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NOTE

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Studies on Terpenoids Produced by Actinomycetes

5-Dimethylallylindole-3-carboxylic Acid and A80915G-8″-acid Produced by Marine-derived *Streptomyces* sp. MS239

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Abstract As a result of screening for terpenoids produced by marine-derived *Streptomyces* sp. MS239, two new terpenoids named 5-dimethylallylindole-3-carboxylic acid and A80915G-8″-acid were isolated and their structures were determined mainly by NMR analyses.

Keywords terpenoid, *Streptomyces* sp. MS239, 5-dimethylallylindole-3-carboxylic acid, A80915G-8″-acid

All Actinomycetes including *Streptomycetes* utilize only the MEP (2-*C*-methyl-D-erythritol-4-phosphate) pathway for the formation of the primary metabolite IPP (isopentenyl diphosphate), the starting material for the biosynthesis of terpenoids and steroids. On the other hand, some Actinomycetes strains are equipped with the mevalonate pathway as well, and use it for the production of terpenoids as secondary metabolites [1]. For example, terpentecin, BE-40644, napyradiomycin, naphterpin, furaquinocin, oxaloterpin, furanonaphthoquinone and endophenazine were biosynthesized via the mevalonate pathway (for structures, see references [2, 3]). Since these terpenoids show interesting biological properties such as antitumor, antibacterial, or antioxidative activities, novel terpenoids produced by Actinomycetes were expected to be promising candidates for drug discovery.

Marine microorganisms, marine Actinomycetes in particular, have been studied as one of the most important resources for new biologically active metabolites [4]. Marine organism-derived terpenoids, marinone [5], neomarinone [6], BU-4664L (identical with diazepinomicin) [7, 8], glaciapyrroles [9], prenylated naphthoquinones [10], (5*S*,8*S*,9*R*,10*S*)-selina-4(14),7(11)-diene-8,9-diol [11], azamerone [12] and amorphane sesquiterpenes [13] were products of Actinomycetes. These terpenoids showed antitumor and/or antibacterial properties. Stimulated by these findings, we carried out a screening of terpenoids from marine Actinomyces equipped with the mevalonate pathway.

In previous papers, we reported isolation of several terpenoids produced by *Streptomyces* species that possessed the mevalonate pathway genes [2, 3]. As a result of further screening for *Streptomyces* strains possessing the mevalonate pathway genes, we selected *Streptomyces* sp. MS239 that gave a positive result to PCR using the HMG-CoA reductase gene as a probe [14]. Detailed investigation of the metabolites of this strain resulted in isolation of two new terpenoids named 5-dimethylallylindole-3-carboxylic acid (**1**) and A80915G-8″-acid (**2**) (Fig. 1), together with the known compounds A80195G [15], napyradiomycins A1 and B1 [16], and naphthomevalin [17].

Streptomyces sp. MS239 was cultured at 28°C for 7 days by rotary shaking in 500-ml baffled Erlenmeyer flasks

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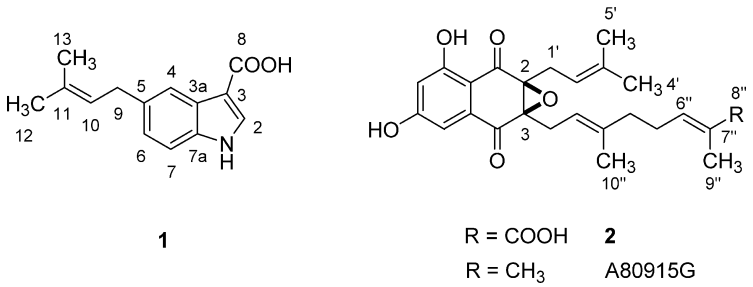

Fig. 1 Structures of 5-dimethylallylindole-3-carboxylic acid (**1**), A80915G-8″-acid (**2**) and A80915G.

Table 1 Physicochemical properties of 5-dimethylallylindole-3-carboxylic acid (**1**) and A80915G-8″-acid (**2**)

	1	**2**
Appearance	Colorless oil	Yellow oil
$[\alpha]_D^{25}$		+54° (*c* 0.085 $CHCl_3$)
Molecular formula	$C_{14}H_{15}NO_2$	$C_{25}H_{28}O_7$
HRESITOF-MS (*m/z*)	Found: 230.1175	Found: 441.1907
	Calcd: 230.1176 $(M+H)^+$	Calcd: 441.1907 $(M+H)^+$
UV λ_{max} nm (ε) (MeOH)	285 (7900), 221 (16200)	363 (4300), 266 (9900), 248 (12000)
IR (KBr) cm^{-1}	1680	1690, 1640

containing 100 ml of culture medium. The filtrate prepared from the fermentation broth was extracted with EtOAc and the residue obtained after removal of the solvent was analyzed by TLC developed with *n*-hexane - EtOAc = 1 : 1 or $CHCl_3$ - MeOH = 20 : 1 followed by staining with vanillin - H_2SO_4. Spots that changed to bright purple or violet were selected as candidates for terpenoids, and semi-preparative purification of these spots were carried out by silica gel column chromatography and reversed-phase C-18 HPLC. The samples thus prepared were subjected to ^{1}H-NMR analysis and fractions showing methyl proton singlets at around δ_H 1.0 in their ^{1}H-NMR spectra were assumed to contain terpenoids. As a result of this screening, two fractions showing 2∼4 methyl proton singlets at around δ_H 1.0 in their ^{1}H-NMR spectra were selected as plausible terpenoids. NMR studies on these components including COSY, HSQC and HMBC, HR-MS proved them to be terpenoids with structures **1** and **2**. Their physicochemical properties are summarized in Table 1.

The molecular formula of **1** was defined as $C_{14}H_{15}NO_2$ based on the HRESITOF mass spectrum which showed the molecular ions at *m/z* 230.1175 $[M+H]^+$ and 252.1005 $[M+Na]^+$. The ^{13}C-NMR spectrum of **1** displayed 14 signals consisting of one carbonyl carbon, five quaternary sp^2 carbons, five protonated sp^2 carbons, one methylene carbon and two methyl carbons (Table 2). Further structural information on **1** was obtained by analyzing HSQC, HMBC and COSY spectra (Fig. 2).

Singlet methyl protons H-12 and H-13 (δ_H 1.74 and 1.76) showed ^{1}H-^{13}C long range couplings to an olefinic methine carbon (C-10, δ_C 124.1) and a quaternary sp^2 carbon (C-11, δ_C 131.9) in the HMBC spectrum. These data together with the proton spin system between the methylene H-9 (δ_H 3.46) and methine H-10 (δ_H 5.38) revealed by the COSY spectrum proved the presence of a dimethylallyl moiety.

The remaining signals of **1** were as follows; one carbonyl carbon at δ_C 170.6 (C-8), four quaternary sp^2 carbons at δ_C 136.2 (C-5), 134.7 (C-7a), 126.2 (C-3a) and 107.8 (C-3), four protonated sp^2 carbons at δ_C 132.4 (C-2), 124.2 (C-6), 120.7 (C-4) and 111.4 (C-7). The ^{13}C-NMR spectrum displayed eight aromatic carbon signals, and the pattern of the aromatic region in the ^{1}H-NMR spectrum showed the presence of *ortho*-coupled protons δ_H 7.32 (H-7) and 7.11 (H-6), a broad singlet proton δ_H 8.03 (H-4), a doublet proton δ_H 7.97 (H-2), and a broad singlet at δ_H 8.58 corresponding to an NH signal. The relation between H-2 and NH was confirmed by the COSY spectrum. These data suggested the presence of a disubstituted indole nucleus.

HMBC correlations from H-10 to C-5, from H-9 to C-4,

C-5 and C-6, from H-6 to C-7a, from H-4 to C-3, C-6 and C-7a indicated that the dimethylallyl residue was attached to the indole nucleus at C-5. Finally, the sp^2 proton H-2 was coupled to four aromatic carbons at C-3, C-3a, C-7a and C-8 in the HMBC spectrum. The carbonyl carbon at C-3 was assigned to a carboxylic acid based on the molecular formula of **1**.

The ^{13}C-NMR data of the indole-3-carboxylic acid moiety in **1** was in good agreement with those of an authentic sample except for C-5 and C-8 (Table 2). Difference of the ^{13}C chemical shifts of C-8 in **1** ($CDCl_3$) and indole-3-carboxylic acid (DMSO-d_6) may be ascribed to different solvents used for these two compounds. The structure of **1** is similar to 6-isoprenylindole-3-carboxylic acid (for NMR data, see Table 2) isolated from *Colletotrichum* sp., an endophytic fungus in *Artemisia annua* [18]. They differ only at the position of the dimethylallyl substituent on the aromatic ring. In view of the structural similarity between these two compounds, the C-3 chemical shift reported for 6-isoprenylindole-3-carboxylic acid may be an error.

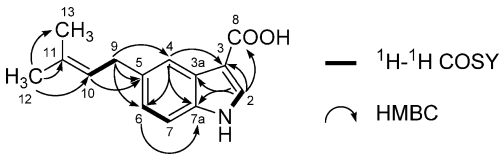

Fig. 2 HMBC and COSY correlations observed for 5-dimethylallylindole-3-carboxylic acid (**1**).

A80915G-8″-acid (**2**) was isolated as an optically active yellow oil ($[\alpha]_D$ +54, *c* 0.085, $CHCl_3$). The molecular formula of **2** was determined to be $C_{25}H_{28}O_7$ by the HRESITOF mass spectrum, which showed the molecular ions at *m/z* 441.1907 $[M+H]^+$ and 463.1635 $[M+Na]^+$.

Most of the NMR spectral data for **2** were very similar to those of synthetic (±)-A80915G [19] expect for C-6″, C-7″, C-8″ and C-9″ (Table 3). In the ^{13}C-NMR spectrum of (±)-A80915G, however, the methyl carbon (δ_C 25.5 for C-8″) was replaced by a quaternary carbon (δ_C 173.9 for C-8″) in **2**. Thus, the structure of **2** was proved to be a carboxylic acid derivative of A80915G at the 8″ position. The geometry of the C-6″ and C-7″ double bond in **2** was established to be (*E*) based on the ^{13}C chemical shift of the methyl signal appearing at a higher field (δ_C 12.2) due to the γ-effect.

The absolute configurations at C-2 and C-3 of **2** were established to be as shown in Fig. 1 based on its same positive optical rotation value as that of A80915G [17]. [for A80915G: $[\alpha]_D^{25}$ +98.7 (*c* 0.2, $CHCl_3$)].

A80915G-8″-acid showed weak antibacterial activity against *Bacillus subtilis* ATCC6633 (64 μg/ml) and

Table 2 NMR spectral data of 5-dimethylallylindole-3-carboxylic acid (**1**) and 6-dimethylindole-3-carboxylic acid in $CDCl_3$ and indole-3-carboxylic acid in DMSO-d_6

No.	**1**		6-Dimethylallylindole-3-carboxylic acid [18]		Indole-3-carboxylic acid
	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C
2	132.4, (d)	7.97, d (2.8)	131.7, (d)	7.97, d (2.7)	132.9, (d)
3	107.8, (s)		124.1, (s)		107.7, (s)
3a	126.2, (s)		129.0, (s)		126.3, (s)
4	120.7, (d)	8.03, br s	121.4, (d)	8.13, d (8.0)	121.0, (d)[a]
5	136.2, (s)		121.5. (d)	7.16, br d (8.0)	121.7, (d)[a]
6	124.2, (d)	7.11, d (8.4)	137.5, (s)		122.8, (d)[a]
7	111.4, (d)	7.32, d (8.4)	110.6, (d)	7.24, br s	112.8, (d)
7a	134.7, (s)		137.5, (s)		132.9, (s)
8	170.6, (s)		—[b]		166.7, (s)
9	34.6, (t)	3.46, d (7.2)	34.4, (t)	3.48, d (7.6)	
10	124.1, (d)	5.38, t (7.2)	123.5, (d)	5.39, t (7.6)	
11	131.9, (s)		132.5, (s)		
12	25.8, (q)	1.74, s	25.8, (q)	1.78, s	
13	17.8, (q)	1.76, s	17.9, (q)	1.77, s	
NH		8.58, br s		8.54, br s	

[a] May be exchangeable. [b] Not reported.

Table 3 NMR spectral data of A80915G-8″-acid (**2**) in $CDCl_3$

Position	^{13}C	^{1}H (*J* in Hz)	Position	^{13}C	^{1}H (*J* in Hz)
1	194.8 (s)		4′	25.9 (q)	1.66, s
2	67.7 (s)		5′	18.2 (q)	1.67, s
3	67.0 (s)		1″	25.8 (t)	2.74, dd (14.9, 6.6),
4	191.8 (s)				2.62, dd (14.9, 6.6)
4a	133.9 (s)		2″	116.5 (d)	5.18, t (6.6)
5	107.8 (d)	6.94, d (2.0)	3″	136.5 (s)	
6	163.7 (s)		4″	37.7 (t)	2.17, m
7	108.6 (d)	6.55, d (2.0)	5″	26.3 (t)	2.32, m
8	164.3 (s)		6″	144.9 (d)	6.73, t (6.6)
8a	108.6 (s)		7″	126.7 (s)	
1′	25.2 (t)	3.28, dd (15.1, 6.6),	8″	173.9 (s)	
		2.32, m	9″	12.2 (q)	1.76, s
2′	116.5 (d)	5.04, t (6.6)	10″	16.2 (q)	1.71, s
3′	135.4 (s)		8-OH		11.70, s

6-OH proton was not observed.

was inactive to *Staphylococcus aureus* 209P JC-1 (MIC >64 μg/ml) and *Enterococcus faecium* ATCC19434 (>64 μg/ml). [Mueller Hinton agar medium (Difco)]. Although 6-isoprenylindole-3-carboxylic acid was reported to show weak antibacterial activity against *Bacillus subtilis* (25 μg/ml) [18], 5-dimethylallylindole-3-carboxylic acid did show not antibacterial activity (MIC >64 μg/ml).

Experimental

General Experimental Procedures

Optical rotation was recorded on a JASCO DIP-140 polarimeter. UV and IR spectra were recorded on a HITACHI U-3310 spectrophotometer, and a Shimadzu 8300 FTIR spectrometer, respectively. Both 1D ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL Alpha 400 NMR spectrometer. Two-dimensional ^{1}H-^{1}H COSY, NOESY, ^{1}H-^{13}C HSQC and HMBC spectra were recorded on a Varian Inova 500 NMR spectrometer. Samples were dissolved in $CDCl_3$ or DMSO-d_6 and the solvent peak was used for internal standard (δ_H 7.24 and δ_C 77.0, for $CDCl_3$), (δ_C 39.5, for DMSO-d_6). High-resolution ESI TOF mass spectra were obtained using an Applied Biosystems MDS SCIEX Q-STAR LC-MS. HPLC purifications were carried out using a Senshu Pak PEGASIL ODS column (20 i.d.×250 mm, at flow rate of 14.0 ml/minute) equipped with a Hitachi High Technologies L-2450 diode array detector. Merck silica gel 60 F_{254} plastic-backed sheets were used for TLC analysis.

Producing Organism and Phylogenetic Analysis

The producing organism MS239 was isolated from saline mud collected at Tokushima, Japan. The 16S rRNA gene of the strain was compared with the bacterial sequence data stored in DDBJ database by using BLAST algorithm. The 16S rRNA sequencing of this strain revealed high sequence identity with *Streptomyces chromofuscus* NRRL B-12175 (99.8%), *Streptomyces variegatus* LMG 20315 (99.8%), *Streptomyces brasiliensis* NBRC 101283 (99.8%). From these characteristics, the strain MS239 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. MS239.

Fermentation, Extraction and Isolation

Streptomyces sp. MS239 was inoculated into a 15 ml test tube containing 5.0 ml of a preliminary seed medium consisting of soluble starch 1.0%, polypeptone 1.0% (Nihon Seiyaku Co.), molasses 1.0% (Oji Seito) and meat extract 1.0% ('Lab-Lemco' powder, Oxoid Ltd.) (pH 7.2) and was cultured at 28°C for 3 days on a rotary shaker at 170 rpm. One milliliter of the seed culture was inoculated in 500-ml Erlenmeyer flasks containing 100 ml of the medium consisting of starch 2.5%, soybean meal 1.5% (Nisshin Oillio Group Ltd.), dry yeast 0.2% (Ebiosu, Tanabe Seiyaku Co.) and $CaCO_3$ 0.4%, pH 6.2. After 7 days of the productive fermentation at 28°C, the broth was separated into mycelial cake and filtrate by suction filtration. The mycelial cake was extracted with 60% Me_2CO and the extract was evaporated *in vacuo* to remove the acetone, and the aqueous residue was extracted with

EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to remove the EtOAc. The filtrate was extracted with an equal amount of EtOAc. The organic layer was separately dried over anhydrous Na_2SO_4 and evaporated to remove the EtOAc.

The combined EtOAc extract was subjected to silica gel column chromatography developed with *n*-hexane - EtOAc=1 : 1. Fractions containing **2** and the known compounds, A80915G, napyradiomycin A1, B1, and naphthomevalin were eluted first being separated from fractions containing **1**. After washing the column with the same solvent, the column was further eluted with EtOAc - MeOH=3 : 1 to give **1**. TLC plates (developed with $CHCl_3$ - MeOH=20 : 1) were visualized by UV light and color reaction with vanillin - H_2SO_4 . Fractions containing **2** and the known compounds (Rf 0.5～0.9) were purified by ODS HPLC (20×250 mm, Senshu Pak PEGASIL ODS) with a PDA detector eluted with CH_3CN in H_2O (70%) at a flow rate of 14 ml/minute to yield **2** (0.1 mg/liter, 14.2 minutes), naphthomevalin (6.5 minutes), napyradiomycin A1 (10.5 minutes), napyradiomycin B1 (11.1 minutes) and A80915G (11.8 minutes).

The combined fraction containing **1** (Rf 0.3, $CHCl_3$ - MeOH=20 : 1, silica gel TLC) was purified by ODS HPLC column chromatography. The column was eluted with CH_3CN in H_2O (50%) containing 0.1% HCOOH at a flow rate of 14 ml/minute to give a pure sample of **1** (0.2 mg/liter, 14.1 minutes).

Antibacterial Activity

According to the CLSI recommendation [20], MIC were determined by the agar dilution method with 2-fold serial dilutions of antibiotics.

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