Isolation of New Hexapeptides—JBIR-39 and JBIR-40—from a Marine Sponge-Derived *Streptomyces* sp. Sp080513SC-24

Ikuko Kozone1, Miko Izumikawa1, Keiichiro Motohashi2, Aya Nagai3, Masahito Yoshida4, Takayuki Doi5, Motoki Takagi6** and Kazuo Shin-ya7**

1Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan
2Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aza-aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan
3Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan

**Corresponding authors: Dr. K Shin-ya, Biomedicinal Information Research Centre (BIRC), Japan National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan, E-mail: k-shinya@aist.go.jp
Dr. M Takagi, Biomedicinal Information Research Centre (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan, E-mail: motoki-takagi@aist.go.jp

Published April 21, 2011


Copyright: © 2011 Kozone I, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

*Streptomyces* sp. Sp080513SC-24 was isolated from a marine sponge, *Haliclona* sp., which is inhabited by diverse *Actinobacteria*, and then its culture was comprehensively searched for secondary metabolites. New two hexapeptides, JBIR-39 (1), and -40 (2), were isolated from the fermentation broth of Sp080513SC-24. The structures of 1 and 2 were elucidated on the basis of 1D and 2D NMR spectroscopy and MS analyses.

Keywords: *Haliclona*; Hexapeptide; Marine sponge; Piperazic acid; *Streptomyces*

Abbreviations: HPLC: High-performance liquid chromatography; MPLC: Medium-performance liquid chromatography; HR-ESI-MS: High-resolution-electrospray ionization-mass spectrometry; DQF-COSY: Double quantum filtered-correlation spectroscopy; HSQC: Heteronuclear single quantum coherence; HMBC: Heteronuclear multiple bond correlation

Introduction

Marine microorganisms, particularly marine *Actinobacteria*, have attracted considerable attention as one of the most important resources for new biologically active metabolites [1]. For example, new compounds have been isolated from *Actinobacteria* of sponge origin [2-5]. Our group was recently engaged in the isolation of *Actinobacteria* from marine sources. Some of the isolated *Actinobacteria* have been found to produce new compounds, namely, the teleocidin analog [2-5]. Our group was recently engaged in the isolation of new compounds, termed JBIR-39 (1) and JBIR-40 (2) (Figure 1), from the fermentation broth of *Streptomyces* sp. strain Sp080513SC-24 isolated from a marine sponge, *Haliclona* sp. This paper describes the fermentation, isolation, and structure elucidation of 1 and 2.

Materials and Methods

General experimental procedures

Optical rotations were obtained on an SEPA-300 polarimeter (Horiba, Kyoto, Japan). UV and IR spectra were measured on a DUV 370 UV/Vis spectrophotometer (Beckman Coulter, CA, USA) and an FT-720 spectrophotometer (Horiba), respectively. NMR spectra were recorded on a Varian NMR System 600 NB CL (Varian, Palo Alto, CA, USA) in DMSO-<sup>d6</sup> (2.50 ppm for <sup>1</sup>H, 39.5 ppm for <sup>13</sup>C) with the residual solvent peak as the internal standard. HR-ESI-MS data were recorded on an LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA). MPLC was performed using a Purif-pack ODS-100 column (100µm, Shoko Scientific, Yokohama, Japan). Analytical reversed-phase HPLC was carried out using an L-column2 ODS column (5.0µm, 4.6 i.d. × 150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan) equipped 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 (5.0µm, 20 i.d. × 150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan).

Microorganism

*Streptomyces* sp. strain Sp080513SC-24 has been reported as a new species of genus *Streptomyces* isolated from *Haliclona* sp. and was termed *Streptomyces spongie* NBRC 106415<sup>T</sup> [11].

Fermentation

*Streptomyces* sp. Sp080513SC-24 was cultivated in 50-ml test tubes, each containing 15 ml of a seed medium consisting of starch (Kosokagaku, Tokyo, Japan) 1.0%, polypeptide (Nihon Pharmaceutical, Tokyo, Japan) 1.0%, molasses (Dai-Nippon Meiji Sugar, Tokyo, Japan) 1.0%, and meat extract (Extract Ehlrich, Wako Pure Chemical Industry, Osaka, Japan) 1.0% before sterilization with pH adjusted to 7.2. The test tubes were shaken on a reciprocal shaker (355 r.p.m.) 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 (Nissin Oilio, Tokyo, Japan) 1.5%, dry yeast (Mitsubishi Tanabe Pharma, Osaka, Japan) 0.2%, CaCO<sub>3</sub> (Kozaki Pharmaceutical, Tokyo, Japan) 0.4%, and Diaion HP-20 resin (Mitsubishi Chemical, Tokyo, Japan) 0.1% before sterilization.

Page 2 of 4

with pH adjusted to 7.0. The fermentation was carried out on a rotary shaker (180 r.p.m.) at 27°C for 5 days.

Isolation

The supernatant of whole broth (2 l) collected by centrifugation was successively partitioned with AcOEt (1 l × 3) and n-BuOH (1 l × 2). The n-BuOH layer was evaporated to dryness. The dried residue (413 mg) was fractionated by reversed-phase MPLC (Purif-Pack ODS-100) with a MeOH-water gradient system (0–100% MeOH) and fractions including major metabolites were collected by LC-MS monitoring. The eluate was subjected to preparative reversed-phase HPLC using an L-column2 ODS column developed with 60% aqueous MeOH containing 0.1% formic acid (flow rate: 9.5 ml min⁻¹) to give JBIR-39 (1, 5.3 mg; Retention time (Rt), 19 min) and JBIR-40 (2, 3.1 mg; Rt, 13 min).

JBIR-39 (1): colorless oil; [α]D₂⁰ = -11.0 (c: 0.1, MeOH); UV (MeOH) λmax (ε) 233nm (3,100); IR (KBr) νC=O 3340, 1720, 1640 cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) and ¹³C NMR (150 MHz, DMSO-d₆), see Table 1; HR-ESI-MS m/z 698.3828 [M + H⁺] (calcd. for C₂₃H₂₃NO₂O₂ 698.3837).

JBIR-40 (2): colorless oil; [α]D₂⁰ = -7.8 (c: 0.4, MeOH); UV (MeOH) λmax (ε) 235nm (2,800); IR (KBr) νC=O 3400, 1730, 1640 cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) and ¹³C NMR (150 MHz, DMSO-d₆), see Table 1; HR-ESI-MS m/z 684.3681 [M + H⁺] (calcd. for C₂₅H₂₄NO₂O₂ 684.3680).

Results and Discussion

Structure Elucidation of 1

Compound 1 was isolated as a colorless oil and its HR-ESI-MS was consistent with a molecular formula of C₇₈H₇₃NO₂₀. The ¹H and ¹³C NMR spectral data for 1 are shown in Table 1. From IR absorption (νC=O 3430, 1720, and 1640 cm⁻¹) and ¹³CNMR spectral data, 1 was considered to be a peptide compound. The structural information for 1 was further obtained by a series of 2D NMR analyses, including HSQC, HMBC, and DQF-COSY spectra (Figure 2). The ¹H-¹³C long-range couplings from singlet methyl proton 4-H (δH 1.29) to a carbonyl carbon C-1 (δC 147.7), a quaternary carbon C-2 (δC 59.5), and aethylene carbon C-3 (δC 62.8), from a doublet methyl proton 3-H (δH 3.89) to C-1, C-2, and C-4 (δC 20.6), and from an amide proton 2-NH (δH 7.90) to C-2 revealed an α-methylserine (α-MeSer) moiety. The sequence from a typical α-methylene proton 6-H (δH 4.78, 48.5) to an amide proton 9-NH (δH 5.28) through methylene protons 7-H (δH 1.25, 1.94), an oxymethylene proton 8-H (δH 3.65), and amethylene proton 9-H (δH 2.79) was established by the DQF-COSY spectrum. In the HMBC spectrum, ¹H-¹³C long-range couplings from the α-methylene proton 6-H and methylene proton 7-H to an amide carbonyl carbon C-5 (δC 170.1), and from the amine proton 9-NH to an amide carbonyl carbon C-10 (δC 172.9) were established. These data revealed that C-6 (δH 48.5) and C-9 (δC 52.7) are connected to the different nitrogen atoms and by taking into consideration the peptidyl structure of 1, and the existence of a γ-hydroxyl piperazic acid (γ-OPip) as an amino acid unit, but not a proline residue, was suggested. In the same manner, the 2 identical sequences from an α-methine protons 11-/16-H (δH 5.60, δH 48.4) to amine protons 14-NH/19-NH (δH 5.15) through methylene protons 12-/17-H (δH 1.99, 1.78), 13-/18-H (δH 1.52, 1.42), and 14-/19-H (δH 2.98, 2.60), and another resembled sequence from an α-m ethine proton 21-H (δH 5.60, δH 48.4) to an amine proton 24-NH (δH 5.00) through methylene protons 22-H (δH 1.99, 1.78), 23-H (δH 1.52, 1.42), and 24-H (δH 2.98, 2.60) were also established by the analyses of the DQF-COSY spectrum of 1. The ¹H-¹³C long-range couplings from 21-H to an amide carbonyl carbon C-20 (δC 170.7) and C-25 (δC 174.9), and from 24-NH to C-25 proved a piperazic acid (Pip) moiety. From the molecular formula and ¹H and ¹³C NMR chemical shift values, other 2 units were also determined as Pip moieties. The sequence from an oxymethylene proton 26-H (δH 4.43, δH 71.8) to a triplet methine proton 29-H (δH 0.79) through a methine proton 27-H (δH 1.71), which was in turn coupled to a doublet methyl proton 30-H (δH 0.87), and a methylene proton 28-H (δH 1.23, 1.00) was established by the DQF-COSY spectrum. The ¹H-¹³C long-range couplings from 26-H and 27-H to a carbonyl carbon C-25 (δC 174.9) established an isoleucic acid moiety. The connectivity of these partial structures was determined by ¹H-¹³C long-range couplings from 6-H, 7-H, and 2-NH to an amide carbonyl carbon C-5, from 9-NH and 11-H to an amide carbonyl carbon C-10, from 14-NH and 16-H to an amide carbonyl carbon C-15 (δC 172.2), from 19-NH and 21-H to an amide carbonyl carbon C-20, and from 24-NH and 26-H to an amide carbonyl carbon C-25.
(Waters, SYNAPT G2 HDMS) data, as shown in Figure 3.

**Structure elucidation of 2**

Compound 2 was isolated as a colorless oil and its HR-ESI-MS was consistent with a molecular formula of $C_{30}H_{32}O_8$. In the NMR spectral data for 2, the triplet methyl protons 29-H had disappeared and instead, 2 doublet methyl protons ($\delta_1$ 0.88 and 0.70) were observed. The $^1^H$ NMR spectrum of 2 also showed the disappearance of the methyl signal at C-29 in 1, which indicated the existence of a valinic acid residue. In the same way as in 1, the amino acid sequence of 2 was supported by HR-ESI-MS/MS data (Figure 3). These collective spectroscopic data proved that the isoleucic acid moiety in 1 was displaced by a valinic acid residue in 2.

**Absolute configuration of 1**

The absolute configuration of 1 was defined by Marfey’s method [14] applied for the acid hydrolysate of 1 in comparison with standard amino acids. Compound 1 (1.0 mg) was hydrolyzed in 6N HCl at 110°C for 12h. After the compound was concentrated to dryness, the residue was dissolved in 10 ml of EtOAc-H$_2$O (1: 1). The amino acid mixture recovered in the aqueous layer was dried in vacuo and was added to 5% NaHCO$_3$ (500μl) and 0.2 mg N-((5-fluoro-2,4-dinitrophenyl)-l-alaninamide (FDAA) in acetone (500μl). The solution was heated in an oil bath at 80°C for 3 h. The reaction products were analyzed by the ultra performance liquid chromatography (UPLC) system (Waters) as follows: column, Acquity UPLC BEH C$_18$ column (2.1 i.d. × 50 mm, Waters); flow rate, 0.3 ml min$^{-1}$; solvent, 25% aqueous MeCN containing 0.1% formic acid to detect α-MeSer. The retention times of FDAA derivatives were determined in LC-MS monitoring using negative mode (m/z = 370). Retention times of the standard FDAA derivatives were as follows: (R)-α-MeSer, 1.30 min and (S)-α-MeSer, 1.53 min. The chromatogram of the hydrolysate derivatives showed a peak corresponding to (R)-α-MeSer. To determine the absolute configuration of three piperazic acids, the reaction products were analyzed by the UPLC system using 10-100% (7 min) aqueous MeCN containing 0.1% formic acid. Retention times of the standard FDAA derivatives of (R) - and (S)-piperazic acid were 2.36 min and 2.55 min, respectively. The chromatogram of the hydrolysate derivatives showed that the ratio of peaks corresponding to (R)-piperazic acid and (S)-piperazic acid are 2: 1. Although two (R)-piperazic acids and a (S)-piperazic acid were determined in 1, the sequence of three piperazic acids is unknown. The analysis of the order of each piperazic acid unit is presently underway.

The isoleucic acid moiety dissolved in the organic layer was dried in vacuo and was added to 0.2 mg p-bromophenacyl bromide and trace amounts of potassium fluoride in DMF (100μl). The mixture was heated in an oil bath at 50°C for 12h. The reaction product was analyzed by HPLC as follows: column, Daicel CHIRAL PAK IC column (4.6 i.d. × 250 mm, Daicel, Osaka, Japan); flow rate, 1.0 ml min$^{-1}$; solvent, hexane–isopropanol (9: 1). The retention times of p-bromophenacyl adducts were analyzed by UV (254 nm) monitoring. The retention times of the standard p-bromophenacyl derivatives were as follows: l-isoleucic acid, 25 min; p-isoleucic acid, 40 min; allo-l-isoleucic acid, 28 min; and allo-p-isoleucic acid, 49 min. The retention time of the p-bromophenacyl adduct of the hydrolysate in the chromatogram showed a corresponding peak to l-isoleucic acid.

The relative configuration of the γ-hydroxyl piperazic acid ring was established from $J_{H-H}$ coupling constants. Since the both spin coupling constants between the α-methine proton 6-H and methylene protons 7-H were relatively small ($J_{4H_1-4H_2} = 2.3$ Hz and $J_{4H_1-4H_2} = 5.5$ Hz, respectively), 6-H was deduced to be in the equatorial location. The
higher-fielded 1H chemical shift of 7-Hb (δ 2.94) comparing with that of lower-fielded 1H chemical shift of 7-Ha (δ 2.25), together with an NOE between 7-Hb and 9-H, supported that 7-H is in the axial location and the conformation of this six-membered ring structure is chair form. In addition, both relatively small coupling constants between 7-Ha and 8-H (J 7Ha-8H = 2.6 Hz) and between 7-Hb and 8-H (J 7Hb-8H = 6.5 Hz) suggested that 8-H is in the equatorial location. Thus, the relative configurations were established to be 6S* and 8S*, respectively as shown in Figure 4.

Conclusion

We isolated two novel hexapeptide compounds, 1 and 2, from the culture broth of sponge-derived Streptomyces sp. Sp080513SC-24. The structures of 1 and 2 possessed an α-methylserine moiety, a γ-hydroxyl piperazic acid moiety, three piperazic acid moieties, and an isoleucic acid moiety or a valinic acid moiety. The structures of 1 and 2 were found to be related to piperdiamycin F and D isolated from the artificially streptomycin or rifampicin resistant strains of a soil-isolated Streptomyces species [15]. However, Sp080513SC-24 showed sensitivity to streptomycin (MIC = 1μg/ml) or rifampicin (MIC = 30μg/ml). Furthermore, piperdiamycins possessing the Pip and γ-OH-Pip moieties have been isolated from a marine-organized Streptomyces [16]. These compounds have been reported to show antimicrobial and cytotoxic activities [15,16]. Therefore, we attempted to investigate the cytotoxic and antimicrobial activities of 1 and 2. The results showed that 1 and 2 did not exhibit cytotoxic activity against several cancer cell lines nor did they show antibacterial activities against Micrococcus luteus and Escherichia coli (data not shown). 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.

Acknowledgements

This work was supported by a grant from the New Energy and Industrial Technology Department Organization (NEDO) of Japan, a Grant-in-Aid for Scientific Research (20380070 to K.S.) from the Japan Society for the Promotion of Science (JSPS), and the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

References