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Isolation of Spirastrellolides A and B from a Marine Sponge *Epipolasis* sp. and Their Cytotoxic Activities

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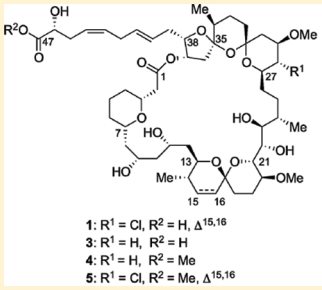
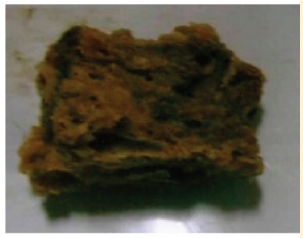
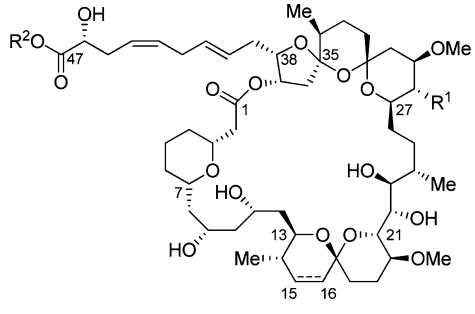
Supporting Information

ABSTRACT: Spirastrellolides A (**1**) and B (**3**) have been isolated as free acids from a marine sponge *Epipolasis* sp. collected in the East China Sea. These compounds had been isolated from the Caribbean marine sponge *Spirastrella coccinea* after conversion to the methyl ester. We examined the cytotoxic activities of **1** and **3** and found that the activities of the free acids are comparable to those of the corresponding methyl esters.




Epipolasis sp.

Spirastrellolides are antimitotic macrolides isolated from the Caribbean marine sponge *Spirastrella coccinea*.[1−4] Their isolation was accomplished only after conversion to methyl esters. Even though potent inhibitory activity of spirastrellolide A (**1**) against protein phosphatase 2A was reported,[3] neither the cytotoxic activities nor spectroscopic data of the free acids for this class of metabolites have been reported. Intensive synthetic efforts have culminated in the total syntheses of the methyl esters of spirastrellolides A (**1**) and F (**2**),[5−8] but not the free acids. In the course of our search for cytotoxic agents from marine sponges, we found activity in a marine sponge *Epipolasis* sp. collected in the East China Sea. From this sponge we have isolated spirastrellolides A (**1**) and B (**3**) as the predominant cytotoxic constituents. The isolation, structural assignment, and cytotoxic activities of **1** and **3** are reported below.



1: R^1 = Cl, R^2 = H, $\Delta^{15,16}$
2: R^1 = Cl, R^2 = H
3: R^1 = H, R^2 = H
4: R^1 = H, R^2 = Me
5: R^1 = Cl, R^2 = Me, $\Delta^{15,16}$

The MeOH extract of the marine sponge *Epipolasis* sp. was subjected to a solvent partitioning scheme, from which the active $CHCl_3$ fraction was subjected to ODS flash chromatography, followed by RP-HPLC purification with C_{18} (Cosmosil AR-II), Phenyl-Hexyl (Luna 5u), and C_{18} (Cosmosil MS-II) stationary phases to afford spirastrellolides A (**1**, 3.9×10^{-8} % yield, wet weight) and B (**3**, 5.4×10^{-8} % yield, wet weight).

We first studied the structure of compound **3**, which was more abundant. The molecular formula of **3** was established as $C_{52}H_{84}O_{17}$ on the basis of HRESIMS data. The ^{1}H NMR spectrum in CD_3OD exhibited three methyl doublets (δ 0.94, 1.00, and 1.04) and two methoxy signals (δ 3.29 and 3.38). Additionally 20 aliphatic methylene carbons, 16 aliphatic methine carbons including 13 oxymethines, and four olefinic carbons were observed in the HSQC spectrum. The olefinic protons were conspicuously broad. Even though the molecular formula of **3** matched that of spirastrellolide B, one aliphatic methylene and one oxymethine carbon were lacking in our assignment. Interpretation of the COSY and TOCSY data in conjunction with the HSQC data revealed the presence of four partial structures (C-2 to C-16, C-18 to C-30, C-31 to C-34, and C-36 to C-43). The HMBC spectrum demonstrated the following correlations: H_2-18, H-21/C-17; H_2-30, H_2-32/C-31; H-33a, 34-CH_3, H-36b, and H-37/C-35. These correlations suggested that the carbon skeleton from C-2 to C-43 of **3** was identical with that of spirastrellolide B. Because we envisioned that the lack of correlation between H-43 and H-44 and the disappearance of H_2-45 and H-46 signals were due to signal

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1 and 3 (600 MHz, CD_3OD)

	spirastrellolide A (1)			spirastrellolide B (3)		
position	δ_C,[a] type	δ_H (J in Hz)	HMBC	δ_C,[a] type	δ_H (J in Hz)	HMBC
1	171.5, C			171.2, C		
2	43.3, CH_2	2.43, m	1, 3	42.1, CH_2	2.36, m	1, 3
		2.59, m	1		2.63, m	1
3	75.0, CH	3.85, m	1	75.2, CH	3.71, m	
4	32.2, CH_2	1.34, m		30.9, CH_2	1.51, m	3
		1.52, m			1.51, m	3
5	24.9, CH_2	1.53, m		24.4, CH_2	1.51, m	7
		1.84, m			1.82, m	
6	33.5, CH_2	1.21, m		32.4, CH_2	1.18, m	
		1.53, m			1.50, m	
7	76.4, CH	3.66, m		76.0, CH	3.57, m	3
8	45.8, CH_2	1.39, m		44.8, CH_2	1.40, m	7
		1.55, m	7		1.54, m	
9	65.9, CH	4.07, t (10.5)		65.4, CH	4.10, m	
10	47.8, CH	1.32, m		46.9, CH_2	1.38, m	11
		1.38, m			1.38, m	11
11	64.8, CH	41.8, m		65.4, CH	4.10, m	
12	42.5, CH_2	1.39, m		41.6, CH_2	1.29, m	
		1.73, m			1.67, m	11, 13
13	71.9, CH	3.68, m		72.7, CH	3.59, m	11, 12, 15
14	35.4, CH	1.97, m	15	35.5, CH	1.23, m	13
15	135.6, CH	5.69, dd (9.7, 1.3)	13, 14, 17	29.4, CH_2	1.49, m	13, 14
					1.49, m	13, 14
16	130.0, CH	5.59, dd (9.7, 2.7)	14, 15, 17	35.3, CH_2	1.49, m	15, 17
					1.66, m	17
17	95.1, C			96.1, C		
18	35.2, CH_2	1.72, m		35.4, CH_2	1.52, m	17
		1.72, m			1.68, m	17, 20
19	24.9, CH_2	1.76, m		24.8, CH_2	1.71, m	20
		2.09, m			2.00, m	
20	75.4, CH	3.39, m		75.2, CH	3.34, m	
21	72.5, CH	4.03, d (9.8)	19, 20	70.9, CH	3.85, d (9.4)	17, 19, 22, 23
22	69.3, CH	3.83, d (10.1)	23	68.8, CH	3.88, d (9.4)	20, 21, 23
23	75.3, CH	3.57, d (10.1)	22, 25, 24-Me	75.4, CH	3.63, m	21, 22, 25, 24-Me
24	34.5, CH	1.85, m		34.0, CH	1.86, m	25
25	26.5, CH_2	1.10, m		26.6, CH_2	1.10, m	
		1.90, m			1.96, m	
26	31.7, CH_2	1.28, m		35.3, CH_2	1.30, m	
		2.16, m			1.57, m	
27	76.4, CH	3.66, m		70.8, CH	3.67, m	25
28	66.3, CH	3.34, m	26, 27, 29	38.8, CH_2	0.96, m	29
					2.03, m	
29	79.8, CH	3.63, m		74.1, CH	3.67, m	
30	44.3, CH_2	1.34, m	28, 29, 31	43.8, CH_2	1.08, m	29, 31, 32
		2.18, m	28, 29, 31		1.96, m	28, 29, 31
31	98.7, C			98.8, C		
32	37.1, CH_2	1.66, m		37.5, CH_2	1.60, m	31, 33, 34
		1.79, m			1.72, m	31
33	24.6, CH_2	1.39, m		24.2, CH_2	1.34, m	31, 35
		1.92, m			1.94, m	31, 34
34	39.4, CH	1.75, m		38.8, CH	1.68, m	
35	110.1, C			109.2, C		
36	47.8, CH_2	2.19, m	34, 37	47.5, CH_2	2.07, m	34, 37, 38
		2.44, m	35		2.33, m	35, 38
37	74.2, CH	5.50, m		73.5, CH	5.33, m	35
38	84.2, CH	4.16, m	37	83.4, CH	4.07, m	
39	31.2, CH_2	2.33, m		30.5, CH	2.33, m	38, 40, 41
		2.79, m			2.67, m	38
40	126.4, CH	5.35, m		126.4, CH	5.33, m	42

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Table 1. continued

	spirastrellolide A (**1**)			spirastrellolide B (**3**)		
position	δ_C,[a] type	δ_H (J in Hz)	HMBC	δ_C,[a] type	δ_H (J in Hz)	HMBC
41	132.7, CH	5.65, m		132.2, CH	5.63, m	
42	31.7, CH_2	2.78, m		31.2, CH_2	2.72, m	
		2.78, m			2.82, m	
43	131.2, CH	5.50, m		130.7, CH	5.45, m	
44	126.5, CH	5.51, m		126.5, CH	5.48, m	
45	ND[b]	ND		ND	ND	
46	ND	ND		ND	ND	
47	ND	ND		ND	ND	
14-Me	17.0, CH_3	0.94, d (6.9)	13, 14, 15	17.6, CH_3	0.81, d (5.5)	13, 14, 15
24-Me	18.5, CH_3	1.31	23, 24, 25	18.4, CH_3	1.04, d (6.8)	23, 24, 25
34-Me	17.3, CH_3	1.16	33, 34, 35	16.6, CH_3	1.00, d (6.2)	33, 34, 35
20-OMe	56.8, CH_3	3.49, s	20	56.4, CH_3	3.38, s	20
29-OMe	55.3, CH_3	3.41, s	29	54.8, CH_3	3.29, s	29

[a]Chemical shifts were assigned from the HSQC and HMBC data. [b]ND means "not detected".

broadening arising from unfavorable equilibrium at ambient temperature, we heated the sample to 40 °C or cooled it to 0 °C, both of which did not extend TOCSY correlations from H-43. Next we anticipated that neutralization of the carboxylic acid would alter the equilibrium and measured the TOCSY spectrum in pyridine-d_5. Unexpectedly the ^{1}H NMR spectrum gave two sets of cross-peaks in the C-12 to C-34 region, indicating the presence of an unidentified equilibrium in the corresponding portion of the molecule. However, no additional cross-peak was observed from H-43. The compound did not decompose under these conditions. From the above-mentioned experiments we considered it highly likely that **3** was spirastrellolide B itself. In order to confirm this idea, we converted **3** to the methyl ester **4** and measured the ^{1}H NMR, COSY, and TOCSY spectra in benzene-d_6. Even though the ^{1}H signals of H-44, H_2-45, and H-46 were clearly observed in the methyl ester **4**, chemical shifts of ^{1}H signals in the region between C-2 and C-12 did not perfectly coincide with those in the literature.[2] It was reported that the ^{1}H NMR spectrum of spirastrellolide F methyl ester exhibited a strikingly dynamic behavior due to the presence of an intricate hydrogen-bonding network, whereas the ^{13}C chemical shifts did not fluctuate.[6] We therefore considered that the ^{1}H NMR chemical shift discrepancies observed in our case arose from the different degree of hydrogen-bond formation, which should be concentration-dependent. We changed the concentration of **4** from 700 μg/mL to 1.3 mg/mL and observed the ^{1}H NMR spectrum. The data at the higher concentration matched better with those in the literature.[2] Additionally, and importantly, the ^{13}C chemical shifts determined by the HSQC spectrum of **4** coincided well with those in the literature, permitting us to conclude that **3** was the free acid of spirastrellolide B (Supporting Information).

The molecular formula of **1**, $C_{52}H_{81}ClO_{17}$, as deduced from the HRESIMS data, was identical with that of spirastrellolide A. The structure of **1** was studied by interpretation of 2D NMR data, revealing the C-2 to C-43 partial structure identical with that of spirastrellolide A. However, no further cross-peaks were observed from H-43, as observed for **3**. However, because of the coincidence of the molecular formula and the partial structure, we considered **1** to be spirastrellolide A itself. Therefore, we converted **1** to the methyl ester **5** and measured the ^{1}H NMR, COSY, and TOCSY spectra at a concentration of 300 μg/mL. Significant ^{1}H NMR chemical shift discrepancies with those in the literature were observed for **5**.[3] Then we measured the NMR spectrum at a concentration of 900 μg/mL and found that the chemical shifts became closer to those reported for spirastrellolide A methyl ester in the literature; ^{13}C chemical shifts of **5** coincided well with those in the literature.[1] Therefore, we concluded that **1** was the free acid of spirastrellolide A.[3]

We evaluated the cytotoxicities of **1** and **3**–**5** against HeLa cells. The acids, **1** and **3**, exhibited IC_{50} values of 20 and 40 nM, respectively, whereas the methyl esters, **4** and **5**, exhibited comparable activities: IC_{50} values of 70 and 30 nM, respectively.

In this study, we successfully isolated spirastrellolides A and B as the free acids from a marine sponge for the first time. Even though the amounts of the isolated spirastrellolides were small, we were able to acquire their spectroscopic data, confirm their identity, and conduct cytotoxicity tests.

Unexpectedly, NMR signals for H-45, H-46, and C-45 to C-47 were not detectable with our samples. Also unexpectedly, the cytotoxicities of spirastrellolides A and B were comparable to those of the corresponding methyl esters.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectra were measured on a Shimadzu Biospec 1600 DNA/protein/enzyme analyzer. NMR spectra were recorded on a JEOL alpha 600 NMR spectrometer at 300 and 273 K. Chemical shifts were referenced to solvent peaks: δ_H 3.31 and δ_C 49.15 for MeOH-d_4; δ_H 8.74 and δ_C 150.35 for pyridine-d_5; δ_H 7.16 and δ_C 128.39 for benzene-d_6. ESI mass spectra were measured on a JEOL JMS-T100LC mass spectrometer. HPLC were carried out on a Shimadzu LC 20 AT with a SCL-10Avp controller and a SPD-Avp detecter.

Animal Material. The sponge *Epipolasis* sp. was collected by dredging off Nagannu Island (26°14.06′ N, 127°33.12′ E), East China Sea, at a depth of 42 m, during a cruise of *R/V Toyoshio-maru*, on May 25, 2007. The sponge was a large mass of 11 kg wet weight. Sponge description: subspherical mass; surface covered by sand except oscular region; color ocher in ethanol; consistency compressible. The precise observation of the ectosomal region could not be conducted because the surface area of the sponge was used for extraction of chemical compounds. Choanosome cavernous. Choanosomal skeleton confused, with a few vague tracts of oxeas. Spicules, oxeas in a large size range and raphidae. Oxeas fusiform, slightly arched at middle part; 565.5 ± 63.5 (450–720) μm in length, 17.9 ± 4.8 (10.0–27.5) μm in width (N = 30). Trichodragmas straight or slightly arched, 84.8 ± 6.1 (75.0–92.5) μm in length, less than 2 μm in width (N = 20). Two

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species, *E. suluensis* (Wilson, 1925) from the Philippines and *E. maraensis* Kang & Sim, 2008 from Korea, have a geographical distribution close to the present species. Of these, the large category of oxea of *E. marensis* is much larger than those of the present species. The raphidae length of the present species falls within the range of the longest raphidae of *E. marensis* and the shortest raphidae of *E. suluensis*, and the external morphology of *E. suluensis* is lamellated, but the present species is subspherical. Furthermore the present species is distinct by lacking the small category of oxea present in the two species. Trichodragmas straight or slightly arched. The specimen used for identification (NSMT-Po-1976) was deposited at the National Museum of Nature and Science, Tokyo.

Extraction and Isolation. The frozen specimens (11 kg) were cut into pieces and extracted with MeOH (25 L × 3) at room temperature (rt). The combined MeOH extracts were evaporated to dryness and partitioned between $CHCl_3$ (2 L × 3) and H_2O (2 L). The $CHCl_3$ layer was partitioned between 90% MeOH (1 L) and *n*-hexane (1 L × 3). The active 90% MeOH fraction was further partitioned between 60% MeOH and $CHCl_3$ (1 L × 4). The $CHCl_3$ layer was subjected to ODS flash column chromatography using 50%, 70%, 90%, and 100% MeOH and $CHCl_3$/MeOH/H_2O (7:3:0.5) as eluents. The fraction eluted with 90% MeOH was purified by ODS HPLC (Cosmosil AR-II; 20 × 250 mm) with 70−100% MeOH containing 0.2% acetic acid to afford 15 fractions. The seventh fraction was separated by RP-HPLC (Phenomenex Phenyl-Hexyl; 21.2 × 250 mm) with 55−75% MeCN to afford 22 fractions. Fractions 11 to 15 were combined and purified by ODS HPLC (Cosmosil MS-II; 10 × 250 mm) with 70−85% MeOH containing 0.2% acetic acid to afford **1** (0.43 mg). The ninth fraction from the Cosmosil AR-II column HPLC was separated by HPLC (Phenomenex Phenyl-Hexyl; 21.2 × 250 mm) with 55−75% MeCN to afford 20 fractions. Fractions 8 to 11 were combined and purified by ODS HPLC (Cosmosil MS-II; 10 × 250 mm) with 70−85% MeOH containing 0.2% acetic acid to afford **3** (0.59 mg).

*Spirastrellolide A (**1**):* $[\alpha]^{25}_{D}$ +14 (*c* 0.03, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 210 nm (3.67); ^{1}H NMR data (CD_3OD), see Table 1; ^{13}C NMR data (CD_3OD, 600 MHz), see Table 1; HRESIMS *m*/*z* 1035.5097 $[M + Na]^+$ (calcd for $C_{52}H_{81}ClO_{17}Na$, 1035.5060).

*Spirastrellolide B (**3**):* $[\alpha]^{25}_{D}$ +31 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 213 nm (3.54); ^{1}H NMR data (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR data (CD_3OD), see Table 1; HRESIMS *m*/*z* 1003.5628 $[M + Na]^+$ (calcd for $C_{52}H_{84}O_{17}Na$, 1003.5606).

Methylation of Spirastrellolides. To a solution of **1** (0.05 mg) in MeOH (1.0 mL) was added 100 μL of 2.0 M trimethylsilyldiazomethane in hexane. The solution was left at rt for 30 min, and the excess trimethylsilyldiazomethane was removed by a stream of N_2. The reaction mixture was purified by ODS HPLC to afford spirastrellolide A methyl ester (**5**, 0.05 mg). A 0.1 mg portion of **3** was treated in the same manner to afford spirastrellolide B methyl ester (**4**, 0.1 mg).

*Spirastrellolide A methyl ester (**5**):* UV (MeOH) λ_{max} (log ε) 203 nm (3.67); ^{1}H NMR (C_6D_6, 600 MHz), see Table S2; HRESIMS *m*/*z* 1049.5263 $[M + Na]^+$ (calcd for $C_{53}H_{83}ClO_{17}Na$, 1049.5217).

*Spirastrellolide B methyl ester (**4**):* UV (MeOH) λ_{max} (log ε) 205 nm (3.77); ^{1}H NMR (C_6D_6, 600 MHz), see Table S1; HRESIMS *m*/*z* 1017.5800 $[M + Na]^+$ (calcd for $C_{53}H_{86}O_{17}Na$, 1017.5763).

MTT Assay against HeLa Cells. The cytotoxicities of **1** and **3**−**5** against HeLa cells were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa human cervical cancer cells (Cell Resorce Center for Biomedical Research, Tohoku University) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 μg/mL antibiotic-antimycotic (Gibco) at 37 °C under an atmosphere of 5% CO_2. After 24 h preincubation, to each well of a 96-well microplate containing 200 μL of tumor cell suspension (1×10^4 cells/mL) was added the sample in duplicate, and the plate was incubated for 72 h. After addition of (MTT) saline solution (1 mg/mL, 50 μL) to each well, the plate was incubated for 3 h. After the incubation, the supernatant was discarded and DMSO (150 μL) was added. The absorbance was measured to determine IC_{50} values. In this assay, adriamycin was used as a positive control (IC_{50} value of 0.05 μM).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR data for **1** and **3**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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