New Ceramide from Marine Sponge *Haliclona koremella* and Related Compounds as Antifouling Substances against Macroalgae

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A new ceramide *N*-docosanoyl-D-*erythro*-(2*S*,3*R*)-16-methyl-heptadecasphing-4(*E*)-enine (C_{22} ceramide) was isolated from the marine sponge *Haliclona koremella* as an antifouling substance against macroalgae. The structure of this substance was elucidated by spectral means. Antifouling activity of several related compounds was also examined.

Biofouling organisms such as blue mussel, barnacle, and macroalgae cause serious problems to ships' hulls, the cooling system of power plants, and aquaculture nets.[1,2] Organotin compounds such as TBTO [bis(*n*-tributyltin)oxide] have been used as antifouling agents against these invertebrates. Stern warnings however, have been issued regarding the toxic effects of such heavy-metal compounds on the marine environment.[3,4] Therefore, it is necessary to discover or develop antifouling substances with reduced or no toxicity. It is well known that many marine invertebrates such as sponges and corals remain remarkably free from settlement by fouling organisms. It has been suggested that they have biologically active compounds that prevent other marine organisms from settling and attaching to their bodies. In fact, several compounds with such activity have been found among marine invertebrates.[5−9] These compounds are considered to play an important roll in the antifouling mechanism of marine organisms. Our screening method[10] for antifouling activity against the macroalga *Ulva conglobata* enabled us to isolate an antifouling substance from *Haliclona koremella* collected in Palau. Here we report the structure of this compound.

C_{22} ceramide (**1**) was obtained as a colorless powder, and its molecular formula was determined to be $C_{40}H_{79}NO_3$ by negative ion HRFABMS. The IR spectrum showed absorptions at 3418 and 1060 cm^{-1} (hydroxy); 1634 cm^{-1} (amide); and 2922, 2854, and 1386 cm^{-1} (aliphatic). The ^{1}H NMR spectrum (see Table 1) exhibited a large methylene envelope at δ 1.23. The presence of a sphingosine moiety was indicated by signals for a nonequivalent methylene proton signal [δ 3.68 (dd, $J =$ 3.0, 11.0 Hz) and 3.94 (dd, $J =$ 3.8, 11.0 Hz)], two methine proton signals [δ 3.89 (m) and 4.30 (dd, $J =$ 3.4, 6.6 Hz)], and two olefinic proton signals [δ 5.51 (dd, $J =$ 6.6 15.4 Hz) and 5.77 (dt, $J =$ 7.2, 15.4 Hz)]. The large coupling constant revealed that the double bond had the (*E*) configuration. These data, including coupling constants, coincided well with those of *N*-palmitoylsphingosine (C_{16} ceramide) except for the large integration of the methylene envelope signal [δ 1.23] and a doublet signal [δ 0.85 (6H)]. This signal indicated the presence of an isopropyl group. By COSY, a cross peak was observed between an amide proton (δ 6.20) and the H2 methine (δ 3.89), which, in turn, was coupled to three protons at δ 4.30, 3.68, and 3.94.

Table 1. NMR Data for C_{22} Ceramide (**1**) in $CDCl_3$

position	δ_H[a] (mult., J in Hz)	δ_C[b] (multi.)
1	3.68 (dd, 3.0, 11.0) 3.94 (dd, 3.8, 11.0)	62.58 (t)
2	3.89 (m)	54.57 (d)
3	4.30 (dd, 3.4, 6.6)	74.76 (d)
4	5.51 (dd, 6.6, 15.4)	128.87 (d)
5	5.77 (dt, 7.2, 15.4)	134.34 (d)
6	2.04 (m)	32.29 (t)
7	1.83 (m)	31.94 (t)
8−13	1.23 (m)	29.72 (t)
14	1.23 (m)	29.96 (t)
15	2.50 (m)	39.10 (t)
16	1.56 (m)	27.99 (d)
17, 18	0.85 (d, 6.6)	22.67 (q)
1′		173.80 (s)
2′	2.26 (t, 7.5)	36.88 (t)
3′	1.63 (m)	25.78 (t)
4′−21′	1.23 (m)	29.72 (t)
21′	1.23 (m)	22.70 (t)
22′	0.89 (t, 6.8)	14.10 (q)
NH	6.20 (d, 7.3)	

[a] Data were recorded at 500 MHz. [b] Data were recorded at 125 MHz. Multiplicity is given in DEPT. Assignments were made by HSQC, NOESY, TOCSY, and COSY studies.

To determine the structure of the sphingosine unit, the antifouling substance was hydrolyzed with 1M HCl−MeOH for 18 h at 76 °C and separated into petroleum ether and MeOH layers. The MeOH layer provided a C_{18} sphingosine with an isopropyl terminus [δ 0.85 (6H, d, $J =$ 6.6 Hz)] identified by ^{1}H NMR spectral features[11] and FABMS (M$^+$ at m/z =300). A methyl ester (0.8 mg) of *n*-docosanoic acid was obtained from the petroleum ether layer, confirmed by GC−EIMS spectral analysis (M$^+$ m/z 354) comparison with an authentic sample.

Comparison of optical rotation values between **1** ($[\alpha]^{25}_D$ −6.0°) and the literature[12] value ($[\alpha]^{25}_D$ −8.0°) for a similar gorgonian-derived ceramide allowed the assignment of D-*erythro*-(2*S*,3*R*) stereochemistry. This compound was thus determined as *N*-docosanoyl-D-*erythro*-(2*S*,3*R*)-16-methyl-heptadecasphing-4(*E*)-enine (C_{22} ceramide), a new ceramide composed of a branched sphingosine and an unbranched fatty acid chain.

The antifouling activity of **1** and related compounds in Figure 1 against *U. conglobata* spores is shown in

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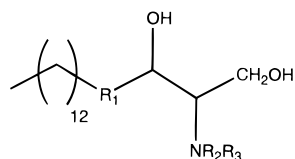


sphingolipid	R_1	R_2	R_3
sphingosine	CH=CH	H	H
dihydrosphingosine	CH_2-CH_2	H	H
dimethylsphingosine	CH=CH	CH_3	CH_3
C_2 ceramide (*N*-acetylsphingosine)	CH=CH	$CO-CH_3$	H
C_6 ceramide (*N*-hexanoylsphingosine)	CH=CH	$CO-(CH_2)_4-CH_3$	H
C_8 ceramide (*N*-octanoylsphingosine)	CH=CH	$CO-(CH_2)_6-CH_3$	H
C_{16} ceramide (*N*-palmitoylsphingosine)	CH=CH	$CO-(CH_2)_{14}-CH_3$	H

Figure 1. Chemical structures of the assayed compounds.

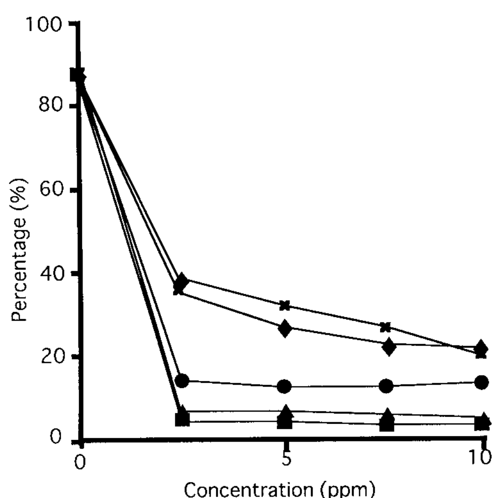
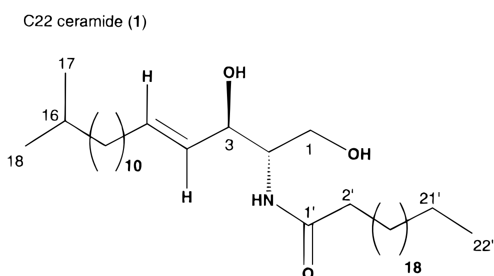


Figure 2. Effect of concentration of **1** and several ceramide compounds on the germination rate of spores of *Ulva conglobata*. The symbols indicate: ●,C_2 ceramide; ♦, C_6 ceramide; ×, C_8 ceramide; ▲, C_{16} ceramide; ■, C_{22} ceramide from *Haliclona koremella*.

Figures 2 and 3. IC_{50} values of attachment inhibition were higher than ones of germination inhibition for all kinds of ceramides. The C_2 ceramide showed stronger attachment inhibition than other kinds of ceramides. Consequently, the length of the acyl residue was found to be important for the antifouling activity of the ceramides.



In the case of the sphingosines, the effect at various concentrations on the attachment rate was similar to that on the germination rate. As the concentration of each sphingosine was increased, the germination rate and attachment rate decreased to about 15% at 1.0 ppm.

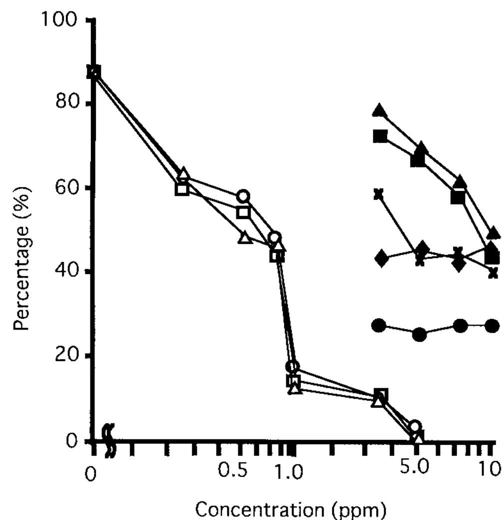


Figure 3. Effect of concentration of **1**, several ceramide compounds, and sphingosines on the attachment rate of spores of *Ulva conglobata*. The symbols indicate: ●, C_2 ceramide; ♦, C_6 ceramide; ×, C_8 ceramide; ▲, C_{16} ceramide; ■, C_{22} ceramide from *Haliclona koremella*; ○, sphingosine; □, dihydrosphingosine; △, dimethylsphingosine.

Moreover, the germination rate and the attachment rate were about 0 % at 5.0 ppm (Figure 3). These results suggest that related sphingosines inhibit the germination and attachment of *U. conglobata* spores similarly. But germination inhibition of *U. conglobata* spores was affected by lower concentrations of ceramides than was attachment. It seems that the bioactivity of sphingosines differs from that of ceramides. None of these compounds however, showed any toxicity toward *Ulva conglobata* fronds at 50 ppm. The sphingosines showed antimicroalgal activity against *Oscillatoria amphibia* (Cyanophyceae), *Skeletonema costatum* (Diatomophyceae), *Brachiomonas submaria* (Chlorophyceae), and *Prorocentrum micans* (Dinophyceae) at 1–5 ppm, except for dihydrosphingosine against *S. costatum*. No antimicroalgal activity of the ceramides was recognized at 1–5 ppm (Table 2).

Several other ceramides and sphingolipids have been reported from marine sponges,[11,13,14] and sphingolipid (including sphingosine and dihydrosphingosine) phytotoxicity against duckweed has been reported.[15] These results expand the possible ecological roles of ceramides and sphingosines in marine sponges and their potential industrial applications.

Experimental Section

General Experimental Procedures. NMR experiments were carried out with a Varian Unity 500 NMR spectrometer using $CDCl_3$ as the solvent for ^1H NMR and ^{13}C NMR. FABMS and EIMS data were measured with a JEOL JMS–SX102 mass spectrometer. GC–EIMS was measured using a JEOL JMS–SX102 mass spectrometer equipped with a HP5890 gas chromatography. Optical rotation was determined with a HORIBA SEPA-300 high sensitive polarimeter. IR spectra were measured on a JASCO FT/IR-7000 spectrometer. UV spectra were recorded on a Shimadzu UV-2200A spectrometer in MeOH.

Assay for Antifouling Activity against Spores of *Ulva conglobata*. A test sample was dissolved in 0.1

Table 2. Antimicroalgal Activity of the Sphingosines and the Ceramides

	ceramide[a] (ppm)			sphingosine (ppm)			dihydrosphingosine (ppm)			dimethylsphingosine (ppm)		
	0.5	1.0	5.0	0.5	1.0	5.0	0.5	1.0	5.0	0.5	1.0	5.0
O. amphibia	−	−	−	−	−	+[b]	−	+	+	−	+	+
B. submaria	−	−	−	−	−	+	−	+	+	−	−	+
P. micans	−	−	−	−	−	+	−	−	+	−	−	+
S. costatum	−	−	−	−	−	+	−	−	−	−	−	+

[a] The ceramides used as test samples were C_2, C_6, C_8, C_{16}, and C_{22} ceramides described in the Experimental Section. [b] A "+" indicates toxicity equal to 10 μg/mL of DCMU.

mL of MeOH and dried in a polystyrene Petri dish (35 mm in diameter) at 25 °C, before 5 mL PES medium[16] was added. A total of 1500−3000 spores of *Ulva* were incubated in each Petri dish, triplicate dishes being prepared for each set of experiments. After a 5-day incubation at 20−25 °C under a light intensity of 40 μE/m^2/s with a 14L:10D photoperiod, the numbers of germinated spores, ungerminated spores, and unattached spores on 1 cm^2 of the Petri dish were counted, and the germination rate and attachment rate were calculated.[10]

Assay for Lethal Activity against *Ulva* Fronds. The toxic effects of the new compound (**1**) and related ceramides and sphingosines were examined to test the safety toward the marine environment. An *Ulva* frond (about 2.0 × 2.0 cm) was incubated in the presence of 1−5 ppm of a test sample under the same conditions as those already described, triplicate dishes being prepared. The lethal activity was evaluated by direct visual observation.

Assay for Antimicroalgal Activity. A test sample was dissolved in 10 μL of MeOH and dried in 96-well polystyrene microplates. F/2 medium (200 μL) and *Oscillatoria amphibia*, *Skeletonema costatum*, *Brachiomonas submaria*, or *Prorocentrum micans* were incubated in each well at 25 °C under a light intensity of 28 μE/m^2/s with a 12L:12D photoperiod in the presence of 0.5−5 ppm of a test sample. 3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea (DCMU) 10 μg/mL and 500 μg/mL was used as the positive control, and duplicate dishes were prepared for each set of experiments. The activity against *P. micans* and *B. submaria* was evaluated with an optical microscope, while that against *O. amphibia* and *S. costatum* was evaluated by direct visual observation.[17]

Sponge Material. A specimen of *Haliclona koremella* de Laubenfels (family Chalinidae; order Haplosclerida) was collected at Palau and identified by Prof. P. Bergquist (University of Auckland). It was kept frozen until used. A voucher specimen (coll. no. 96P-1) has been deposited in Marine Biotechnology Institute, Shimizu Laboratories, Japan.

Preparation of the Chemicals. The ceramides and sphingosines tested, except C_{22} ceramide (**1**), were purchased from Biomol Research Laboratories, Inc. Their structures are shown in Figure 1.

Extraction and Isolation. *Haliclona koremella* (2.3 kg) was soaked in EtOH for 48 h at room temperature, and the extract was evaporated and separated into H_2O-soluble and Et_2O-soluble fractions. Antifouling activity was recognized in the Et_2O-soluble fraction. This fraction was chromatographed on an ODS column (Cosmosil 140 C18−OPN, Nacalai Tesque) with MeOH−H_2O (50−100%). Further purification was performed by HPLC on an ODS column (TSK gel ODS-80Ts, 20 mm × 25 cm, Tosoh) with 100% MeOH to afford **1** (10.6 mg), 0.00046% from *Haliclona koremella*.

C_{22} ceramide (1): colorless powder, $[\alpha]^{25}_D$ −6.0° (*c* 0.01, MeOH); IR (KBr) ν_{max}: 3418, 2950, 2922, 2854, 1634, 1386, and 1060 cm^{-1}; UV (MeOH) λ_{max} 206 nm (ϵ 1990); FABMS (NBA) *m/z* [M − H]$^-$ 621; HRFABMS (NBA) *m/z* [M − H]$^-$ 621.6134 (calcd for $C_{40}H_{79}NO_3$, 621.6138); ^{1}H and ^{13}C NMR data are shown in Table 1.

Methanolysis of 1. Compound **1** (2.0 mg) was refluxed with 1M HCl−MeOH (5 mL) for 18 h at 76 °C. The reaction mixture was separated into petroleum ether and MeOH layers. The MeOH layer was concentrated and purified on ODS short column to afford C_{18} sphingosine (0.7 mg): $[\alpha]^{25}_D$ +2.7° (*c* 0.01, MeOH); FABMS (NBA) *m/z* [M − H]$^-$ 298; ^{1}H NMR ($CDCl_3$) δ 5.82 (1H, dt, J = 6.9, 15.7 Hz), 5.42 (1H, dd, J = 6.4, 15.7 Hz), 4.33 (1H, dd, J = 3.2, 6.4 Hz), 3.88 (1H, m), 4.10 (1H, dd, J = 3.6, 10 Hz), 3.62 (1H, dd, J = 3.6, 10 Hz), 2.01 (2H, m), 1.63 (2H, m), 1.24 (br, s), 0.85 (6H, d, J = 6.4 Hz). The petroleum ether layer was evaporated under reduced pressure to yield fatty acid methyl ester (*n*-docosanoic acid methyl ester) (0.8 mg); GC−EIMS (NBA) *m/z* 354 [M$^+$]; ^{1}H NMR ($CDCl_3$) δ 3.64 (s, OCH_3), 2.28 (2H, t), 1.56 (2H, m), 1.23 (br s), 0.86 (3H, t, J = 7.1 Hz).

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References and Notes

(1) Anil, A. C.; Chiba, K.; Okamoto, K. *Biofouling* **1990**, *2*, 137−150.
(2) Characklis, W. G. In *Biofouling and Biocorrosion in Industrial Water Systems*; Flemming, H. C., Geesey, G. G. Ed.; Springer: Berlin, 1991; pp 7−27.
(3) Leitch, E. G.; Puzzuoli, F. V. *J. Protect. Coat. Linings* **1992**, 28−41.
(4) Davis, A. R.; Targett, N. M.; McConnell, O. J.; Young, C. M. In *Bioorganic Marine Chemistry*; Springer: Berlin, 1989; Vol. 3, pp 86−114.
(5) Mizobuchi, S.; Shimidzu, N.; Katsuoka, M.; Adachi, K.; Miki, W. *Nippon Suisan Gakkaishi* **1993**, *59*, 1195−1199.
(6) Kawamata, M.; Kon-ya, K.; Miki, W. *Fisheries Sci.* **1994**, *60*, 485−486.
(7) Kon-ya, K.; Shimidzu, N.; Adachi, K.; Miki, W. *Fisheries Sci.* **1994**, *60*, 773−775.
(8) Kon-ya, K.; Shimidzu, N.; Otaki, N.; Yokoyama, A.; Adachi, K.; Miki, W. *Experientia* **1995**, *51*, 153−155.
(9) Tsukamoto, S.; Kato, H.; Hirota, H.; Fusetani, N. *J. Nat. Prod.* **1997**, *60*, 126−130.
(10) Hattori T.; Shizuri, Y. *Fisheries Sci.* **1996**, *62*, 955−958.
(11) Li, H.; Matsunaga, S.; Fusetani, N. *Tetrahedron* **1995**, *51*, 2273−2280.

(12) Shin, J.; Seo, Y. *J. Nat. Prod.* **1995**, *58*, 948−953.
(13) Nagle, D. G.; McClatchey, W. C.; Gerwick, W. H. *J. Nat. Prod.* **1992**, *55*, 1013−1017.
(14) Garg, H. S.; Agrawal, S. *J. Nat. Prod.* **1995**, *58*, 442−445.
(15) Tanaka, T.; Abbas, H. K.; Duke, S. O. *Phytochemistry* **1993**, *33*, 779−785.
(16) Provasoli, L. In *Proceedings of US-Japan Conference, Culture and Collection of Algae*; Watanabe, A., Hattori, A. Ed.; Japan Society of Plant Physiology: Tokyo, 1968; pp 63−75.
(17) Chen, C.; Imamura, N.; Nishijima, M.; Adachi, K.; Sakai, M.; Sano, H. *J. Antibiot.* **1996**, *10*, 998−1005.

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