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NOTE

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A Phenylacetylated Peptide, JBIR-96, Isolated from *Streptomyces* sp. RI051-SDHV6

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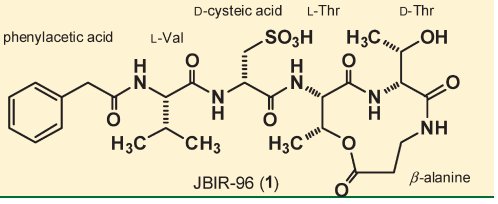
Supporting Information

ABSTRACT: Searching for metabolites from *Streptomyces* sp. RI051-SDHV6 resulted in the discovery of a novel peptide, JBIR-96 (**1**). The structure of **1** was established as an *N*-phenylacetylated pentapeptide involving a cysteic acid and a peptide lactone structure by extensive NMR and MS analyses. In addition, the absolute configuration of **1** was established by Marfey's and modified Mosher's methods.



Members of the class Actinobacteria have been extensively studied for their ability to produce pharmaceutically useful compounds. However, the rate of discovery of novel compounds from these bacteria has decreased significantly. In undertaking this work, our goal was to show that new species have the capability to produce unique metabolites. For this purpose, we isolated new species of actinomycetes from a variety of samples including soils, lichens, and marine organisms and then examined cultures of these strain. For screening metabolites using this approach, we have discovered novel compounds including the anthracyclines tetracenoquinocin and 5-iminoaranciamycin,[1] tetrapeptides JBIR-34 and JBIR-35,[2] salicylamide JBIR-58,[3] diterpene JBIR-65,[4] angucycline JBIR-88,[5] butenolide JBIR-89,[5] and JBIR-66,[6] all produced by the new species of actinomycetes. Further screening has resulted in the isolation of a novel phenylacetylated peptide, JBIR-96 (**1**, Figure 1), from the culture of *Streptomyces* sp. RI051-SDHV6 found in a soil sample collected from Rishiri Island, Hokkaido Prefecture, Japan. This paper describes the production, isolation, and structure elucidation of **1**.

Streptomyces sp. RI051-SDHV6 was cultured in 500 mL baffled Erlenmeyer flasks each containing 100 mL of a production medium and incubated for 5 days at 27 °C with rotary shaking. The mycelial cake was extracted with 80% aqueous acetone and concentrated *in vacuo*. The aqueous concentrate was partitioned with EtOAc, and the aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was concentrated *in vacuo* and subjected to sequential reversed-phase ODS flash column chromatography. The pure form of **1** was obtained by reversed-phase HPLC.

Compound **1** was obtained as a colorless powder ($[\alpha]^{24}_{D}$ −42.5, *c* 0.8, in MeOH; UV λ_{max} (ε) 258 (3100) nm, in MeOH). The molecular formula of **1** was determined by HRESIMS to be $C_{27}H_{39}N_5O_{11}S$ ($[M - H]^-$, *m/z* 640.2286, calcd for $C_{27}H_{38}N_5O_{11}S$, 640.2289). Absorptions assignable to ester and amide carbonyl groups (ν_{max} 1740, 1650, 1180 cm^{-1}) as well as sulfonyl group (ν_{max} 1240, 1050 cm^{-1}) were observed in the IR spectrum of the compound. The direct connectivity between each proton and carbon was established from the HSQC spectrum, and the tabulated ^{13}C and ^{1}H NMR spectroscopic data for **1** are shown in Table 1. The structure of **1** was established mainly by double-quantum-filtered (DQF)-COSY and constant time HMBC[7] spectra, together with tandem MS (MS/MS) analysis as follows.

The ^{1}H and ^{13}C NMR spectra strongly suggested **1** as being a peptide. The sequence from the amide proton 11-NH (δ_H 8.42) to the methyl proton H-26 (δ_H 0.87) through α-methine proton H-11 (δ_H 4.10, δ_C 58.5) and methine proton H-25 (δ_H 2.10), which in turn coupled to methyl proton H-27 (δ_H 0.82), was observed (Figure 2). In addition to these $^{1}H-^{1}H$ correlations, long-range couplings from H-11 and H-25 to carbonyl carbon C-10 (δ_C 171.4) indicated the presence of a valine unit.

The sequence from amide proton 9-NH (δ_H 8.78) to methylene protons H-24 (δ_H 3.03, 2.91) through α-methine proton H-9 (δ_H 4.44, δ_C 51.7), which in turn long-range-coupled to carbonyl carbon C-8 (δ_C 170.9), indicated a β-substituted alanine substructure. The presence of a 2-amino-3-sulfopropanoic acid (cysteic acid) unit was proved as follows. The sulfonyl group, the presence of which was suggested by HRESIMS/MS fragmentation analysis (*m/z* 561.2794, $[M + H - HSO_3]^+$, calcd for $C_{27}H_{39}N_5O_8$, 561.2799; *m/z* 559.2634, $[M - H - HSO_3]^-$, calcd for $C_{27}H_{37}N_5O_8$, 559.2642), was determined to

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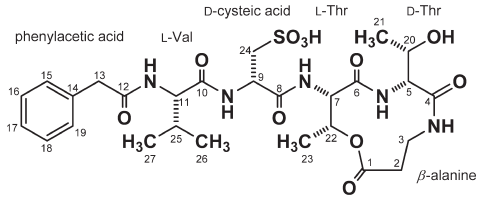

Figure 1. Structure of JBIR-96 (**1**).

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Spectral Data for 1 in DMSO-d_6

position	δ_C	δ_H (multiplicity, *J* in Hz)
1	170.2	
2	34.0	2.37 (ddd, 12.9, 7.6, 5.3)
		2.16 (dt, 12.9, 2.4)
3	36.3	3.38 (dddd, 14.4, 7.9, 5.3, 2.4)
		2.85 (dddd, 14.4, 7.9, 7.6, 2.4)
3-NH		7.32 (d, 7.9)
4	170.5	
5	64.6	3.68 (dd, 7.0, 6.5)
5-NH		8.05 (d, 6.5)
6	171.0	
7	56.9	4.36 (dd, 9.4, 1.8)
7-NH		8.13 (d, 9.4)
8	170.9	
9	51.7	4.44 (q, 5.4)
9-NH		8.78 (d, 5.4)
10	171.4	
11	58.5	4.10 (dd, 8.2, 6.6)
11-NH		8.42 (d, 8.2)
12	171.4	
13	42.4	3.57 (d, 14.4)
		3.53 (d, 14.4)
14	136.5	
15, 19	129.2	7.24 (m)
16, 18	128.3	7.23 (m)
17	126.5	7.13 (t, 6.2)
20	65.0	3.92 (sextet, 6.5)
20-OH		5.08 (d, 5.3)
21	19.9	1.10 (d, 6.5)
22	71.6	5.49 (dq, 6.5, 1.8)
23	16.2	1.05 (d, 6.5)
24	50.5	3.03 (dd, 13.9, 5.4)
		2.91 (dd, 13.9, 5.4)
25	30.3	2.10 (dq, 6.8, 6.6, 6.2)
26	20.1	0.87 (d, 6.8)
27	18.3	0.82 (d, 6.8)

be substituted at the β-methylene carbon C-24 by its chemical shift (δ_C 50.5). The $^{1}H-^{1}H$ correlations between amide proton 7-NH (δ_H 8.13) and α-methine proton H-7 (δ_H 4.36, δ_C 56.9) and between oxymethine proton H-22 (δ_H 5.49) and methyl proton H-23 (δ_H 1.05) were observed. The $^{1}H-^{13}C$ long-range couplings from the methine proton H-7 to carbonyl carbon C-6 (δ_C 171.0) and the oxymethine carbon C-22 (δ_C 71.6) as well as from the methyl proton H-23 to the α-methine carbon C-7

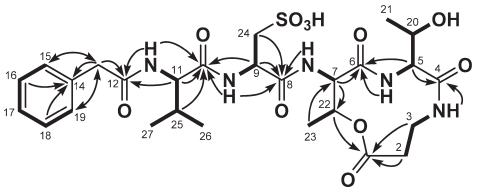

Figure 2. Key correlations observed in DQF-COSY (bold lines) and HMBC (arrows) spectra of **1**.

(δ_C 56.9) were observed (Figure 2). The preceding data demonstrated the presence of a threonine moiety. It is important to note that the extremely downfield shifted ^{1}H resonance at H-22 compared with that of H-20 suggested the acylated shift at H-22, *vide infra*.

A second threonine moiety was established as follows. The sequence from amide proton 5-NH (δ_H 8.05) to methyl proton H-21 (δ_H 1.10) through α-methine proton H-5 (δ_H 3.68, δ_C 64.6) and oxymethine proton H-20 (δ_H 3.92), which in turn coupled to hydroxy proton 20-OH (δ_H 5.08), was observed in the DQF-COSY spectrum of **1**, as shown in Figure 2. In addition to these $^{1}H-^{1}H$ correlations, long-range coupling from H-5 to amide carbonyl carbon C-4 (δ_C 170.5) was observed.

The final amino acid residue was deduced as follows: the sequence from methylene protons H-2 (δ_H 2.37, 2.16), which in turn long-range-coupled to a carbonyl carbon C-1 (δ_C 170.2), through methylene protons H-3 (δ_H 3.38, 2.85) to an amide proton 3-NH (δ_H 7.32), which in turn long-range-coupled to a carbonyl carbon C-4, established a 3-aminopropanoic acid (β-alanine) unit (Figure 2).

The remaining unit with the exception of amino acid residues was determined as phenylacetic acid. The sequence from aromatic protons H-15,19 (δ_H 7.24) to H-17 (δ_H 7.13) through H-16,18 (δ_H 7.23) and $^{1}H-^{13}C$ long-range correlations from H-16,18 to aromatic quaternary carbon C-14 (δ_C 136.5), as well as from methylene protons H-13 (δ_H 3.57, 3.53) to carbonyl carbon C-12 (δ_C 171.4), and the presence of aromatic carbons C-14 and C-15,19 (δ_C 129.2) established the partial structure as a phenylacetic acid unit.

Finally, the connectivity among these amino acid units was elucidated by the $^{1}H-^{13}C$ long-range couplings between 3-NH and C-4, H-5 and C-6, 7-NH and C-8, H-9 and C-10, and H-11 and C-12. The $^{1}H-^{13}C$ long-range coupling from the oxymethine proton H-22 to the carbonyl carbon C-1, together with the acylated shift at H-22, proved that threonine and β-alanine residues were connected through an ester bond.

The amino acid sequence of **1** was also confirmed by the HRESIMS/MS analysis for a partial hydrolysate of **1**. Compound **1** treated with 0.1 N LiOH at 25 °C for 1 h gave its hydrolysate (**2**, Figure 3) with the formula $C_{27}H_{41}N_5O_{12}S$ (*m/z* 660.2553, $[M + H]^+$, calcd for $C_{27}H_{42}N_5O_{12}S$, 660.2550). In the positive ion mode of the molecular ion of **2** ($[M + H]^+$, $C_{27}H_{42}N_5O_{12}S$) as a precursor ion, the fragment ions *m/z* 571.2067 $[M - \beta\text{-Ala}]^+$, *m/z* 470.1593 $[M - \beta\text{-Ala} - \text{Thr}]^+$, *m/z* 369.1129 $[M - \beta\text{-Ala} - \text{Thr} \times 2]^+$, *m/z* 218.1177 $[M - \beta\text{-Ala} - \text{Thr} \times 2 - \text{cysteic acid}]^+$, *m/z* 190.1225 $[M - \beta\text{-Ala} - \text{Thr} \times 2 - \text{cysteic acid} - \text{CO}]^+$, *m/z* 443.1294 $[M + H - PhCH_2CO - Val]^+$, *m/z* 292.1506 $[M + H - PhCH_2CO - Val - \text{cysteic acid}]^+$, *m/z* 191.1036 $[M + H - PhCH_2CO - Val - \text{cysteic acid} - \text{Thr}]^+$, and *m/z* 90.0551 $[M + H - PhCH_2CO - Val - \text{cysteic acid} - \text{Thr} \times 2]^+$ were observed, as shown in Figure 3. Thus, **2** was

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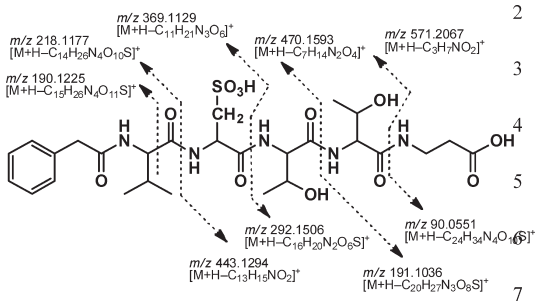

Figure 3. Fragmentation pattern of the hydrolysate (**2**) of **1** in the positive ion mode of HRESIMS/MS.

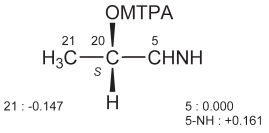

Figure 4. Absolute configuration of C-20 in **1** revealed by the modified Mosher's method. The differences in chemical shift values were obtained by subtracting the (*R*)-MTPA ester values from (*S*)-MTPA ester values ($\delta\Delta = \delta(S)$-MTPA $- \delta(R)$-MTPA).

determined to be an *N*-phenylacetylated linear pentapeptide arranged in the order β-alanine, two threonines, cysteic acid, and valine.

The absolute configuration of the two threonines, the cysteic acid, and the valine residues in **1** was clarified by Marfey's method[8] applied for the acid hydrolysate of **1** in comparison with standard amino acids. Retention times of the standard N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) derivatives were as follows: L-threonine, 6.1 min; L-allo-threonine, 7.7 min; D-allo-threonine, 8.7 min; D-threonine, 10.7 min; L-valine, 6.3 min; D-valine, 12.2 min. Retention times of the standard N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-valinamide (FDVA) derivatives were as follows: D-cysteic acid, 6.6 min; L-cysteic acid, 7.3 min. The chromatogram of the FDAA or FDVA derivatives of the acid hydrolysate showed peaks corresponding to L-threonine (6.3 min), D-threonine (10.8 min), L-valine (6.2 min), and D-cysteic acid (6.6 min). Since both L-threonine (6.3 min) and D-threonine (10.8 min) were observed in the hydrolysate, we adopted the modified Mosher's method.[9]

The proton chemical shift of the methyl proton H-21 in the (*R*)-MTPA ester derivative of **1** appeared at a lower field than that of the (*S*)-MTPA ester derivative (Figure 4). On the other hand, the proton chemical shift of the α-methine proton H-5 and the amide proton 5-NH in the (*R*)-MTPA ester derivative was observed at a higher field than that of the (*S*)-MTPA ester derivative. Thus, the absolute configuration at the C-20 position was deduced to be *S*, indicating that this threonine moiety is D-threonine. Conclusively, the absolute structure of **1** was established, as shown in Figure 1.

In conclusion, we herein isolated a unique peptide designated JBIR-96 from *Streptomyces* sp. RI051-SDHV6. It has been reported that antibiotic YF 044P-D,[10] antibiotic L 174580,[11] and antibiotic YM 47690[12] are isolated from *Streptomyces* sp. as phenylacetyl *N*-terminal masked peptides. Antibiotic YF 044P-D, antibiotic L 174580, and antibiotic YM 47690 show inhibition of proteases, aspartyl protease, rennin, and cathepsin L, respectively. In addition, the cysteic acid of **1** is identical to those of stenothricin[13] and imacidinic acid,[14] which show antibacterial activity. However, to the best of our knowledge, **1** is the first example of the *N*-phenylacetylated pentapeptide involving the cysteic acid and lactone structures. Contrary to the characteristic structure of **1**, it did not show cytotoxic or antimicrobial activity. The unique structure of **1** may have been biosynthesized by a novel nonribosomal peptide synthetase gene. Thus, studies on the detailed biosynthesis of **1** are now underway.

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. HRESIMS/MS data were recorded on a Waters SYNAPT G2. UV spectra were measured on a Beckman Coulter 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 DMSO-d_6, and the solvent peak was used for spectra calibration (δ_C 39.7, δ_H 2.49 ppm). Reverse-phase medium-pressure liquid chromatography (MPLC) was performed on a Purif-Pack ODS 100 column (Shoko Scientific, Yokohama, Japan). Analytical reversed-phase HPLC was carried out using a CAPCELL PAK C_{18} MGII column (5.0 μm, 4.6 i.d. × 150 mm; Shiseido, Tokyo, Japan) with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. Preparative reversed-phase HPLC was carried out using a CAPCELL PAK C_{18} MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido) 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. RI051-SDHV6. *Streptomyces* sp. RI051-SDHV6 was isolated from a soil sample collected in Rishiri Island, Hokkaido Prefecture, Japan, using the sodium dodecyl sulfate-yeast extract (SDS-YE) method.[15] To identify the genus of the strain RI051-SDHV6, we compared the 16S rRNA gene sequences of RI051-SDHV6 to those available in the DNA Data Bank of Japan using the basic local alignment search tool (BLAST) search and identified it as the genus *Streptomyces*.

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, and 0.4% $CaCO_3$ (pH 7.2 before sterilization). Strain RI051-SDHV6 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 (2.5 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. The fermentation broth (2 L) of RI051-SDHV6 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 first washed with EtOAc and extracted with *n*-BuOH, successively. The *n*-BuOH layer was concentrated *in vacuo*. The dried residue (770 mg) was subjected to reversed-phase MPLC using a H_2O−MeOH stepwise solvent system (0%, 20%, 40%, and 60% MeOH). The 40% MeOH eluate (38 mg) was further purified by preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column developed with 50% MeOH−H_2O containing 0.1% formic acid (flow rate: 10 mL/min) monitored by LC-MS to yield **1** (25.3 mg, t_R = 15.9 min).

Determination of Amino Acid Configurations. Compound **1** (1.0 mg) was hydrolyzed in 0.2 mL of 6 N HCl at 110 °C for 14 h. After the reaction mixture was concentrated *in vacuo*, the residue was added to 0.1 M $NaHCO_3$ (200 μL) with 0.2 mg of FDAA or FDVA. The solution

was heated at 75 °C for 30 min. The FDAA derivatives of threonine and valine were analyzed by the LC-MS system as follows: column, CAPCELL PAK C_{18} MGII column; flow rate, 1 mL/min; solvent, 45% (for threonine) or 60% (for valine) MeOH−H_2O containing 0.1% formic acid. The FDVA derivative of cysteic acid was analyzed by using a CROWNPAK CR(+) column (4.0 i.d. × 150 mm; DAICEL Chemical Industries, Osaka, Japan) developed with 20% CH_3CN−H_2O containing 0.4% TFA (flow rate, 1 mL/min).

Compound **1** (1.0 mg) was reacted with (+)- or (−)-MTPA chloride (30 μL) in pyridine (200 μL) at room temperature for 14 h. The reaction mixture was concentrated to dryness, and the residue was dissolved in 10 mL of EtOAc−H_2O (1:1). The (*R*)- or (*S*)-MTPA ester recovered in the organic layer was dried *in vacuo* and was purified by preparative reversed-phase HPLC using an XBridge C_{18} column (5.0 μm, 10 i.d. × 150 mm; Waters, Milford, MA, USA) with 60% MeOH−H_2O containing 0.1% formic acid (flow rate: 4 mL/min) to yield (*R*)- or (*S*)-MTPA ester (t_R = 9.6 min).

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR, DQF-COSY, CT-HMBC, HRESIMS, and MS/MS spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Motohashi, K.; Takagi, M.; Shin-ya, K. *J. Nat. Prod.* **2010**, *73*, 755–758.
(2) Motohashi, K.; Takagi, M.; Shin-ya, K. *J. Nat. Prod.* **2010**, *73*, 226–228.
(3) Ueda, J.; Khan, S. T.; Takagi, M.; Shin-ya, K. *J. Antibiot.* **2010**, *63*, 267–269.
(4) Takagi, M.; Motohashi, K.; Khan, S. T.; Hashimoto, J.; Shin-ya, K. *J. Antibiot.* **2010**, *63*, 401–403.
(5) Motohashi, K.; Takagi, M.; Yamamura, H.; Hayakawa, M.; Shin-ya, K. *J. Antibiot.* **2010**, *63*, 545–548.
(6) Takagi, M.; Motohashi, K.; Izumikawa, M.; Khan, S. T.; Hwang, J.-H.; Shin-ya, K. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 2355–2357.
(7) Furihata, K.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7337–7340.
(8) Bhushan, R.; Brückner, H. *Amino Acids* **2004**, *27*, 231–247.
(9) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.
(10) Sato, T.; Shibazaki, M.; Yamaguchi, H.; Abe, K.; Matsumoto, H.; Shimizu, M. *J. Antibiot.* **1994**, *47*, 588–590.
(11) U.S. Pat. 4 874 745, 1989.
(12) Japan Pat. 96 151 394, 1996.
(13) Hasenböhler, A.; Kneifel, H.; König, W. A.; Zähner, H.; Zeiler, H.-J. *Arch. Microbiol.* **1974**, *99*, 307–321.
(14) Brecht-Fischer, A.; Zähner, H.; Laatsch, H. *Arch. Microbiol.* **1979**, *122*, 219–229.
(15) Hayakawa, M.; Nonomura, H. *Actinomycetologica* **1989**, *3*, 95–104.