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Note

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JBIR-94 and JBIR-125, Antioxidative Phenolic Compounds from *Streptomyces* sp. R56-07

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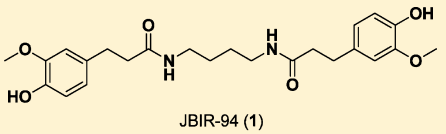
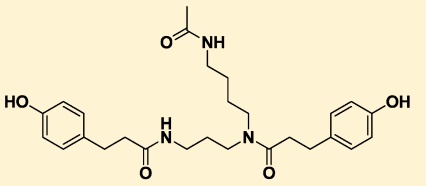
Supporting Information

ABSTRACT: Two phenolic compounds, JBIR-94 (**1**) and JBIR-125 (**2**), were isolated from the fermentation broth of strain R56-07, which was identified by phylogenetic methods as a novel species of *Streptomyces*. The structures of **1** and **2** were assigned on the basis of 1D and 2D NMR spectroscopy and MS analyses. Compounds **1** and **2** exhibited 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity with an IC_{50} value of 11.4 and 35.1 μM, respectively. These compounds are the first examples of hydroxycinnamic acid amides containing putrescine or spermidine produced by actinomycetes.

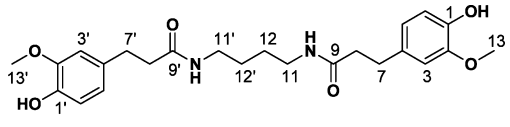




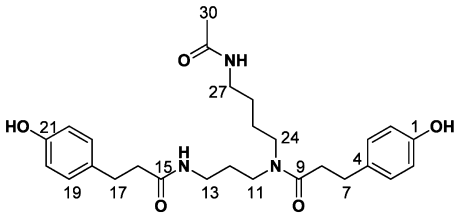
Actinomycetes are commonly recognized for their ability to produce chemically diverse and pharmaceutically useful compounds. However, in recent years, the rate of discovery of novel compounds from these bacteria has significantly decreased.[1,2] One approach to reverse this trend is to isolate actinomycetes from a wide variety of environmental sources and to employ diverse isolation methods to obtain new bioactive compounds.[3] Applying these principles, in our efforts to discover novel secondary metabolites, we isolated two phenolic compounds, JBIR-94 (**1**) and JBIR-125 (**2**), from the culture broth of a new species of *Streptomyces* (strain R56-07) using the rehydration and centrifugation method.[4] Herein, the fermentation, isolation, structure elucidation, and biological activity of **1** and **2** are described.

The strain was cultured in baffled Erlenmeyer flasks containing 100 mL of production medium incubated at 27 °C for 5 days with rotary shaking. Compounds **1** and **2** were obtained from the ethyl acetate extract of the fermentation broth, after which they were subjected to a series of purifications by MPLC, gel filtration, and HPLC.

JBIR-94 (**1**) was obtained as a colorless amorphous solid that was found to have the molecular formula $C_{24}H_{32}N_2O_6$ upon positive ion HRESIMS analysis. The IR spectrum of **1** indicated the presence of an amide carbonyl function (1652 cm^{-1}). A symmetric structure of **1** was deduced by interpreting the



JBIR-94 (**1**)



JBIR-125 (**2**)

molecular formula and the NMR spectroscopic data of **1** (Table 1). These data also showed the presence of 24 carbons, which were classified into two similar sets comprising a carbonyl carbon, two oxygenated aromatic carbons, an aromatic quaternary carbon, three aromatic methine carbons, a nitrogen-bearing methylene carbon, a methoxy carbon, and three

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data for JBIR-94 (1) in CD_3OD

position	type	δ_C	δ_H (J in Hz)
1, 1′	C	145.9	
2, 2′	C	148.9	
3, 3′	CH	113.2	6.81, d (1.6)
4, 4′	C	133.6	
5, 5′	CH	121.9	6.66, dd (1.6, 8.0)
6, 6′	CH	116.2	6.73, d (8.0)
7, 7′	CH_2	32.7	2.85, t (7.8)
8, 8′	CH_2	39.4	2.47, t (7.8)
9, 9′	C	175.4	
10, 10′	NH		
11, 11′	CH_2	39.9	3.11, br s
12, 12′	CH_2	27.6	1.31, br s
13, 13′	CH_3	56.3	3.86, s

methylene carbons. The typical ABX-type coupling pattern among aromatic protons H-3 (δ_H 6.81, d, J = 1.6), H-5 (δ_H 6.66, dd, J = 1.6, 8.0), and H-6 (δ_H 6.73, d, J = 8.0) indicated the presence of a 1,2,4-trisubstituted benzene ring. The HMBC spectrum showed the ^{1}H−^{13}C long-range couplings from methylene protons H_2-7 (δ_H 2.85) to aromatic carbons C-3 (δ_C 113.2), C-4 (δ_C 133.6), and C-5 (δ_C 121.9), as well as the coupling from the aromatic proton H-6 to aromatic carbons C-2 (δ_C 148.9) and C-4 at the *meta*-position. In addition, the coupling from H-5 and H-3 to C-1 (δ_C 145.9) and from the methoxy proton H_3-13 (δ_H 3.86) to C-2 were observed. Taken together, these findings revealed the structure of 2,4-substituted phenol moieties, as shown in Figure 1a. The HMBC spectrum

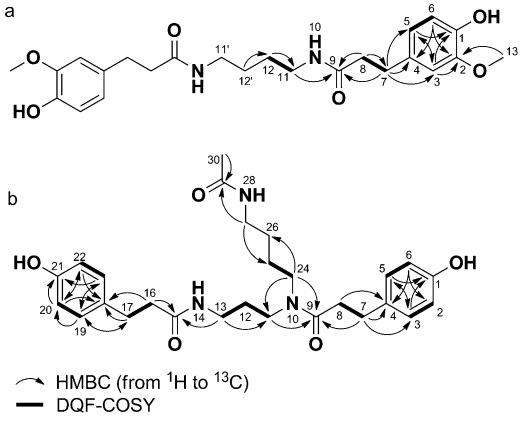

Figure 1. Key correlations observed in 2D NMR spectra of **1** (a) and **2** (b) by DQF-COSY (bold lines) and HMBC (arrows).

also revealed correlations from the methylene protons of H_2-7 (δ_H 2.85) and H_2-8 (δ_H 2.47), which were spin coupled to each other, to amide carbonyl carbon C-9 (δ_C 175.4), indicating a consecutive connection from C-7 to C-9. Furthermore, ^{1}H−^{1}H spin coupling between methylene protons H_2-11 (δ_H 3.11) and H_2-12 (δ_H 1.31) was observed in the DQF-COSY spectrum. Because the HMBC correlations from H_2-12 to C-12′ (δ_C 27.6) and from H_2-11 to C-9 were observed, it is concluded that **1** has the symmetrical structure illustrated in Figure 1a.

JBIR-125 (**2**) gave a molecular formula of $C_{27}H_{37}N_3O_5$ by positive ion HRESIMS. The IR spectrum of **2** displayed an absorption band at 1652 cm^{-1}, indicating the presence of an amide moiety. Although the analytical HPLC chromatograph of **2** allowed a single peak, the ^{13}C NMR spectrum of **2** showed two sets of 27 carbon signals. These findings suggested the existence of a set of equilibrating rotamers (**2a** and **2b**, 1:1) in CD_3OD at 25 °C. We next measured the ^{1}H NMR spectrum of **2** at 80 °C in DMSO-d_6. The resulting spectrum demonstrated that the individual signals assigned for the same positions moved closer together (Supporting Information), which confirmed our hypothesis. Full NMR measurements of inseparable rotamers **2a** and **2b** were performed in CD_3OD at 25 °C. The tabulated ^{1}H and ^{13}C assignments revealed by HSQC are listed in Table 2. The DQF-COSY spectrum of **2a**

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic Data for JBIR-125 (2a and 2b) in CD_3OD

		2a		**2b**	
position	type	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	C	156.81		156.81	
2/6	CH	116.19	6.72,[b] d (7.8)	116.19	6.71,[b] d (7.8)
3/5	CH	130.39	7.06,[c] d (7.8)	130.39	7.03,[c] d (7.8)
4	C	133.04		133.04	
7	CH_2	32.08	2.81, t (7.6)	32.16	2.85, t (7.6)
8	CH_2	36.14	2.63, t (7.6)	36.14	2.58, t (7.6)
9	C	175.00		174.74	
10	N				
11	CH_2	44.78	3.24, t (7.2)	47.06	3.15, ovl[a]
12	CH_2	28.41	1.62, ovl[a]	29.82	1.62, ovl[a]
13	CH_2	37.74	3.09, t (6.0)	37.67	3.13, (6.6)
14	NH				
15	C	175.33		175.49	
16	CH_2	39.43	2.47, t (7.8)	39.28	2.42, t (7.6)
17	CH_2	32.08	2.86, t (7.8)	32.08	2.82, t (7.6)
18	C	132.83		132.83	
19/23	CH	130.49	7.06,[c] d (7.8)	130.49	7.06,[c] d (7.8)
20/22	CH	116.24	6.72,[b] d (7.8)	116.24	6.72,[b] d (7.8)
21	C	156.81		156.81	
24	CH_2	49.0[d]	3.20, ovl[a]	46.91	3.30, t (7.2)
25	CH_2	27.22	1.47, ovl[a]	26.06	1.53, ovl[a]
26	CH_2	27.72	1.48, ovl[a]	27.60	1.45, ovl[a]
27	CH_2	39.91	3.18, ovl[a]	40.11	3.20, ovl[a]
28	NH				
29	C	173.25		173.25	
30	CH_3	22.57	1.95, s	22.57	1.97, s

[a]Overlapped with other signals. [b,c]Interchangeable. [d]Chemical shift was assigned from the HSQC cross-peak.

exhibited two sets of AB-type spin couplings between two sets of four equivalent aromatic protons, H-3/H-5/H-19/H-23 (δ_H 7.06, d, J = 7.8 Hz) and H-2/H-6/H-20/H-22 (δ_H 6.72, d, J = 7.8 Hz), which indicates two 1,4-disubstituted benzene ring substructures.

In the HMBC spectrum, the correlations from the aromatic protons H-3/H-5/H-19/H-23 to oxygenated aromatic carbons C-1/C-21 (δ_C 156.81 × 2) and aliphatic methylene carbons C-7/C-17 (δ_C 32.08 × 2) were observed, and the aromatic protons H-2/H-6/H-20/H-22 were correlated to quaternary carbons C-4 (δ_C 133.04) and C-18 (δ_C 132.83). These results determined the ^{1}H and ^{13}C NMR assignments of two *para*-substituted phenol motifs in **2a**. The HMBC spectrum of **2a** also revealed the following correlations: from the methylene protons H_2-7 (δ_H 2.81) to the aromatic quaternary carbon C-4, to C-3/C-5 (δ_C 130.39), and to an amide carbonyl carbon C-9 (δ_C 175.00); from the methylene protons H_2-16 (δ_H 2.47) to

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an amide carbonyl carbon C-15 (δ_C 175.33); and from methylene protons H_2-17 (δ_H 2.86) to C-18 and C-19/23. By using the DQF-COSY correlations of H-7/H-8 and H-16/H-17, it was found that the above resulted from two sets of 3-(4-hydroxyphenyl)propanamide moieties. The proton−proton spin couplings among methylene protons H_2-11 (δ_H 3.24), H_2-12 (δ_H 1.62), and H_2-13 (δ_H 3.09) indicated the presence of a propyl substructure. The nitrogen-bearing methylene protons H_2-11 (δ_C 44.78) and H_2-13 (δ_C 37.74) showed HMBC correlations to the amide carbonyl carbons C-9 and C-15, respectively. Taken together, these data indicate that the two 3-(4-hydroxyphenyl)propanamide moieties were interconnected by the propyl chain linker.

The remaining substructure was established as follows. $^1H-^1H$ spin couplings between methylene protons H_2-24 (δ_H 3.20) and H_2-25 (δ_H 1.47) and between H_2-26 (δ_H 1.48) and H_2-27 (δ_H 3.18) were observed in the DQF-COSY spectrum. Because the ^1H NMR shifts at H_2-25 and H_2-26 overlapped in the DQF-COSY spectrum, assignments of these units were established by $^1H-^{13}C$ long-range couplings. A $^1H-^{13}C$ long-range coupling from the methylene protons H_2-27 to methylene carbon C-25 (δ_C 27.22) revealed the sequence from C-24 to C-27 through C-25 and C-26. Both the methylene protons H_2-27 and a singlet methyl proton H_3-30 (δ_H 1.95) were long-range-coupled to an amide carbonyl carbon C-29 (δ_C 173.25). In addition to these correlations, the ^{13}C chemical shift at C-24 suggested the presence of an *N*-(3-aminopropyl)acetamide moiety. Finally, long-range couplings from H_2-24 to the amide carbonyl carbon C-9 and the methylene carbon C-11 (δ_C 44.78) established the structure of **2a**, as shown in Figure 1b. These results suggested that **2b** is a rotational isomer of **2a** because of the presence of a tertiary amine.

The hydroxycinnamic acid-derived amides have broad structure variations from simple phenolic amides to complex macrocyclic polyamine alkaloids and have been found in many higher plants.[5−8] Putrescine, spermidine, and spermine are metabolites that are produced by many microorganisms.[9] However, to the best of our knowledge, **1** and **2** are the first examples of hydroxycinnamic acid amides containing putrescine or spermidine produced by actinomycetes.

Due to the presence of phenolic hydroxy groups, both **1** and **2** were expected to exert radical-scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. α-Tocopherol was utilized as a positive control, showing an IC_{50} value of 9.0 μM. Compound **1** exhibited DPPH radical-scavenging activity with almost the same IC_{50} value (IC_{50} = 11.4 μM) as that of α-tocopherol, while **2** showed weaker activity, with an IC_{50} value of 35.1 μM.

In conclusion, we isolated two new polyamine hydroxycinnamic acid conjugates, JBIR-94 (**1**) and JBIR-125 (**2**), from the culture broth of a new species of *Streptomyces*, R56-07, isolated from a soil sample from Rishiri Island in a subpolar climate with the rehydration and centrifugation method, which allows the selection of actinomycete zoospores, unlike traditional methods. Recently we also isolated **1** from two strains (RL23 and RL66) of *Streptomyces* derived from mangrove soil samples collected on Ishigaki Island, which experiences a humid temperate climate, with an original isolation media containing 50% artificial seawater.[3] We have already reported that it is important to isolate actinomycetes from a wide variety of environmental substrates by employing various isolation methods for obtaining new bioactive compounds.[3] This study supports our hypothesis.

EXPERIMENTAL SECTION

General Experimental Procedures. UV and IR spectra were measured on a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were recorded on a Varian NMR System 600 NB CL, Varian NMR System 500 NB CL, or a JEOL alpha-500. Chemical shifts were calibrated internally against the residual signal of the solvent in which the sample was dissolved (CD_3OD: δ_C 49.00, δ_H 3.35; DMSO-d_6: δ_H 2.50). HRESIMS data were recorded using a Waters LCT-Premier XE mass spectrometer. MPLC was carried out on a Purif-Pack SI-30 column (Shoko Scientific). Gel filtration was performed using a Sephadex LH-20 column (GE Healthcare BioSciences AB). RP-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. All other reagents and solvents used were of the highest grade available.

Microorganisms. *Streptomyces* sp. R56-07 was isolated from a soil sample collected at Rishiri Island, Hokkaido Prefecture, Japan, by the rehydration and centrifugation method.[4] The basic local alignment search tool (BLAST) was used to compare the 16S rRNA gene sequence (GenBank: AB649055) of R56-07 with 16S rRNA gene sequences available in the EzTaxon server[10] to identify the species of strain R56-07. *Streptomyces* sp. R56-07 was expected to be a new species of the genus *Streptomyces*, and its 16S rRNA gene sequence exhibited a low sequence similarity of 98.2% with *Streptomyces paucisporeus*. The detailed identification of this strain will be reported elsewhere.

Fermentation. *Streptomyces* sp. R56-07 was cultivated in 50 mL test tubes containing 15 mL of seed medium composed of 1.0% starch (Kosokagaku), 1.0% Polypepton (Nihon Pharmaceutical), 1.0% molasses (Dai-Nippon Meiji Sugar), and 1.0% meat extract (Extract Ehlrich, Wako Pure Chemical Industry) (pH 7.2, adjusted before sterilization). The test tubes were shaken on a reciprocal shaker at 27 °C for 2 days (320 rpm). Aliquots (2.5 mL) of the broth were then transferred to 500 mL baffled Erlenmeyer flasks containing 100 mL of a production medium composed of 1.0% starch, 1.0% glucose, 1.0% glycerol, 0.5% Polypepton, 0.2% yeast extract (Mitsubishi Tanabe Pharma), 0.1% NaCl, 0.32% $CaCO_3$, and 1.0 mL of corn steep liquor (pH 7.4 prior to sterilization). The samples were cultured on a rotary shaker (180 rpm) at 27 °C for 5 days.

Isolation. The fermentation broth (2 L) was separated into the supernatant and mycelial cake by centrifugation. The mycelial cake was extracted with acetone (500 mL) and concentrated *in vacuo*. The aqueous concentrate was then extracted with EtOAc (100 mL × 3) and evaporated to dryness. The supernatant was extracted with EtOAc (2 L × 3), after which the organic phase was evaporated. The combined extracts (0.74 g) were then subjected to silica gel MPLC eluted with a gradient system of hexane−EtOAc (0−25% EtOAc) and a stepwise solvent system of $CHCl_3$−MeOH (100:0, 99:1, 98:2, 95:5, 90:10, 80:20, 70:30, 0:100, successively). The fraction eluted with 10% MeOH (122.6 mg) was subjected to a Sephadex LH-20 column and eluted with $CHCl_3$−MeOH (1:1). Next, the fraction containing **1** was purified by RP-HPLC developed with 40% aqueous MeOH containing 0.1% formic acid (flow rate: 10 mL/min) to yield **1** (1.3 mg, t_R = 16.3 min). On the other hand, the 20% MeOH (99.4 mg) eluate was then subjected to Sephadex LH-20 column chromatography ($CHCl_3$−MeOH, 1:1) to obtain crude **2**. Final purification was carried out by RP-HPLC under the same conditions described above to give **2** (2.3 mg, t_R = 33.8 min).

*JBIR-94 (**1**):* colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 230 (3.77), 280 (3.41) nm; IR (KBr) ν_{max} 1652 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; HRESIMS *m/z* 445.2304 $[M + H]^+$ (calcd for $C_{24}H_{33}N_2O_6$, 445.2339).

*JBIR-125 (**2a** and **2b**):* colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 223 (4.32), 279 (3.77) nm; IR (KBr) ν_{max} 1652 cm^{-1}; ^1H

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NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 2; HRESIMS m/z 484.2777 $[M + H]^+$ (calcd for $C_{27}H_{38}N_3O_5$, 484.2811).

DPPH Radical-Scavenging Activity. A 384-well plate was used for the 1,1-diphenyl-2-picrylhydrazyl radical-scavenging assay.[11,12] Briefly, **1**, **2**, and α-tocopherol as a positive control were dissolved in MeOH. Next, 10 μL of 400 μM DPPH dissolved in EtOH and 10 μL of sample were mixed in a 384-well microplate. After 30 min of incubation at room temperature, the absorbance was measured at 525 nm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HR-ESIMS, ^1H NMR, ^{13}C NMR, and 2D NMR spectra in CD_3OD for JBIR-94 (**1**) and JBIR-125 (**2**), and ^1H NMR spectrum for **2** at 80 °C in DMSO-d_6 are available free of charge via the Internet at http://pubs.acs.org.

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