JOURNAL OF NATURAL PRODUCTS

ARTICLE

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JBIR-56 and JBIR-57, 2(1*H*)-Pyrazinones from a Marine Sponge-Derived *Streptomyces* sp. SpD081030SC-03

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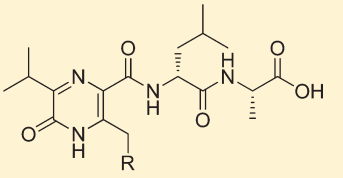
Supporting Information

ABSTRACT: Strain SpD081030SC-03, representing a novel species of *Streptomyces*, was isolated from a marine sponge. Two 3,5,6-trisubstituted 2(1*H*)-pyrazinones, JBIR-56 (**1**) and JBIR-57 (**2**), were isolated from a culture of SpD081030SC-03. The planar structures of **1** and **2** were assigned on the basis of extensive NMR and MS analyses. In addition, analyses of the methylated derivative of **1** confirmed a 3,5,6-trisubstituted 2(1*H*)-pyrazinone moiety. The absolute configurations of the amino acid residues were determined by application of Marfey's method. Because **1** did not appear to comprise the normal connection of amino acid units, we confirmed its structure by the total synthesis of **1**. Biosynthetically, **1** consists of a unique skeleton connected to the peptide chain at C-5 of the pyrazinone ring.



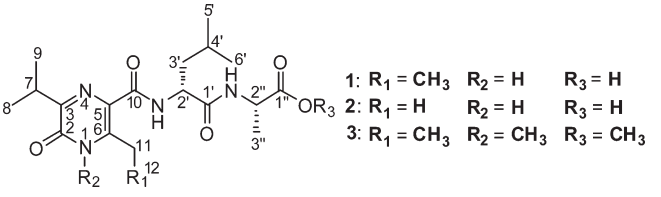
JBIR-56 (**1**): R = CH_3

JBIR-57 (**2**): R = H

Microorganisms from marine habitats,[1,2] especially actinobacteria, constitute a promising untapped resource of novel compounds; therefore, these organisms are currently receiving a great deal of attention. When compared to higher organisms, microorganisms can be easily maintained under laboratory conditions, ensuring a constant and inexpensive supply of their secondary metabolites. Moreover, many compounds originally believed to be produced by marine organisms have been found to be produced by host-associated microorganisms.[3] A significant body of work on the isolation of actinobacteria from marine habitats has emerged in the last 10 years, and screening of these organisms has yielded several novel bioactive compounds.[4−6] Our group has recently engaged in the isolation of microorganisms from marine sources, including fungi and actinobacteria. We have discovered novel compounds produced by newly identified species of marine-derived actinomycetes including the anthracyclines, tetracenoquinocin and 5-iminoaranciamycin,[7] tetrapeptides JBIR-34 and JBIR-35,[8] a novel salicylamide, JBIR-58,[9] a new diterpene, JBIR-65,[10] and JBIR-66.[11] In the course of our screening for new metabolites, we successfully isolated two new peptides, JBIR-56 (**1**) and JBIR-57 (**2**), from the culture broth of a new species (SpD081030SC-03) of *Streptomyces* associated with an unidentified marine sponge. Here, the fermentation, isolation, and structure elucidation of **1** and **2** and the total synthesis of **1** are described.

RESULTS AND DISCUSSION

Streptomyces sp. SpD081030SC-03 isolated from a marine sponge was identified as a new species of the genus *Streptomyces*



1: $R_1 = CH_3$ $R_2 = H$ $R_3 = H$
2: $R_1 = H$ $R_2 = H$ $R_3 = H$
3: $R_1 = CH_3$ $R_2 = CH_3$ $R_3 = CH_3$

based on its 16S rRNA gene sequence.[12] Comparison of almost the entire length (1507 bp) of the 16S rRNA with sequences in the Eztaxon-type strain database[13] revealed that the closest phylogenetic neighbor of the strain was *Streptomyces lucensis* NBRC 13056^T (AB184280), with a sequence identity of only 97.6%. The low identity with previously reported sequences indicates that this strain is a novel species in the genus *Streptomyces*.[12]

The strain was cultured at 27 °C for five days by rotary shaking in baffled 500 mL Erlenmeyer flasks containing 100 mL of production medium. Compounds **1** and **2** were then recovered from the supernatant of the fermentation broth using HP-20 resin, after which they were purified by sequential chromatography utilizing MPLC and HPLC.

The structures of **1** and **2** were primarily elucidated by spectroscopic methods, including 2D NMR. The molecular formula of JBIR-56 (**1**) was established as $C_{19}H_{30}N_4O_5$ based on the HRESIMS data ($[M + H]^+$, m/z 395.2294). The peptide structure of **1** was evident from the ^1H and ^{13}C NMR data recorded in DMSO-d_6 as shown in Table 1. The absorptions at

Received: May 6, 2011
Published: July 05, 2011

3380 and 1660 cm^{-1} in the IR spectrum of **1** revealed the presence of hydroxy, amide, and carbonyl functional groups. Together with these IR absorptions, typical NMR chemical shifts for amide and α-methine resonances (δ_C 174.0 and 171.4, $\delta_{C/H}$ 50.7/4.52 and 48.1/4.12, respectively) indicated that **1** is a peptide compound. Detailed structural information was obtained from the HSQC, HMBC, and DQF-COSY spectra of **1** (Figure 1a). The sequence from an amino proton 2′-NH (δ_H 8.18) to the doublet methyl protons H_3-5′ (δ_H 0.89) through an α-methine proton H-2′ (δ_H 4.52, δ_C 50.7), methylene protons H_2-3′ (δ_H 1.52), and a methine proton H-4′ (δ_H 1.56), which was in turn coupled to doublet methyl protons H_3-6′ (δ_H 0.88), was established on the basis of the DQF-COSY spectrum. Long-range couplings of both H-2′ and H-3′ to a carbonyl carbon C-1′ (δ_C 171.4) observed in the HMBC spectrum indicated the presence of a leucine residue. Similarly, an alanine unit was assigned on the basis of interpretation of the DQF-COSY and HMBC spectroscopic data. Long-range couplings from the amide proton 2″-NH (δ_H 8.34) to the carbonyl carbon C-1′ and the α-methine carbon C-2″ (δ_C 48.1) proved a Leu-Ala unit as shown in Figure 1a.

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic Data for JBIR-56 (1) and JBIR-57 (2) in DMSO-d_6

	JBIR-56 (**1**)			JBIR-57 (**2**)	
		^{1}H			^{1}H
position	^{13}C	(*J* in Hz)	position	^{13}C	(*J* in Hz)
2	155.9		2	155.8	
3	158.7		3	158.3	
5	120.3		5	120.9	
6	145.9		6	140.8	
7	29.5	3.22, q (6.6)	7	29.5	3.21, q (6.8)
8	19.9	1.15, d (6.6)	8	19.9	1.15, d (6.8)
9	19.8	1.15, d (6.6)	9	19.8	1.15, d (6.8)
10	163.3		10	163.6	
11	23.2	2.99, dq (12.6, 7.2)	11	16.7	2.53, s
		2.93, dq (12.6, 7.2)			
12	14.0	1.13, t (7.2)			
Leu			Leu		
1′	171.4		1′	171.6	
2′	50.7	4.52, dd (13.8, 8.4)	2′	50.6	4.53, dd (14.8, 8.4)
3′	42.6	1.52, dd (13.8, 6.0)	3′	42.6	1.52, dd (14.8, 6.2)
4′	24.7	1.56, q (6.0)	4′	24.7	1.56, q (6.2)
5′	23.1	0.89, d (6.0)	5′	23.3	0.89, d (6.2)
6′	22.4	0.88, d (6.0)	6′	22.4	0.88, d (6.2)
2′-NH		8.18, d (8.4)	2′-NH		8.16, d (8.4)
Ala			Ala		
1″	174.0		1″	174.1	
2″	48.1	4.12, dq (7.2, 6.6)	2″	47.8	4.14, dq (7.0, 6.2)
3″	17.7	1.23, d (7.2)	3″	17.7	1.24, d (7.0)
2″-NH		8.34, br s	2″-NH		8.41, br s

The leucine residue was further connected to two carbons (δ_C 163.3 and 120.3) on the basis of HMBC correlations with the 2′-NH. The δ_C 163.3 chemical shift suggested that leucine was involved in an amide bond with a conjugated acid. Additional ethyl (δ_H 2.99, 2.93, 1.13) and isopropyl (δ_H 3.22, 1.15) spin systems were also apparent from the DQF-COSY spectrum. The methylene protons of the ethyl group had HMBC correlations to carbons at δ_C 145.9 and 120.3, whereas the methyl protons showed correlations to carbons at δ_C 145.9 and 23.2. These correlations suggested a 2,3-disubstituted pentenoic acid fragment connected to the leucine. The isopropyl group had HMBC correlations to two carbons (δ_C 158.7 and 155.9) (Figure 1a). The remaining atoms required by the molecular formula were two nitrogens, an oxygen, and a hydrogen. While there were several ways to combine these elements, a weak correlation from the methylene protons of the ethyl group to the carbon at δ_C 155.9 was consistent with only two reasonable structures, a 3,5,6-trisubstituted 2(1*H*)-pyrazinone or a 3,5,6-trisubstituted 4(1*H*)-pyridazinone. Carbon chemical shift predictions were in much better agreement with the pyrazinone structure.

In order to provide additional support for this structure, chemical modification was conducted as follows: **1** was treated with methyl iodide and potassium carbonate in DMF to yield 1-methyl-JBIR-56 methyl ester (**3**) as the main derivative. The molecular formula of **3** was established as $C_{21}H_{34}N_4O_5$ based on the HRESIMS data ($[M + H]^+$, *m/z* 423.2601). The appearance of two new signals ($\delta_{C/H}$ 30.6/3.60 and 52.7/3.70 ppm) compared to **1** was observed in the ^{1}H and ^{13}C NMR spectra of **3**. The structure of **3**, including signals of a methoxy group, 1″-*O*-CH_3 ($\delta_{C/H}$ 52.7/3.70), and an *N*-methyl group, 1-CH_3 ($\delta_{C/H}$ 30.6/3.60), was confirmed by analysis of the DQF-COSY, HSQC, and HMBC spectra (Figure 1b). Each ^{1}H−^{13}C long-range correlation from the 1″-*O*-CH_3 to an ester carbonyl carbon, C-1″ (δ_C 174.7), from the *N*-methyl proton, 1-CH_3, to an amide carbonyl carbon, C-2 (δ_C 156.4), and an aromatic quaternary carbon, C-6 (δ_C 147.2), confirmed the 3,5,6-trisubstituted 2(1*H*)-pyrazinone moiety (Figure 1b). Taken together, the structure of **1** was proposed.

The molecular formula of JBIR-57 (**2**) was determined to be $C_{18}H_{28}N_4O_5$ on the basis of the HRESIMS data ($[M + H]^+$, *m/z* 381.2130), which showed the disappearance of a methyl or a methylene group from **1**. The IR and UV spectra of **2** were similar to those of **1**. In addition, most of the NMR spectroscopic data for **2** were closely related to those of **1**; however, the ethyl moiety consisting of the triplet methyl protons H_3-12 and the methylene protons H_2-11 in **1** was replaced by a singlet methyl signal (δ_H 2.53, δ_C 16.7). These collective spectroscopic data demonstrated that **2** was a C-6 methyl analogue of **1**.

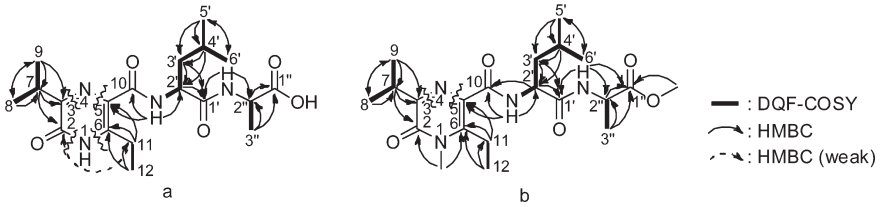
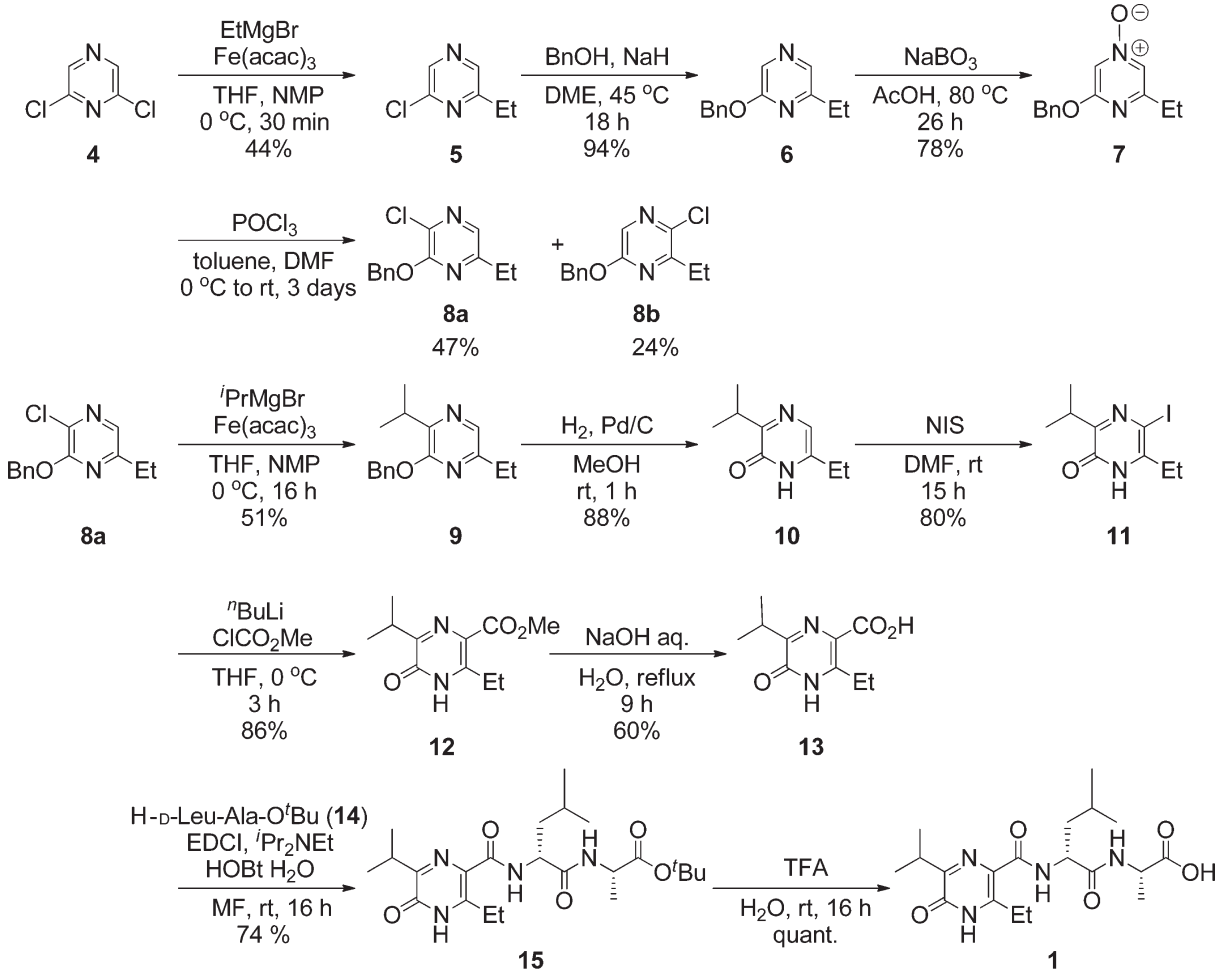


Figure 1. Key correlations observed in 2D NMR spectra of **1** (a) and **3** (b) (bold lines show ^{1}H−^{1}H DQF-COSY results, and arrows show HMBC results).

dx.doi.org/10.1021/np200386c |*J. Nat. Prod.* 2011, 74, 1630–1635

Scheme 1. Total Synthesis of JBIR-56



The absolute configuration of **1** was defined by Marfey's method.[14] The acid hydrolysate of **1** was reacted with FDAA (Marfey's reagent) and then analyzed on the basis of the UV absorption at 340 nm and positive mode ESI-LC-MS monitoring using an analytical reversed-phase UPLC system equipped with an ODS column. All derivatives were identified on the basis of comparison of their retention times, molecular formulas, and UV spectra with those of standard amino acids derived from FDAA conjugated compounds. The configurations of the Ala and Leu residues were determined to be L and D, respectively. Overall, the absolute configuration of **1** was assigned as 2′*R*,2″*S*. Similarly, the absolute configuration of **2** was determined to be the same as that of **1**.

TOTAL SYNTHESIS OF JBIR-56

Because **1** was not composed of typical amino acid units and this skeleton is the first example in both natural and synthetic compounds, we confirmed its structure by the total synthesis of **1**. The synthetic route for **1** is illustrated in Scheme 1. Initially, monoalkylation of a 2,6-dichloropyrazine (**4**) and the subsequent introduction of a benzyloxy group at the C-2 position were conducted to afford 2-benzyloxy-6-ethylpyrazine (**6**).[15] *N*-Oxidation at the C-4 position, followed by chlorination, gave a mixture of regioisomers **8a** and **8b**. These isomers could be separated by silica gel column chromatography. The structures of both isomers **8a** and **8b** were confirmed by 2D NMR techniques such as HMBC. Further transformations were then conducted by employing isomer **8a**. An isopropyl group was introduced to the 3-position of **8a** and the benzyl group was removed to yield 6-ethyl-3-isopropyl-2(1*H*)-pyrazinone (**10**).[15] Iodination of compound **10** at the 5-position gave **11**. Compound **11** was treated with two equivalents of *n*-BuLi to generate a dianion. The *in situ* generated dianion was subsequently treated with chloroformic acid methyl ester to give the 5-acylated product.[16] Hydrolysis of the methyl ester **12** provided the desired 6-ethyl-3-isopropyl-2(1*H*)-pyrazinone-5-carboxylic acid (**13**). Coupling of the carboxylic acid **13** and the dipeptide **14** was conducted in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl). Acidic removal of the *tert*-butyl group gave the desired product **1** in good yield. The structure of synthetic **1** was confirmed by the NMR spectra and HRESIMS data. Although the NMR data of synthetic **1** were in close agreement with those of **1** produced by *Streptomyces* sp. SpD081030SC-03, there was a minute difference in the chemical shifts of the Ala residue between the synthetic and natural product (see Supporting Information, Table S1). Thus, a mixture (1:1) of synthetic and natural products was analyzed by NMR. The NMR data showed that the chemical shifts of the synthetic and natural product were indistinguishable. In addition, other resulting spectroscopic data,

dx.doi.org/10.1021/np200386c |*J. Nat. Prod.* 2011, 74, 1630–1635

including the (−) sign and magnitude of the specific rotation of synthetic **1**, were identical to those of natural **1**. On the basis of these findings, we validated the structure of **1**.

CONCLUSION

We isolated two new peptides, JBIR-56 (**1**) and JBIR-57 (**2**), from the culture broth of a new sponge-derived species of *Streptomyces*, SpD081030SC-03. The methylated derivative (**3**) of **1** helped to confirm the part of these compounds that was most intriguing structurally, the 3,5,6-trisubstituted 2(1*H*)-pyrazinone moiety. Although 3,6-disubstituted 2(1*H*)-pyrazinone derivatives with an isopropyl group at the C-3 position such as argvalin[17] and phevalin[18] have been isolated from *Streptomyces* spp., there have been no reports of 2(1*H*)-pyrazinones with a peptide chain connected at the C-5 position, as in **1**. Accordingly, the unique structures of **1** and **2** may have been biosynthesized by a novel nonribosomal peptide synthetase. Thus, investigations of the detailed biosynthesis of **1** are now underway.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Horiba SEPA-300 polarimeter. UV and IR spectra were measured with a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were collected using a JEOL model EX-270, JEOL model ECP-400, or a Varian NMR System 600 NB CL in DMSO-d_6 (δ_C 39.7, δ_H 2.49 ppm), CD_3OD (δ_C 49.3, δ_H 3.30 ppm), or $CDCl_3$ (δ_C 77.0, δ_H 7.25 ppm), with the residual solvent peak serving as the internal standard. HRESIMS data were recorded using a Waters LCT-Premier XE mass spectrometer. Reversed-phase MPLC was conducted on a Purif-Pack ODS-100 column (Shoko Scientific). Analytical reversed-phase HPLC was conducted using an L-column2 ODS column (4.6 i.d. × 150 mm; Chemical Evaluation and Research Institute) in conjunction with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. Analytical reversed-phase UPLC (Waters, ACQUITY) was performed using a BEH ODS column (2.1 i.d. × 50 mm; Waters) in conjunction with a Waters ACQUITY UPLC photodiode array eλ detector and an LCT-Premier XE mass spectrometer. Preparative reversed-phase HPLC was conducted using an L-column2 ODS column (20 i.d. × 150 mm) or a PEGASIL ODS (20 i.d. × 150 mm, Senshu Scientific) in conjunction with a Hitachi High Technologies L-2455 photodiode array detector. Other reagents and solvents were of the highest grade available.

Isolation and Identification of the Bacterium. *Streptomyces* strain SpD081030SC-03 was isolated from a marine sponge, Demospongiae, collected in Ishigaki City, Okinawa Prefecture, Japan. This strain was isolated using starch casein nitrate agar plates and a simple dilution plating method.[19] To identify the strain, almost complete 16S rRNA gene sequences were determined (DDBJ accession number AB539002) and compared to previously published sequences using EzTaxon.[13]

Fermentation. *Streptomyces* sp. SpD081030SC-03 was cultivated in 15 mL test tubes that each contained 5 mL of a seed medium consisting of 1.0% starch (Kosokagaku), 1.0% Polypepton (Nihon Pharmaceutical), 1.0% molasses (Dai-Nippon Meiji Sugar), and 1.0% meat extract (Extract Ehlrich, Wako Pure Chemical Industry) (pH 7.2). The test tubes were shaken on a reciprocal shaker (355 rpm) at 27 °C for two days. Aliquots (2.5 mL) of the broth were then transferred to 500 mL baffled Erlenmeyer flasks containing 100 mL of a production medium consisting of 2.5% starch, 1.5% soybean meal (Nisshin Oillio), 0.2% dry yeast (Mitsubishi Tanabe Pharma), 0.4% $CaCO_3$ (Kozaki Pharmaceutical), and 1.0% Diaion HP-20 resin (Mitsubishi Chemical) (pH 6.2) and cultured on a rotary shaker (180 rpm) at 27 °C for five days.

Isolation. The fermentation broth (2 L) was separated into the mycelial cake and supernatant by centrifugation. The supernatant was subsequently applied to a Diaion HP-20 column, and the column was then washed with 20% aqueous MeOH and eluted with 100% MeOH. After evaporation of the 100% MeOH eluent *in vacuo*, the resultant residue (611 mg) was subjected to reversed-phase MPLC (Purif-Pack ODS-100) using an aqueous MeOH linear gradient system (20−100% MeOH), and fractions including major metabolites were collected by LC-MS monitoring. The 80% MeOH eluate (9.32 mg) was then subjected to preparative reversed-phase HPLC using an L-column2 ODS column (20 i.d. × 150 mm) developed with 65% aqueous MeOH containing 0.1% formic acid (flow rate 10 mL/min) to give JBIR-56 (**1**, t_R = 13.7 min, 2.41 mg) and JBIR-57 (**2**, t_R = 11.3 min, 0.21 mg).

*JBIR-56 (**1**):* colorless oil; $[\alpha]^{25}_D$ −11 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 262 (3.97), 312 (3.66) nm; IR (KBr) ν_{max} 3380, 1660 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6), see Table 1; HRESIMS *m*/*z* 395.2294 [M + H]$^+$ (calcd for $C_{19}H_{31}N_4O_5$, 395.2294).

*JBIR-57 (**2**):* colorless oil; $[\alpha]^{25}_D$ −12 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 263 (4.00), 310 (3.67) nm; IR (KBr) ν_{max} 3380, 1650 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) see Table 1; HRESIMS *m*/*z* 381.2130 [M + H]$^+$ (calcd for $C_{18}H_{29}N_4O_5$, 381.2138).

Methylation of 1. Compound **1** (4.0 mg) from a large-scale culture (4 L) was dissolved in 400 μL of DMF and mixed with 0.4 mg of K_2CO_3 and 50 μL of methyl iodide. The solution was then allowed to stand at 50 °C for 12 h. Next, the mixture was diluted with MeOH, evaporated *in vacuo*, and subjected to preparative reversed-phase HPLC using a PEGASIL ODS column (20 i.d. × 150 mm) developed with 70% aqueous MeOH (flow rate 10 mL/min) to give 1-methyl-JBIR-56 methyl ester (**3**, t_R = 11.6 min, 2.2 mg). The structure of **3** was confirmed by LC-MS and NMR analyses: colorless oil; UV (MeOH) λ_{max} (log ε) 266 (3.98), 322 (3.68) nm; ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.17 (1H, d, *J* = 7.2 Hz, 2′-N*H*), 6.94 (1H, d, *J* = 7.1 Hz, 2″-N*H*), 4.57 (1H, m, H-2″), 4.52 (1H, m, H-2′), 3.70 (3H, S, 1″-O-C*H*$_3$), 3.60 (3H, s, 1-N-C*H*$_3$), 3.42, 3.34 (2H, m, H-11), 3.41 (1H, m, H-7), 1.71 (1H, m, H-4′), 1.85, 1.67 (2H, m, H-3′), 1.41 (3H, d, *J* = 7.2 Hz, H-3″), 1.30 (3H, t, *J* = 7.2 Hz, H-12), 1.23 (3H, d, *J* = 6.5 Hz, H-8), 1.22 (3H, d, *J* = 6.5 Hz, H-9), 0.98 (3H, d, *J* = 6.5 Hz, H-5′), 0.95 (3H, d, *J* = 6.5 Hz, H-6′); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 173.5 (C, C-1″), 171.9 (C, C-1′), 165.4 (C, C-10), 158.4 (C, C-3), 156.4 (C, C-2), 147.2 (C, C-6), 121.7 (C, C-5), 52.7 (CH_3, 1″-O-CH_3), 51.9 (CH, C-2′), 48.3 (CH, C-2″), 40.5 (CH_2, C-3′), 30.9 (CH, C-7), 30.6 (CH_3, 1-N-CH_3), 25.3 (CH, C-4′), 23.3 (CH_3, C-5′), 22.4 (CH_2, C-11), 22.2 (CH_3, C-6′), 20.2 (CH_3, C-8), 20.1 (CH_3, C-9), 18.6 (CH_3, C-3″), 12.9 (CH_3, C-12); HRESIMS *m*/*z* 423.2601 [M + H]$^+$ (calcd for $C_{21}H_{35}N_4O_5$, 423.2607).

Determination of Amino Acid Configuration. A sample of **1** or **2** (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 amino acid mixture was then recovered in the aqueous layer. After drying the aqueous layer *in vacuo*, it was dissolved in 5% $NaHCO_3$ (500 μL), and FDAA (Marfey's reagent, 0.2 mg) in acetone (500 μL) was added. The mixture was then heated in an oil bath at 80 °C for 3 h. The reaction products were subsequently analyzed by UPLC (Waters, ACQUITY) as follows: Waters BEH ODS column (2.1 i.d. × 50 mm, flow rate of 0.3 mL/min) developed with an aqueous MeCN containing 0.1% formic acid linear gradient system (10−100% MeCN, 7 min). The retention times of the FDAA derivatives were determined by monitoring UV absorption at 340 nm and the positive mode of ESIMS. The retention times of the standard FDAA derivatives were as follows: L-Leu 3.37 min, D-Leu 3.73 min, L-Ala 2.47 min, and D-Ala 2.70 min. The retention times of the FDAA derivatives of **1** and **2** are Ala unit (2.47 min) and Leu unit (3.73 min).

2-Chloro-6-ethylpyrazine (5). A 1.0 M THF solution of ethyl magnesium bromide (16.1 mL, 16.1 mmol) was slowly added to 2,6-dichloropyrazine (**4**) (2.0 g, 13.4 mmol) and iron acetylacetonate (237 mg, 0.671 mmol) in a mixed solution of THF (50 mL) and

N-methyl pyrrolidone (NMP, 5.0 mL) at 0 °C. After 30 min, the reaction was quenched with saturated $NaHCO_3$(aq), and the product was then extracted with EtOAc three times. The combined organic extracts were subsequently washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Next, the residue was purified by column chromatography (hexane−EtOAc) to give **5** (836 mg, 5.86 mmol) in 44% yield as a colorless powder: mp >300 °C; IR (neat) ν_{max} 3057, 2930, 1726, 1520, 1375 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ_H 8.41 (1H, s), 8.36 (2H, s), 2.83 (2H, q, J = 7.6 Hz), 1.33 (3H, t, J = 7.6 Hz); ^{13}C NMR (67.5 Hz, $CDCl_3$) δ_C 159.0, 148.6, 141.8, 141.4, 28.2, 13.2; HRESIMS m/z $[M + H]^+$ 143.0372 (calcd for $C_6H_8ClN_2$, 143.0376).

2-Benzyloxy-6-ethylpyrazine 4-Oxide (7). A 60% oil dispersion of NaH (50 mg, 1.26 mmol) and benzyl alcohol (0.130 mL, 1.26 mmol) in dimethoxyethane (DME, 4.0 mL) were added to a DME (4.0 mL) solution of the pyrazine **5** (100 mg, 0.701 mmol) at 45 °C. The mixture was then stirred for 18 h, after which it was quenched with saturated $NaHCO_3$(aq). The product was subsequently extracted and purified using the above procedure to give **6** (142 mg, 0.662 mmol) in 94% yield as a colorless oil. Next, sodium perborate monohydrate (413 mg, 4.14 mmol) was added to an acetic acid (30 mL) solution of the pyrazine **6** (740 mg, 3.45 mmol) at 80 °C. The mixture was then stirred for 26 h, after which the reaction mixture was quenched with saturated NaOH(aq), and the product was extracted and purified using the procedure described above to give **7** (621 mg, 2.70 mmol) in 78% yield as a colorless oil: IR (neat) ν_{max} 3446, 3089, 2973, 1599, 1521, 1446 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ_H 7.70 (1H, s), 7.68 (1H, s), 7.40−7.43 (2H, m), 7.36−7.39 (3H, m), 5.42 (2H, s), 2.69 (2H, q, J = 7.6 Hz), 1.29 (3H, t, J = 7.6 Hz); ^{13}C NMR (67.5 Hz, $CDCl_3$) δ_C 162.5, 158.5, 135.8, 128.5, 128.3, 128.3, 126.4, 120.3, 68.7, 28.4, 12.4; HRESIMS m/z $[M + H]^+$ 231.1129 (calcd for $C_{13}H_{15}N_2O_2$, 231.1134).

2-Benzyloxy-3-chloro-6-ethylpyrazine (8a). Phosphorus oxychloride (0.130 mL, 1.37 mmol) was added to a toluene (5.0 mL) and DMF (0.50 mL) solution at 0 °C. After 15 min, a DMF (3 mL) solution of pyrazine **7** (158 mg, 0.686 mmol) was added, and the mixture was stirred for three days at 0 °C. The reaction was then quenched with saturated $NaHCO_3$(aq). This procedure afforded **8a** (113 mg, 0.454 mmol) in 66% yield as a colorless oil. Regioisomer **8b** was obtained in 16% yield (27.8 mg, 0.112 mmol) as a colorless oil. **8a**: IR (neat) ν_{max} 2974, 2939, 1531, 1413, 1351, 1098 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ_H 7.79 (1H, s), 7.48−7.51 (2H, m), 7.30−7.42 (3H, m), 5.48 (2H, s), 2.72 (2H, q, J = 7.6 Hz), 1.29 (3H, t, J = 7.6 Hz); ^{13}C NMR (67.5 Hz, $CDCl_3$) δ_C 155.0, 154.0, 136.2, 134.6, 133.7, 128.4, 128.1, 128.0, 68.5, 27.4, 13.1; HRESIMS m/z $[M + H]^+$ 249.0790 (calcd for $C_{13}H_{13}ClN_2O$, 249.0795). **8b**: IR (neat) ν_{max} 2977, 2939, 1538, 1392, 1325, 1196 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ_H 7.88 (1H, s), 7.44−7.47 (2H, m), 7.30−7.40 (3H, m), 5.39 (2H, s), 2.88 (2H, q, J = 7.6 Hz), 1.30 (3H, t, J = 7.6 Hz); ^{13}C NMR (67.5 Hz, $CDCl_3$) δ_C 158.4, 152.8, 138.8, 136.3, 131.1, 128.5, 128.3, 128.2, 68.4, 27.7, 11.5; HRESIMS m/z $[M + H]^+$ 249.0789 (calcd for $C_{13}H_{14}ClN_2O$, 249.0795).

6-Ethyl-3-isopropyl-2-pyrazinone (10). A 0.75 M THF solution of isopropylmagnesium bromide (5.20 mL, 3.88 mmol) was slowly added to pyrazine **8a** (483 mg, 1.94 mmol) and iron acetylacetonate (34.3 mg, 0.0970 mmol) in a mixed solution of THF (20 mL) and NMP (2.0 mL) at 0 °C. After 16 h, the reaction was quenched with 1.0 M HCl(aq) and the product was extracted and purified using the above procedure to give **9** (417 mg, 1.62 mmol) in 51% yield as a colorless oil. Palladium on carbon (25.0 mg) was added to a MeOH (4.0 mL) solution of the pyrazine **9** (100 mg, 0.390 mmol) under a H_2 atmosphere at room temperature. The mixture was stirred for 1 h, after which the reaction mixture was filtered through a short pad of Celite, and the filtrate was concentrated under reduced pressure. The residue was then purified by preparative TLC (hexane−EtOAc, 7:3) to give **10** (57.3 mg, 0.345 mmol) in 88% yield as a white powder: mp 92−94 °C; IR (neat) ν_{max} 2974, 2085, 1460, 1219 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ_H 7.16 (1H, s), 3.30−3.38 (1H, m), 2.51 (2H, q, J = 7.8 Hz), 1.23 (1H, t, J = 7.8 Hz), 1.18 (6H, d, J = 6.8 Hz); ^{13}C NMR (100 Hz, CD_3OD) δ_C 160.5, 157.2, 141.3, 120.7, 29.5, 23.3, 19.2, 12.0; HRESIMS m/z $[M + H]^+$ 167.1191 (calcd for $C_9H_{15}N_2O$, 167.1184).

6-Ethyl-5-iodo-3-isopropyl-2-pyrazinone (11). *N*-Iodosuccinimide (67.0 mg, 0.297 mmol) was added to a DMF (2.0 mL) solution of **10** (45.0 mg, 0.270 mmol) at room temperature. After the reaction mixture was stirred for 15 h, the reaction was quenched with H_2O and the product was extracted with EtOAc and concentrated under reduced pressure. The residue was then purified by preparative TLC (hexane−EtOAc, 7:3) to give **11** (62.9 mg, 0.215 mmol) in 80% yield as a white powder: mp 167 °C; IR (neat) ν_{max} 2946, 1869, 1636, 1446, 1276 cm^{-1}; ^1H NMR (270 MHz, CD_3OD) δ_H 3.20−3.34 (1H, m), 1.19 (1H, t, J = 7.6 Hz), 1.17 (6H, d, J = 6.6 Hz); ^{13}C NMR (100 Hz, CD_3OD) δ_C 161.5, 158.5, 146.0, 90.1, 31.0, 30.2, 20.5, 13.0; HRESIMS m/z $[M + H]^+$ 293.0153 (calcd for $C_9H_{14}IN_2O$, 293.0151).

6-Ethyl-3-isopropyl-2-pyrazinon-5-ylcarboxylic Acid (13). A 1.67 M hexane solution of *n*-butyllithium (0.260 mL, 0.428 mmol) was added to a THF solution (3.0 mL) of **11** (50.0 mg, 0.171 mmol) at −78 °C. After 20 min, methyl choloroformate (32.0 μL, 0.410 mmol) was added, and the mixture was stirred for 25 min at −78 °C. The reaction was then quenched with saturated NH_4Cl(aq), after which the product was extracted with EtOAc. The organic extract was subsequently washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was then used without purification because it was difficult to separate the desired ester moiety **12** from the protonated byproduct **10**. A 1.0 M sodium hydroxide solution (2.0 mL, 2.00 mmol) was then added to the crude mixture containing **12** (45.4 mg) at reflux temperature. After 9 h, the reaction was quenched with 1.0 M hydrochloric acid. The product was then extracted with EtOAc, concentrated under reduced pressure, and purified by preparative TLC ($CHCl_3$−MeOH, 9:1) to give carboxylic acid **13** (16.2 mg, 0.0771 mmol) in 60% yield (2 steps) as a white powder: mp 244 °C; IR (neat) ν_{max} 2967, 1655, 1562, 1366 cm^{-1}; ^1H NMR (270 MHz, CD_3OD) δ_H 3.26 (1H, sep, J = 1.6 Hz), 2.91 (2H, q, J = 7.8 Hz), 1.18−1.23 (9H, m); ^{13}C NMR (100 Hz, CD_3OD) δ_C 169.9, 160.9, 157.9, 146.6, 125.4, 31.6, 25.0, 20.1, 14.0; HRESIMS m/z $[M + H]^+$ 211.1076 (calcd for $C_{10}H_{15}N_2O_3$, 211.1083).

Peptide 15. EDCI·HCl (13.7 mg, 0.0714 mmol), 1-hydroxybenzotriazole hydrate (9.60 mg, 0.0714 mmol), and *N*,*N*-diisopropylethylamine (22.0 μL, 0.157 mmol) were added to a DMF (2.0 mL) solution of **13** (10.0 mg, 0.0476 mmol) at room temperature. After 10 min, H-D-Leu-Ala-O^{t}Bu (**14**, 15.0 mg, 0.0571 mmol) was added, and the mixture was stirred for 16 h at room temperature. The reaction was then quenched with H_2O, and the product was extracted with EtOAc, concentrated under reduced pressure, and subjected to preparative TLC (hexane−EtOAc, 4:6) to give **15** (16.7 mg, 0.0371 mmol) in 74% yield as a white powder: mp 172 °C; IR (neat) ν_{max} 3299, 2935, 1650, 1731, 1506, 1370 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ_H 4.57 (1H, t, J = 6.4 Hz), 4.24 (1H, q, J = 7.8 Hz), 3.29−3.31 (1H, m), 3.05 (2H, q, J = 7.8 Hz), 1.68−1.69 (3H, m), 1.42 (9H, s), 1.35 (3H, d, J = 7.3 Hz), 1.22−1.26 (9H, m), 0.97 (6H, t, J = 5.8 Hz); ^{13}C NMR (100 Hz, CD_3OD) δ_C 174.5, 173.3, 165.9, 160.6, 158.0, 147.4, 82.6, 52.8, 50.4, 43.0, 31.1, 28.2, 26.3, 24.6, 23.4, 22.5, 20.2, 17.3,14.1; HRESIMS m/z $[M + H]^+$ 451.2920 (calcd for $C_{23}H_{39}N_4O_5$, 451.2920).

Synthesis of 1. TFA (9.0 mL) was added to 1.0 mL of an aqueous solution of **15** (16.7 mg, 0.0371 mmol) at room temperature. After 16 h, the TFA and H_2O were removed by reduced pressure. The residue was then purified by preparative TLC ($CHCl_3$−MeOH, 9:1) to give **1** (14.6 mg, 0.0370 mmol) in quantitative yield as a white powder: colorless oil; $[\alpha]^{25}_D$ −11 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 262 (3.97), 312 (3.66) nm; ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) see Supporting Information (Table S1); HRESIMS m/z $[M + H]^+$ 395.2285 (calcd for $C_{19}H_{31}N_4O_5$, 395.2294).

■ ASSOCIATED CONTENT

Supporting Information. NMR spectra of compounds **1**, **2**, and synthetic **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This work was supported by a grant from the New Energy and Industrial Technology Department Organization (NEDO). The authors thank EIWEISS Chemical Corporation for generously providing EDCI·HCl .

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