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Three new depsipeptides, JBIR-113, JBIR-114 and JBIR-115, isolated from a marine sponge-derived *Penicillium* sp. fS36

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Fungi found in marine habitats constitute a rarely exploited but promising resource for the discovery of novel bioactive substances,[1] such as diketopiperazine alkaloids,[2] trichodermatides[3] and carbonarones.[4] We recently discovered novel compounds, namely, aspochracin derivative JBIR-15,[5] glycosyl benzenediols JBIR-37 and -38,[6] sorbicillinoids JBIR-59[7] and -124,[8] xanthoquinodin JBIR-97, -98 and -99,[9] and JBIR-74 and -75,[10] which were obtained from marine sponge-derived fungi. Therefore, we attempted to isolate fungi from abyssal sea marine sponges to obtain novel substances of fungal origin. In this study, we isolated three new depsipeptides termed JBIR-113 (**1**), JBIR-114 (**2**) and JBIR-115 (**3**) from a culture of *Penicillium* sp. fS36 that was obtained from a marine sponge (Figure 1a). This paper describes the fermentation, isolation and structural elucidations of **1–3**.

Penicillium sp. fS36 was isolated from an unidentified marine sponge collected at a depth of 195 m from a position (i.e., 28° 53′ 35.8″ N, 129° 29′ 57.0″ E) near Takarajima Island, Kagoshima Prefecture, Japan. The sponge was ground using a mortar and pestle. These pieces were suspended in sterile seawater and the suspension spread on manila clam extract agar.[11] The isolated strain was maintained on potato dextrose agar medium (BD Biosciences, San Jose, CA, USA). The sequence analysis of ribosomal DNA and internal transcribed spacer region of the producing fungus showed high sequence similarities with *Penicillium namyslowskii* (AB028190, 100%). The strain was cultivated in 50 ml test tubes containing 15 ml of potato dextrose medium (24 g l^{-1} potato dextrose; BD Biosciences). The test tubes were shaken using a reciprocal shaker (320 r.p.m.) at 27 °C for 2 days. Aliquots (5 ml) of the culture were transferred to 500 ml Erlenmeyer flasks containing brown rice (15 g, Hitomebore, Miyagi, Japan), Bacto-yeast extract (30 mg, BD Bios-

ciences), sodium tartarate (15 mg), dipotassium hydrogenphosphate (15 mg) and water (45 ml) and were incubated in a static culture at 27 °C for 14 days.

The culture (10 flasks) was extracted with 80% aqueous Me$_2$CO (1 l) and the extract was filtered. After concentration *in vacuo*, water (600 ml) was added to the residual aqueous solution followed by extraction with EtOAc (700 ml×3). The organic layer was dried over anhydrous Na$_2$SO$_4$ and evaporated. The residual portion (2.5 g) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-30, Shoko Scientific Co., Yokohama, Japan) and developed with a gradient system of *n*-hexane–EtOAc (0–25% EtOAc) followed by a stepwise solvent system comprising CHCl$_3$–MeOH (0, 2, 5, 10, 20, 30 and 100% MeOH). The fractions including **1–3** were collected by LC-MS monitoring. The 5% MeOH eluate (230 mg) was applied on a Sephadex LH-20 column (GE Healthcare BioSciences AB, Uppsala, Sweden) and developed with CHCl$_3$–MeOH (1:1) to obtain the crude material (38.1 mg). Compounds **1** (9.9 mg), **2** (1.7 mg) and **3** (1.7 mg) were obtained by preparative reversed-phase HPLC on a CAPCELL PAK C18 MGII column (5.0 μm, internal diameter 20×150 mm; Shiseido, Tokyo, Japan) with 55% aqueous MeOH containing 0.1% formic acid (flow rate: 10 ml min^{-1}; retention times: 25.4 min for **1**; 17.4 min for **2**; 12.8 min for **3**).

Compound **1** is a colorless amorphous solid with the following properties: $[\alpha]_D^{22}$–87.7 (MeOH; *c* 0.15); UV λ_{max} (ε) in MeOH: 223 (17 000) and 270 (900) nm; and IR absorption (ν_{max}) 1654 and 1751 cm^{-1}. The molecular formula of **1** was determined to be C$_{31}$H$_{41}$N$_5$O$_7$ using high resolution-ESI-MS (*m/z* 596.3098 [M+H]$^+$; calculated for C$_{31}$H$_{42}$N$_5$O$_7$ *m/z* 596.3084). Its peptidic nature was evident from the abundance of resonances corresponding to amide

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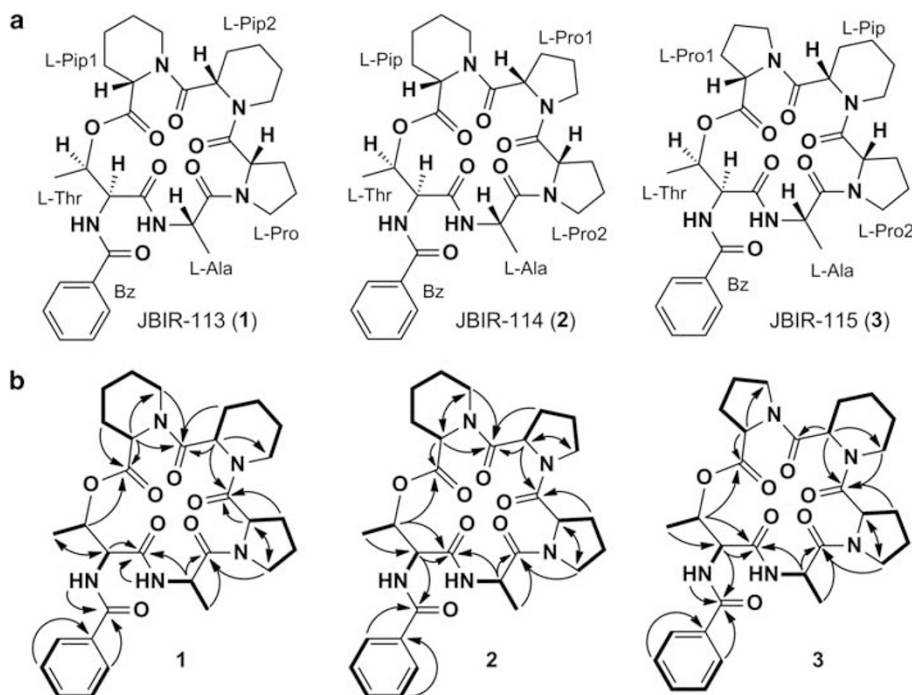E-mail: k-shinya@aist.go.jp

Figure 1 (a) Structures of JBIR-113 (1), JBIR-114 (2), and JBIR-115 (3). (b) Key correlations of double quantum-filtered (DQF)-COSY (bold lines) and HMBC (arrows, proton to carbon) of 1.

NH protons (δ_H 7.14–7.52) and the resonances corresponding to the carbonyl carbons (δ_C 167.8–174.3) in the ^{1}H and ^{13}C NMR spectra of 1, respectively (Table 1). The direct connectivity between protons and carbons was established via a heteronuclear single quantum coherence spectrum; the ^{13}C and ^{1}H NMR spectroscopic data of 1 is shown in Table 1. The ^{1}H sequences and ^{1}H–^{13}C long-range couplings from the α-methine protons to the corresponding amide carbonyl carbons, which were elucidated using double quantum-filtered (DQF)-COSY and constant time heteronuclear multiple bond correlation (CT-HMBC)[12] spectra, respectively, proved the presence of a Pro, an Ala, a Thr and two pipecolic acids (Pip) residues, as shown in Figure 1. The residual benzoyl (Bz) group in 1 was revealed by consecutive COSY correlations from aromatic proton Bz-3/7-H (δ_H 7.85) to Bz-5-H (δ_H 7.52) through Bz-4/6-H (δ_H 7.45) and by the HMBC correlations from Bz-4/6-H to aromatic carbon Bz-C-2 (δ_C 134.2) and from Bz-3/7-H to amide carbonyl carbon Bz-C-1 (δ 167.8). The amino-acid sequence and substituted position of the Bz moiety in 1 were determined via the ^{1}H-^{13}C long-range correlations from an α-methine proton Pip1-2-H (δ_H 4.43) and a ε-methylene proton Pip1-6a-H (δ_H 4.19) to a carbonyl carbon Pip2-C-1 (δ_C 174.3), from an α-methine proton Pip2-2-H (δ_H 4.68) to a carbonyl carbon of Pro (δ_C 170.9), from δ-methylene protons Pro-5a-H (δ_H 3.66) and Pro-5b-H (δ_H 3.59) to a carbonyl carbon Ala-C-1 (δ_C 172.6), from an α-methine proton Ala-2-H (δ_H 4.25) and an amide proton Ala-NH (δ_H 7.33) to a carbonyl carbon Thr-C-1 (δ_C 168.0), and from an amide amine proton Thr-NH (δ_H 7.14) to the carbonyl carbon Bz-C-1. Additionally, an HMBC correlation from an oxymethine proton Thr-3-H (δ_H 5.28) to a carbonyl carbon Pip1-C-1 (δ_C 169.3) established that Thr and Pip-1 are connected through an ester bond. Thus, the planar structure of 1 shown in Figure 1a was established.

The absolute configurations of the amino-acid residues in 1 were determined using Marfey's method.[13] Accordingly, 1 (0.4 mg) was hydrolyzed in 6 N HCl (200 μl) at 110 °C for 12 h. After drying the reaction solution under an N$_2$ gas flow, 0.5 M NaHCO$_3$ (40 μl) and 10 mM *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in Me$_2$CO (60 μl) were added. The mixture was then reacted at 60 °C for 20 min. The resultant products were analyzed using a UPLC-TOF-MS system (Waters, ACQUITY) as follows: a Waters BEH ODS column (internal diameter 2.1×50 mm) was developed with an aqueous MeCN-containing 0.1% formic acid linear gradient system (10–40% MeCN, 10 min; flow rate, 0.6 ml min^{-1}). The retention times of the FDAA derivatives were determined by monitoring the UV absorption at 340 nm in the positive and negative mode of HR-ESI-MS. By comparing the retention times of standard amino-acid FDAA derivatives and FDAA derivatives of 1, the amino-acid residues in 1 were established to be in the L form (i.e., standard amino-acid FDAA derivatives: L-Pip (6.82 min), D-Pip (6.33 min), L-Pro (4.49 min), D-Pro (4.96 min), L-Ala (3.97 min), D-Ala (5.08 min), L-Thr (3.08 min), and D-Thr (3.62 min); target FDAA derivatives: Pips (6.82 min), Pro (4.49 min), Ala (3.97 min) and Thr (3.08 min)). Therefore, the structure of 1 including the absolute configuration was determined, as shown in Figure 1a.

Compound 2 features the following properties: $[\alpha]_D^{22}$ −26.4 (MeOH; *c* 0.08); UV λ_{max} (ε) in MeOH: 210 (26 000) nm; HR-ESI-MS: *m/z* 582.2928 [M+H]$^+$, calculated for C$_{30}$H$_{40}$N$_5$O$_7$ *m/z* 582.2928; and IR absorption (v_{max}) 1656 and 1751 cm^{-1}. The properties of 3 are as follows: $[\alpha]_D^{22}$ −37.8 (MeOH; *c* 0.08); HR-ESI-MS: *m/z* 582.2905 [M+H]$^+$, calculated for C$_{30}$H$_{40}$N$_5$O$_7$ *m/z* 582.2928; UV λ_{max} (ε) in MeOH: 210 (16 000) and 271 (2200) nm; and IR absorption (v_{max}) 1652 and 1751 cm^{-1}. NMR spectroscopic analysis of 2 and 3 revealed that both compounds comprise a Thr, Ala, Pip and two Pro residues (Table 1).

The sequence of amino-acid residues in 2 was established via the following HMBC correlations (Figure 1b): from an α-methine

Table 1 ^{13}C and ^{1}H NMR spectroscopic data for JBIR-113 (1), JBIR-114 (2) and JBIR-115 (3).

		1[a]				**2**[a]				**3**[b]	
Unit	Position	δ_C	δ_H, mult (J in Hz)	Unit	Position	δ_C	δ_H, mult (J in Hz)	Unit	Position	δ_C	δ_H, mult (J in Hz)
Pip1	1	169.3		Pip	1	169.1		Pro1	1	170.4	
	2	53.9	4.43, d (6.0)		2	54.2	4.64, ovl[c]		2	58.4	4.28, dd (1.2, 7.2)
	3a	27.2	1.89, ovl[c]		3a	27.5	1.93, ovl[c]		3a	31.4	1.63, ovl[c]
	3b		1.77, ovl[c]		3b		1.78, ovl[c]		3b		1.47, ovl[c]
	4	18.7	1.63, ovl[c]		4	18.9	1.63, ovl[c]		4a	22.2	2.00, m
	5a	24.16	1.81, ovl[c]		5a	24.2	1.81, ovl[c]		4b		1.18, m
	5b		1.37, m		5b		1.37, m		5a	46.4	3.82, ddd (3.2, 9.1, 12.0)
	6a	39.1	4.19, dd (3.5, 13.0)		6a	39.4	4.28, ovl[c]		5b		3.28, ddd (7.9, 7.9, 12.0)
	6b		3.29, ddd (3.5, 13.0, 13.0)		6b		3.37, ddd (3.0, 13.5, 13.5)				
Pip2	1	174.3		Pro1	1	173.3		Pip	1	171.8	
	2	50.9	4.68, dd (3.5, 7.0)		2	56.5	4.65, ovl[c]		2	51.5	5.03, dd (2.4, 7.1)
	3a	26.1	1.94, ovl[c]		3a	28.9	2.22, m		3a	26.3	2.08, ovl[c]
	3b		1.81, ovl[c]		3b		1.91, ovl[c]		3b		1.68, ovl[c]
	4a	18.8	1.69, ovl[c]		4a	25.8	2.23, m		4	19.3	1.50, ovl[c]
	5a	24.22	1.82, ovl[c]		4b		2.07, m		5a	24.9	1.41, ovl[c]
	5b		1.68, ovl[c]		5a	46.8	3.68, ovl[c]		5b		1.36, ovl[c]
	6a	43.2	3.70, ovl[c]		5b		3.63, ovl[c]		6a	43.0	3.80, dd (4.1, 12.6)
			3.68, ovl[c]						6b		3.13, br d (12.6)
Pro	1	170.9		Pro2	1	167.9		Pro2	1	171.0	
	2	59.1	4.57, dd (6.5, 6.5)		2	59.2	4.46, dd (5.5, 8.0)		2	58.8	4.00, dd (5.6, 8.6)
	3a	30.0	2.37, ddd (6.5, 13.5, 13.5)		3a	29.9	2.38, m		3a	30.0	1.63, ovl[c]
	3b		2.06, ddd (6.5, 13.5, 13.5)		3b		2.12, ovl[c]		3b		1.55, m
	4a	23.1	1.85, ovl[c]		4a	23.1	1.90, ovl[c]		4a	22.9	1.28, dd (6.8, 12.9)
	4b		1.79, ovl[c]		4b		1.80, ovl[c]		4b		1.10, dd (7.1, 12.9)
	5a	47.4	3.66, ovl[c]		5a	47.0	3.69, ovl[c]		5a	47.7	3.58, ddd (7.1, 7.1, 12.4)
	5b		3.59, ddd (5.4, 6.8, 12.0)		5b		3.50, dd (6.5, 12.0)		5b		3.51, ddd (6.8, 6.8, 12.4)
Ala	1	172.6		Ala	1	172.2		Ala	1	172.5	
	2	47.3	4.25, dq (6.5, 8.5)		2	47.4	4.24, q (6.5)		2	47.5	4.50, dq (1.8, 6.7)
	3	19.8	1.25, d (6.5)		3	19.8	1.26, d (6.5)		3	20.0	1.36, d (6.7)
	NH		7.33, d (8.5)		NH		6.86, br s		NH		8.16, br s
Thr	1	168.0		Thr	1	167.6[d]		Thr	1	168.5	
	2	55.6	4.94, d, (9.0)		2	55.7	4.86, d (8.5)		2	55.8	5.37, br d (8.6)
	3	73.4	5.28, q (6.4)		3	73.2	5.25, q (6.5)		3	74.8	5.74, q (6.5)
	4	17.2	1.33, d (6.4)		4	17.1	1.32, d (6.5)		4	17.9	1.51, d (6.5)
	NH		7.14, d (9.0)		NH		7.07, d (8.5)		NH		7.52, d (8.6)
Bz	1	167.8		Bz	1	167.6[d]		Bz	1	166.9	
	2	134.2			2	134.2			2	134.8	
	3/7	127.3	7.85, d (7.3)		3/7	127.3	7.85, d (7.5)		3/7	127.4	7.90, d (7.5)
	4/6	128.5	7.45, dd (7.3)		4/6	128.6	7.46, dd (7.5, 7.5)		4/6	128.2	6.92, dd (7.5, 7.5)
	5	131.7	7.52, dd (7.3, 7.3)		5	131.8	7.52, dd (7.5, 7.5)		5	131.1	6.98, dd (7.5, 7.5)

[a]Measured on a 500 NB CL NMR instrument (Varian, Palo Alto, CA, USA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C with the residual solvent peak as the internal standards (in CDCl$_3$, 7.25 p.p.m. for ^{1}H and 77.0 p.p.m. for ^{13}C).
[b]Measured on a 600 NB CL NMR instrument (Varian) at 600 MHz for ^{1}H and 150 MHz for ^{13}C with the residual solvent peak as the internal standard (in C$_6$D$_6$, 7.15 p.p.m. for ^{1}H and 128.0 p.p.m. for ^{13}C).
[c]Overlapped with other signals.
[d]Interchangeable.

proton Pip-2-H (δ_H 4.64) to a carbonyl carbon of Pro1 (δ_C 173.3), from an α-methine proton Pro1-2-H (δ_H 4.65) to a carbonyl carbon of Pro2 (δ_C 167.9), from a δ-methylene proton Pro2-5a-H (δ_H 3.69) to a carbonyl carbon of Ala (δ_C 172.2), from an α-methine proton Ala-2-H (δ_H 4.24) to carbonyl carbon of Thr (δ_C 167.6), from Thr-3-H (δ_H 5.25) to carbonyl carbon of Pip1

(δ_C 169.1), and from Thr-2-H (δ_H 4.86) to Bz-C-1 (δ_C 167.6) (Figure 1b).

The ^{1}H and ^{13}C NMR spectra of **3** in CDCl$_3$ were very similar to those of **2**. As the signals observed for the ^{1}H NMR spectrum of **3** in CDCl$_3$ significantly overlapped, we determined the NMR spectra of **3** in C$_6$D$_6$. ^{1}H–^{13}C long-range correlations from an α-methine proton

Pip-2-H (δ_H 5.03) and ε-methylene proton Pip-6b-H (δ_H 3.13) to carbonyl carbon Pro2-C-1 (δ_C 171.0), from δ-methylene proton Pro2-5a-H (δ_H 3.58) to carbonyl carbon of Ala (δ_C 172.5), from an α-methine proton Ala-2-H (δ_H 4.50) to a carbonyl carbon Thr-C-1 (δ_C 168.5), from an oxymethine proton Thr-3-H (δ_H 5.74) to a carbonyl carbon Pro1-C-1 (δ_C 170.4), from an amide amine proton of Thr (δ_H 7.52) and an α-methine proton Thr-2-H (δ_H 5.37) to a carbonyl carbon Bz-C-1 (δ_C 166.9) were observed (Figure 1b). According to the index of hydrogen deficiency of 14 that was calculated from the molecular formula of **3**, the nitrogen atom in Pro1 should form a peptide bond with carbonyl carbon Pip-1-C (δ_C 171.8). Thus, the planar structure of **3** was elucidated.

The absolute configurations of the amino acids in **2** and **3** were determined to be L-Pip, L-Pro (×2), L-Ala and L-Thr using the same methods as for **1** (Figure 1a).

We evaluated the cytotoxic and antimicrobial activities of **1-3**, but **1-3** did not show cytotoxicity to human cervical carcinoma HeLa cells lines (IC$_{50}$ > 100 μM) or antimicrobial activity against *Micrococcus luteus*, or *Escherichia coli*.

The elucidated structures of **1–3** are structurally related to petrosifungins that were isolated from a marine sponge-derived fungus.[14] Although other peptides containing pipecolic acid have been reported including neamphamide A[15] from a marine sponge and microsporins A and B[16] from a marine sponge-derived fungus, these peptides are very rare in natural products and are all of marine origin. The results of this study suggest that fungi isolated from marine sponges in abyssal sea possess the desirable ability to produce unique compounds.

ACKNOWLEDGEMENTS

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