Notes

Tetrapeptides Possessing a Unique Skeleton, JBIR-34 and JBIR-35, Isolated from a Sponge-Derived Actinomycete, *Streptomyces* sp. Sp080513GE-23

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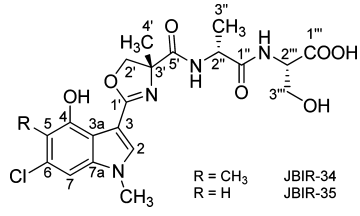
Received December 14, 2009

Two new modified indole-containing peptides, JBIR-34 (**1**) and JBIR-35 (**2**), were isolated from the fermentation broth of a sponge-derived actinomycete identified by phylogenetic methods as a *Streptomyces* sp. (strain Sp080513GE-23). The planar structures of **1** and **2** were assigned on the basis of 1D and 2D NMR spectroscopy and MS analyses. Further, the absolute configurations of the amino acid residues were determined using Marfey's method.

Marine microorganisms, particularly marine actinomycetes, have attracted considerable attention as some of the most important resources for new biologically active metabolites.[1] Of interest to us, new compounds have been isolated from actinomycetes of sponge origin.[2−5] There is much controversy, however, about whether new species of *Streptomyces* can frequently produce novel compounds. Thus, our intention was to support the idea that new species are cable of producing unique metabolites. For this purpose, we isolated novel species of *Streptomyces* from a marine sponge, *Haliclona* sp., which is inhabited by diverse actinomycetes, and then searched for secondary metabolites in the cultures of the isolated strains. *Streptomyces* sp. Sp080513GE-23 isolated from the sponge was identified as a new species of the genus *Streptomyces* by performing a homology search of its 16S rRNA gene sequence and was found to possess several unique nonribosomal peptide synthetase (NRPS) genes by screening for biosynthetic genes of secondary metabolites.[6] The presence of these biosynthetic genes suggested that this strain produces nonribosomal peptides. In the course of our screening program for novel metabolites of Sp080513GE-23, we succeeded in isolating two peptides with unique skeletons from the culture broth, designated as JBIR-34 (**1**) and JBIR-35 (**2**). This paper describes the fermentation, isolation, and structure elucidation of **1** and **2**.



Strain Sp080513GE-23 was cultured at 27 °C on a rotary shaker. The supernatant of a fermentation broth (1 L) was successively partitioned with EtOAc and *n*-BuOH. The *n*-BuOH layer was evaporated to dryness, and the dried residue (563.4 mg) was subjected to repeated reversed-phase chromatography to yield **1** (0.39 mg) and **2** (0.44 mg).

Compound **1** was isolated as a colorless oil. The molecular formula ($C_{21}H_{25}ClN_4O_7$) of **1** was obtained from its HRESIMS spectrum, which showed a molecular ion at *m*/*z* 481.1492 [M + H]$^+$.

The peptide structure of **1** was evident from the ^{1}H and ^{13}C NMR data, recorded in DMSO-d_6, as shown in Table 1. Diagnostic resonances for three carbonyl carbons (δ_C 172.9, 172.0, and 172.0) and methine resonances for two α-amino acids (δ_C/δ_H 54.9/3.86 and 48.9/4.35) indicated the peptide structure of **1**. Absorptions at 3430 and 1640 cm^{-1} in the IR spectrum of **1** were also characteristic of hydroxy, amide, and carbonyl functional groups.

Further structural information of **1** was obtained by analyzing its DQF-COSY, HSQC, and HMBC spectra (Figure 1). The ^{13}C NMR spectrum showed eight aromatic carbon signals representing six quaternary sp^2 carbons, C-3 (δ_C 101.4), C-3a (δ_C 113.9), C-4 (δ_C 150.3), C-5 (δ_C 113.0), C-6 (δ_C 131.0), and C-7a (δ_C 137.6), and two protonated sp^2 carbons, C-2 (δ_C 134.1) and C-7 (δ_C 102.4). The ^{1}H−^{13}C long-range couplings from the singlet methyl protons CH_3-N (δ_H 3.74) to C-2 and C-7a; from a singlet aromatic methine proton H-2 (δ_H 7.89) to C-3, C-3a, and C-7a; from the singlet methyl protons CH_3-5 to C-4, C-5, and C-6; from a phenolic hydroxy proton OH-4 (δ_H 12.90) to C-4 and C-5; and from a singlet aromatic methine proton H-7 (δ_H 7.11) to C-3a, C-5, and C-6 together with the UV absorption at a particular wavelength revealed the presence of a pentasubstituted indole nucleus. Furthermore, ^{1}H−^{13}C long-range couplings from the singlet methyl protons CH_3-4′ (δ_H 1.48) to an oxymethylene carbon C-2′ (δ_C 77.9), a quaternary carbon C-3′ (δ_C 73.1), and a carbonyl carbon C-5′ (δ_C 172.9) and from doublet methylene protons H-2′ (δ_H 4.62 and 4.36) to a quaternary sp^2 carbon C-1′ (δ_C 164.7), a methyl carbon C-4′ (δ_C 25.9), C-3′, and C-5′ suggested the presence of a trisubstituted oxazoline moiety. The long-range coupling between H-2 and C-1′ revealed that C-1′ in the oxazoline moiety was connected to the indole moiety at the position of C-3. Similarly, Ser and Ala residues were confirmed. The connectivity of these amino acid residues was established by ^{1}H−^{13}C long-range couplings from an α-methine proton H-2″ (δ_H 4.35), an amide amine proton NH-2″ (δ_H 7.66), H-2′, and H-4′ to an amide carbonyl carbon C-5′; from a broad singlet amide amino proton NH-2‴ (δ_H 7.82), methyl protons H-3″

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10.1021/np900810r
Published on Web 02/10/2010

Table 1. ^{13}C (125 MHz) and ^{1}H NMR (500 MHz) Spectroscopic Data for **1** and **2** (DMSO-d_6)

	JBIR-34 (**1**)		JBIR-35 (**2**)	
position	^{13}C	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)
indole				
2	134.1, CH	7.89, s	134.1, CH	7.92, s
3	101.4, C		101.9, C	
3a	113.9, C		114.0, C	
4	150.3, C		152.8, C	
5	113.0, C		107.5, CH	6.48, s
6	131.0, C		129.9, C	
7	102.4, CH	7.11, s	102.3, CH	7.08, s
7a	137.6, C		140.0, C	
CH_3-N	33.9, CH_3	3.74, s	34.1, CH_3	3.77, s
OH-4		12.90, s		12.82, s
CH_3-5	13.1, CH_3	2.22, s		
oxazoline				
1′	164.7, C		164.6, C	
2′	77.9, CH_2	4.62, d (8.6), 4.36[a]	77.9, CH_2	4.61, d (8.7), 4.38, d (8.7)
3′	73.1, C		73.0, C	
4′	25.9, CH_3	1.48, s	26.0, CH_3	1.48, s
5′	172.9, C		172.9, C	
Ala				
1″	172.0, C		172.4, C	
2″	48.9, CH	4.35[a]	48.7, CH	4.39, m
3″	19.2, CH_3	1.23, d (7.1)	19.4, CH_3	1.25, d (7.3)
NH-2″		7.66, d (7.4)		7.66, d (7.5)
Ser				
1‴	172.0, C		172.5, C	
2‴	54.9, CH	3.86, m	55.2, CH	4.19, m
3‴	62.6, CH_2	3.52, 3.35, m	62.1, CH_2	3.62, 3.55, m
NH-2‴		7.82, br s		8.13, d (7.9)

[a] Overlapping.

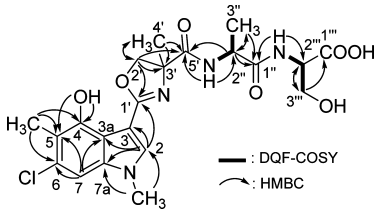

Figure 1. HMBC and DQF-COSY correlations observed for **1**.

(δ_H 1.23), and H-2″ to an amide carbonyl carbon C-1″ (δ_C 172.0); and from NH-2‴ to an α-methine carbon C-2‴ (δ_C 54.9), as shown in Figure 1.

Compound **2** was obtained as a colorless oil. Its molecular formula was determined as $C_{20}H_{23}ClN_4O_7$ on the basis of HRESIMS data (m/z 467.1305 $[M + H]^+$), which showed the disappearance of a methyl unit from **1**. The IR and UV spectra of **2** were similar to those of **1**. Most of the NMR spectroscopic data for **2** were also similar to those of **1** (Table 1). In the ^{1}H NMR spectrum of **2**, however, the singlet signal due to the methyl group at CH_3-5 in **1** was replaced with an aromatic proton (δ_H 6.48). The ^{13}C NMR spectrum of **2** also showed the disappearance of the methyl signal, as was the case with **1**. Thus, the quaternary carbon in **1** was replaced with an aromatic methine carbon (δ_C 107.5 for C-5) in **2**. These collective spectroscopic data proved **2** to be the 5-desmethyl analogue of **1**.

The absolute configuration of **1** was defined by acid hydrolysis and Marfey's method, using standard amino acids (Table 2).[7] The absolute configurations of the Ser and Ala residues were determined as L (2‴*S*) and D (2″*R*), respectively. In addition, acid hydrolysis of the remaining oxazoline residue converted it into α-methylserine. The application of Marfey's method led to the assignment of the absolute configuration of α-methylserine as *R*. Thus, the absolute configuration of the oxazoline residue was established as *R* (Table 2). In the same manner, the absolute configuration of **2** was also defined as 3′*R*, 2″*R*, 2‴*S* by Marfey's method.

Compounds **1** and **2** exhibited DPPH radical scavenging activity with IC_{50} values of 1.0 and 2.5 mM, respectively, which was weak

Table 2. Retention Times for Amino Acids Obtained from **1** and **2** as Their N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide Derivatives

residue	standards retention time (min)	acid hydrolysate of **1** retention time (min)	acid hydrolysate of **2** retention time (min)
L-Ser[a]	20.5	20.5	20.5
D-Ser[a]	22.2		
L-Ala[a]	23.8		
D-Ala[a]	27.1	27.1	27.1
(*S*)-α-MeSer[b]	1.53		
(*R*)-α-MeSer[b]	1.30	1.30	1.30

[a] Ammonium formate (10 mM) mobile phase consisting of 1% MeOH in both A and B with 5% MeCN in A and 60% MeCN in B (linear gradients started with 0% B and finished with 100% B in 45 min, Senshu PAK PEGASIL ODS column, 4.6 i.d. × 250 mm, flow rate of 1.0 mL/min, UV 340 nm). [b] Isocratic mobile phase consisting of 25% MeCN containing 0.1% formic acid (Waters UPLC system, Acquity UPLC BEH C_{18} column, 2.1 i.d. × 50 mm, flow rate of 0.3 mL/min, MS negative ion mode m/z 370).

activity compared with that of α-tocopherol (IC_{50} = 50 μM). In addition, **1** and **2** exhibited no cytotoxic activity against several cancer cell lines and no antibacterial activity against *Micrococcus luteus* and *Escherichia coli*.

In conclusion, we isolated two novel tetrapeptide compounds, **1** and **2**, from the culture broth of a sponge-derived *Streptomyces* sp. Sp080513GE-23. The structures of **1** and **2** possessed a modified indole moiety, a trisubstituted oxazoline moiety, and L-Ser and D-Ala residues. The trisubstituted oxazoline moieties of **1** and **2** are identical to residues in BE-32030 A−E isolated from *Nocardia* sp. A32030.[8] There has been no report of the indole skeleton modified by chlorine, methyl, and hydroxy functional groups such as **1**. The unique structures of **1** and **2**, which contain nonproteinogenic amino acids, may have been biosynthesized by a novel NRPS gene detected in Sp080513GE-23. The results of this study confirm that this sponge contains undiscovered microorganisms that possess the ability to produce new substances. We anticipate that this study will convince chemists that new species of *Streptomyces*

can produce compounds containing unique skeletal structures and also encourage them to investigate such species.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a Horiba SEPA-300 polarimeter. UV and IR spectra were measured on a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were taken on Varian NMR System 500 NB CL in DMSO-d_6 (2.50 ppm for ^{1}H, 39.5 ppm for ^{13}C) with the residual solvent peak as internal standard. HRESIMS data were recorded on a Waters LCT-Premier XE mass spectrometer. Reversed-phase medium-pressure liquid chromatography (MPLC) was performed on a Purif-Pack ODS-100 (Moritex). Analytical reversed-phase HPLC was carried out using an L-column2 ODS (4.6 i.d. × 150 mm; Chemical Evaluation and Research Institute) equipped with a 2996 photodiode array detector (Waters) and a 3100 mass detector (Waters). Analytical reversed-phase UPLC (Waters, ACQUITY) was carried out using a BEH ODS (2.1 i.d. × 50 mm; Waters) equipped with a Waters ACQUITY UPLC photodiode array eλ detector and an LCT-Premier XE mass spectrometer. Preparative reversed-phase HPLC was carried out using an L-column2 ODS (20 i.d. × 150 mm) equipped with a Hitachi High Technologies L-2455 photodiode array detector.

Microorganism. The producing *Streptomyces* designated as Sp080513GE-23 was isolated from a marine sponge, *Haliclona* sp., collected from Tateyama city (E 139°49′; N 34°59′), Chiba Prefecture, Japan. The basic local alignment search tool search of the 16S rRNA gene sequence (GenBank: AB498636) from Sp080513GE-26 showed that this strain belonged to the genus *Streptomyces*.

Fermentation. *Streptomyces* sp. Sp080513GE-23 was cultivated in 15 mL test tubes each containing 5 mL of a preliminary seed medium consisting of starch (Kosoukagakuyakuhin, Tokyo, Japan) 1%, polypeptone (Nihon Pharmaceutical, Tokyo, Japan) 1%, molasses (Dai-Nippon Meiji Sugar, Tokyo, Japan) 1%, and meat extract (Wako, Osaka, Japan) 1% (pH 7.2). The test tubes were shaken on a reciprocal shaker (355 rpm) at 27 °C for 2 days. Aliquots (2.5 mL) of the broth were transferred to 500 mL baffled Erlenmeyer flasks containing 100 mL of a production medium consisting of starch 2.5%, soybean meal (Nisshin Oillio, Tokyo, Japan) 1.5%, dry yeast (Mitsubishi Tanabe Pharma, Osaka, Japan) 0.2%, $CaCO_3$ (Kozaki Pharmaceutical, Tokyo, Japan) 0.4%, and Diaion HP-20 resin (Mitsubishi Chemical, Tokyo, Japan) 1.0% (pH 6.2 before sterilization). The fermentation was carried out on a rotary shaker (180 rpm) at 27 °C for 5 days.

Isolation. The fermentation broth (1 L) was centrifuged, and the supernatant was successively partitioned with EtOAc (1 L × 3) and *n*-BuOH (1 L × 2). The *n*-BuOH layer was evaporated to dryness. The dried residue (563.4 mg) was fractionated by reversed-phase MPLC with a MeOH−water gradient system (0−100% MeOH), and fractions including major metabolites were collected by LC-MS monitoring. This eluate was subjected to preparative reversed-phase HPLC using an L-column2 ODS developed with 65% aqueous MeOH containing 0.1% formic acid to give JBIR-34 (**1**, t_R 19.6 min, 0.39 mg) and JBIR-35 (**2**, t_R 10.5 min, 0.44 mg).

JBIR-34 (1): colorless oil; $[\alpha]^{25}_D$ −140 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 304 (3.83) nm; IR (KBr) ν_{max} 3430, 1640 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see Table 1; HRESIMS *m*/*z* 481.1492 [M + H]$^+$ (calcd for $C_{21}H_{26}ClN_4O_7$, 481.1490).

JBIR-35 (2): colorless oil; $[\alpha]^{25}_D$ −140 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 299 (3.91) nm; IR (KBr) ν_{max} 3430, 1650 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see Table 1; HRESIMS *m*/*z* 467.1305 [M + H]$^+$ (calcd for $C_{20}H_{24}ClN_4O_7$, 467.1334).

Determination of Amino Acids Configuration. A sample of **1** or **2** (1.0 mg) was hydrolyzed in 6 N HCl at 110 °C for 12 h. After concentration to dryness, the residue was dissolved in 10 mL of EtOAc−H_2O (1:1). The amino acid mixture recovered in the aqueous layer was dried *in vacuo*, and 5% $NaHCO_3$ (500 μL) and 0.2 mg of N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in acetone (500 μL) were added. The mixture was heated on a bath at 80 °C for 3 h. The conditions for HPLC analyses and the retention times for standard and hydrolysate FDAA derivatives are provided in Table 2.

DPPH Radical Scavenging Activity. A 96-well multiplate was used for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.[9,10] Compounds **1**, **2**, and α-tocopherol as a positive control were dissolved in MeOH. The 90 μL of 200 μM DPPH dissolved in MeOH and 10 μL of sample were mixed in a microplate. After 1 h incubation at room temperature, the absorbance was measured at 540 nm.

Acknowledgment. This work was supported by a grant from the New Energy and Industrial Technology Department Organization (NEDO) of Japan.

Supporting Information Available: NMR spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP900810R