Callyspongins A and B: Novel Polyacetylene Sulfates from the Marine Sponge *Callyspongia truncata* That Inhibit Fertilization of Starfish Gametes

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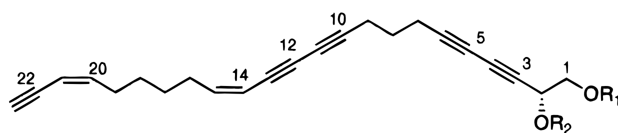
Bioassay-guided fractionation of the fertilization-inhibitory constituents of the marine sponge *Callyspongia truncata* yielded two novel polyacetylene sulfates designated callyspongins A (**1**) and B (**2**). The structures were determined on the basis of spectroscopic data. Compounds **1** and **2** inhibited fertilization of starfish gametes with minimum inhibitory concentrations of 6.3 and 50 μM, respectively, but affected neither maturation of oocytes nor cell division of embryos. These findings indicate that the sulfate group plays an important role in specific inhibition of starfish fertilization.

In the process of fertilization, the egg and sperm find each other and fuse together to create a new individual with genetic potentials derived from both parents. There are few, if any, chemicals[1−3] that inhibit the fertilization events without affecting cell division. In the course of our continuing studies of bioactive metabolites from marine organisms, we have collected a sponge, *Callyspongia truncata* (Lindgren) (order Haplosclerida, family Callyspongiidae), off Sada-misaki, Ehime Prefecture, Japan, which showed antifertilization activities against starfish gametes but did not affect embryonic cell division during initial screening. Bioassay-guided fractionation of the crude extract resulted in the isolation of two novel polyacetylene sulfates that we have named callyspongins A (**1**) and B (**2**). To our knowledge, this is the first isolation of bioactive polyacetylene sulfates from marine organisms. Here, we describe the isolation, structure elucidation, and biological activities of these compounds.

The methanolic extract of the frozen marine sponge *Callyspongia truncata* was partitioned between EtOAc and water. The aqueous layer was further extracted with BuOH. Both BuOH and EtOAc layers showed inhibitory activity against starfish fertilization. The BuOH layer was separated by gel filtration on Toyopearl HW40 using water as eluent to yield callyspongin A (**1**) (141 mg) as a viscous oil. The EtOAc layer was chromatographed on a silica gel column using $CHCl_3$−Me_2CO (1:1 in volume) as eluent to afford callyspongin B (**2**) (50 mg) as a viscous oil.



1 $R_1=R_2=SO_3Na$
2 $R_1=SO_3Na$, $R_2=H$
3 $R_1=R_2=H$

Callyspongin A (**1**) is a sulfate, which was deduced by the observation of an IR absorption (measured as a film) at 1220 cm^{-1} and the presence of a prominent peak at *m/z* 97 $[HSO_4]^-$ in the negative mode fast-atom bombardment mass spectrum (FABMS). The presence of an ion peak at *m/z* 115 ([matrix + Na]$^+$) in the positive mode secondary ion mass spectrum (SIMS) (matrix: glycerol) indicated that the sulfate was present as a sodium salt. Compound **1** had a molecular formula of $C_{23}H_{22}O_8S_2Na_2$, which was established by high-resolution FABMS data (*m/z* 513.0659 [M − Na]$^-$, Δ +0.5 mmu). The IR absorption bands implied the presence of acetylene bonds (2260 cm^{-1}) and vinyl groups (1640 cm^{-1}) in addition to the sulfate group. The UV spectrum showed absorption maxima at 267 and 287 nm, with the ϵ values of 8900 and 7100, respectively, suggesting the presence of an ene-diyne.[4] Both ^1H and ^{13}C NMR spectra (Table 1) together with a DEPT spectrum revealed that the 23 carbons in **1** consisted of two 1,2-disubstituted double bonds, an oxygenated methylene, an oxygenated methine, seven methylene, a methine, and nine quaternary sp carbons. Assignments of all the protonated carbons were made by the analysis of the $^{13}C-^1H$ COSY spectrum as shown in Table 1. In the $^1H-^1H$ COSY spectrum, two oxygenated methylene protons (H-1) at δ 4.14 were coupled to a low-field oxygenated methine proton (H-2) at δ 5.20. Connectivities from C-7 to C-9 and from C-14 to C-21 were readily inferred from the $^1H-^1H$ COSY spectrum. Construction of the gross structure was achieved by HMBC experiments.[5] Correlations of H-1 to C-3, of H-2 to C-3, C-4, and C-5 (four-bonded coupling), and of H-7 to C-4 (four-bonded coupling), C-5, and C-6 supported the connectivities from C-1 to C-7. The methylene protons (H-9) showed correlations to C-10, C-11, and C-12 (four-bonded coupling), and C-12 also had correlation with H-14, thus determining the connectivities from C-9 to C-14. Thus, the connectivities from C-1 to C-21 were revealed by assembly of the partial structural units described above. The coupling constant (J = 11.0 Hz) in the ^1H NMR spectrum and clear crosspeaks between olefinic protons (H-14/H-15 and H-20/H-21) in the NOESY experiment indicated the Z geometry of $\Delta^{14,15}$- and $\Delta^{20,21}$-double bonds. The connection from C-23 to C-21 via the C-22 quaternary sp carbon was established on the basis of the long-range ^1H coupling between H-23

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Table 1. NMR Spectral Data for Compounds **1** and **2**

	1[a]		**2**[b]	
carbon no.	δ_C and multiplicity	δ_H and multiplicity[c]	δ_C and multiplicity	δ_H and multiplicity[c]
1	70.2 t	4.14 d (2H, 5.5)	69.1 t	3.68 m (2H)
2	68.6 d	5.20 t (5.5)	60.5 d	4.43 m
3	72.0 s		77.1 s	
4	73.3 s[d]		68.6 s[e]	
5	66.3 s[d]		65.2 s[e]	
6	83.2 s		80.5 s	
7	19.5 t	2.44 t (2H, 6.9)	17.7 t	2.38 t (2H, 6.9)
8	28.3 t	1.75 quin (2H, 6.9)	26.4 t	1.67 quin (2H, 6.9)
9	19.8 t	2.45 t (2H, 6.9)	18.0 t	2.42 t (2H, 6.9)
10	85.3 s		84.5 s	
11	67.3 s		65.5 s	
12	79.4 s		78.0 s	
13	74.2 s		72.5 s	
14	109.6 d	5.52 d (11.0)	108.0 d	5.56 d (11.0)
15	150.0 d	6.14 dt (11.0, 7.3)	148.3 d	6.12 dt (11.0, 7.8)
16	31.7 t	2.31 m (2H)	30.0 t	2.22 m (2H)
17	29.5 t	1.44 m (2H)	27.6 t	1.35 m (2H)
18	29.4 t	1.44 m (2H)	27.6 t	1.35 m (2H)
19	31.3 t	2.32 m (2H)	29.5 t	2.23 m (2H)
20	147.6 d	6.04 dt (11.0, 7.8)	145.2 d	5.99 dt (11.0, 7.8)
21	109.8 d	5.48 brd (11.0)	108.6 d	5.46 dd (11.0, 1.5)
22	82.3 s		80.2 s	
23	83.8 d	3.46 brs	84.6 d	3.95 d (1.5)
2-OH				5.68 brs

[a] Measured in CD_3OD-D_2O (1:1, v/v). [b] Measured in DMSO-d_6. [c] Coupling constants, J_{H-H} (in Hz), are given in parentheses. [d,e] Signals with identical letters may be interchanged.

and H-21 and by the HMBC crosspeaks of H-20 to C-22, of H-21 to C-23, and of H-23 to C-21 and C-22. Consequently, the structure of callyspongin A was determined as **1**.

Callyspongin A (**1**) was hydrolyzed with *Helix pomatia* sulfatase (50 °C, 12 h) to afford the diol derivative (**3**). Its ^{1}H and ^{13}C NMR spectral data were identical to those of siphonodiol,[6,7] which is an antifungal polyacetylene diol isolated from the marine sponge, *Siphonochalina truncata*. The specific rotation of **3** [$[\alpha]^{25}_D$ −6.6° (*c* 0.09, MeOH)] was the same as that of siphonodiol reported by the Shionogi group.[6] Because the configuration at C-2 of siphonodiol was established to be *R* by Fusetani *et al.*,[7] the absolute configuration at C-2 in **1** was determined to be *R*.

Callyspongin B (**2**) had a molecular formula of $C_{23}H_{23}O_5SNa$, as established by the high-resolution FABMS data (*m/z* 411.1264 [M − Na]$^-$, Δ −0.2 mmu). The UV and IR spectra were nearly identical to those of **1**, suggesting that **2** is desulfated compound of **1**. ^{1}H and ^{13}C NMR spectra (Table 1) were similar to those of **1** except that the ^{13}C signal assigned to C-2 and the proton signal assigned to H-2 shifted to a higher magnetic field as compared with those of **1**. Further, the hydroxyl proton signal at δ 5.68 was coupled to the oxygenated methine proton (H-2) signal at δ 4.43 in the ^{1}H−^{1}H COSY of **2**. These findings indicated that C-2 of **2** was hydroxylated and C-1 was sulfated. Although the absolute configuration of **2** has not been determined experimentally, it is highly possible that the C-2 configuration is the same as that of **1**, since **2** and **1** were derived from the same origin.

We have treated starfish (*Asterias amurensis*) gametes with **1**, **2**, and **3** to investigate the effects of these compounds on fertilization and egg activation. As shown in Table 2, concentrations at or higher than 50 μM of **1** were necessary to inhibit sperm motility, whereas those at or above 6.3 μM prevented fertilization as revealed by the egg's incapability of forming the fertilization envelope by insemination. Concentrations at or above 50 μM of **2** blocked fertilization whereas those at or above 100 μM inhibited sperm motility. On the other hand, fertilization was unaffected by **3** even at the concentration of 200 μM, although sperm motility was inhibited at 13 μM. Maturation of starfish oocytes as induced by 1-methyladenine, the maturation-inducing substance of starfishes,[8] was unaffected by any of these compounds at 100 μM. When fertilized eggs were cultured in the presence of the **1** or **2** at 100 μM, they developed up to the blastula stage. On the other hand, embryos lysed in sea water containing 100 μM of **3**, showing that the diol exerts nonselective toxicity against gametes and embryos. These findings indicated that the sulfate group of **1** and **2** plays an important role in specific inhibition of starfish fertilization.

Table 2. Inhibitory Effects of Compounds **1**−**3** on Starfish Gametes

	minimum inhibitory concentration (μM)		
	1	**2**	**3**
sperm motility	50	100	13
egg fertilization	6.3	50	>200

Relatively specific inhibition of fertilization by **1** deserves further experimentation to clarify its mode of action during the fertilization process.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX500 (500 MHz for ^{1}H, 125 MHz for ^{13}C). NMR chemical shifts were referenced to solvent peaks: δ_H 3.30 (residual CHD_2OD) and δ_C 49.8 for CD_3OD-D_2O (1:1), δ_H 2.50 (residual CHD_2SOCD_3) and δ_C 39.5 for DMSO-d_6. The HMBC spectra were optimized for a $^nJ_{CH}$ of 8 Hz. A spectral width of approximately 3000 Hz was employed with the HMBC spectra acquired as 512 data points in t_2 for 256 data points in t_1 increments and zero-filled to 1024 and 512 points in t_2 and t_1, respectively, prior to

Fourier transformation. NOESY experiments were performed with mixing times of 0.75 s. Multiplicities of the ^{13}C signals were determined by DEPT experiments. FABMS and HRFABMS were obtained on a JEOL SX102A spectrometer (glycerin for **1** and **2**, diethanolamine for **3** as a matrix). SIMS was obtained on a Hitachi M-80B spectrometer (glycerin as a matrix). UV and IR spectra were recorded with a Shimadzu UV-160A and a JASCO FT/IR-5300 spectrometer, respectively. Optical rotations were measured with a JASCO DIP-370 digital polarimeter.

Bioassays. Gametes of the starfish *A. amurensis* were used in these experiments. Oocytes and sperm were removed from ovarian and testicular fragments, respectively. Experiments were performed at 20 °C, and artificial sea water (Jamarin Laboratory, Osaka, Japan) was used throughout. Oocytes were induced to mature by the treatment with 1 μM 1-methyladenine (Sigma, St. Louis, MO). Maturing oocytes were fertilized at 40 min after the start of 1-methyladenine treatment.

Stock solutions of compounds to be tested in methanol were added to the suspensions of oocytes, eggs, sperm, or fertilized eggs to give final concentrations of methanol less than 0.2% in sea water. Methanol had no effects on the phenomena observed at the concentrations tested. To assay for loss of sperm motility in sample solutions, concentrated semen was suspended in serially diluted sample solutions, and the sperm were examined 30 min after the start of the sample treatment. To assay for oocyte maturation and fertilization, oocytes were placed in serially diluted sample solutions for 15 min before addition of 1-methyladenine. Insemination was carried out at 40 min after the start of 1-methyladenine treatment, and the oocytes were examined for the presence of the germinal vesicle and the fertilization envelope at 5 min after insemination. To assay for developmental arrest of embryos, a small number of fertilized eggs were added to serially diluted sample solutions within 30 min after insemination. They were periodically observed for any cytological changes.

Material, Extraction, and Isolation. The marine sponge *C. truncata* (420 g, wet weight) was collected off the coast of Sada-misaki, Ehime Prefecture, Japan. The marine sponge was identified by Professor Patricia R. Bergquist, The University of Auckland, New Zealand. A voucher specimen is kept in the laboratory of one of the authors (S.O.). The methanolic extract (23.7 g) was partitioned between EtOAc and water. Then, the aqueous layer was extracted with BuOH. The BuOH-soluble material (3.0 g) and the EtOAc-soluble material (3.3 g) exhibited an inhibitory activity against fertilization of starfish eggs at the minimum inhibitory concentration of 31 and 62 μg/mL, respectively. The BuOH-soluble portion was separated by gel filtration on Toyopearl HW40 using water as eluent to afford callyspongin A (**1**) (141 mg; 0.034% wet weight) as a viscous oil. The EtOAc-soluble portion was chromatographed on a silica gel column using $CHCl_3-Me_2CO$ (1:1 in volume) as eluent to afford callyspongin B (**2**) (50 mg; 0.012% wet weight) as a viscous oil.

Callyspongin A (1): $[\alpha]^{25}_D$ −40.3° (*c* 1.08, H_2O); UV (H_2O) λ_{max}207 (ϵ 25 100), 214 (26 800), 253 (7400), 267 (8900), 287 nm (7100); IR (film) ν_{max} 2260, 1640, 1220 cm^{-1}; ^1H and ^{13}C NMR Table 1; (−)-HRFABMS *m/z* 513.0659 $[M - Na]^-$, calcd for $C_{23}H_{22}O_8S_2Na$, Δ +0.5 mmu; (−)-FABMS *m/z* 513 $[M - Na]^-$, 97 $[HSO_4]^-$.

Callyspongin B (2): $[\alpha]^{25}_D$ +3.1° (*c* 0.41, DMSO); UV (DMSO) λ_{max}270 (ϵ 4800), 285 nm (4100); IR (film) ν_{max} 3440, 2260, 1630, 1285 cm^{-1}; ^1H and ^{13}C NMR Table 1; (−)-HRFABMS *m/z* 411.1264 $[M - Na]^-$, calcd for $C_{23}H_{23}O_5S$, Δ −0.2 mmu; (−)-FABMS *m/z* 411 $[M - Na]^-$, 97 $[HSO_4]^-$.

Enzymatic Hydrolysis of 1. A solution of **1** (88 mg) in 2 mL of H_2O was added to 10 mL of β-glucuronidase-sulfatase solution (*Helix pomatia*, 1 × 10^6 RU/mL, L'Industrie Biologique Francaise) and left at 50 °C for 12 h with occasional shaking. After the reaction mixture was extracted with $CHCl_3$, the $CHCl_3$ extract was chromatographed on a silica gel column using EtOAc−hexane (EtOAc: 20−40%) as eluent to afford **3** (9 mg): $[\alpha]^{25}_D$ −6.6° (*c* 0.09, MeOH) (lit.[6] $[\alpha]^{25}_D$ −6.7°); (+)-FABMS *m/z* 438 $[MH + \text{diethanolamine}]^+$. ^1H and ^{13}C NMR spectral data were identical with those of siphonodiol.[6]

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