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Odobromoamide, a Terminal Alkynyl Bromide-Containing Cyclodepsipeptide from the Marine Cyanobacterium *Okeania* sp.

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The bioassay-guided fractionation of the Okinawan marine cyanobacterium *Okeania* sp. led to the isolation of the novel cyclodepsipeptide odobromoamide (**1**). The gross structure of **1** was determined by spectroscopic analyses, and its absolute stereochemistry was determined using a variety of different methods, including chemical derivatization and degradation followed by HPLC analysis. In addition, odobromoamide (**1**) exhibited broad-spectrum of cytotoxicity against human cancer cell line panel.

Marine cyanobacteria are well known as prolific producers of structurally interesting and biologically active secondary metabolites.[1–3] Furthermore, some of these compounds have potential as therapeutic agents.[4–6] During the past 12 years, several structurally unique compounds containing a halogen atom as part of an unusual alkynyl halide moiety have been isolated from marine cyanobacteria. Jamaicamide A is a highly functionalized lipopeptide with several intriguing structural features, including an acetylenic bromide moiety, which was isolated from *Lyngbya majuscula*.[7] The cyclic depsipeptide veraguamides were isolated from the marine cyanobacterium cf. *Oscillatoria margaritifera*.[8] As part of our ongoing efforts to identify novel bioactive marine cyanobacterial metabolites, we recently isolated odoamide[9] and odobromoamide (**1**, Figure 1), a cyclodepsipeptide containing a terminal alkynyl bromide, from the Okinawan marine cyanobacterium *Okeania* sp. In this report, we describe the isolation, structural determination and biological activity of odobromoamide (**1**).

Results and Discussion

A large sample of the marine cyanobacterium *Okeania* sp. (1.2 kg, wet weight) was collected from Odo, Okinawa Prefecture, Japan, and extracted with methanol. The extract was filtered and the filtrate was concentrated *in vacuo* to give a residue, which was partitioned between EtOAc and H_2O. The organic layer was collected and washed sequentially with 90% aqueous MeOH and *n*-hexane. The material obtained from the 90% aqueous MeOH portion was fractionated by ODS column chromatography, followed by reversed-phase HPLC to give odobromoamide (**1**) as a colorless oil.

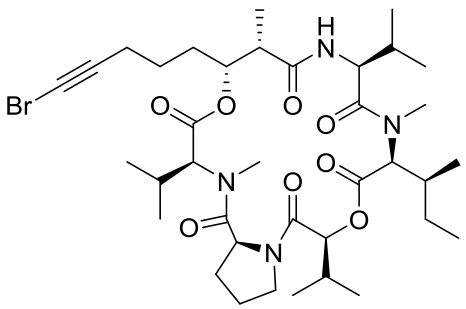


Figure 1. Structure of odobromoamide (**1**).

ESIMS analysis of odobromoamide (**1**) showed a 1:1 doublet ion peaks with *m/z* values of 767 and 769, indicating the presence of a bromine atom. The molecular formula of **1** was determined to be $C_{37}H_{59}BrN_4O_8$ by HRESIMS (*m/z* 767.3577 $[M+H]^+$, calcd 767.3595). The details of the ^1H and ^{13}C NMR spectra of **1** are summarized in Table 1. The ^1H NMR spectrum of compound **1** revealed the presence of one NH group (δ_H 6.26) and two *N*-methylamide groups (δ_H 2.91, 2.96). The ^{13}C NMR spectrum indicated the presence of six carbonyl carbons (δ_C 165.8, 169.7, 170.6, 170.8, 172.1, 173.5). Two of the ten degrees of unsaturation in this compound were accounted for by a terminal alkynyl bromide moiety based on its characteristic carbon chemical shifts (δ_C 79.4, 38.4).[7] The remaining two degrees of unsaturation were assigned to two rings.

Two-dimensional analyses including COSY, HSQC and HMBC experiments revealed the presence of proline (Pro), valine (Val), *N*-methylvaline (*N*-Me-Val) and *N*-methylisoleucine (*N*-Me-Ile). COSY correlations between H-13/H-14, H-14/H-15 and H-14/H-16 suggested the presence of a valine-like unit. Furthermore, the HSQC spectrum of **1** indicated that H-13 was attached to an oxymethine carbon (δ_C 77.5). This residue was therefore characterized as 2-hydroxy-3-methylbutanoic acid (Hmba). The sequence of these amino acids and Hmba was determined by HMBC analysis, which indicated correlations between H-6/C-2, H-6/C-7, H-8/C-7, H-8/C-12, H-13/C-12, H-13/C-17, H-18/C-17, H-23/C-18, H-23/C-24 and H-25/C-24 (Figure 2).

The structure of the remaining $C_9H_{11}BrO_2$ unit was determined as follows. COSY analysis connected the methine proton at H-30 which showed correlation to the methyl group at C-37 to the oxymethine at H-31. Furthermore, oxymethine at H-31 was connected to the methylene protons at H_2-34, via the two methylene protons at H_2-32 and H_2-33. In addition, HMBC correlations between H_2-34/C-35 and H_2-34/C-36 and the characteristic chemical shifts ($\delta_{C\text{-}35}$ 38.4, $\delta_{C\text{-}36}$ 79.4) led to this unit being identified as 8-bromo-3-hydroxy-2-methyloct-7-ynoic acid (Br-Hmoya) (Figure 2).

The connections between the amino or hydroxy acid units and Br-Hmoya were determined by HMBC analysis, and the final degree of unsaturation indicated that odobromoamide (**1**) was cyclic. The NH proton of Val showed a cross peak to the

Table 1. NMR spectra data for odobromoamide (**1**) in $CDCl_3$

Unit	No.	δ_C [a]	δ_H, mult (J in Hz) [b]
N-Me-Val	1	170.6	
	2	64.9	3.90, d (10.8)
	3	28.3	2.27, m
	4	19.5	0.89, d (6.6)
	5	19.6	0.95, d (7.1)
	6	28.7	2.96, s
Pro	7	172.1	
	8	57.2	4.90, dd (8.4, 5.0)
	9a	29.5	2.26, m
	9b		1.76, m
	10	24.9	1.98, m
	11a	47.3	3.79, m
	11b		3.57, m
Hmba	12	165.8	
	13	77.5	4.77, d (8.3)
	14	29.7	2.14, m
	15	18.6	0.92, d (6.4)
	16	18.1	1.01, d (6.4)
N-Me-Ile	17	169.7	
	18	65.3	4.14, d (9.5)
	19	34.9	1.95, m
	20a	26.5	1.52, m
	20b		1.04, m
	21	11.7	0.94, t (7.1)
	22	16.3	1.02, d (6.3)
	23	29.9	2.91, s
Val	24	173.5	
	25	52.8	4.65, dd (8.3, 5.6)
	26	32.1	1.91, m
	27	17.3	0.83, d (6.8)
	28	20.3	0.91, d (6.4)
	NH		6.26, d (8.3)
Br-Hmoya	29	170.8	
	30	42.3	3.04, m
	31	76.4	4.81, m
	32a	27.5	2.06, m
	32b		1.57, m
	33a	25.0	1.58, m
	33b		1.40, m
	34	19.2	2.18, m
	35	79.4	
	36	38.4	
	37	14.6	1.22, d (7.4)

a) Recorded at 100 MHz. b) Recorded at 400 MHz.

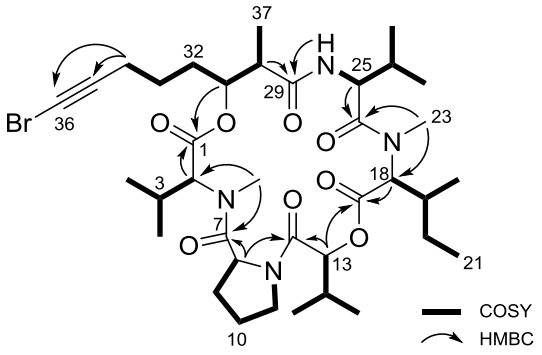
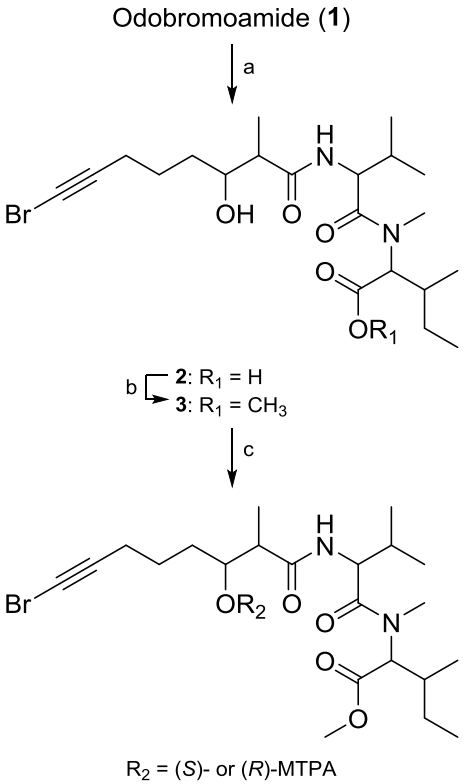


Figure 2. Key 2D NMR correlations for **1**.

C-29 carbonyl carbon of Br-Hmoya, whereas the oxymethine proton (H-31) of Br-Hmoya correlated with the C-1 carbonyl carbon of *N*-Me-Val. Thus, the gross structure of **1** was determined as shown in Figure 2.

To determine the absolute configurations of its amino acid components, we hydrolyzed odobromoamide (**1**) under acidic conditions and separated the resulting mixture by HPLC to yield the different amino acid moieties and Hmba. The stereochemistries of the Pro, Val, *N*-Me-Val and *N*-Me-Ile units in **1** were determined to be L, L, L and L, respectively, following their derivatization with Marfey's reagant[10] and subsequent analysis by HPLC. The absolute configuration of Hmba was determined by chiral HLPC analysis following its derivatization with phenacyl bromide.[11] A comparison of the retention time of this phenacyl ester with that of an authentic standards[12] established an L configuration at the stereocenter of Hmba.

The acid hydrolysis of **1** failed to access to Br-Hmoya, and compound **1** was therefore hydrogenated with Pd/C prior to being hydrolyzed to remove the bromine atom and reduce the terminal alkyne functionality to the corresponding alkane. The hydrogenation product was then hydrolyzed under acidic conditions, and the resulting mixture was separated by silica gel column chromatography to give 3-hydroxy-2-methyloctanoic acid (Hmoaa). The chemical shifts and coupling constants of the protons of the Hmoaa derived from natural **1** were identical to those of *syn*-3-hydroxy-2-methyloctanoic acid,[13] proving that Hmoaa adopted a *syn* configuration. To determine the absolute configurations of the Hmoaa moiety, **1** was subjected to methanolysis, resulting in the isolation of fragment **2** (Scheme 1). This product was treated with trimethylsilyldiazomethane to give the corresponding methyl ester **3**, which was reacted with Mosher's reagent. The Δδ values of the MTPA esters revealed that C-3 existed as the *R* configuration,[14] which demonstrated that the absolute configuration of the Hmoaa moiety in **1** was



Scheme 1. Preparation of MTPA esters. *Reagents and conditions*: (a) KOH, MeOH-H_2O, rt; (b) trimethylsilyldiazomethane, MeOH-benzene, rt; (c) (*R*)- or (*S*)- MTPACl, DMAP, pyridine, rt.

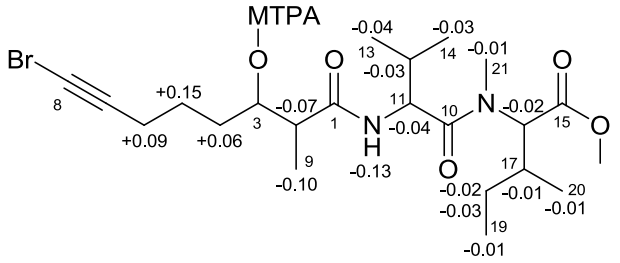


Figure 3. Δδ values (δ_S-δ_R) in ppm for the MTPA esters of **1**.

2*S*, 3*R* (Figure 3). The absolute stereostructure of odobromoamide (**1**) was therefore deduced to be 2*S*, 8*S*, 13*S*, 18*S*, 19*S*, 25*S*, 30*S* and 31*R*, as shown in Figure 1.

The biological activity of odobromoamide (**1**) was evaluated using assays for brine shrimp toxicity and in vitro cytotoxicity against HeLa S3 cells. Compound **1** showed moderate toxicity against brine shrimp with an LD_{50} value of 13.0 μM and exhibited potent cytotoxic activity against HeLa S3 cells with an IC_{50} value of 0.31 μM. In addition, a trypan blue dye exclusion assay revealed that **1** induced cell death in HeLa cells, and that this activity was suppressed in the presence of Z-VAD-FMK, an irreversible and cell-permeable inhibitor of caspases (Figure 4). This result indicated that the cytotoxicity associated with **1** was dependent on the caspase family of proteins. Odobromoamide (**1**) was also evaluated against a panel of 39 human cancer cell lines (HCC panel) at the Japanese Foundation for Cancer Research (see Supporting Information). The average 50% growth inhibition (GI_{50}) value across all of the cells tested was 28 nM.

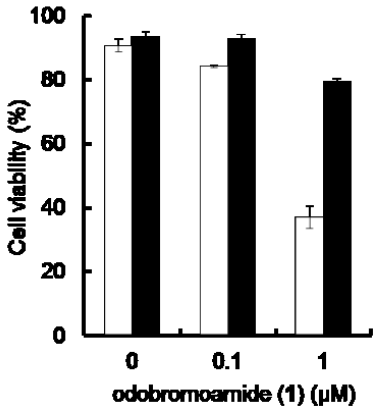


Figure 4. Result of the trypan blue dye exclusion assay of **1**. HeLa S3 cells were preincubated (solid column) or not (open column) with 50 μM of Z-VAD-FMK and then treated with the indicated concentrations of **1**. After further incubation for 48 h, cell viability was determined. Values are the mean ± SD of quadruplicate determinations.

Conclusion

In conclusion, cyclodepsipeptide odobromoamide (**1**), which is a novel analog of veraguamides, was isolated from the Okinawan marine cyanobacterium *Okeania* sp. The structure of **1** was established by stereoscopic analysis, HPLC analysis and derivatization reaction. Structurally, **1** consists of a unique C8 alkynoate unit containing a terminal alkynyl bromide, which is rarely observed in nature. Odobromoamide (**1**) displayed cytotoxic activity against HeLa S3 cells.

Experimental

General Experimental Pricedures. Optical rotation was measured on a JASCO P-1010 polarimeter. UV and IR spectra were measured on a JASCO V-660 UV visible spectrophotometer and a JASCO FT/IR-6100 spectrometer, respectively. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker AVANCE 400 MHz NMR spectrometer. The chemical shifts were reported in relative to the residual solvent signals (δ_H 7.26, δ_C 77.0) in $CDCl_3$. ESIMS data were obtained using a Waters Quattro micro API mass spectrometer, HRESIMS was performed on a Waters Micromass Q-TOF spectrometer. HPLC isolation of odobromoamide (**1**) was conducted on a JASCO PU-2080 Plus Intelligent HPLC pump and a JASCO UV-2075 Plus Intelligent UV/VIS detector. Cell viability in 96-well plates was measured using a BioTek ELx800 absorbance microplate reader.

Marine Cyanobacterial Samples. Samples of the marine cyanobacterium, *Okeania* sp. were collected by hand from the coast of Odo, Okinawa Prefecture, Japan in May 2009. The cyanobacterium was identified by 16S rRNA sequence analysis (see Supporting Information).

Extraction and Isolation. Approximately 1.2 kg (wet weight) of the cyanobacterial samples were extracted with MeOH (2.0 L). The extract was concentrated, and the residue was partitioned between H_2O/EtOAc (1:1). The material obtained from the organic layer was further partitioned between MeOH/H_2O (90:10) and *n*-hexane. The aqueous MeOH fraction (3.0 g) was separated by column chromatography on ODS (30.0 g) using 40% aqueous MeOH, 60% aqueous MeOH, 80% aqueous MeOH and MeOH. The fraction (976.4 mg) eluted with 80% aqueous MeOH was subjected to reversed-phase HPLC [Develosil ODS-HG-5 (20×250 mm), 85% MeOH at 5.0 mL/min, UV detection at 215 nm] to yield odobromoamide (**1**, 437.4 mg, t_R=40.0 min). The purity of **1** was determined as >95% by HPLC analysis.

Odobromoamide (**1**): colorless oil; $[\alpha]_D^{26}$ −38.6 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 202 nm (4.26); IR (neat) 3333, 2962, 1646, 1540, 1204, 1096 cm^{-1}; For ^{1}H NMR (400.13 MHz, $CDCl_3$) and ^{13}C NMR (100.61 MHz, $CDCl_3$) data, see Table 1; HRESIMS *m/z* $[M+H]^+$ 767.3577 (calcd for $C_{37}H_{60}BrN_4O_8$, 767.3595).

Absolute Configurations of Amino Acid Residues in 1. Odobromoamide (**1**, 1.2 mg) was treated with 5M HCl (0.5 mL) at 105 °C for 12 h. The hydrolysate was concentrated to dryness and partitioned between H_2O and EtOAc. The aqueous layer was subjected to HPLC [Cosmosil HILIC (4.6×250 mm), MeCN/10 mM $AcONH_4$=85:15 at 1.0 mL/min, UV detection at 215 nm] to yield *N*-Me-Ile, *N*-Me-Val, Hmba, Val and Pro. Each amino acid expect for Hmba was added with 0.1% solution of Nα-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA, Marfey's reagent, 200 μL) in acetone and 0.5M $NaHCO_3$ (100 μL) followed by heating at 40 °C for 90 min. After cooling to room temperature, the reaction mixture was neutralized with 2M HCl (25 μL) and diluted with MeOH (300 μL). The solution was subjected to reversed-phase HPLC [Cosmosil 5C_{18}-AR-II (4.6×250 mm), MeOH/20 mM AcONa=60:40 (solvent A) or 50:50 (solvent B) at 1.0 mL/min, UV detection at 340 nm]. The L-FDAA derivatives of standard amino acids were prepared by the same procedure. The retention times (min) of the authentic standards were as follows: L-*N*-Me-Ile (6.6), L-allo-*N*-Me-Ile (6.9), D-*N*-Me-Ile (11.6), D-allo-*N*-Me-Ile (12.2), L-*N*-Me-Val (5.3) and D-*N*-Me-Val (8.5) in solvent A, L-Val (6.8), D-Val (18.3), L-Pro (4.9) and

D-Pro (6.5) in solvent B. The retention times and ESIMS product ions (*m/z* $[M+Na]^+$) of the L-FDAA derivatives of *N*-Me-Ile and *N*-Me-Val from the hydrolyzate were 6.6 min (420.1) and 5.3 min (406.1) in solvent A, respectively, proving the configurations of *N*-Me-Ile and *N*-Me-Val were L. The retention times and ESIMS product ions (*m/z* $[M+Na]^+$) of the L-FDAA derivatives of Val and Pro from the hydrolyzate were 6.8 min (392.1) and 4.9 min (390.1) in solvent B, respectively, proving the configurations of Val and Pro were L.

Absolute Configuration of Hmba Unit of 1. The absolute configuration of the Hmba unit in **1** was determined by derivatization with HPLC labeling reagent and chiral HPLC analyses of the derivatives. Hmba from the acid hydrolyzate of **1** was treated with 80 μL of a phenacyl bromide solution (12 mg/mL in acetone) and 80 μL of a triethylamine solution (10 mg/mL in acetone), and the mixture was stirred at 50 °C for 6 h. The reaction mixture was subjected to chiral HPLC [DAICEL CHIRALPAK IC (4.6×250 mm), *n*-hexane/EtOH=90:10 at 1.0 mL/min, UV detection at 254 nm]. The phenacyl esters of authentic standards were prepared by the same procedure. The retention times (min) of the authentic standards were L-Hmba (12.2 min) and D-Hmba (21.2 min). The retention time and ESIMS product ion (*m/z* $[M+Na]^+$) of Hmba in **1** was 12.2 min (259.1), proving the configuration of Hmba was L.

Isolation of Hmoaa Moiety. A solution of **1** (5.4 mg) in MeOH (1.0 mL) was treated with 10% Pd/C catalyst and stirred under an atmosphere of hydrogen at room temperature for 24 h. The reaction mixture was filtered, concentrated *in vacuo* and subjected to HPLC [COSMOSIL $5C_{18}$-AR-II (10×250 mm), 85% MeOH at 4.0 mL/min, UV detection at 215 nm] to yield the hydrogenation product of **1**. The product was treated with 3M HCl (0.5 mL) at 105 °C for 24 h. The hydrolysate was concentrated to dryness and partitioned between H_2O and EtOAc. The EtOAc layer was separated by column chromatography on silica gel using *n*-hexane/EtOAc (3:1 to 0:1) to give Hmoaa.

Hmoaa: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.95 (m, 1H), 2.63 (dq, *J* = 7.2, 3.6 Hz, 1H), 1.54–1.29 (m, 8H), 1.22 (d, *J* = 7.2 Hz, 3H), 0.90 (t, *J* = 6.9 Hz, 3H); HRESIMS *m/z* $[M+Na]^+$ 197.1126 (calcd for $C_9H_{18}O_3Na$, 197.1148).

Preparation of MTPA Esters. A solution of **1** (6.9 mg) in 5% methanolic KOH (0.5 mL) was stirred for 24 h at room temperature. The reaction mixture was concentrated, and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was subjected to reversed-phase HPLC [Cosmosil $5C_{18}$-AR-II (10×250 mm), 65% MeOH with 0.1% TFA at 4.0 mL/min, UV detection at 215 nm] to yield compound **2**. The product was treated with trimethylsilyldiazomethane (10 μL) in benzene/MeOH (2:1, 60 μL), and the mixture was stirred for 2 h at room temperature, to give methyl ester **3**. This compound was divided into two equal portions (0.3 mg each), and one portion was reacted with *R*-MTPACl (10 μL) and DMAP (0.5 mg) in pyridine (50 μL), and the mixture was stirred for 20 h at room temperature. The reaction mixture was concentrated, and the residue was partitioned between EtOAc/0.1M $NaHCO_3$ (1:1). The extract obtained from the organic layer was subjected to reversed-phase HPLC [Cosmosil $5C_{18}$-AR-II (10×250 mm), 85% MeOH at 4.0 mL/min, UV detection at 215 nm] to yield *S*-MTPA ester. Using the same procedure as described above, *R*-MTPA ester was obtained from the other portion of **3**.

S-MTPA ester: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.16 (d, *J* = 8.4 Hz, 1H), 5.29 (q, *J* = 6.2 Hz, 1H), 5.01 (d, *J* = 10.5 Hz, 1H), 4.70 (dd, *J* = 8.5, 7.2 Hz, 1H), 3.69 (s, 3H), 3.06 (s, 3H), 2.47 (quin, *J* = 7.0 Hz, 1H), 2.17 (t, *J* = 7.0 Hz, 2H), 2.00 (m, 1H), 1.95 (m, 1H), 1.70 (m, 2H), 1.47 (m, 2H), 1.33 (m, 1H), 1.09 (d, *J* = 6.9 Hz, 3H), 0.99 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.91 (d, *J* = 6.8 Hz, 3H), 0.85 (d, *J* = 6.7 Hz, 3H), 0.84 (t, *J* = 7.4 Hz, 3H); HRESIMS *m/z* $[M+Na]^+$ 727.2180 (calcd for $C_{32}H_{44}BrF_3N_2O_7Na$, 727.2176).

R-MTPA ester: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.29 (d, *J* = 8.7 Hz, 1H), 5.28 (q, *J* = 6.0 Hz, 1H), 5.03 (d, *J* = 10.4 Hz, 1H), 4.74 (dd, *J* = 8.6, 7.0 Hz, 1H), 3.69 (s, 3H), 3.07 (s, 3H), 2.54 (quin, *J* = 6.6 Hz, 1H), 2.08 (td, *J* = 7.1, 2,6 Hz, 2H), 2.01 (m, 1H), 1.98 (m, 1H), 1.64 (m, 2H), 1.35 (m, 1H), 1.32 (m, 2H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.02 (m, 1H), 0.96 (d, *J* = 6.7 Hz, 3H), 0.94 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 3H); HRESIMS *m/z* $[M+Na]^+$ 727.2170 (calcd for $C_{32}H_{44}BrF_3N_2O_7Na$, 727.2176).

Brine Shrimp Toxicity Assay. The brine shrimp (*Altemia salina*) toxicity assay was performed using a slight modification of the original method. The samples were dissolved in MeOH and transferred to 0.7 cm squares of filter paper. The squares were dried and added to test vials of artificial seawater (2.0 mL). Approximately 10 hatched brine shrimp were transferred to the vials. After 24 h at room temperature, the brine shrimp were counted and the percentage of live verses total shrimp was calculated to determine the LD_{50} values.

MTT Assay. The cytotoxicity against human cell lines HeLa S3 was measured by means of the MTT assay. HeLa S3 cells were seeded at 4×10^3 cells/well in 96-well plates and cultured overnight at 37 °C with 5% CO_2. Various concentrations of compound were added and the culture plates were kept for 72 h. MTT solution (20 μL, 5 mg/mL in DMSO) was added to each well and the plate was further incubated for 4 h. After the incubation, all remaining supernatant were removed and DMSO (150 mL) was added to each well to dissolve the resultant formazan crystal. Absorbance was measured by using a microplate reader at a wavelength of 540 nm, using 630 nm as the reference wavelength. The IC_{50} values were calculated by curve fitting method.

Trypan Blue Dye Exclusion Assay. HeLa S3 cells were seeded at 8×10^4 cells/well in 24-well plates, cultured overnight and then preincubated with or without 50 μM Z-VAD-FMK for 30 min. The cells were then treated with various concentrations of compound for 48 h. They were then stained with trypan blue, and the number of stained cells was counted.

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Supporting Information

1D and 2D NMR spectra and HCC panel data for odobromoamide, and gene screening. This material is available electronically on J-STAGE.

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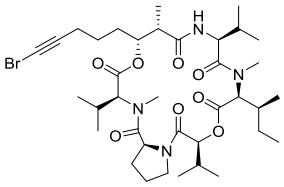
Graphical Abstract

<Title>
Odobromoamide, a Terminal Alkynyl Bromide-Containing Cyclodepsipeptide from the Marine Cyanobacterium *Okeania* sp.
<Authors' names>
Kosuke Sueyoshi, Takafumi Kudo, Aki Yamano, Shimpei Sumimoto, Arihiro Iwasaki, Kiyotake Suenaga, Toshiaki Teruya
<Summary>
Odobromoamide, a novel depsipeptide containing an alkynyl bromide, was isolated from the Okinawan marine cyanobacterium *Okeania* sp. The structure was established by stereoscopic analysis, HPLC analysis and synthetic methods. Odobromoamide displayed cytotoxic activity against HeLa S3 cells and broad-spectrum cytotoxicity against human cancer cell line panel.
<Diagram>



Odobromoamide