISP-2 medium (ISP-2M, ISP2; International *Streptomyces* Project)[24] prepared in 50% artificial seawater (Sealife, Marinetech, Tokyo, Japan).

Determination of HMGR Gene. The HMGR gene was amplified using primers (pHMGF, 5′-GGGCATCGCCGCGGACCCTCCTCG-ACGAGCG-3′, and pHMGR, 5′-GCGATGACGGCGAGGCGGCG-GGCGTTCTC-3′), and PCR parameters (4 min at 95 °C for primary denaturation, 30 cycles consisting of 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C, and 10 min at 72 °C for extension).[25] Amplified fragments were purified using a QIAquick PCR purification kit (Qiagen, CA), and purified PCR fragments were cloned using a TOPO TA cloning kit (Invitrogen, CA) according to the instructions of the manufacturer. Cloned fragments were then sequenced using plasmid-based M13 primers.

RT-PCR. Total RNA was extracted from cells cultured for 48 h at 28 °C using the modified Kirby mix protocol.[26] For reverse transcriptase reaction (RT-reaction), a SMART Scribe reverse transcriptase kit (Clonetech, Mountain View, Canada) was used. The reaction was carried out at 50 °C for 30 min using 0.2 μg of total RNA template and the above-mentioned primers. The HMGR gene from the RT-reaction products was amplified according to the protocol described above.

Fermentation. The seed medium was composed of 1% starch, 1% polypepton, 1% molasses, 1% meat extract, and 1.75% Sealife (pH 7.2 before sterilization). The production medium consisted of 2% glycerol, 1% molasses, 0.5% casein, 0.1% polypepton, 0.4% $CaCO_3$, 1% HP-20 (Mitsubishi Chemical, Tokyo, Japan), and 1.75% Sealife (pH 7.2 before sterilization). Strain SpC080624SC-11 was cultivated in 50 mL test tubes containing 15 mL of the seed medium. The test tubes were shaken on a reciprocal shaker (355 rpm) at 27 °C for 3 days. Aliquots (5 mL) of the culture were transferred to 500 mL Erlenmeyer flasks containing 100 mL of the production medium and cultured on a rotary shaker (180 rpm) at 27 °C for 5 days.

Purification of 1−3. The fermentation broth (2 L) of SpC080624SC-11 was centrifuged, and the collected mycelial cake was extracted with acetone (500 mL). The extract was concentrated *in vacuo*, and the residual aqueous concentrate was extracted with *n*-hexane and evaporated to dryness. The dried residue (58 mg) was separated by preparative TLC (silica gel 60; Merck, NJ) using *n*-hexane−EtOAc (5% EtOAc). A fraction (R_f value 0.5, 18.0 mg) was subjected to preparative reversed-phase HPLC using an L-column2 ODS column (20 i.d. × 150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) with a photodiode array detector and a mass detector developed with 90% aqueous MeOH containing 0.1% formic acid (flow rate 10 mL/min) to yield **1** (2.0 mg, retention time t_R = 11.9 min). Another fraction (R_f value 0.8, 6.2 mg) was purified using preparative reversed-phase HPLC with 95% aqueous MeOH containing 0.1% formic acid to yield **2** (2.1 mg, t_R = 14.4 min) and **3** (2.4 mg, t_R = 21.0 min).

Feeding Experiments. Strain SpC080624SC-11 was grown at 27 °C in 500 mL Erlenmeyer flasks containing 50 mL of medium consisting of 2% glycerol, 1% molasses, 0.5% casein, 0.1% polypepton, and 0.4% $CaCO_3$. Sodium [1-^{13}C]acetate (0.1%) was added at 30 h after the inoculation of the seed culture. The fermentation broth (1 L) was centrifuged, and the collected mycelial cake was extracted with acetone (250 mL). The extract was concentrated *in vacuo*, and the residual aqueous concentrate was extracted with EtOAc and evaporated to dryness. The dried residue (251 mg) was chromatographed by normal-phase MPLC using an *n*-hexane−EtOAc stepwise system (0%, 5%, and 10% EtOAc). The eluate (5% EtOAc, 84 mg) was rechromatographed by normal-phase MPLC with a $CHCl_3$−MeOH−NH_4OH system (200:20:1). The fraction including **2** and **3** (32 mg) was subjected to preparative reversed-phase HPLC using an L-column2 ODS column developed with 93% aqueous MeOH containing 0.1% formic acid (flow rate 10 mL/min) to yield **2** (5.9 mg, t_R = 14.2 min) and **3** (6.3 mg, t_R = 25.6 min). Relative enrichments for all carbon atoms of the labeled **2** and **3** were obtained by comparison of ^{13}C NMR integrals with those of natural-abundance standards. The resonance intensities at 108.9 ppm in the ^{13}C NMR spectra for these compounds from the culture in the presence or absence of sodium [1-^{13}C]acetate were aligned. The signals were assigned to C-2 and C-7 in **2** and **3**. These signals were used as standards for the evaluation of isotopic abundance.

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Supporting Information Available: Spectroscopic methods, HR-ESIMS, and ^{1}H and ^{13}C NMR, DQF-COSY, CT-HMBC, and NOE spectra of **1**−**3** are available free of charge via the Internet at http://pubs.acs.org.

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