NOTE

JBIR-83 and JBIR-84, new promothiocin derivatives, isolated from *Streptomyces* sp. RI19

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Members of the class Actinobacteria are known to produce pharmaceutically useful compounds and have been extensively studied. Soil has thus far been the primary source of *Actinobacteria*. However, the rate of novel compound discovery from these terrestrial strains has significantly decreased recently. Therefore, we wanted to explore the possibility of isolating unusual *Actinobacteria* capable of producing new metabolites by using clearly devised methods instead of general selection methods using antibiotics. For this purpose, we isolated *Actinobacteria* from soil samples using the membrane filter (MF) method,^{1,2} which allows for selection of *Actinobacteria* without using antibiotics. In fact, we have already reported the isolation of a new species of *Streptomyces* by the MF method.² Our attempts to discover novel compounds from these actinobacterial origin resulted in the successful isolation of new promothiocin derivatives designated as JBIR-83 (1) and JBIR-84 (2) from the culture broth of *Streptomyces* sp. RI19 (Figure 1a). In this paper, we report the fermentation procedure to obtain 1 and 2 and their subsequent isolation, structure elucidation and in brief, their biological activities.

Strain RI19 was isolated by the MF method from a soil sample collected at Shuri, Okinawa Prefecture, Japan. The detailed method of strain isolation has been already reported.² To identify which genus strain RI19 belongs to, we compared its 16S rRNA gene sequences with those available in the DNA Data Bank of Japan using the basic local alignment search tool.³ The strain was identified as the genus *Streptomyces* because its 16S rRNA gene sequence exhibited a high sequence similarity of 99% with *Streptomyces setonensis*.

The strain was cultivated in 50 ml test tubes, each containing 15 ml of a seed medium consisting of starch (Kosokagaku, Tokyo, Japan) 1.0%, polypeptone (Nihon Pharmaceutical, Tokyo, Japan) 1.0%, molasses (Dai-Nippon Meiji Sugar, Tokyo, Japan) 1.0% and meat extract 1.0% (Extract Ehlich; Wako Pure Chemical Industry, Osaka, Japan), pH 7.2 (adjusted before sterilization). The test tubes were agitated 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 β -cyclodextrin (Kanto Chemical, Tokyo, Japan) 4.0%, glycerin 0.5%, pharmamedia (Traders Protein, Lubbock, TX, USA) 2.0%, CuSO₄ 5 mg, MnCl₂ 5 mg and ZnSO₄ 5 mg (pH 7.2; adjusted before sterilization) and cultured on a rotary shaker (180 r.p.m.) at 27 °C for 5 days.

The mycelial cake in the fermentation broth (21) was separated from the supernatant by centrifugation followed by extraction with acetone. The extract was evaporated *in vacuo* to remove the acetone, and the aqueous residue was extracted with EtOAc. The organic layer was dried over Na₂SO₄ and then evaporated to dryness. The residue (0.74 g) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60; Moritex, Tokyo, Japan) and developed with a gradient system of *n*-hexane–EtOAc (0–30% EtOAc) followed by CHCl₃–MeOH (0–50% MeOH). The 3% MeOH-eluted fraction (60.9 mg) was further purified by preparative reversed-phase HPLC using a Pegasil ODS column (20 i.d. × 150 mm; Senshu Chemical, Tokyo, Japan) with 65% MeOH–H₂O containing 0.1% formic acid (flow rate: 10 ml min^{−1}) to yield 1 (1.75 mg; retention time, 8.74 min) and 2 (3.02 mg; retention time, 23.7 min).

Compounds 1 and 2 were obtained as colorless oils ($[\alpha]_D^{25} +15.0$ (c 0.02) for 1 and $+38.6$ (c 0.14) for 2, in MeOH). The molecular formulas of 1 and 2 were determined by HR-electrospray ionization-MS to be C₃₄H₃₆N₁₀O₇S₂ (found: 761.2272 (M+H)⁺, calcd: 761.2288) and C₃₇H₃₉N₁₁O₈S₂ (found: 830.2524 (M+H)⁺, calcd: 830.2503), respectively. In addition, 1 and 2 showed the same IR absorption ($\nu_{\max}$ (KBr) 3400, 1660 cm^{−1}), which indicated the presence of an amide functional group. UV absorptions were also identical between 1 and 2 (1: $\lambda_{\max}$ (ϵ), 313 nm (sh, 9400) and 226 nm (sh, 36 000); 2: $\lambda_{\max}$ (ϵ), 313 nm (sh, 9000) and 226 nm (sh, 38 000) in MeOH).

The structures of 1 and 2 were mainly determined by NMR spectral analyses and the tabulated ¹³C and ¹H NMR spectral data for 1 and 2,

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Figure 1 (a) Structures of JBIR-83 (1), JBIR-84 (2) and promothiocin A. Key correlations in double quantum filtered COSY (bold lines) and HMBC (arrows) spectra of **1** (b) and **2** (c).

which were obtained by analyses of heteronuclear single quantum coherence spectra, are shown in Table 1. Comparison of the ^{13}C and ^1H NMR data of **1** with those of promothiocin A⁴ together with 2D NMR spectral analyses, such as ^1H - ^1H double quantum filtered (DQF)-COSY and heteronuclear multiple bond correlation (HMBC) spectra (key correlations are summarized in Figure 1b) revealed that **1**

has a similar skeleton as that of promothiocin A. In the ^1H NMR spectrum of **1**, proton signals corresponding to amino-acid-derived partial structures, 2 thiazoles, 2 methyloxazols and a valine residue, were observed. The most characteristic difference in the ^1H NMR spectra between **1** and promothiocin A was the presence of a dehydroalanine unit. The other difference was the appearance of a

Table 1 ^1H and ^{13}C NMR spectral data for JBIR-84 (1) and JBIR-84 (2)

Position	1		2		Promothiocin A ^a
	^{13}C	^1H (J in Hz)	^{13}C	^1H (J in Hz)	^{13}C
<i>Thiazole (1)</i>					
1	164.5		164.0		163.7
2	150.1		149.6		149.0
3	127.2	8.38, s	126.6	8.40, s	126.9
4	160.4		160.0		159.7
<i>Valine</i>					
5-NH		7.98, d (7.6)		8.14, d (7.5)	
5	58.0	4.41, m	57.6	4.41, m	57.4
6	32.3	2.37, m	31.8	2.40, m	31.2
7	20.5	1.00, d (6.9)	20.1	1.00, d (6.8)	18.3
8	18.5	0.98, d (6.9)	18.1	1.00, d (6.8)	19.4
9	170.8		170.4		170.6
<i>Methyloxazol (1)</i>					
10-NH		8.71, d (7.3)		8.83, d (7.3)	
10	43.6	4.89, m	43.3	4.87, m	35.2
11	18.6	1.42, d (6.7)	18.2	1.42, d (6.5)	
12	162.9		162.6		158.2
13	128.7		128.1		128.6
14	151.2		152.8		152.6
14-CH ₃	12.0	2.51, s	11.4	2.50, s	11.2
15	160.4		160.0		160.1
<i>Thiazole (2)</i>					
16-NH		7.70, d (8.3)		7.70, d (8.1)	
16	45.6	5.46, m	45.2	5.46, m	45.2
17	21.3	1.60, d (6.7)	20.9	1.60, d (6.3)	19.8
18	172.3		171.9		171.8
19	149.4		148.7		148.5
20	125.8	8.28, s	125.4	8.31, s	125.0
21	160.4		160.0		159.8
<i>Methyloxazol (2)</i>					
22-NH		7.89, br s		7.88, br s	
22	43.6	5.10, m	42.9	5.12, m	43.1
23	19.1	1.42, d (6.7)	18.2	1.42, d (6.8)	18.0
24	161.8		161.4		161.8
25	133.6		132.9		132.3
26	149.7		149.6		149.2
26-CH ₃	12.0	2.49, s	11.9	2.64, s	11.5
<i>Pyridine</i>					
27	151.2		148.7		148.0
28	130.2		130.2		130.3
29	141.0	8.42, d (8.1)	141.6	8.47, d (8.0)	141.2
30	121.8	8.12, d (8.1)	120.7	8.17, d (8.0)	120.6
31	149.4		149.2		149.0
32	165.9		161.4		161.2
32-NH		8.01, br s		10.65, s	
<i>Dehydroalanine</i>					
33			133.8		133.6
34			165.1		164.9
35			102.7	6.57, s, 5.84, s	102.4

C (150 MHz) and ^1H (600 MHz) NMR spectra were obtained on an NMR System 600 NB CL (Varian, Palo Alto, CA, USA) in DMSO-*d*₆, and the solvent peak was used as an internal standard (δ_{C} 39.5, δ_{H} 2.50).

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methyl proton signal at H-11 (δ_{H} 1.42), which indicated that a glycine-originated residue was replaced by alanine in **1**. The sequence from an amide proton 10-NH (δ_{H} 8.71) to H-11 through methine proton H-10 (δ_{H} 4.89) in the DQF-COSY spectrum together with the ^1H - ^{13}C long-range couplings from H-11 to C-10 (δ_{C} 43.6) and C-12 (δ_{C} 162.9) confirmed the connectivity of the methyl group. Furthermore, the connectivities of all partial structures were confirmed by HMBC experiments as shown in Figure 1b. From these observations, the structure of **1** was established as shown in Figure 1a. It is a methyl derivative of the antibiotic 10381T.⁵

The ^1H and ^{13}C NMR signals of **2** resemble those of promothiocin A. The only difference between **2** and promothiocin A was the presence of a methyl doublet at C-11 (δ_{H} 1.42, δ_{C} 18.2), which was also recognized in **1**. Thus, these results show that **2** is a methyl derivative of promothiocin A at C-10 as shown in Figure 1a.

We evaluated the biological activities, namely, the cytotoxic, antimicrobial and free radical scavenging activities of **1** and **2**. The results showed that **1** protected neuronal hybridoma N18-RE-105 cells from L-glutamate toxicity, which provides an assessment of their free radical scavenging activity,^{6–8} with EC_{50} values of 74 μM , whereas **2** hardly showed the protective activity. The protective effect of **1** was weaker than the representative antioxidant, α -tocopherol (EC_{50} =6.3 μM). Although compounds such as phenazostatins,⁶ benzozostatins,⁹ aestivophoenins¹⁰ and carbazomadurasins¹¹ isolated from actinomycete have been previously reported to show protective activity against L-glutamate toxicity, their structures are not similar to that of **1**. In addition, because radical scavenging activity could not be predicted from the chemical structures of **1**, it would be interesting to clarify the mode of action of **1**. Moreover **1** and **2** did not exhibit cytotoxic activity against several cancer cell lines nor did they show antibacterial activities against *Micrococcus luteus*, *Escherichia coli* and *Candida albicans*.

In conclusion, we obtained new promothiocin derivatives, namely, JBIR-83 and JBIR-84, from the culture broth of *Streptomyces* strain

RI18 isolated using the MF method. We believe that this study will convince chemists that Actinobacteria isolated by the MF method can produce new bioactive compounds and also encourage them to investigate the isolation of Actinobacteria through a new method.

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