JOURNAL OF NATURAL PRODUCTS

Article

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Biseokeaniamides A, B, and C, Sterol *O*-Acyltransferase Inhibitors from an *Okeania* sp. Marine Cyanobacterium

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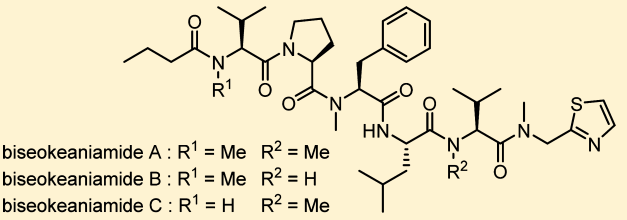
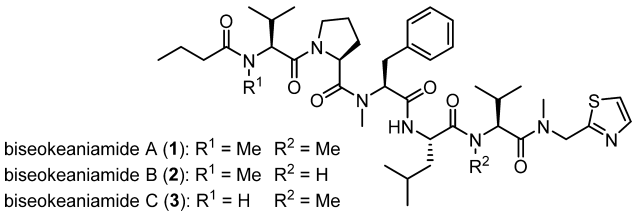
Supporting Information

ABSTRACT: Biseokeaniamides A, B, and C (**1**−**3**), structurally novel sterol *O*-acyltransferase (SOAT) inhibitors, were isolated from an *Okeania* sp. marine cyanobacterium. Their structures were elucidated by spectroscopic analyses and degradation reactions. Biseokeaniamide B (**2**) exhibited moderate cytotoxicity against human HeLa cancer cells, and compounds **1**−**3** inhibited both SOAT1 and SOAT2, not only at an enzyme level but also at a cellular level. Biseokeaniamides (**1**−**3**) are the first linear lipopeptides that have been shown to exhibit SOAT-inhibitory activity.



Marine cyanobacteria produce a variety of compounds possessing interesting biological activities, such as cytoskeletal disruptors, protease inhibitors, and neurotoxic membrane channel blockers.[1] The discovery of these molecules can sometimes encourage innovation in the fields of biology and pharmacy.[2] The bioactive secondary metabolites of marine cyanobacteria have been studied by researchers in the United States, and several important compounds, including curacin A[3] and apratoxin A,[4] have been discovered. In our continuing search for new bioactive substances from marine cyanobacteria,[5] we investigated the constituents of an *Okeania* sp. cyanobacterium collected in Okinawa, Japan, and discovered thiazole-containing lipopeptides, biseokeaniamides A, B, and C (**1**−**3**). Although several peptides possessing a terminal thiazole have been isolated from marine cyanobacteria, the amino-acid sequences of **1**−**3** are significantly different from those of known compounds.[6] Here, we report the isolation, structure determination, biological activities, and structure−activity relationships of biseokeaniamides (**1**−**3**).



RESULTS AND DISCUSSION

An *Okeania* sp. marine cyanobacterium (2700 g, wet weight) was collected at Bise, Okinawa, Japan, and extracted with MeOH. The extract was filtered, concentrated, and partitioned between EtOAc and H_2O. The EtOAc-soluble material was further partitioned between 90% aqueous MeOH and hexane. The material obtained from the aqueous MeOH portion was subjected to fractionation with reversed-phase column chromatography (ODS silica gel, MeOH−H_2O) and repeated reversed-phase HPLC to give biseokeaniamides A (**1**, 200 mg), B (**2**, 10.5 mg), and C (**3**, 2.6 mg).

Biseokeaniamide A (**1**) was obtained as a colorless oil. In CD_3OD, **1** existed as a 3:1 mixture of rotamers. The NMR data for the major rotamer of **1** are summarized in Table 1. The molecular formula of **1** was found to be $C_{42}H_{65}N_7O_6S$ by HRESIMS. The ^1H and ^{13}C NMR data suggested that **1** was a peptidic compound, with seven deshielded methine protons (δ_H 5.16, 5.14, 5.13, 4.98, 4.79, 4.64, and 4.55) and seven carbonyl carbons (δ_C 176.2, 175.7, 175.2, 171.8, 171.6, 170.0, and 168.0). In addition, one pair of deshielded methines with a small coupling constant, δ_H 7.74 (d, J = 3.4) and 7.59 (d, J = 3.4), suggested the presence of a thiazole ring, which was supported by the carbon signals (δ_C 143.0, 121.7, and 168.0).[7] Further analyses of COSY, HMBC, HMQC, and NOESY data revealed that **1** was composed of *N*-methyl-2-thiazolemethaneamine (Thz-*N*-Me-Gly), butanoic acid (Ba), and five amino acid residues, including two *N*-Me-valines, proline, *N*-Me-phenylalanine, and leucine. On the basis of the two NOESY correlations, H-6 (*N*-Me-Val1)/H-2 (Leu) and H-5 (pro)/H-2 (*N*-Me-Val2), and the five HMBC correlations, H-4 (Thz-*N*-Me-Gly)/C-1 (*N*-Me-Val1), *N*-H (Leu)/C-1 (*N*-Me-Phe), H-

Received: February 14, 2017

DOI: 10.1021/acs.jnatprod.7b00137
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. NMR Data for Biseokeaniamides A (1), B (2), and C (3) in CD_3OD

		biseokeaniamide A (1)		biseokeaniamide B (2)		biseokeaniamide C (3)	
residue	position	δ_C[a], type	δ_H[b] (J in Hz)	δ_C[a], type	δ_H[b] (J in Hz)	δ_C[a], type	δ_H[b] (J in Hz)
Thz-*N*-Me-Gly	1	143.0, CH	7.74, d (3.4)	143.0, CH	7.73, d (3.5)	143.0, CH	7.74, d (3.4)
	2	121.7, CH	7.59, d (3.4)	121.8, CH	7.57, d (3.5)	121.7, CH	7.59, d (3.4)
	3	168.0, C		168.1, C		168.0, C	
	4a	49.8, CH_2	5.13, d (15.7)	50.0, CH_2	4.92, m	49.8, CH_2	5.08, d (15.7)
	4b		4.64, d (15.7)		4.89, m		4.70, d (15.7)
	5	36.1, CH_3	3.11, s	36.5, CH_3	3.26, s	36.2, CH_3	3.15, s
N-Me-Val1/Val	1	171.6, C		173.9, C		171.6, C	
	2	59.8, CH	5.16, d (11.1)	55.7, CH	4.72, d (8.0)	59.8, CH	5.19, m
	3	27.9, CH	2.37, m	31.9, CH	2.13, m	28.2, CH	2.37, m
	4	19.9, CH_3	0.96, m	19.8, CH_3	0.97, m	20.0, CH_3	0.94, m
	5	20.9, CH_3	0.92, m	18.6, CH_3	0.96, m	18.9, CH_3	0.90, m
	6	31.1, CH_3	3.08, s			31.0, CH_3	3.12, s
Leu	1	175.7, C		175.3, C		175.6, C	
	2	49.9, CH	4.79, m	53.9, CH	4.42, m	50.2, CH	4.79, m
	3a	40.7, CH_2	1.79, m	41.5, CH_2	1.77, m	40.7, CH_2	1.80, m
	3b		1.30, m		1.53, m		1.32, m
	4	26.0, CH	1.74, m	25.9, CH	1.71, m	26.0, CH	1.66, m
	5	23.9, CH_3	0.94, m	23.8, CH_3	0.94, m	21.3, CH_3	0.92, m
	6	23.9, CH_3	0.94, m	21.0, CH_3	0.92, m	23.8, CH_3	0.90, m
	*N*H		9.07, d (7.5)		8.92, d (7.4)		8.96, brd
N-Me-Phe	1	171.8, C		172.0, C		171.8, C	
	2	64.3, CH	5.14, m	64.4, CH	5.17, m	63.8, CH	5.17, m
	3a	34.6, CH_2	3.22, m	34.6, CH_2	3.22, m	34.9, CH_2	3.13, m
	3b		2.99, m		3.00, m		2.97, m
	4	139.3, C		139.4, C		139.2, C	
	5/9	130.6, CH	7.21, m	130.6, CH	7.20, m	130.7, CH	7.21, m
	6/8	129.9, CH	7.32, m	129.9, CH	7.32, m	130.0, CH	7.31, m
	7	128.0, CH	7.25, m	128.0, CH	7.25, m	128.0, CH	7.25, m
	10	30.1, CH_3	2.84, s	30.1, CH_3	2.84, s	30.0, CH_3	2.83, s
Pro	1	175.2, C		175.4, C		175.4, C	
	2	56.9, CH	4.55, m	56.9, CH	4.57, m	56.8, CH	4.49, m
	3	29.7, CH_2	0.83, m	29.8, CH_2	0.84, m	29.9, CH_2	0.87, m
	4a	26.2, CH_2	1.83, m	26.2, CH_2	1.81, m	26.2, CH_2	1.85, m
	4b		1.58, m		1.55, m		1.58, m
	5a	48.4, CH_2	3.79, m	49.6, CH_2	3.79, m	49.2, CH_2	3.86, m
	5b		3.51, m		3.54, m		3.53, m
N-Me-Val2/Val	1	170.0, C		170.3, C		171.6, C	
	2	60.4, CH	4.98, d (11.0)	60.5, CH	4.98, d (11.0)	57.7, CH	4.33, d (9.2)
	3	28.9, CH	2.14, m	29.0, CH	2.16, m	32.0, CH	1.99, m
	4	19.46,[c] CH_3	0.86, m	19.6, CH_3	0.89, m	18.8, CH_3	0.90, d (6.6)
	5	18.8, CH_3	0.79, d (6.8)	18.9, CH_3	0.81, d (6.9)	19.5, CH_3	0.87, d (6.8)
	6	30.8, CH_3	2.93, s	31.4, CH_3	2.96, s		
butanoic acid	1	176.2, C		176.2, C		175.8, C	
	2a	36.4, CH_2	2.36, m	36.4, CH_2	2.37, m	38.6, CH_2	2.19, m
	2b		2.33, m		2.33, m		2.17, m
	3	19.49,[c] CH_2	1.61, m	19.5, CH_2	1.61, m	20.4, CH_2	1.60, m
	4	14.2, CH_3	0.95, m	14.2, CH_3	0.95, m	14.1, CH_3	0.92, m

[a]Measured at 100 MHz. [b]Measured at 400 MHz. [c]These carbons are interchangeable.

10 (*N*-Me-Phe)/C-1 (Pro), H-2 (Pro)/C-1 (*N*-Me-Val2), and H-6 (*N*-Me-Val2)/C-1 (Ba), the following sequence was clarified: Ba-*N*-Me-Val-Pro-*N*-Me-Phe-Leu-*N*-Me-Val-Thz-*N*-Me-Gly (Figure 1, Table S1).

Biseokeaniamides B (**2**) and C (**3**) existed as 5:1 and 3:1 mixtures of rotamers in CD_3OD, respectively. Their NMR data for the major rotamers are summarized in Table 1. The molecular formulas of **2** and **3** were found to be $C_{41}H_{63}N_7O_6S$ by HRESIMS. The ^1H and ^{13}C NMR features of both compounds were analogous to those of **1** except that they did not contain two *N*-methyl groups. Detailed analyses of the spectroscopic data revealed that **2** had a Val in place of the *N*-Me-Val after the Leu residue of **1**: Ba-*N*-Me-Val-Pro-*N*-Me-Phe-Leu-Val-Thz-*N*-Me-Gly (Figure 1, Table S2). Meanwhile, we could not directly confirm the existence of a thiazole ring in **3** because of the lack of HMBC correlations, H-1/C-3 or H-2/C-3. However, the similarity of the chemical shifts for **3** and those for the other compounds (**1** and **2**) indicated the presence of a thiazole ring at the C-terminus. In addition, although no HMBC or NOESY correlations were observed

DOI: 10.1021/acs.jnatprod.7b00137
J. Nat. Prod. XXXX, XXX, XXX−XXX

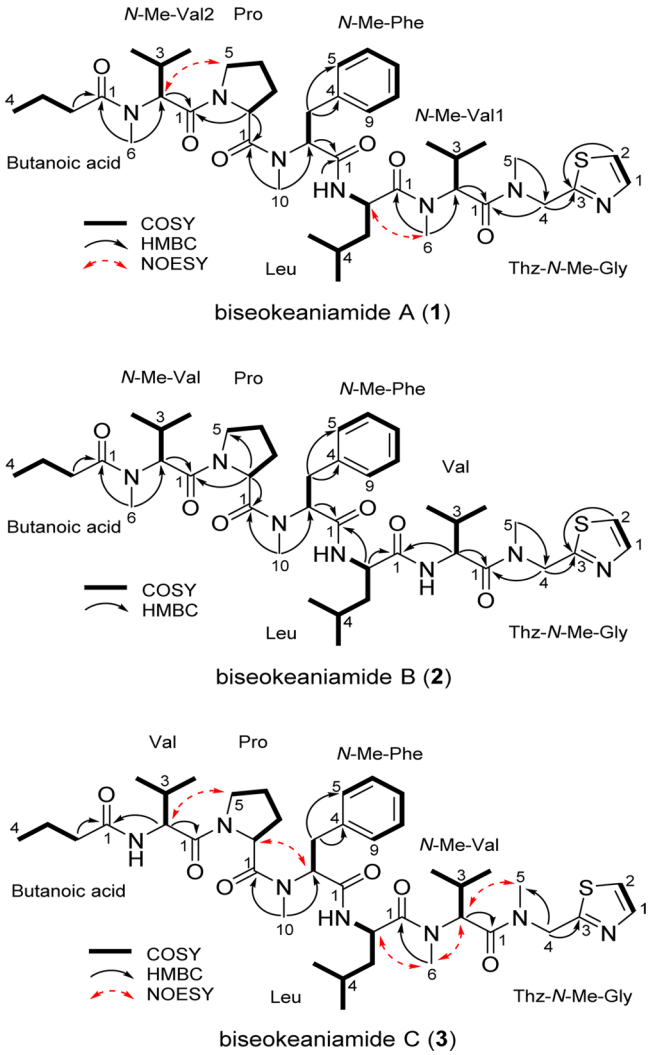


Figure 1. Gross structures of biseokeaniamides A (**1**), B (**2**), and C (**3**) based on 2D NMR analyses.

between *N*-Me-Phe and Leu in **3**, the connectivity of these residues was established based on the molecular formula. Finally, **3** was also determined to be an *N*-demethyl analogue of **1**, with the N-terminal *N*-Me-Val replaced by a Val: Ba-Val-Pro-*N*-Me-Phe-Leu-*N*-Me-Val-Thz-*N*-Me-Gly (Figure 1, Table S3). As a result, although biseokeaniamides B (**2**) and C (**3**) are both *N*-demethyl analogues of **1**, the demethylated position differs between them (Figure 1).

The absolute configuration of biseokeaniamide A (**1**) was determined by acid hydrolysis of **1** followed by a combination of chiral-phase HPLC analyses and advanced Marfey's method.[7] The results clarified that the absolute configurations of all of the amino acid moieties in **1** were the L-form. The absolute configurations of **2** and **3** were also determined by the same procedures as described above, and every amino acid moiety in both compounds was established to be the L-form.

To evaluate the growth-inhibitory activities of biseokeaniamides (**1**–**3**) against human cancer cells, MTT assays were performed with HeLa and HL60 cells. The cells were placed in 96-well plates and treated with various concentrations of the compounds for 72 h. The data from these assays revealed that only biseokeaniamide B (**2**) showed growth-inhibitory activity against HeLa cells but was inactive against HL60 cells (Table 2). On the basis of a comparison of the IC_{50} values of these compounds, biseokeaniamide B (**2**) has 10 times stronger activity against HeLa cells than **1** and **3**. Structurally, these compounds differ by the loss of an *N*-methyl group in the valine residue near the C-terminus. Therefore, the absence of this *N*-methyl group is important for the growth-inhibitory activity against HeLa cells. Next, we evaluated the cell-killing activity of **2** by using the trypan blue dye exclusion assay. The results clarified that biseokeaniamide B (**2**) induced cell death in HeLa cells. Additionally, this cytocidal activity was suppressed in the presence of Z-VAD-FMK, a pan-caspase inhibitor (Figure 2A). Furthermore, **2** activated caspase 3 dose-dependently (Figure 2B). These results indicated that **2** induced apoptosis in HeLa cells. Finally, we evaluated the inhibitory activity of biseokeaninamides A–C (**1**–**3**) toward sterol *O*-acyltransferase (SOAT). SOAT is an ER membrane protein that is responsible for esterification of cholesterol for storage inside cells, and excessive accumulation of cholesteryl esters produced by SOAT causes hypercholesterolemia and related diseases, such as atherosclerosis. To date, two types of SOAT isozymes, SOAT1 and SOAT2, have been recognized with distinct functions.[8] SOAT1 is expressed ubiquitously, while SOAT2 is expressed predominantly in the liver (hepatocytes) and intestine. Therefore, it is important to determine the selectivity of inhibitors against SOAT1 and SOAT2 isozymes for their development as new antihypercholesterolemia and antiatherosclerotic agents. Against this background, the SOAT-inhibitory activities of **1**–**3** were evaluated by using the cell-based and enzyme-based assay.[9] The results clarified that **1**–**3** inhibited both SOAT1 and SOAT2 not only at an enzyme level but also at a cellular level, and their activity was potent and comparable with other known compounds, such as purpactin A[10] (Table 2). Compounds **1**–**3** are the first linear lipopeptides possessing inhibitory activity against both SOAT isozymes. On the basis of a comparison of the IC_{50} values of these compounds, biseokeaniamide C (**3**) has

Table 2. Biological Activities of Biseokeaninamides A (1), B (2), and C (3)

	IC_{50} (μM)					
	cell growth-inhibitory activity against human cancer cells		SOAT-inhibitory activity			
			cell-based assay		enzyme-based assay	
sample	HeLa	HL60	SOAT1	SOAT2	SOAT1	SOAT2
biseokeaniamide A (**1**)	29	30	1.8	1.3	1.8	9.6
biseokeaniamide B (**2**)	4.5	19	6.9	2.5	6.8	9.9
biseokeaniamide C (**3**)	43	>100	>12	9.6	11	>32
purpactin A[a]			2.5	1.5	0.9	1.8

[a]SOAT inhibitor control.

DOI: 10.1021/acs.jnatprod.7b00137
J. Nat. Prod. XXXX, XXX, XXX–XXX

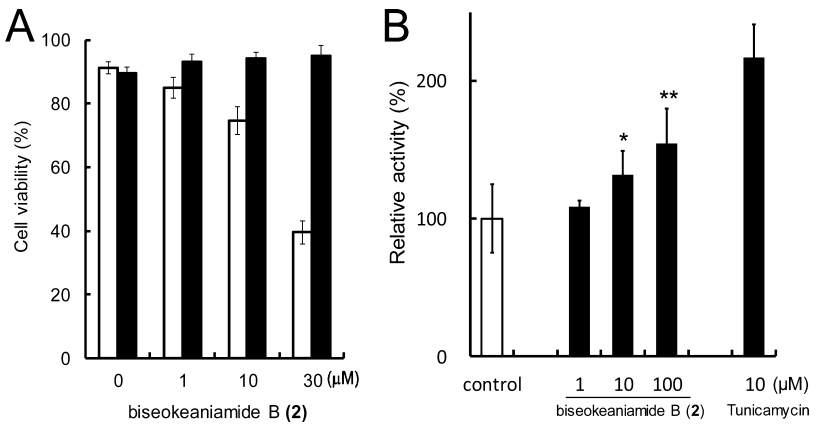


Figure 2. Induction of apoptosis by biseokeaniamide B (**2**) in HeLa cells. (A) HeLa cells were preincubated (solid column) or not (open column) with 50 μM Z-VAD-FMK. They were then treated with the indicated concentrations of **2**. After further incubation for 48 h, cell viability was determined based on trypan blue dye exclusion. Values are the mean ± SD of quadruplicate determinations. (B) HeLa cells were treated with the indicated concentrations of **2** for 24 h at 37 °C. Cell extracts were then tested for caspase 3 activity. Tunicamycin, a known apoptosis inducer, was used as a positive control. Values are the means ± SD of triplicate determinations. *p < 0.10 versus control, **p < 0.05 versus control.

relatively weaker activity than **1** and **2**. Therefore, the presence of the *N*-methyl group of the N-terminal *N*-Me-Val is important for the SOAT-inhibitory activity.

In summary, biseokeaniamides A (**1**), B (**2**), and C (**3**) were isolated from an *Okeania* sp. marine cyanobacterium. Their structures were established using a combination of spectroscopic analyses, degradation reactions, and HPLC analyses. Biseokeaniamide B (**2**) exhibited growth-inhibitory activity against HeLa cells and induced apoptosis. In addition, **1**−**3** inhibited both SOAT1 and SOAT2, not only at an enzyme level but also at a cellular level. Although several kinds of SOAT inhibitors, such as purpactins[10] and beauveriolides,[11] have been reported to date, biseokeaniamides (**1**−**3**) are the first linear lipopeptides possessing inhibitory activity against both SOAT isozymes. Further research, including studies on their structure−activity relationships, may reveal the detailed potency of biseokeaniamides (**1**−**3**) as antihypercholesterolemic agents.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-1000 polarimeter. IR spectra were recorded on a JASCO RT/IR-4200 instrument. All NMR spectral data were recorded on a JEOL JNM-ECX400 spectrometer for ^{1}H (400 MHz) and ^{13}C (100 MHz). ^{1}H NMR chemical shifts (referenced to residual CHD_2OD observed at δ_H 3.31) were assigned using a combination of data from COSY and HMQC experiments. Similarly, ^{13}C NMR chemical shifts (referenced to CD_3OD observed at δ_C 49.0) were assigned based on HMBC and HMQC experiments. ESI mass spectra were obtained on an LCT Premier EX spectrometer (Waters). All chemicals and solvents used in this study were the best grade and available from a commercial source (Nacalai Tesque).

Identification of the Marine Cyanobacterium. A cyanobacterial filament was isolated under a microscope and crushed with compressing. The 16S rDNA genes were PCR-amplified from isolated DNA using the primer set CYA106F, a cyanobacterial-specific primer, and 23S30R, a cyanobacterial-specific primer. The PCR reaction contained DNA derived from a cyanobacterial filament, 0.5 μL of KOD FX Neo (Toyobo), 1.0 μL of each primer (0.4 μM), 12.5 μL of 2× PCR buffer for KOD FX Neo, 5.0 μL of dNTPs (0.4 mM), and H_2O for a total volume of 25 μL. The PCR reaction was performed as follows: initial denaturation for 10 min at 94 °C, amplification by 40 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C, and final elongation for 7 min at 72 °C. PCR products were analyzed on agarose gel (1%) in TBE buffer and visualized by ethidium bromide staining. The obtained DNA was sequenced with CYA106F and 16S1541R primers. This sequence is available in the DDBJ/EMBL/GenBank databases under the accession number LC164723. A phylogenetic tree including 1267 bp of the 16S rRNA gene sequence revealed that the present cyanobacterium formed a clade with *Okeania* spp. Therefore, the cyanobacterium was classified into the genus *Okeania*. A voucher specimen of this sample, named 1504-34, has been deposited at Keio University.

Collection, Extraction, and Isolation. The cyanobacterium, *Okeania* sp. (sample 1504-34), was collected at Bise, Okinawa Prefecture, Japan, at a depth of 0−1 m in April 2015. The collected cyanobacterium (2700 g) was extracted with MeOH (2 L) for 1 week. The extract was filtered, and the filtrate was concentrated. The residue was partitioned between EtOAc (4 × 0.3 L) and water (1.0 L). The material obtained from the organic layer was partitioned between 90% aqueous MeOH (0.3 L) and hexane (3 × 0.3 L). The aqueous MeOH fraction (880 mg) was first separated by column chromatography on ODS (10 g) eluted with 40% MeOH, 60% MeOH, 80% MeOH, and MeOH. The fraction (355 mg) eluted with 80% MeOH was subjected to HPLC [Cosmosil Cholester (ϕ 20 × 250 mm); flow rate 5 mL/min; detection, UV 215 nm; solvent 65% MeCN] to give biseokeaniamide A (**1**, 200 mg, t_R = 26.0 min) and a fraction that contained biseokeaniamides B (**2**) and C (**3**) (t_R = 24.3 min). The fraction containing **2** and **3** was further separated by HPLC [Cosmosil 5PE-MS (ϕ 20 × 250 mm); flow rate 5 mL/min; detection, UV 215 nm; solvent 83% MeOH] to give biseokeaniamides B (**2**, 10.5 mg, t_R = 32.0 min) and C (**3**, 2.6 mg, t_R = 36.7 min).

Biseokeaniamide A (1): colorless oil; $[\alpha]^{30}_D$ −209 (c 0.64, MeOH); IR (neat) 3275, 2962, 2868, 1653, 1644, 1625, 1453, 1434, 1089, 753 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, and NOESY data, Tables 1 and S1; HRESIMS m/z 796.4798 $[M + H]^+$ (calcd for $C_{42}H_{66}N_7O_6S$, 796.4795).

Biseokeaniamide B (2): colorless oil; $[\alpha]^{30}_D$ −130 (c 0.08, MeOH); IR (neat) 3285, 2962, 2871, 1653, 1642, 1625, 1453, 1433, 1089, 750 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, and NOESY data, Tables 1 and S2; HRESIMS m/z 782.4618 $[M + H]^+$ (calcd for $C_{41}H_{64}N_7O_6S$, 782.4639).

Biseokeaniamide C (3): colorless oil; $[\alpha]^{30}_D$ −157 (c 0.025, MeOH); IR (neat) 3278, 2963, 2869, 1653, 1643, 1625, 1448, 1409, 1093, 750 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, and NOESY data, Tables 1 and S3; HRESIMS m/z 782.4590 $[M + H]^+$ (calcd for $C_{41}H_{64}N_7O_6S$, 782.4639).

Determination of the Configuration of Biseokeaniamide A (1). Biseokeaniamide A (**1**, 0.5 mg) was treated with 6 M HCl (100 μL) for 24 h at 110 °C. The hydrolyzed product was evaporated to dryness and could be separated into each component [conditions for HPLC separation: column, Cosmosil 5C_{18}-PAQ (ϕ 4.6 × 250 mm);

DOI: 10.1021/acs.jnatprod.7b00137
J. Nat. Prod. XXXX, XXX, XXX−XXX

flow rate, 1.0 mL/min; detection at 215 nm; solvent H_2O; retention times (min) of components: Pro (3.2), *N*-Me-Val (3.7), Leu (4.8), *N*-Me-Phe (12.5)].

Each fraction was dissolved in H_2O (50 μL) and analyzed by chiral-phase HPLC, and the retention times were compared to those of authentic standards [column, DAICEL CHIRALPAK MA(+) (ϕ 4.6 × 50 mm) or DAICEL CHIRALPAK WH (ϕ 4.6 × 50 mm); flow rate, 1.0 mL/min; detection at 254 nm; solvent, several conditions]. With DAICEL CHIRALPAK MA(+) by using 2 mM $CuSO_4$ as a solvent, the retention times for authentic standards were 2.5 min (*N*-Me-D-Val) and 3.6 min (*N*-Me-L-Val). The retention time of *N*-Me-Val in the hydrolysate was 3.6 min, indicating the presence of *N*-Me-L-Val. With DAICEL CHIRALPAK WH by using 3.0 mM $CuSO_4$, the retention times for authentic standards were 18.0 min (D-Pro) and 10.5 min (L-Pro). The retention time of Pro in the hydrolysate was 10.5 min, indicating the presence of L-Pro. With DAICEL CHIRALPAK WH by using 10.0 mM $CuSO_4$–MeCN (80:20), the retention times for authentic standards were 7.4 min (*N*-Me-D-Phe) and 24.6 min (*N*-Me-L-Phe). The retention time of *N*-Me-Phe in the hydrolysate was 24.6 min, indicating the presence of *N*-Me-L-Phe.

The Leu fraction was dissolved in H_2O (50 μL). A 1.0% 1-fluoro-2,4-dinitrophenyl-5-L-leucineamide (L-FDLA) solution in acetone (100 μL) and 25 μL of 1 M $NaHCO_3$ were added, and the mixture was heated at 80 °C for 3 min. The solution was cooled to room temperature, neutralized with 1 M HCl, and evaporated to dryness. The residue was resuspended in 50 μL of MeCN–H_2O (50:50), and the solution was analyzed by reversed-phase HPLC [Cosmosil Cholester (ϕ 4.6 × 250 mm); flow rate, 1.0 mL/min; detection at 340 nm; solvent 0.02 M NaOAc–MeCN (60:40)]. The retention times for authentic standards were 9.0 min (D-Leu) and 4.3 min (L-Leu). The retention time of Leu in the hydrolysate was 4.3 min, indicating the presence of L-Leu.

Determination of the Configurations of the Amino Acids in Biseokeaniamides B (2) and C (3). Biseokeaniamides B (**2**, 0.3 mg) and C (**3**, 0.3 mg) were treated with 6 M HCl (100 μL) for 24 h at 110 °C, respectively. Each hydrolyzed product was evaporated to dryness and could be separated into each component [conditions for HPLC separation: column, Cosmosil 5C_{18}-PAQ (ϕ 4.6 × 250 mm); flow rate, 1.0 mL/min; detection at 215 nm; solvent H_2O; retention times (min) of components: Pro (3.2), Val (3.4), *N*-Me-Val (3.7), Leu (4.8), *N*-Me-Phe (12.5)].

Each fraction was dissolved in H_2O (50 μL). A 1.0% L-FDLA solution in acetone (100 μL) and 25 μL of 1 M $NaHCO_3$ were added, and the mixture was heated at 80 °C for 3 min. The solution was cooled to room temperature, neutralized with 1 M HCl, and evaporated to dryness. The residue was resuspended in 50 μL of MeCN–H_2O (50:50), and the solution was analyzed by reversed-phase HPLC [Cosmosil 5C_{18}-AR-II (ϕ 4.6 × 250 mm); flow rate, 1.0 mL/min; detection at 340 nm; solvent 0.02 M NaOAc–MeCN (65:35) or 0.02 M NaOAc–MeCN (75:25)]. With 0.02 M NaOAc–MeCN (65:35), the retention times for authentic standards were 5.9 min (D-Pro), 3.8 min (L-Pro), 16.3 min (D-Val), 4.2 min (L-Val), 16.7 min (*N*-Me-D-Val), 5.5 min (*N*-Me-L-Val), 26.0 min (D-Leu), and 4.9 min (L-Leu). The retention times of the amino acids in the hydrolysate were 3.8, 4.2, 5.5, and 4.9 min, indicating the presence of L-Pro, L-Val, *N*-Me-L-Val, and L-Leu in each hydrolysate of both compounds **2** and **3**. With 0.02 M NaOAc–MeCN (75:25), the retention times for authentic standards were 4.7 min (*N*-Me-D-Phe) and 3.6 min (*N*-Me-L-Phe). The retention time of the amino acid in the hydrolysate was 3.6 min, indicating the presence of *N*-Me-L-Phe in each hydrolysate of both compounds **2** and **3**.

Cell Growth Analysis. HeLa cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, 300 μg/mL L-glutamine, and 2.25 mg/mL $NaHCO_3$. HL60 cells were cultured at 37 °C with 5% CO_2 in RPMI (Nissui) supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, 300 μg/mL L-glutamine, and 2.25 mg/mL $NaHCO_3$. HeLa cells were seeded at 2 × 10^4 cells/well in 96-wells plates (Iwaki) and cultured overnight. HL60 cells were seeded at 1 × 10^5 cells/well in 96-well plates. Various concentrations of compounds were then added, and cells were incubated for 72 h. Cell proliferation was measured by the MTT assay.

Trypan Blue Dye Exclusion Assay. HeLa cells were seeded at 4 × 10^4 cells/well in 24-well plates (Iwaki), cultured overnight, and then preincubated with or without 50 μM Z-VAD-FMK (Promega) for 30 min. The cells were then treated with various concentrations of compounds for 48 h. They were then stained with 0.8 mg/mL trypan blue (Sigma-Aldrich), and the cell viability was determined by counting the number of stained (killed) cells.

Caspase 3 Activity Assay. Caspase 3 (DEVDase) activity of the cell lysate was measured using the CaspACE assay system, colorimetric (Promega). Exponentially growing HeLa cells were seeded on a 60 mm dish at 2 × 10^6 cells. After cells were incubated for 12 h at 37 °C, compounds were added. After 24 h, cells were washed with ice-cold phosphate-buffered saline (PBS) and collected. The cell pellet was resuspended in cell lysis buffer (included in the above kit, 30 μL). After a freeze–thawing process (three times) and incubation on ice for 15 min, the supernatant was collected by centrifugation (15000*g*, 4 °C, 20 min). The lysate was used for enzymatic assays (37 °C, 4 h) with Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) as a substrate, according to the manufacturer's protocol. The liberated *p*-nitroaniline was measured using a spectrophotometer at 405 nm. The relative activity was calculated by comparison with a control experiment (1% MeOH) based on the average of three assays.

Cell Culture of SOAT1- and SOAT2-CHO Cells. Two cell lines, CHO cells expressing SOAT1 and SOAT2 isozymes of African green monkey (SOAT1- and SOAT2-CHO cells, respectively),[12] were kind gifts from Dr. L. L. Rudel (Wake Forest University). Briefly, both cell lines were maintained at 37 °C in 5% CO_2 in Ham's F-12 medium supplemented with MEM vitamins, Geneticin (300 μg/mL), and 10% heat-inactivated FBS (hereafter referred to as medium A).

Assay for Neutral Lipid Synthesis in SOAT1- and SOAT2-CHO Cells. The assay for the synthesis of neutral lipids ([^{14}C]-cholesteryl ester (CE), [^{14}C]triglyceride (TG), and [^{14}C]phospholipid (PL)) from [^{14}C]oleic acid in SOAT1- or SOAT2-CHO cells was carried out by our established method.[9] Briefly, SOAT1- or SOAT2-CHO cells (1.25 × 10^5 cells in 250 μL of medium A) were cultured in a 48-well plastic microplate in the culture medium described above and allowed to recover overnight at 37 °C in 5% CO_2. The assays were done with cells that were at least 80% confluent. Following the overnight recovery, test sample (2.5 μL; 0, 0.01, 0.1, and 1 mg/mL in MeOH) and [1-^{14}C]oleic acid (5 μL of 10% EtOH–PBS solution, 1 nmol, 1.85 KBq) were added to each culture. After a 6 h incubation at 37 °C in 5% CO_2, the medium was removed, and the cells in each well were washed twice with PBS. The cells were lysed by adding 0.25 mL of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by the method of Bligh and Dyer.[13] After concentrating the organic solvent, the total lipids were separated on a thin-layer chromatography plate (silica gel F254, 0.5 mm thick, Merck, Darmstadt, Germany) and analyzed with an FLA7000 analyzer (Fuji Film). In this cell-based assay, [^{14}C]CE was produced by the reaction of SOAT1 or SOAT2. SOAT-inhibitory activity (%) is defined as ([1-^{14}C]CE-drug/[^{14}C]CE-control) × 100. The IC_{50} value is defined as the drug concentration causing 50% inhibition of a biological activity and is calculated from two times duplicated experiments (n = 3).

Preparation of Crude Enzymes from SOAT1- and SOAT2-CHO Cells. Crude enzyme from SOAT1- and SOAT2-CHO cells using a Potter-type homogenizer was prepared by our established method.[9] Briefly, the SOAT1 or SOAT2-CHO cells (2.0 × 10^8 cells) were homogenized in 5 mL of cold buffered sucrose solution (pH 7.2, 100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, and 30 mM EDTA, hereafter referred to as buffer B) including protease inhibitors (Complete mini (Roche)) in a Potter-type homogenizer. The microsomal fraction was pelleted by centrifugation at 100000*g* for 1.0 h at 4.0 °C (TLA110, Beckman Coulter), resuspended in the same buffer at a concentration of 5.0 mg protein/mL, and stored at −80 °C until use.

DOI: 10.1021/acs.jnatprod.7b00137
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Assay for SOAT1 and SOAT2 Activity in the Microsome Fraction. SOAT1 and SOAT2 activities were determined by using fractions prepared as described above as crude enzyme.[9] Briefly, an assay mixture was made containing 500 μg of Bovine Serum Albumin (BSA) (fatty acid free) and [1-^{14}C]oleoyl-CoA (20 μM, 3.7 kBq). A test sample (5.0 μL in MeOH solution) and the microsome fraction of SOAT1- and SOAT2-CHO cells in a total volume of 200 μL of buffer B were incubated at 37 °C. The SOAT reaction was started by adding [1-^{14}C]oleoyl-CoA. After 5 min of incubation, the reaction was stopped by adding $CHCl_3$−MeOH (2:1, 1.2 mL). The product [^{14}C]CE was extracted by the method of Bligh and Dyer.[13] After the organic solvent was removed by evaporation, lipids were separated on a TLC plate and the radioactivity of [^{14}C]CE was measured by our established method.[9] SOAT-inhibitory activity (%) is defined as ([1-^{14}C]CE-drug/[^{14}C]CE-control) × 100. The IC_{50} value is defined as the drug concentration causing 50% inhibition of a biological activity and is calculated from two duplicated experiments (n = 3).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00137.

NMR spectra for biseokeaniamides (**1**−**3**); HPLC chromatograms for determination of the absolute configurations (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Prof. L. L. Rudel (Wake Forest University, Winston-Salem, NC, USA) for kindly providing SOAT1-CHO and SOAT2-CHO cells. This work was supported by JSPS KAKENHI Grant Number 16H03285 and the Naito Foundation.

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DOI: 10.1021/acs.jnatprod.7b00137
J. Nat. Prod. XXXX, XXX, XXX−XXX