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Calyxamides A and B, Cytotoxic Cyclic Peptides from the Marine Sponge *Discodermia calyx*

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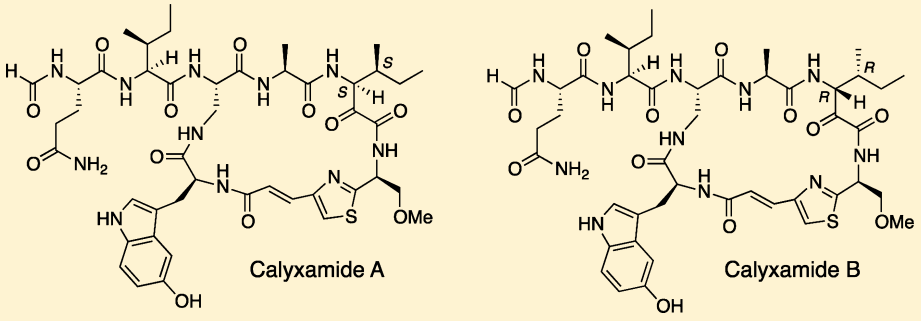
Supporting Information



ABSTRACT: Cyclic peptides containing 5-hydroxytryptophan and thiazole moieties were isolated from the marine sponge *Discodermia calyx* collected near Shikine-jima Island, Japan. The structures of calyxamides A (**1**) and B (**2**), including the absolute configurations of all amino acids, were elucidated by spectroscopic analyses and degradation experiments. The structures are similar to keramamides F and G, previously isolated from *Theonella* sp. The analysis of the 16S rDNA sequences obtained from the metagenomic DNA of *D. calyx* revealed the presence of Candidatus *Entotheonella* sp., an unculturable *δ*-proteobacterium inhabiting the *Theonella* genus and implicated in the biosynthesis of bioactive peptides.

The lithistid sponges are exceptionally rich sources of structurally unique and biologically active natural products.[1] In particular, the members of the family Theonellidae, including the *Theonella* and *Discodermia* genera, contain potent cytotoxic macrolides, such as the swinholides[2] from the *Theonella* genus and discodermolide[3] from *Discodermia dissoluta*. Strongly bioactive peptides have also been isolated, such as cyclotheonamide[4] and keramamides[5] from the *Theonella* genus and discodermins[6] from the *Discodermia* genus. *Discodermia calyx* collected around Japan contains high concentrations of calyculins,[7] which are the PKS and NRPS hybrid products that exhibit potent and specific inhibition against protein phosphatases 1 and 2A. Recently, Jung and coworkers reported C-21 furanoterpenes, bisindole alkaloids, and bromohistidine derivatives isolated from a Korean specimen of *D. calyx*.[8] However, there has been no report regarding the isolation of polypeptides from *D. calyx*, despite the presence of bioactive peptides, such as discodermin[6] and discobahamins,[9] in sponges of the same genus. The presence of the abundant and potent calyculins (calyculin A; 0.15% in wet weight)[7] could mask the presence of other minor cytotoxic compounds. Therefore, a further detailed investigation of *D. calyx*, collected off of Shikine-jima Island, Japan, led to the isolation of the cytotoxic cyclic peptides calyxamides A and B, which are structurally related to the keramamides[5] isolated from *Theonella* sp. Herein, we report the isolation and structural elucidation of calyxamides A and B, as well as the analysis of symbiotic bacteria in *D. calyx*.

The sponge *D. calyx* was collected in the ocean near Shikine-jima Island, Japan. The concentrated MeOH extract of the frozen sponge (2.5 kg) was successively partitioned between hexane and H_2O, and then the latter layer was repartitioned between EtOAc and H_2O. The EtOAc extract was subjected to chromatography on a silica gel column, followed by reversed-phase HPLC on ODS to yield calyxamides A (**1**, 3.0×10^{-4} % wet weight) and B (**2**, 1.0×10^{-4} % wet weight) as yellow solids.

The molecular formula of calyxamide A (**1**) was established as $C_{45}H_{61}N_{11}O_{12}S$ by positive ion ESI-TOFMS. The ^{1}H NMR spectrum (DMSO-d_6) of **1** exhibited *α*- and amide protons, thereby implying its peptide nature. In addition, a minor set of resonance signals existed along with the major one in the ^{1}H NMR spectrum in CD_3OD. An extensive analysis of the 1D and 2D NMR data of **1** (Table 1), including ^{1}H−^{1}H COSY, HMQC, and HMBC spectra in DMSO-d_6, suggested the presence of a *trans*-olefin and alanine (Ala), isoleucine (Ile), and 2,3-diaminopropionic acid (Dpr) residues. Furthermore, there was a modified Ile with a downfield proton (δ_H = 5.17) that showed an HMBC correlation with the carbonyl carbon (δ_C = 198.2), which was consistent with an AKMH

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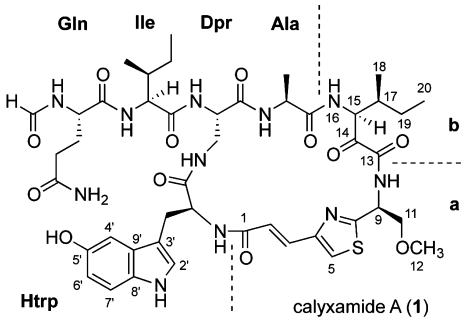

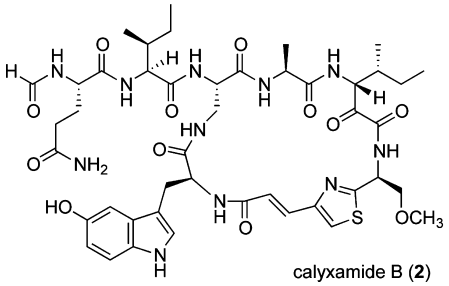

(3-amino-2-keto-4-methylhexanoic acid) residue. In addition, the presence of a thiazole ring conjugated to the *trans*-olefin was indicated by the HMBC spectroscopic data of **1** [δ_H = 7.81 (s, 1H); δ_C = 168.0, 132.4, and 149.9]. The amino acid composition, the α-ketoamide functionality, and the thiazole ring system were reminiscent of those of keramamide F,[5b] a cytotoxic cyclic heptapeptide from the marine sponge *Theonella* sp. A comparison of the NMR data with those reported in the literature readily revealed that **1** had the same partial structure (Ile-Dpr-Ala-**a**-**b**) of the keramamide F skeleton, which contains modified amino acid residues. However, the remaining two portions, corresponding to the isoserine and tryptophan residues of keramamide F, were different. Three aromatic protons (δ_H = 6.53, 6.83, and 7.03) and an exchangeable proton (δ_H = 10.41) coupled to another aromatic proton (δ_H = 6.80) were consistent with a 5-hydroxy-3-substituted indole, which was supported by the UV absorption maximum at 278 nm. The HMBC correlations of the methylene protons (δ_H = 3.02 and 3.21), which were no longer olefinic, indicated the presence of 5-hydroxytryptophan (Htrp),[10] in place of the α,β-dehydrotryptophan in keramamide F. The last amino acid residue exhibiting characteristic ^{1}H NMR signals [δ_H = 1.67 (m, 1H), 1.83 (m, 1H), 2.05 (m, 2H), 6.74 (brs, 1H), and 7.26 (brs, 1H)] was identified as glutamine (Gln), which was connected to Ile. The HMBC correlation between a formyl proton and the α-methine carbon (δ_C 51.2) of Gln indicated the presence of a formyl group (δ_H 7.98; δ_C 161.4) attached to the N-terminus. Evidence for the amino acid sequence of **1** was provided by the NOESY and HMBC correlations and established the cyclic portion of this peptide **1**. On the basis of these NMR data, the gross structure of calyxamide A (**1**) was established. There was an unassignable ^{13}C signal at 99.7 ppm in CD_3OD, which, together with the presence of a minor ion peak at m/z 1034 $[M + CH_3OH + Na]^+$ in the ESI-TOFMS spectrum, suggested hemiacetal formation with MeOH at the α-ketoamide portion in **1**, as the minor component.[4]

A chiral-phase GC analysis of the *N*-trifluoroacetyl/methyl ester derivatives of the hydrolysate of **1** clarified that all of the Ala, Gln, Ile, and Dpr residues in **1** were the L-form. Treatment of **1** with ozone led to the degradation of the (*O*-methylseryl)thiazole moiety and the Htrp moiety, to yield *O*-methylserine and aspartic acid, respectively. Treatment of **1** with H_2O_2/NaOH transformed the AKMH into isoleucine. The *O*-methylserine, aspartic acid, and isoleucine residues thus obtained were also determined to be the L-forms by the chiral-phase GC method, indicating the L-configuration of all amino acids in **1**. Therefore, the complete structure of calyxamide A was concluded to be **1**.

The molecular formula of calyxamide B (**2**) was established as $C_{45}H_{61}N_{11}O_{12}S$, by the positive ion ESI-TOFMS, which was the same as that of calyxamide A (**1**). Extensive analysis of the NMR data of **2** (Table 1), by comparison with those of **1**, confirmed that the planar structure of **2** was the same as that of **1**. The differences between **1** and **2** were found for the ^{1}H and ^{13}C chemical shifts at the β-NH of Dpr (**1**, δ_H = 7.17; **2**, δ_H = 7.69), the α-proton of Ala (**1**, δ_H = 4.36; **2**, δ_H = 4.62), H-13 (**1**, δ_H = 5.17; **2**, δ_H = 5.42), H-9 (**1**, δ_H = 5.30; **2**, δ_H = 4.73), C-13 (**1**, δ_C = 61.6; **2**, δ_C = 57.5), C-16 (**1**, δ_C = 16.5; **2**, δ_C = 14.9), and C-17 (**1**, δ_C = 23.7; **2**, δ_C = 27.3). These differences between **1** and **2** were similar to those between keramamides F and G. Thus, these data implied that **2** is the diastereomer of **1**, which was confirmed by the stereochemical assignment. The chiral-phase GC analysis, after the oxidation of **2** with H_2O_2/NaOH followed by acid hydrolysis and derivatization, furnished D-Ile, as expected. In addition, all of the other amino acid residues in **2** were the L-form, as in the case of **1**. Therefore, the complete structure of calyxamide B was concluded to be **2**.

Calyxamides A (**1**) and B (**2**) showed moderate cytotoxicity against P388 murine leukemia cells, with IC_{50} values of 3.9 and 0.9 μM, respectively. The overall structures are closely related to those of keramamides F, G, H, J, and K, isolated from the Okinawan marine sponge *Theonella* sp.,[5] but the calyxamides are the first cytotoxic cyclic peptides isolated from the Japanese marine sponge, *D. calyx*.

The α-ketoamide functionality was originally found in the cyclotheonamides[4] isolated from the Japanese sponge *Theonella* sp., collected near Hachijo-jima Island by Fusetani and coworkers in 1990. Subsequently, the keramamides[5] were isolated from an Okinawan *Theonella* sp. Although these α-ketoamide-containing cyclic peptides appeared to be unique secondary metabolites for the genus *Theonella*, similar peptides, the discobahamins from a *Discodermia* sp., collected from the deep sea off the Bahamas have been reported.[9] The structural resemblance between the secondary metabolites from *Theonella* and *Discodermia* sponges is not limited to the cyclic peptides, but also includes some closely related polyketides, such as the calyculin-related metabolite swinhoeiamide A[11] and theopederins.[12,13] These findings suggest the existence of various chemotypes in the Theonellidae family of sponges and the possibility of secondary metabolite production by symbiotic bacteria.[14,15] A correlation has been demonstrated between the presence of filamentous bacteria and the isolation of cyclic peptides.[18] In particular, a filamentous bacterium associated with *Theonella swinhoei* has been shown to be the Candidatus *Entotheonella palauensis*, producing the cyclic peptide theopalauamide.[16,17] On the other hand, only two groups have independently reported the presence of *Entotheonella* sp. in the genus *Discodermia*.[18,19] The presence of the calyxamides in *D. calyx*, which are closely related to peptides from *Theonella*, raised the possibility that *D. calyx* also harbors *Entotheonella* as symbiotic bacteria, although the bacterial community associated with *D. calyx* has remained unexplored. To obtain general insights into the bacterial community in *D. calyx*, a microscopic analysis of the symbiosis was conducted. Consequently we

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Table 1. ^{1}H and ^{13}C NMR Chemical Shifts of Calyxamides A (1) and B (2) in DMSO-d_6

position		calyxamide A (1)		calyxamide B (2)	
		δ_C[a]	δ_H[b] (J in Hz)	δ_C[a]	δ_H[b] (J in Hz)
CHO		161.4	7.98, s	161.5	7.99, s
Gln	CO	172.0		171.4	
	α-NH		8.24, d (8.5)		8.22, d (7.9)
	α	51.2	4.33, m[c]	51.2	4.36, m[c]
	β	28.7	1.67, m[c]	28.8	1.68, m[c]
			1.83, m		1.85, m
	γ	31.9	2.05, m	31.9	2.06, m
	δ	174.2		174.3	
	δ-NH_2		6.74, brs		6.71, brs
			7.26, brs		7.27, brs
Ile	CO	171.3		171.2	
	NH		7.92, d (8.5)		7.92, d (9.1)
	α	57.3	4.16, t (8.2)	57.2	4.18, t (7.9)
	β	37.2	1.67, m[c]	37.3	1.69, m[c]
	γ-CH_3	15.9	0.79, m[c]	15.9	0.80, d (6.2)
	γ-CH_2	24.8	1.03, m	24.8	1.05, m
			1.37, m		1.39, m
	δ-CH_3	11.5	0.78, m[c]	11.6	0.78, t (7.4)
Dpr	CO	170.9		169.9	
	α-NH		8.14, d (6.8)		7.95, m[c]
	α	52.0	4.30, m[c]	51.9	4.37, m[c]
	β	41.0	2.71, m	40.7	2.78, m
			3.78, m		3.57, m
	β-NH		7.17, dd (6.2, 9.1)		7.69, t (6.2)
Ala	CO	175.4		174.7	
	NH		8.34, d (4.0)		8.07, d (6.8)
	α	49.8	4.36, m[c]	49.1	4.62, t (7.1)
	β	17.7	1.24, d (6.8)	20.3	1.30, d (7.4)
a	1	165.3		165.0	
	2	124.4	6.83, d (15.3)	125.1	6.60, d (15.3)
	3	132.4	7.27, d (15.3)	131.7	7.15, d (15.3)
	4	149.9		149.8	
	5	123.8	7.81, s	123.0	7.73, s
	7	168.0		166.2	
	9	51.9	5.30, dt (6.2, 8.5)	54.3	4.73, q (6.8)
	10		9.21, d (8.5)		9.24, d (6.8)
	11	73.4	3.58, dd (6.8, 9.6)	73.1	3.89, dd (5.7, 9.1)
			3.66, dd (5.7, 9.6)		3.95, t (8.5)
	12	59.0	3.26, s	58.9	3.35, s
b	13	164.6		161.5	
	14	198.2		197.7	
	15	61.6	5.17, dd (2.8, 8.5)	57.5	5.42, dd (2.8, 9.6)
	16		8.58, d (8.5)		8.57, d (9.6)
	17	37.4	2.21, m	37.5	2.49, m[c]
	18	16.5	0.84, d (7.4)	14.9	0.74, d (7.4)
	19	23.7	1.13, qui (7.4)	27.3	1.20, m
	20	12.3	0.73, t (7.4)	12.2	0.83, t (7.4)
Htrp	CO	171.2		172.2	
	α-NH		7.65, d (9.6)		7.97, m[c]
	α	53.6	4.78, dt (3.8, 10.5)	53.8	4.56, dt (3.4, 11.9)
	β	30.0	3.02, dd (11.6, 15.0)	29.5	2.96, dd (11.9, 14.7)
			3.21, m[c]		3.30, m[c]
	1′-NH		10.41, d (1.7)		10.38, d (1.7)
	2′	123.2	6.80, d (1.7)	123.7	6.78, d (1.7)
	3′	110.0		110.7	
	4′	102.7	6.83, d (2.3)	102.5	6.82, d (2.3)
	5′	150.8		150.8	
	5′-OH		8.59, brs		8.54, s
	6′	111.9	6.53, dd (2.3, 8.5)	111.7	6.53, dd (2.3, 8.5)
	7′	112.2	7.03, d (8.5)	112.2	7.04, d (8.5)
	8′	131.1		131.3	
	9′	128.7		128.3	

[a]In 500 MHz. [b]In 125 MHz. [c]Overlapped.

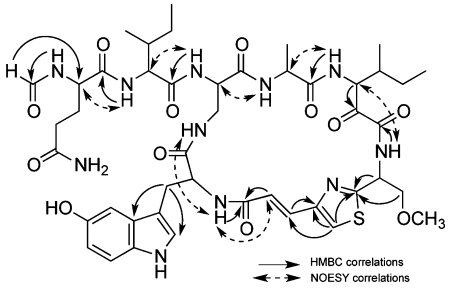

Figure 1. Key HMBC and NOESY correlations of calyxamide A in DMSO-d_6.

identified the presence of filamentous bacteria by light microscopy using separated bacterial cell fractions, as described previously.[16−18] The analysis of the 16S rDNA sequences obtained by PCR using the metagenomic DNA of *D. calyx* as the template revealed the presence of a sequence showing high similarity to those of δ-proteobacteria. A BLAST search showed that the sequence was most similar (97% identity of 1479 alignable bp's) to that of the uncultured Candidatus *Entotheonella* sp. from *Discodermia dissoluta* (GenBank accession no. AY897123), and the Candidatus *Entotheonella palauensis* from *Theonella swinhoei* was also ranked highly (GenBank accession no. AF130847, 96% identity). The common existence of Candidatus *Entotheonella* sp. in both *Theonella* and *Discodermia* may explain the similarity of the secondary metabolites in these two genera.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECX-500 spectrometer in DMSO-d_6, CD_3OD, and CD_3OH. ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million and referenced to solvent peaks: δ_H = 2.46 and δ_C = 40.0 ppm for DMSO-d_6, δ_H = 3.29 and δ_C = 47.8 for both CD_3OD and CD_3OH. HRMS data were obtained from a Bruker Daltonics micro TOF-MS.

Animal Material. The marine sponge *Discodermia calyx* was collected by hand using scuba at a depth of 10 m in the ocean near Shikine-jima Island, 150 km south of Tokyo, and was kept frozen until use. The voucher specimen (S11-001) was deposited at the Laboratory of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, the University of Tokyo. The fresh specimen was minced

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with a knife and suspended in calcium- and magnesium-free artificial seawater, and the suspension was observed by light microscopy.

Isolation. The MeOH (2.0 L × 1, 0.5 L × 3) extract of the sponge *D. calyx* (0.9 kg, wet weight) was partitioned between hexane (350 mL × 2) and H_2O (350 mL). The aqueous layer was further partitioned between EtOAc (350 mL × 2) and H_2O (350 mL). The EtOAc-soluble material (0.72 g) was subjected to open chromatography on a silica gel column, eluting with a stepwise gradient of EtOAc (0−90%) in hexane, and MeOH (15−20%) in $CHCl_3$. The fractions eluted with $CHCl_3$/MeOH were then separated by reversed-phase HPLC on ODS [Cosmosil MS-II column ⌀ 10 × 250 mm; flow rate 4.0 mL/min; 30−100% CH_3CN/H_2O over 30 min; UV detection at 280 nm] to give fraction I and calyxamide B (**2**, 0.9 mg). Fraction I was further purified by reversed-phase HPLC on ODS [Cosmosil MS-II column ⌀ 10 × 250 mm; flow rate 4.0 mL/min; 33% CH_3CN containing 0.05% TFA; UV detection at 280 nm] and cholesterol [Cosmosil Cholester, ⌀ 10 × 250 mm; flow rate 4.0 mL/min; 33% CH_3CN containing 0.05% TFA; UV detection at 280 nm], to yield calyxamide A (**1**, 2.7 mg, t_R = 10.0 min, 13.0 min). Moreover, additional sponge (1.6 kg) was extracted and separated in the same manner as above to afford **1** (4.8 mg) and **2** (1.6 mg).

Calyxamide A (1): yellow solid; $[\alpha]^{27}_D$ −15.4 (*c* 0.62, MeOH); UV_{max} (MeOH) 278 nm; ^1H and ^{13}C NMR (Table 1); ESI-TOFMS *m/z* 1002.4144 $[M + Na]^+$ (calcd for $C_{45}H_{61}N_{11}NaO_{12}S$, 1002.4114).

Calyxamide B (2): yellow solid; $[\alpha]^{32}_D$ −25.3 (*c* 0.28, MeOH); UV_{max} (MeOH) 278 nm; ^1H and ^{13}C NMR (Table 1); ESI-TOFMS *m/z* 1002.4154 $[M + Na]^+$ (calcd for $C_{45}H_{61}N_{11}NaO_{12}S$, 1002.4114).

Amino Acid Analysis by Chiral-Phase GC. Calyxamide A or B (200 μg each) was hydrolyzed with 6 M HCl (500 μL) at 110 °C for 24 h. The reaction mixture was treated with 5−10% HCl/MeOH (500 μL) at 100 °C for 30 min and was then treated with trifluoroacetic anhydride (TFAA)/CH_2Cl_2 (1:1, 500 μL) at 100 °C for 5 min. The chiral-phase GC analysis of the *N*-trifluoroacetyl (TFA)/methyl ester derivatives was performed using a CP-Chirasil-D-Val column (Alltech, 0.25 mm × 25 m; N_2 as the carrier gas; program rate 50−200 at 4 °C/min) and showed peaks at t_R = 4.3, 8.1, 17.6, and 26.6 min. Standard amino acids were also converted to the TFA/Me derivatives by the same procedure. Retention times (min) were as follows: L-Ala (4.3), D-Ala (5.0), L-Ile (8.1), D-Ile (8.8), L-*allo*-Ile (7.6), D-*allo*-Ile (8.4), L-Gln (17.6), D-Gln (18.4), L-Dpr (26.6), D-Dpr (27.1). Thus, the presence of L-Ala, L-Ile, L-Gln, and L-Dpr was confirmed.

Determination of the Configurations of (*O*-Methylseryl)-thiazole and 5-Hydroxytryptophan. A stream of ozone in oxygen was bubbled through a cooled solution of calyxamide A or B (200 μg each) in MeOH (3 mL) at −78 °C for about 15 min. The reaction was quenched with 30% H_2O_2 (15 drops) and allowed to warm to room temperature (rt). After 1 h, the solvent was removed under nitrogen. The reaction mixture was subjected to hydrolysis and TFA/Me derivatization. The chiral-phase GC analysis of the resulting hydrolysate was performed as above and showed peaks at t_R = 4.3, 6.9, 8.1, 12.8, 17.6, and 26.6 min, which established the presence of L-*O*-methylserine and L-aspartic acid. Retention times (min) were as follows: L-*O*-methylserine (6.8), D-*O*-methylserine (7.3), L-Asp (12.8), D-Asp (13.2).

Determination of the Configuration of the C11−C14 Moiety. Calyxamide A or B (200 μg each) in 5% NaOH (500 μL) was treated with 30% H_2O_2 (100 μL) at 65 °C for 40 min. After cooling to rt overnight, the reaction mixture was subjected to hydrolysis, followed by TFA/Me derivatization. The resulting hydrolysate was subjected to the chiral-phase GC analysis as above. The hydrolysate derived from **1** showed peaks at t_R = 4.3, 8.1, 17.6, and 26.6 min. Only the L-form of Ile (t_R = 8.1 min) was observed, and the peak area for Ile was increased, as compared to that derived from the normal hydrolysate from **1**. In contrast, the resulting hydrolysate of **2** showed peaks at t_R = 4.3, 8.1, 8.8, 17.6, and 26.6 min. The D-form of Ile (t_R = 8.8 min) was detected in addition to the L-form of Ile (t_R = 8.1 min).

DNA Isolation. A small piece of frozen sponge material was excised in a manner to include the surface, and the tissue was ground in liquid nitrogen, using a prechilled mortar and pestle. The nitrogen was allowed to boil off, and small aliquots of the powder were dispersed into lysis buffer (8 M urea, 2% sodium dodecyl sulfate, 350 mM NaCl, 50 mM EDTA, 50 mM Tris [pH 7.5]), using 5 mL per g of sponge tissue, for 1 h at 60 °C with gentle mixing. The lysate was extracted with an equal volume of phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and with an equal volume of $CHCl_3$/isoamyl alcohol (24:1). The DNA was then precipitated by 2.5 volumes of EtOH and 1/10 volume of 3 M sodium acetate (pH 5.2) and was washed with 70% EtOH at 4 °C.

PCR Conditions. Amplification of rDNA was performed with the universal eubacterial primers[20] 27f and 1492r, using the metagenomic DNA of *D. calyx*. The PCR cycling conditions were as follows: initial denaturation (95 °C for 5 min), followed by 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 90 s), with a final extension step (72 °C for 10 min). The PCR products were ligated into the pT7Blue vector (Novagen) using a ligation kit (Takara) and were transformed in *Escherichia coli* DH5α. Plasmid DNA was isolated by a Wizard Plus SV Minipreps System (Promega).

Sequencing. DNA sequencing was performed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The M13 universal and reverse primers and the 16S rDNA-specific primer, 514f, were used in the complete sequencing of the 16S rDNA amplicons. The sequence data were analyzed using NCBI BLAST and deposited at DDBJ (accession no. AB683979).

Cytotoxicity Test against P388 Cells. P388 murine leukemia cells were cultured in RPMI 1640 (Wako Chemicals) medium, supplemented with 10 μg/mL of penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (MP Biomedicals), at 37 °C under a 5% CO_2 atmosphere. To each well of 96-well microplates, containing 100 μL of 1 × 10^4 cells/mL tumor cell suspension, was added 100 μL of test solution (samples were dissolved in DMSO), and the plates were incubated for 4 days. After the addition of 50 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide solution (1 mg/mL) to each well, the plates were incubated for 4 h under the same conditions. The mixtures were centrifuged, and the supernatants were removed. The precipitates thus obtained were dissolved in DMSO, and the absorbance at 570 nm was measured with a microplate reader.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectroscopic data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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