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NOTE

Isolation of 2 new metabolites, JBIR-74 and JBIR-75, from the sponge-derived *Aspergillus* sp. fS14

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The marine environment has become an attractive new source of chemical diversity for drug discovery, as new bioactive substances are isolated from marine organisms, including phytoplankton, algae, sponges, tunicates and mollusks.[1] Our group has reported the isolation of azaspiracid-22[2] and JBIR-44[3] from marine sponges. Marine microorganisms, especially fungi,[4] are an untapped resource of novel bioactive substances such as diketopiperazine alkaloids,[5] trichodermatides[6] and carbonarones.[7] We have also reported the discovery of new sesquiterpenes, JBIR-27 and JBIR-28;[8] a new aspochracin derivative, JBIR-15;[9] new glycosyl benzenediols, JBIR-37 and JBIR-38;[10] and a new sorbicillinoid, JBIR-59[11] from marine-derived fungi. The Calcarea class of sponges has been recognized as a reliable source of richly bioactive metabolites possessing a 2-aminoimidazole skeleton.[12–14] However, the isolation of fungi from calcareous marine sponges has not been reported. Therefore, we attempted to isolate marine fungi from an unidentified calcareous marine sponge and then searched for secondary metabolites in the cultures of the isolated strains. In this study, we report the isolation of *Aspergillus* sp. fS14 from this marine sponge, and the fermentation, isolation and structure elucidation of two new compounds, designated JBIR-74 (**1**) and JBIR-75 (**2**) (Figure 1a).

The producing fungus, *Aspergillus* sp. fS14, was isolated from the unidentified marine sponge (class, Calcarea) collected offshore of Taketomikita, Ishigaki Island, Okinawa Prefecture, Japan, according to the previously reported method using jewfish (*Argyrosomus argentatus*) extract agar.[15,16] The sponge was rinsed with sterilized seawater, finely minced with scissors and resuspended in sterilized seawater. A 100 μl aliquot of this suspension was spread on jewfish extract agar plates prepared in 50% (v/v) artificial seawater (Marine art SF-1; Tomita Pharmaceutical, Tokyo, Japan) and supplemented with 35 μg ml^{-1} of nalidixic acid and 50 μg ml^{-1} of cycloheximide. Cycloheximide-resistant fungal colonies grew on the agar plates and were transferred to potato dextrose agar slants, where individual strains were maintained. Strain fS14 was cultivated in a 50 ml test tube containing 15 ml of seed medium (2.4 g l^{-1} Potato Dextrose Broth; BD Biosciences, San Jose, CA, USA). The test tube was shaken on a reciprocal shaker (355 r.p.m.) at 27 °C for 3 days. A 5 ml aliquot of the seed culture was inoculated into a 500 ml Erlenmeyer flask containing 15 g brown rice (Hitomebore, Miyagi, Japan), 30 mg Bacto Yeast Extract (BD Biosciences), 15 mg sodium tartrate, 15 mg potassium hydrogen phosphate and 45 ml water, and incubated in static culture at 27 °C for 14 days.

The culture (one flask) was extracted with 80% aq. Me_2CO (100 ml) and concentrated *in vacuo*. The aqueous concentrate was extracted with EtOAc (50 ml×3). The organic layer was evaporated after drying over Na_2SO_4. The residue (0.31 g) 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) followed by $CHCl_3$-MeOH (0–50% MeOH). The 3% MeOH-eluted fraction (35.9 mg) was further purified by preparative reversed-phase HPLC using a Pegasil ODS column (20 i.d.×150 mm; Senshu Scientific, Tokyo, Japan) with 50% MeOH-H_2O containing 0.1% formic acid (flow rate: 10 ml min^{-1}) to yield **1** (1.92 mg, retention time (Rt) 12.0 min) and **2** (0.40 mg, Rt 5.5 min).

Compounds **1** and **2** were isolated as a white powder ($[\alpha]_D$ **1**; +52.0, *c* 0.1, **2**; +53.2, *c* 0.1). The molecular formulas of **1** and **2** were established as $C_{12}H_{16}N_4O_2$ (*m/z* 249.1352 $[M + H]^+$, calcd for $C_{12}H_{17}N_4O_2$, 249.1352) and $C_{11}H_{14}N_4O_2$ (*m/z* 235.1195 $[M + H]^+$, calcd for $C_{11}H_{15}N_4O_2$, 235.1182), respectively. The presence of amide groups was deduced from their IR absorption (ν_{max} (KBr) **1**: 3400, 1670 cm^{-1}, **2**: 3400, 1640 cm^{-1}). The ^1H and ^{13}C NMR data for **1** and **2** were established by the analyses of heteronuclear single quantum coherence spectra measured in CD_3OD and DMSO-d_6, respectively (Table 1).

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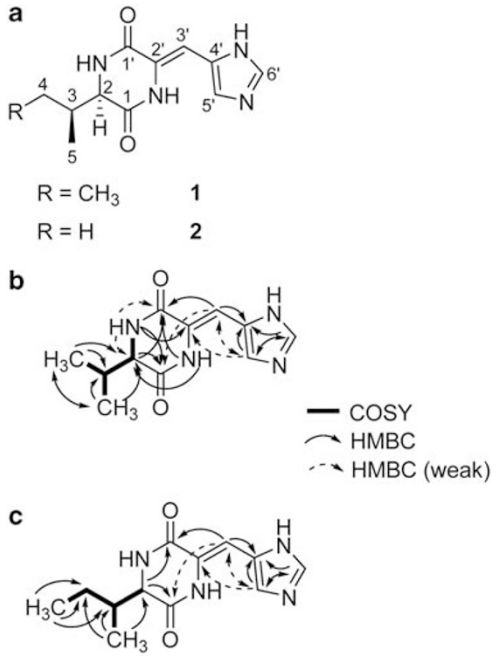

Figure 1 (**a**) Structures of JBIR-74 (**1**) and JBIR-75 (**2**). Key correlations in DQF-COSY (bold lines) and HMBC (arrows) spectra of **1** (**b**) and **2** (**c**).

Table 1 ^{1}H and ^{13}C NMR spectral data for JBIR-74 (1) and JBIR-75 (2)

	1[a]		***2***[b]	
Position	*^{13}C*	*^{1}H (J in Hz)*	*^{13}C*	*^{1}H (J in Hz)*
1	166.8		165.5	
2	59.2	4.16, d (2.3)	60.9	3.94, m
3	40.7	2.04, m	34.0	2.18, m
4	25.4	1.50, m, 1.33, m	18.8	0.94, d (7.0)
5	11.0	0.90, d (7.1)	17.3	0.85, d (7.0)
4-Me	12.8	0.98, t (7.5)		
1′	161.4		160.0	
2′	124.0		125.3	
3′	105.8	6.69, s	104.1	6.51, s
4′	137.0		137.0	
5′	118.6	7.35, s	119.2	7.48, s
6′	136.1	7.79, s	137.0	7.93, s
2-NH				8.28, br s
2′-NH				11.53, s
4′-NH				12.75, br s

^{13}C and ^{1}H NMR spectra were measured with a Varian NMR System 600 or 500 NB CL (Varian, Palo Alto, CA, USA).
[a]CD_3OD (δ_C 49.0, δ_H 3.20), 500 MHz NMR.
[b]DMSO-d_6 (δ_C 39.5, δ_H 2.50), 600 MHz NMR.

The structures of **1** and **2** were clarified by analyses of a series of 2D NMR spectra, such as heteronuclear multiple-bond correlation (HMBC) and double-quantum filtered correlation (DQF-COSY) spectra. As the ^{1}H NMR spectrum of **2** was simpler than that of **1**, compound **2** was analyzed first, to determine the structures of both. The sequence from amide proton 2-NH (δ_H 8.28) to doublet methyl protons 4-H (δ_H 0.94) through an α-methine proton 2-H (δ_H 3.94, δ_C 60.9) and a methine proton 3-H (δ_H 2.18), which in turn ^{1}H spin-coupled to 5-H (δ_H 0.85), was established by the DQF-COSY spectrum of **2**. In addition to this isobutyl moiety, ^{1}H-^{13}C long-range couplings from 2-NH and 2-H to an amide carbonyl carbon C-1 (δ_C 165.5) revealed the presence of a valine moiety. Long-range couplings from a singlet olefinic proton 3′-H (δ_H 6.51) to an aromatic carbon C-4′ (δ_C 137.0) and an aromatic carbon C-5′ (δ_C 119.2), which are long-range coupled to an olefinic proton 6′-H (δ_H 7.93, δ_C 137.0), together with their typical ^{13}C chemical shifts,[17] indicated the presence of an imidazole moiety. Furthermore, ^{1}H-^{13}C long-range couplings from 3′-H to an amide carbonyl carbon C-1′ (δ_C 160.0), and from an amide amino proton 2′-NH (δ_H 11.53) to an olefinic quaternary carbon C-2′ (δ_C 125.3) and the amide carbonyl carbon C-1′, established a 2-amino-3-(1*H*-imidazol-5-yl)acrylic acid moiety. The connectivity between the two partial structures was revealed by the ^{1}H-^{13}C long-range couplings from the α-methine proton 2-H to the amide carbonyl carbon C-1′, and from the olefinic proton 3′-H to the amide carbonyl carbon C-1. In addition, the stereochemistry at C-2′ was identified as *Z* form, according to a W type ^{1}H-^{13}C long-range coupling from 3′-H to C-1. These results proved the structure of **2**, as shown in Figure 1c.

The molecular formula of **1**, together with ^{1}H and ^{13}C NMR data, indicated that **1** consists of the same skeleton as **2,** except for an additional methyl residue. In the DQF-COSY spectrum of **1**, the sequence from a triplet methyl proton 4-CH_3 (δ_H 0.98) to an α-methine proton H-2 (δ_H 4.16) through methylene protons H-4 (δ_H 1.50, 1.33) and a methine proton H-3 (δ_H 2.04), which was in turn coupled to a doublet methyl proton H-5 (δ_H 0.90), was observed. These results indicated the presence of an isoleucine residue. ^{1}H-^{13}C Long-range couplings shown in Figure 1b are identical to those of **2**. Thus, the structure of **1** was identified as the 4-methyl derivative of **2** (Figure 1c).

To determine the configuration of the isoleucine moiety in **1**, we adopted Marfey's method. Compound **1** (1.0 mg) was hydrolyzed in 6 N HCl at 110 °C for 12 h. After concentration to dryness, the residue was dissolved in 10 ml of EtOAc–H_2O (1:1). The mixture recovered in the aqueous layer was dried *in vacuo*. To the dried mixture was added 5% $NaHCO_3$ (500 µl) and 0.2 mg N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in acetone (500 µl), and heated in an 80 °C water bath for 3 h. The reaction product of **1** was analyzed by UPLC system (Waters) as follows: column; Acquity UPLC BEH C_{18} column (2.1 i.d. ×50 mm, Waters); flow rate, 0.8 ml min^{-1}; solvent, 30% CH_3CN aqueous solution containing 0.1% formic acid; detection, 340 nm. The Rts of the standard FDAA derivatives were as follows: L-isoleucine, 2.71 min; D-isoleucine, 5.72 min; L-allo-isoleucine, 2.76 min and D-allo-isoleucine, 5.90 min. The chromatograms of the hydrolysate derivatives of **1** showed a peak corresponding to D-allo-isoleucine (5.90 min). Like **1**, the valine in **2** was determined to be D form (2.50 min) by the UPLC system using CH_3CN aqueous solution (5–100% CH_3CN, 5 min) containing 0.1% formic acid. The Rts of the standard FDAA derivatives of L- and D-valine were 2.23 and 2.49 min, respectively. Thus, the absolute configurations of **1** and **2** were defined as shown in Figure 1a.

The structures of **1** and **2** were found to be structurally related to roquefortine C isolated from *Penicillium roqueforti*.[18] It has been reported that the roquefortine analogues possess cytotoxic and antimicrobial activities.[19,20] We conducted tests to evaluate the cytotoxic and antimicrobial activities of **1** and **2**, and found that neither compound showed cytotoxic activity against several cancer cell lines (IC_{50} > 100 µM), nor did they show antimicrobial activity against *Candida albicans*, *Micrococcus luteus* and *Escherichia coli*. These results suggest that a cyclic tryptophan in roquefortines may be important for biological activity.

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