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NOTE

JBIR-65, a new diterpene, isolated from a sponge-derived *Actinomadura* sp. SpB081030SC-15

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Marine microorganisms, particularly, marine actinomycetes, are one of the most important resources for new biologically active metabolites,[1] and in fact, many new compounds have been isolated from sponge-derived actinomycetes.[2–4] We have recently discovered novel compounds, namely the anthracyclines tetracenoquinocin and 5-iminoaranciamycin,[5] a teleocidin JBIR-31,[6] the tetrapeptides JBIR-34 and JBIR-35,[7] the isoprenoids JBIR-46, JBIR-47, JBIR48[8,9] and a new salicylamide JBIR-58.[10] Our intention was to support the idea that new species are capable of producing unique metabolites. For this purpose, we isolated new species of actinomycetes from marine sponges and then searched for secondary metabolites in the culture of isolated strains. In this study, we isolated a new species (SpB081030SC-15) of *Actinomadura* from an unidentified marine sponge and purified a new diterpene compound designated as JBIR-65 (**1**, Figure 1a) from the fermentation broth of *Actinomadura* sp. SpB081030SC-15. In this study, we report the fermentation procedure to obtain **1** and its subsequent isolation, structure elucidation and, in brief, its biological activities.

Actinomadura sp. SpB081030SC-15 was isolated from an unidentified marine sponge collected offshore of Ishigaki Island, Okinawa Prefecture, Japan. To identify the species of the strain SpB081030SC-15, we compared its 16S ribosomal RNA gene sequences (Accession No. AB123566) with those available in the DNA Data Bank of Japan using a basic local alignment search tool.[11] The strain was identified as a new species of the genus *Actinomadura* because comparison of its 16S ribosomal RNA gene sequence revealed a low sequence similarity of 98% with *Actinomadura nitritigenes* (AB364595).

The strain SpB081030SC-15 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 Ehlrich, Wako Pure Chemical Industry, Osaka, Japan), pH 7.2 (adjusted before sterilization). The seed culture in test tubes was 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 glycerin 2.0%, molasses 1.0%, casein 0.5% and $CaCO_3$ 0.4%, pH 7.2 (adjusted before sterilization) and cultured on a rotary shaker (180 r.p.m.) at 27 °C for 5 days.

The mycelia in the fermentation broth (2 l) was separated by centrifugation and then extracted with 80% 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 Na_2SO_4 and evaporated to dryness. The residue (700 mg) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60, Moritex, Tokyo, Japan) and eluted with a gradient system of *n*-hexane–EtOAc (0–30% EtOAc) and $CHCl_3$-MeOH (0–50% MeOH), successively. The 3% MeOH-eluted fraction (50.1 mg) was further purified by preparative reverse-phase HPLC using an L-column 2 column (20 i.d.×150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) with 60% MeOH-H_2O containing 0.1% formic acid (flow rate: 10 ml·min^{-1}) to yield **1** (0.22 mg, retention time 15.5 min).

Compound **1** was isolated as a colorless oil ($[\alpha]_D^{25}$ −90.0, *c* 0.1, MeOH) that yielded an $[M+H]^+$ ion at *m/z* 317.1736 in HR electrospray ionization-MS spectra consistent with the molecular formula $C_{19}H_{24}O_4$ (calcd for $C_{19}H_{25}O_4$, 317.1753). The IR absorption (KBr) ν_{max} at 3360 and 1660 cm^{-1} showed the presence of hydroxyl and quinone functional groups in **1**. Direct connectivity between protons and carbons was established by analysis of the heteronuclear single quantum coherence spectrum, and the tabulated ^{13}C and ^{1}H NMR spectral data for **1** are shown in Table 1. The analyses of double-quantum filtered (DQF)-COSY and heteronuclear multiple-bond correlation spectra revealed the structure of **1** (Figure 1b). Correlations between olefinic protons 15-H (δ_H 5.75) and 16-H (δ_H 4.85 and 4.92)

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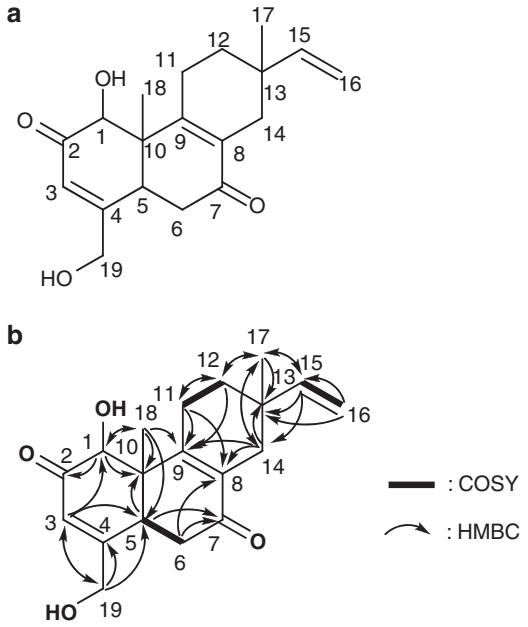

Figure 1 (**a**) Structure of JBIR-65 (**1**). (**b**) Key correlations in double-quantum filtered-COSY (bold lines) and heteronuclear multiple-bond correlation (HMBC) (arrows) spectra of **1**.

Table 1 ^{1}H and ^{13}C NMR spectral data for JBIR-65 (1)

	1	
Position	*^{13}C*	*^{1}H (J in Hz)*
1	78.2	4.28, s
2	199.2	
3	120.4	6.45, br s
4	165.1	
5	42.0	3.39, dd (13.8, 4.5)
6	34.5	2.78, dd (17.9, 4.5), 2.61, dd (13.8, 17.9)
7	197.4	
8	130.1	
9	165.4	
10	49.0	
11	27.0	2.71, m
12	33.2	1.52, m
13	33.9	
14	33.2	2.46, d (17.9), 1.91, d (17.9)
15	146.4	5.75, dd (17.4, 10.8)
16	110.3	4.85[a], 4.92, dd (10.8, 1.0)
17	25.2	1.02, s
18	12.0	0.98, s
19	61.5	4.25, br s

^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectra were obtained on an NMR System 500 NB CL (Varian, Palo Alto, CA, USA) in CD_3OD, and the solvent peak was used as an internal standard (δ_C 49.0, δ_H 3.20).
[a]Overlapping.

and between methylene protons 11-H (δ_H 2.71) and 12-H (δ_H 1.52), respectively, were observed in the DQF-COSY spectrum. The ^{1}H-^{13}C long-range couplings between a singlet methyl proton 17-H (δ_H 1.02) and a quaternary carbon C-13 (δ_C 33.9), methylene carbons C-12 (δ_C 33.2) and C-14 (δ_C 33.2) and an olefinic carbon C-15 (δ_C 146.4) revealed the connectivity between C-11 (δ_C 27.0), C-12, C-13, C-14, C-15 and C-17 (δ_C 25.2). Furthermore, ^{1}H-^{13}C long-range couplings between 11-H and methylene proton 14-H (δ_H 1.91 and 2.46) and olefinic quaternary carbons C-8 (δ_C 130.1) and C-9 (δ_C 165.4) and homoallylic coupling between 11-H and 14-H proved the existence of a 4-methyl-4-vinylcyclohex-1-ene moiety.

A correlation between a methine proton 5-H (δ_H 3.39) and methylene protons 6-H (δ_H 2.61 and 2.78) was observed in the DQF-COSY spectrum. The ^{1}H-^{13}C long-range couplings from a singlet methyl proton 18-H (δ_H 0.98) to an oxymethine carbon C-1 (δ_C 78.2), a quaternary carbon C-10 (δ_C 49.0), C-9 and a methine carbon C-5 (δ_C 42.0), whose methine proton 5-H was in turn long-range coupled to an α,β-unsaturated ketone carbonyl carbon C-7 (δ_C 197.4) and C-8, revealed the presence of an octalone substructure. A hydroxymethyl proton 19-H (δ_H 4.25) was ^{1}H-^{13}C long-range coupled to an olefinic methine carbon C-3 (δ_C 120.4), olefinic quaternary carbon C-4 (δ_C 165.1) and C-5. The ^{1}H-^{13}C long-range couplings between an olefinic methine proton 3-H (δ_H 6.45) and C-1 and between an oxymethine proton 1-H (δ_H 4.28) and an α,β-unsaturated ketone carbonyl carbon C-2 (δ_C 199.2) established the presence of a six-membered moiety, as shown in Figure 1b. The ^{13}C NMR chemical shifts in the case of **1** resembled those in the case of 15-isopimaradien-7-one[12] and 2-oxo-18-hydroxy-10α,17α,19α,20β-(−)-cleroda-3,13(16),14-triene,[13] supporting the planar structure of **1**, as shown in Figure 1a.

To evaluate the biological activity of **1**, we conducted tests to evaluate its cytotoxic, antimicrobial and radical scavenging activities. The results showed that **1** protects neuronal hybridoma N18-RE-105 cells[14–16] from L-glutamate toxicity with an EC_{50} value of 31 μM. However, this protective effect of **1** was weaker than that of the representative antioxidant α-tocopherol (EC_{50} value, 6.3 μM). It would be interesting to clarify the mode of action of **1** because its radical scavenging activity could not be determined on the basis of its chemical structure. The cytotoxic activity of **1** against several cancer cell lines was tested by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) colorimetric assay (Cell Counting Kit, Dojindo, Kumamoto, Japan). However, **1** showed no cytotoxicity even at a concentration of 50 μg ml^{-1} for 48 h. In addition, **1** showed no antimicrobial activity against *Micrococcus luteus*, *Escherichia coli* and *Candida albicans*.

In conclusion, we isolated a new diterpene, JBIR-65 (**1**), from the strain SpB081030SC-15. Diterpenes have been isolated from many fungi and plants, however, few reports of the isolation of diterpenes from actinobacteria are available. To our knowledge, this is the first report of a diterpene compound isolated from the genus *Actinomadura*. This study suggests that new species of actinomycetes isolated from marine organisms have the potential to produce new bioactive compounds.

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