

ORIGINAL ARTICLE

A new angucycline and a new butenolide isolated from lichen-derived *Streptomyces* spp.

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A new 1,1-dichlorocyclopropane-containing angucycline, JBIR-88 (1), and a new butenolide, JBIR-89 (2), respectively, were isolated from the fermentation broths of two lichen-derived actinomycetes identified as a new species of *Streptomyces* (strains RI104-LiC106 and RI104-LiB101) using phylogenetic methods. The structures of 1 and 2 were elucidated on the basis of 1D and 2D NMR spectroscopy and MS analyses. Compound 1 showed cytotoxic activities against cancer cells and antimicrobial activity against *Micrococcus luteus*.

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Keywords: angucycline; antimicrobial activity; butenolide; cytotoxicity; dichlorocyclopropane; lichen; *Streptomyces*

INTRODUCTION

Lichens are composite and symbiotic organisms. Biologically, they often have been interpreted as one organism (fungi and algae associated within a common thallus), but taxonomically, they are classified as a life form of ascomycetous fungi. In all, ~46% of the ascomycota are lichen forming; however, 2–3% of the lichen fungi are basidiomycota. Lichen-forming fungi produce a wide variety of secondary metabolites. The majority of lichen secondary metabolites are aromatic polyketides, and a number of these have been shown to exhibit biological activity.¹ However, few reports of the isolation of actinobacteria from lichens or their products are available, and only one study reports that *Streptomyces* obtained from the surface of a lichen native to British Columbia produces uncialamycin, a new enediyne antibiotic.² Thus, our intention was to provide evidence that new species of *Streptomyces* isolated from lichens are capable of producing unique metabolites. For this purpose, we isolated new species of *Streptomyces* from lichens and then searched for secondary metabolites in the cultures of the isolated strains. The isolated strains RI104-LiC106 and RI104-LiB101 were identified as new species of the genus *Streptomyces* by performing a homology search of their 16S rRNA gene sequences. In the course of screening for novel metabolites of RI104-LiC106 and RI104-LiB101, we succeeded in isolating a new 1,1-dichlorocyclopropane-containing angucycline, designated as JBIR-88 (1), from the culture broth of RI104-LiC106, and a new butenolide, designated as JBIR-89 (2), from RI104-LiB101 (Figure 1). This paper describes the fermentation, isolation, structure elucidation and biological activities of 1 and 2.

RESULTS AND DISCUSSION

Structure elucidation of 1

Strain RI104-LiC106 was cultured at 27 °C on a rotary shaker. The acetone extract of mycelial cake from a fermentation broth (2l) was extracted with EtOAc. After drying over Na₂SO₄, the EtOAc layer was evaporated to dryness, and the dried residue (670 mg) was subjected to normal- and reversed-phase chromatography to yield 1 (1.19 mg) as an orange oil. The molecular formula of 1 was obtained as C₁₉H₉O₆Cl₃ from its HR-electrospray ionization-MS (HR-ESI-MS), which showed a molecular ion at *m/z* 438.9557 [M + H]⁺.

The structure of 1 was clarified by analyses of the 2D NMR spectra, which included heteronuclear single-quantum coherence, heteronuclear multiple-bond correlation and double quantum filtered (DQF)-COSY spectra (Figure 2). Table 1 shows the ¹H and ¹³C NMR data recorded in CDCl₃ from the heteronuclear single-quantum coherence analysis. The *o*-coupling correlation (³J_{H-H}=8.1 Hz) between doublet aromatic protons H-10 (δ_H 7.80) and H-11 (δ_H 7.64) was observed by DQF-COSY. In the heteronuclear multiple-bond correlation spectrum, the aromatic protons H-10 and H-11 were strongly *m*-coupled to aromatic quaternary carbons as follows: H-10 to C-8 (δ_C 157.9) and C-11a (δ_C 133.3), and H-11 to C-7a (δ_C 115.9) and C-9 (δ_C 129.7). In addition, H-11 was ¹H-¹³C long-range coupled to a quinone carbonyl carbon C-12 (δ_C 181.5) at the *peri* position and weakly *W*-type coupled to another quinone carbonyl carbon C-7 (δ_C 192.5). ¹H-¹³C long-range couplings from the phenolic hydroxyl proton 8-OH (δ_H 12.21) to C-8 and C-7a, together with the low-field ¹³C chemical shift at C-7 (δ_C 192.5) compared with C-12 (δ_C 181.5),

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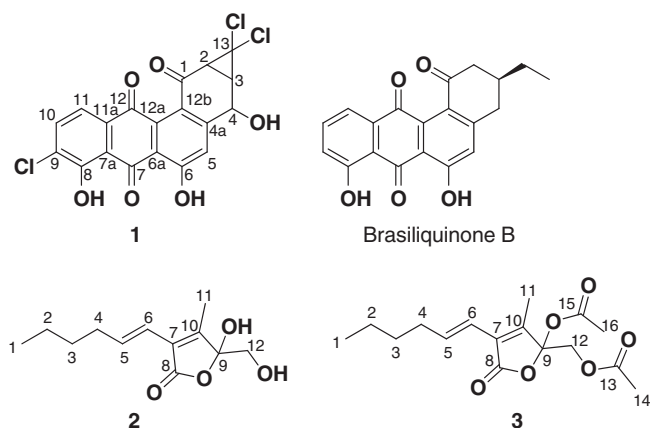


Figure 1 Structures of 1–3 and brasiliquinone.

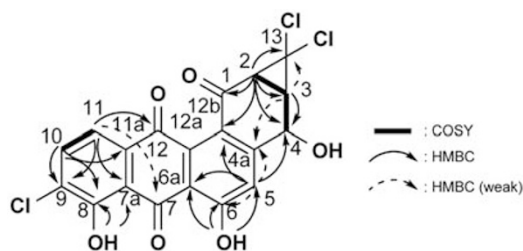


Figure 2 Key correlations between double quantum filtered-COSY (bold lines) and heteronuclear multiple-bond correlation (arrows) spectra of 1.

supported the existence of the hydrogen-bonded phenolic hydroxyl proton with the quinone carbonyl carbon C-12. Taken together, these data indicated the presence of a naphthoquinone moiety in 1 (Figure 2).

The sequence from a doublet methine proton H-2 (δ_{H} 3.13) to an oxymethine proton H-4 (δ_{H} 5.24) through a double doublet methine proton H-3 was observed in the DQF-COSY spectrum. The six-membered ring structure shown in Figure 2 was established by ^1H - ^{13}C long-range couplings from the methine proton H-2 to an α,β -unsaturated carbonyl carbon C-1 (δ_{C} 189.1) and an aromatic quaternary carbon C-12b (δ_{C} 126.6), which in turn strongly *m*-coupled to an aromatic proton H-5 (δ_{H} 7.51), and from H-5 to an oxymethine carbon C-4 (δ_{C} 64.7) and an aromatic quaternary carbon C-4a (δ_{C} 152.1), which in turn was long-range coupled to the methine proton H-3. Both H-2 and H-3 were ^1H - ^{13}C long-range coupled to a quaternary carbon C-13 (δ_{C} 60.4). Thus, C-2, C-3 and C-13 were considered to form a cyclopropane moiety. ^1H - ^{13}C long-range couplings from a phenolic hydroxyl proton 6-OH (δ_{H} 12.18) to C-5 (δ_{C} 120.3), C-6 (δ_{C} 164.9) and C-6a (δ_{C} 117.4), which in turn strongly *m*-coupled to an aromatic proton H-5, established the assignment of these carbons. Although no correlation to a quaternary aromatic carbon C-12a (δ_{C} 136.5) was detected, this carbon was assigned to the remaining naphthoquinone moiety. These assignments are also supported by comparing ^{13}C NMR chemical shifts with those of brasiliquinone B.⁸ The molecular formula of 1 suggests that three hydroxyl groups and three chlorine atoms are substituted at C-4, C-6 and C-8, and at C-9 and C-13, respectively. Therefore, these spectral data prove the formation of a 1,1-dichlorocyclopropane ring comprising C-2, C-3 and C-13. To confirm the 1,1-dichlorocyclopropane ring

Table 1 ^{13}C (150 MHz) and ^1H NMR (600 MHz) spectroscopic data for 1 in CDCl_3

Position	1	
	^{13}C	^1H (J in Hz)
1	189.1	
2	42.4	3.13 d (10.7)
3	38.3	3.03 dd (10.7, 5.9)
4	64.7	5.24 d (5.9)
4a	152.1	
5	120.3	7.51 s
6	164.9	
6a	117.4	
7	192.5	
7a	115.9	
8	157.9	
9	129.7	
10	138.0	7.80 d (8.1)
11	120.6	7.64 d (8.1)
11a	133.3	
12	181.5	
12a	136.5	
12b	126.6	
13	60.4	
6-OH		12.18 s
8-OH		12.21 s

Abbreviation: H NMR, proton nuclear magnetic resonance.

in 1, the $^1J_{\text{C-H}}$ and ^{13}C NMR chemical shifts of 1 were compared with those of 1,1-dichloro-7,7a-dihydro-1*H*-cyclopropa[*b*]naphthalene-2(1*aH*)-one.⁹ Results showed that the $^1J_{\text{C-H}}$ large values of C-2 (178.5 Hz) and C-3 (175.3 Hz) in 1 were almost identical to those of C-2 (172 Hz) and C-3 (169 Hz) in 1,1-dichloro-7,7a-dihydro-1*H*-cyclopropa[*b*]naphthalene-2(1*aH*)-one. In addition, the ^{13}C NMR chemical shifts of the 1,1-dichloropropane ring in 1 were similar to those of C-2 (δ_{C} 37.8), C-3 (δ_{C} 30.7) and C-13 (δ_{C} 60.7) in 1,1-dichloro-7,7a-dihydro-1*H*-cyclopropa[*b*]naphthalene-2(1*aH*)-one, with the exception of the disappearance of the hydroxyl group at C-4. Thus, the structure of 1 was determined as shown in Figure 1.

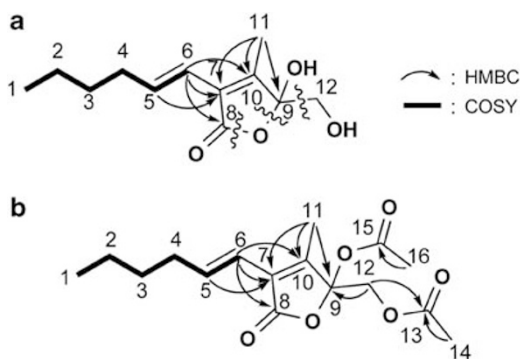
Structure elucidation of 2

Similar to strain RI104-LiC106, strain RI104-LiB101 was cultured and extraction of the mycelial acetone extract was performed using EtOAc. The dried residue of the EtOAc layer (670 mg) then was subjected to normal- and reversed-phase chromatography to yield 2 (1.22 mg) as a colorless oil. The molecular formula of 2 was determined to be $\text{C}_{20}\text{H}_{23}\text{ClN}_4\text{O}_7$ on the basis of HR-ESI-MS data (m/z 467.1305 [$\text{M} + \text{H}]^+$). The ^1H and ^{13}C NMR spectral data for 2 are shown in Table 2. The structural information for 2 was obtained by the series of 2D NMR analyses (Figure 3a). The sequence from a triplet methyl proton H-1 (δ_{H} 0.90) to a doublet olefinic proton H-6 (δ_{H} 6.03) through the methylene protons H-2 (δ_{H} 1.34), H-3 (δ_{H} 1.42), H-4 (δ_{H} 2.18) and an olefinic methine proton H-5 (δ_{H} 6.84) was established by the analyses of the DQF-COSY spectrum. The structural relationships among C-7, C-8, C-9 and C-10 were established by ^1H - ^{13}C long-range couplings from a singlet methyl proton H-11 (δ_{H} 2.03) to the olefinic quaternary carbons C-7 (δ_{C} 126.6), C-10 (δ_{C} 153.0) and the acetal quaternary carbon C-9 (δ_{C} 104.8), and from H-6 to an ester carbonyl carbon C-8 (δ_{C} 170.2) and the

Table 2 ^{13}C (150 MHz) and ^1H NMR (600 MHz) spectroscopic data for **2** in CDCl_3 and ^{13}C (125 MHz) and ^1H NMR (500 MHz) spectroscopic data for **3** in CDCl_3

Position	2		3	
	^{13}C	^1H (J in Hz)	^{13}C	^1H (J in Hz)
1	14.2	0.90 t (7.4)	14.2	0.91 t (7.3)
2	22.5	1.34 m	22.6	1.35 m
3	31.2	1.42 m	31.1	1.43 m
4	33.8	2.18 q (7.1)	33.9	2.19 q (7.0)
5	140.3	6.84 dt (15.8, 7.1)	140.5	6.87 dt (15.8, 7.0)
6	117.2	6.03 d (15.8)	117.0	6.05 d (15.8)
7	126.6		127.2	
8	170.2		168.7	
9	104.8		103.5	
10	153.0		150.4	
11	11.2	2.03 s	10.9	1.99 s
12	65.4	3.75 m	62.8	4.47 d (11.6), 4.37 d (11.6)
13			170.2	
14			20.8	2.03 s
15			167.8	
16			21.7	2.08 s

Abbreviation: H NMR, proton nuclear magnetic resonance.

**Figure 3** Key correlations between double quantum filtered-COSY (bold lines) and heteronuclear multiple-bond correlation (arrows) spectra of **2** (a) and **3** (b).

olefinic quaternary carbons C-7 and C-10. The remaining unit, a hydroxymethyl carbon C-12 (δ_{H} 3.75, δ_{C} 65.4), was not assigned because of the lack of heteronuclear multiple-bond correlation due to signal broadening.

To establish the structure of **2**, the acetylation of **2** was performed to simplify the proton-coupling pattern of the hydroxymethyl C-12 and to determine the cyclized site. The correlation between ^1H and ^{13}C signals for the diacetate derivative of JBIR-89 (**3**) was established by heteronuclear single-quantum coherence analysis (Table 2). In the heteronuclear multiple-bond correlation spectrum of **3**, ^1H - ^{13}C long-range correlations of the methylene proton H-12 (δ_{H} 4.47 and 4.37), the acetal carbon C-9 (δ_{C} 103.5) and the carbonyl carbon C-13 (δ_{C} 170.2), which in turn long-range coupled to a singlet methyl proton H-14 (δ_{H} 2.03), were observed. Other DQF-COSY and ^1H - ^{13}C long-range correlations are identical to those of **2** (Figure 3b). Therefore, the structure of **2** was determined to be 3-(hex-1-en-1-yl)-5-hydroxy-5-(hydroxymethyl)-4-methylfuran-2(5H)-one (Figure 1).

Biological activity

To observe the biological activity of **1** and **2**, cytotoxic and antimicrobial activities were examined. Compound **1** exhibited cytotoxic activity against HeLa and ACC-MESO-1 cells with IC_{50} values of 36 and 52 μM , respectively. However, **2** was inactive against both cells (IC_{50} values > 250 μM). In addition, **1** exhibited antibacterial activity (inhibition zone, 11 mm at a concentration of 25 μg on 6 mm disk) against *Micrococcus luteus*, but showed no antimicrobial activity against *Candida albicans* and *Escherichia coli*. Detailed investigation into the biological activity of **1** is currently underway.

In conclusion, we isolated JBIR-88 (**1**), possessing a unique substructure 1,1-dichlorocyclopropane ring moiety, from the strain RI104-LiC106, and isolated JBIR-89 (**2**) from RI104-LiB101 as a butenolide¹⁰ derivative. The structure of **1** was found to be structurally related to BE-23254 isolated from *Streptomyces* sp. A 23254.¹¹ However, this is the first report of a tetraphene containing the dichlorocyclopropane ring. The results of this study suggest that new species of *Streptomyces* isolated from lichens have the ability to produce new compounds and may serve to encourage chemists to investigate the isolation of actinobacteria from lichens.

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 DU730 UV/Vis spectrophotometer (Beckman Coulter, CA, USA) and an FT-720 spectrophotometer (Horiba), respectively. NMR spectra were recorded on a Varian NMR System 500 or 600 NB CL (Varian, Palo Alto, CA, USA) in CDCl_3 (7.24 p.p.m. for ^1H , 77.0 p.p.m. for ^{13}C) with the residual solvent peak as the internal standard. HR-electrospray ionization-MS (HR-ESI-MS) data were recorded on an LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA). Normal-phase medium-pressure liquid chromatography was performed using a Purif-pack SI-60 column (Moritex, Tokyo, Japan). Analytical reversed-phase HPLC was carried out using an MGII ODS column (4.6 i.d. \times 150 mm; Shiseido, 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 MGII ODS column (20 i.d. \times 150 mm; Shiseido).

Microorganisms

The producing *Streptomyces* designated as RI104-LiC106 and RI104-LiB101 was isolated from the whole body of a lichen collected from Risiri Island, Hokkaido Prefecture, Japan by the moist incubation and desiccation method.³ The basic local alignment search tool search of the 16S rRNA gene sequence of RI104-LiC106 (GenBank: AB552917) and RI104-LiB101 (GenBank: AB552918) showed that the strains belonged to new species of the genus *Streptomyces*.

Fermentation

Streptomyces sp. RI104-LiC106 and RI104-LiB101 were cultivated in 50-ml test tubes each containing 15 ml of a seed medium consisting of starch 1% (Kosokagaku, Tokyo, Japan), polypeptone 1% (Nihon Pharmaceutical, Tokyo, Japan), molasses 1% (Dai-Nippon Meiji Sugar, Tokyo, Japan) and meat extract 1% (Extract Ehlrich, Wako Pure Chemical Industries, Osaka, Japan), pH 7.2 (before sterilization). The test tubes were shaken on a reciprocal shaker (355 r.p.m.) at 27 $^{\circ}\text{C}$ for 2 days. Aliquots (2.5 ml) of the broths were transferred to 500-ml baffled Erlenmeyer flasks containing 100 ml of a production medium corresponding to each strain and cultured on a rotary shaker (180 r.p.m.) at 27 $^{\circ}\text{C}$ for 5 days. The production medium for RI104-LiC106 consisted of β -cyclodextrin 4% (Kanto Chemical, Tokyo, Japan), glycerin 0.5%, pharmaceutical 2% (Traders Protein, Memphis, TN, USA), CuSO_4 5 mg, MnCl_2 5 mg and ZnSO_4 5 mg, pH 7.2 (before sterilization). The production medium for RI104-LiB101 consisted of glycerin 2%, molasses 1%, casein 5% (Kanto Chemical) and CaCO_3 (Kozakai Pharmaceutical, Tokyo, Japan) 0.4%, pH 7.2 (before sterilization).

Isolation of 1

The mycelial cake obtained from the fermentation broth (21) was extracted with Me₂CO (500 ml). The extract was concentrated *in vacuo*, and the aqueous concentrate was extracted with EtOAc (100 ml × 3). After drying over Na₂SO₄, the organic layer was evaporated to dryness. The residue (670 mg) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60, Moritex) and successively eluted with a gradient system of *n*-hexane–EtOAc (0–30% EtOAc) and CHCl₃–MeOH (0–50% MeOH). The 1% MeOH-eluted fraction (52.9 mg) was purified by preparative reversed-phase HPLC using an MGII ODS column (20 i.d. × 150 mm, Shiseido) with 70% MeOH–H₂O containing 0.1% formic acid (flow rate 8.0 ml min⁻¹) to yield 1 (1.19 mg, retention time=13.3 min).

Isolation of 2

The fermentation broth (21) was centrifuged, and the mycelial cake was extracted with Me₂CO (500 ml). After concentration *in vacuo*, the aqueous concentrate was extracted with EtOAc (100 ml × 3). After being dried over Na₂SO₄, the organic layer was evaporated to dryness. The residue (410 mg) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60, Moritex) and successively eluted with a gradient system of *n*-hexane–EtOAc (0–30% EtOAc) and CHCl₃–MeOH (0–50% MeOH). The 1% MeOH-eluted fraction (35.8 mg) was purified by preparative reversed-phase HPLC using an MGII ODS column (20 i.d. × 150 mm, Shiseido) with 60% MeOH–H₂O containing 0.1% formic acid (flow rate 8.0 ml min⁻¹) to yield 2 (1.22 mg, retention time=11.7 min).

JBIR-88 (1): orange oil; [α]_D²⁰ -190 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 438 (4.16) and 271 (4.66) nm; IR (KBr) ν_{max} 3030, 1690, 1670 and 1630 cm⁻¹; ¹H NMR (600 MHz, CDCl₃) and ¹³C NMR (150 MHz, CDCl₃), see Table 1; HR-ESI-MS *m/z* 438.9557 [M + H]⁺ (calcd for C₁₉H₁₀O₆Cl₃, 438.9543).

JBIR-89 (2): colorless oil; [α]_D²⁰ +15 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 261 (3.60) nm; IR (KBr) ν_{max} 3040 and 1760 cm⁻¹; ¹H NMR (600 MHz, CHCl₃) and ¹³C NMR (150 MHz, CHCl₃), see Table 2; HR-ESI-MS *m/z* 227.1286 [M + H]⁺ (calcd for C₁₂H₁₉O₄, 227.1283).

Acetylation of 2

A sample of 2 (1.0 mg) was dissolved in 100 μl of pyridine, mixed with 10 μl of acetic anhydride and stirred at room temperature for 2 h. The mixture was diluted with MeOH, evaporated *in vacuo* and applied to preparative reversed-phase HPLC using an MGII ODS column (20 i.d. × 150 mm, Shiseido) developed with 75% MeOH–H₂O (flow rate 8.0 ml min⁻¹) to yield JBIR-89 diacetate (3; 0.62 mg, retention time=10.6 min).

Biological activity

Cytotoxicity against human cervical carcinoma HeLa cells and human malignant pleural mesothelioma ACC-MESO-1 cells^{4–7} were determined by a colorimetric assay, using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt. Cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries) for HeLa cells or RPMI medium (Nacal Tesque, Kyoto, Japan) for ACC-MESO-1 cells,

supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹) at 37 °C in a humidified incubator under a 5% CO₂ atmosphere. The 384-well plates were seeded with aliquots of a 20-μl medium containing 1 × 10³ cells per well and were incubated overnight before being treated with compounds at various concentrations for 48 h. Plates were incubated for 1 h at 37 °C after the addition of 2 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt reagent solution (Cell Counting Kit; Dojindo, Kumamoto, Japan) per well. The absorption of the formed formazan dye was measured at 450 nm. The vehicle solvent (dimethyl sulfoxide) was used as a negative control. Paclitaxel as a positive control showed cytotoxicity against HeLa and ACC-MESO-1 cells with IC₅₀ values of 30 and 24 nM, respectively.

Antimicrobial activity against *M. luteus* ATCC9341 was measured by a paper disk method. A paper disk (i.d. 6 mm) containing 25 μg of a sample was placed on an LB agar plate consisting of yeast extract 5% (BD Biosciences, San Jose, CA, USA), peptone 1% (BD Biosciences) and NaCl 1%, and the plate was incubated at 27 °C for 24 h. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

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