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Isolation and Structure Determination of Algicidal Compounds from *Ulva fasciata*

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Thirty-seven species of seaweeds including 10 Chlorophyta, 13 Phaeophyta, and 14 Rhodophyta collected from the coast of Nagasaki Prefecture, Japan, were screened for algicidal activity against the red-tide phytoplankton *Heterosigma akashiwo*. The green alga *Ulva fasciata* (Ulvaceae, Chlorophyta) showed the strongest algicidal activity among the seaweeds tested. Bioassay-guided fractionation of the methanol extract of *U. fasciata* led to isolation of three algicidal compounds whose structures were determined to be hexadeca-4,7,10,13-tetraenoic acid (HDTA), octadeca-6,9,12,15-tetraenoic acid (ODTA), and α-linolenic acid on the basis of spectroscopic information. These polyunsaturated fatty acids (PUFAs) showed potent algicidal activity against *H. akashiwo* (LC_{50} 1.35 μg/ml, 0.83 μg/ml, and 1.13 μg/ml for HDTA, ODTA, and α-linolenic acid, respectively), and the result demonstrated the potential of these PUFAs for practical harmful algal bloom control.

Key words: algicidal compounds; *Ulva fasciata*; hexadeca-4,7,10,13-tetraenoic acid; octadeca-6,9,12,15-tetraenoic acid; α-linolenic acid

Harmful algal blooms (HABs) have been a serious problem for public health and fisheries industries in recent years. HAB effects are very noxious and cause significant damage to the ecosystem. Especially toxic algal blooms and red tides have had a severe economic impact on shellfish cultivation. Human illness and even death can result from eating seafood contaminated by some of the red-tide algae.

Several methods to control HAB have been developed that involve the use of chemical and physical agents, *e.g.*, copper sulfate,[1)] clay minerals,[2,3)] yellow loess,[4)] and also biological agents, *e.g.*, virus,[5)] bacteria,[6–8)] planktonic ciliates,[9)] heterotrophic dinoflagellates.[10)] But these strategies have disadvantages such as difficulty of application, high cost, and in some cases, they have the potential of disastrous environmental consequences.

Seaweeds are distributed widely in coastal regions and are indigenous to the marine environment. Some seaweed is known to produce allelopathic substances against the microalgae responsible for red tide. Several studies have been done aimed at development of a novel, low-cost, environmentally benign method to control HAB by utilizing seaweeds. A pioneering study on the allelochemicals of seaweed toxic to microalgae was done by Kakisawa *et al.*[11)] They isolated an allelopathic substance from the brown alga *Cladosiphon okamuranus* and identified it as octadeca-6*Z*,9*Z*,12*Z*,15*Z*-tetraenoic acid (ODTA). After testing it against 37 species of microalgae, they found that ODTA is active against phytoplankton without cell coverings involving the red tide phytoplankton *Heterosigma akashiwo*, *Chattonella antique*, *C. marina*, *Gymnodinium nagasakiense*, and *G. sanguineum*. They also showed that highly unsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid have a potency of algicidal activity similar to ODTA (100% mortality at a dose of 2 ppm) against *H. akashiwo*. Later in 1998, although the structure was not determined, Suzuki *et al.* isolated an allelopathic substance active against *H. akashiwo* and zoospore of the brown alga, *Laminaria religiosa*, from the crustose coralline algae, *Lithophyllum* spp.[12)] In 2000, Korean researches found that the methanol extract of the red alga, *Corallina pilulifera* (Rhodophytae), shows strong growth inhibition to the red tide microalgae *Cochlodinium polykrikoides*, after screening 28 seaweeds collected from the coast of Korea.[13)] They also showed that the powder of the red alga has the ability to inhibit the growth of a further six species of harmful microalgae at a dose of 6.6 mg/ml. Recently, in 2003, Jin and Dong reported that both fresh tissue and the dried powders of both nonsexual and sexual strains of *Ulva pertusa* strongly inhibited the growth of *H. akashiwo* and *Alexandrium tamarense* in coexistence assays.[14)] Very recently, in 2004, Chiang *et al.* explained that the allelopathic substances of *Botryococcus braunii* Kutzing (Chlorophyceae) to various phytoplankton were the fatty acids α-linolenic, oleic, linolenic, and palmitic acid.[15)]

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Abbreviation: PUFA, polyunsaturated fatty acid

We screened 37 species of green, red, and brown algae collected in the coastal region of Nagasaki Prefecture in Japan for algicidal activity against *H. akashiwo*, and found the highest activity in the methanol extract of the green alga *Ulva fasciata*. Here, we describe the isolation and identification of the algicidal principles.

Materials and Methods

Plant materials. Seaweeds were collected from intertidal areas of the coast of Nagasaki Prefecture, Japan in April–July 2004. Ecological damage during harvesting was prevented by not removing the algae stems. All samples were brought to the laboratory in plastic bags containing sea water to prevent evaporation, and then washed with distilled water to separate potential contaminants. For convenient use of the samples, the seaweeds collected were air-dried for 5 d at room temperature and then ground to powder using a blender.

Screening for algicidal activity. Seaweed tissues were subjected to methanol and water extraction following the method previously described by Jeong *et al.*[13] with minor modifications. Each 100 mg of dried sample was soaked in 5 ml of methanol at room temperature for 1 d and filtered through no. 2 filter paper under reduced pressure. This extraction procedure was repeated three times, and the extracts were combined. The residual tissue was dried up and extracted with water for 1 d at room temperature.

Axenic HAB species of *Heterosigma akashiwo* NIES-4, *Olisthodiscus luteus* NIES-15, *Gymnodinium mikimotoi* NIES-680, and *Fibrocapsa japonica* NIES-462 were obtained from the National Institute for Environmental Studies of Japan. All these HAB species were cultured aseptically in f/2 medium (Guillard's, Sigma) at 20 °C, 40 μmol/m^2/s using 40 W white fluorescent lamps (Toshiba) with 12L:12D cycle and subcultured after 30 d. Prior to the experiments, the HAB species were subcultured for 7 d.

Methanol extract solution (10 μl) and water extraction solution (40 μl) were added to each 1 ml of *H. akashiwo*, *O. luteus*, *G. mikimotoi*, and *F. japonica* cell suspension of a cell density of 3×10^4 cells/ml. After 4 h, the survivability and mortality of the cells were calculated under microscopic observation (×20). All experiments in this study were done separately at least in triplicate, and aseptic techniques were employed in all experimental steps. Algicidal activity was calculated using the formula: algicidal activity = (dead cells/living and dead cells) × 100%. All data in this study were tested by means of the ANOVA-test ($p < 0.05$).

Extraction and isolation of algicidal compounds from U. fasciata. U. fasciata collected at Makishima, Nagasaki Prefecture, Japan in July, 2004 (4.36 kg dry wt) was crushed into small pieces using a blender, and extracted twice with MeOH (10.5 L, 4 d and 12.5 L, 2 d). After removing the MeOH under reduced pressure, the residue, containing about 300 ml of water, was partitioned between water (700 ml) and hexane (1,200 ml). The hexane extract was concentrated, taken up in hexane (350 ml), and extracted with 4 M aqueous HCl (100 ml × 3) followed by 50% aqueous MeOH (150 ml × 2 and 300 ml). The hexane layer was died (Na_2SO_4) and concentrated to leave an deep green oily residue (12.95 g), which was chromatographed on silica gel (63–230 μm, 240 g) eluted with a gradient of hexane–EtOAc (9:1, 5:1, 1:1, then 0:1) to give four fractions (FL1–FL4). The active fraction, FL3 (2.35 g), eluted with hexane:EtOAc = 1:1, was then chromatographed on Octadecyl-S (50 μm, 38 g) eluted with 90% MeOH to give four fractions (FL3-1 to FL3-4). FL3-2 (319.4 mg) and FL3-3 (548.8 mg) were active. HPLC purification of FL3-2 (319.4 mg) (Cosmosil 5C18-MS-II, 20 × 250 mm, 80% CH_3CN, flow rate 5 ml/min) yielded compound **1** (73.5 mg). Part of FL3-3 (82 mg) was also separated by HPLC in the same manner to yield compound **1** (13.1 mg), compound **2** (21.4 mg), and compound **3** (22.1 mg).

*Compound **1** (hexadeca-4,7,10,13-tetraenoic acid).* NMR δ_H (400 MHz, $CDCl_3$): 0.97 (t, 3H, $J = 7.6$ Hz, $-CH_3$), 2.08 (m, 2H, $-CH_2-CH_3$), 2.41 (m, 4H, –CH=CH–CH_2–CH_2–COOH), 2.80–2.85 (m, 6H, –CH=CH–CH_2–CH=CH– × 3), 5.32–5.43 (m, 8H, –CH=CH– × 4), 6.10–6.33 (br, 1H, COOH). NMR δ_c (125 MHz, $CDCl_3$): 14.3 (CH_3CH_2–), 20.6 (CH_3CH_2–), 22.5 (CH=CH–CH_2–), 25.5 (CH=CH–CH_2–CH=CH), 25.6 (CH=CH–CH_2–CH=CH), 25.6 (CH=CH–CH_2–CH=CH), 33.8 (–CH_2–COOH), 127.1 (–CH=CH–), 127.5 (–CH=CH–), 127.9 (–CH=CH–), 127.9 (–CH=CH–), 128.4 (–CH=CH–), 128.6 (–CH=CH–), 129.6 (–CH=CH–), 132.1 (–CH=CH–), 178.3 (–COOH). IR ν_{max} (KBr) cm^{-1}: 3200–3400 (OH), 1713 (C=O), 712 (C=C). EIMS *m/z*: 248 (M^+), 226, 219, 205, 119, 108, 105, 93, 91, 79 (base), 67, 55. HRMS *m/z* (M^+): Calcd. for $C_{16}H_{24}O_2$: 248.1776, Found: 248.1768.

*Compound **2** (octadeca-6,9,12,15-tetraenoic acid).* NMR δ_H (400 MHz, $CDCl_3$): 0.97 (t, 3H, $J = 7.6$ Hz, $-CH_2CH_3$), 1.42 (quint., 2H, $J = 7.6$ Hz, 4-H), 1.61–1.71 (m, 2H, 3-H), 2.03–2.12 (m, 4H, –CH=CH–CH_2– × 2), 2.35 (t, 2H, $J = 7.6$ Hz, $-CH_2$–COOH), 2.77–2.87 (m, 6H, –CH=CH–CH_2–CH=CH– × 3), 5.28–5.43 (m, 8H, –CH=CH– × 4), 6.65–7.59 (br, 1H, COOH). NMR δ_c (75 MHz, $CDCl_3$): 14.4 ($-CH_3$), 20.6 ($-CH_2-CH_3$), 24.4 ($-CH_2-CH_2$COOH), 25.6 (CH=CH–CH_2–CH=CH), 25.7 (CH=CH–CH_2–CH=CH), 25.7 (CH=CH–CH_2–CH=CH), 26.9 (CH=CH–CH_2–CH_2–), 29.0 (CH=CH–CH_2–CH_2–), 33.8 (–CH_2–COOH), 127.0 (–CH=CH–), 127.8 (–CH=CH–), 128.0 (–CH=CH–), 128.2 (–CH=CH–), 128.2 (–CH=CH–), 128.5 (–CH=CH–), 129.5 (–CH=CH–), 132.0 (–CH=CH–), 178.8 (COOH). EIMS *m/z*: 276 (M^+, base), 247, 220, 207, 180, 161, 147, 135, 119, 108, 93, 79, 67, 55, 41. HRMS

Table 1. Initial Screening of Algicidal Activity (mortality, %) of Methanol (10 µl/ml) and Water Extract Solution (40 µl/ml) of Seaweeds from Nagasaki Beach (collected April–July 2004) on *H. akashiwo*, *O. luteus*, *G. mikimotoi*, and *F. japonica*

Data are the mean ± SD from at least three independent assays.

	H. akashiwo		*O. luteus*		*G. mikimotoi*		*F. japonica*	
Seaweed species	MeOH	Water	MeOH	Water	MeOH	Water	MeOH	Water
Chlorophyta								
Caulerpa sp.	10.1 ± 3.2	—	—	—	13.5 ± 2.8	—	15.9 ± 1.9	2.6 ± 1.1
Chaetomorpha crassa	6.2 ± 4.1	—	—	—	9.9 ± 2.4	—	—	—
Codium fragile	—	—	1.4 ± 1.3	—	6.7 ± 0.3	—	36.5 ± 4.1	—
Enteromorpha prolifera	23.9 ± 5.6	1.3 ± 0.5	2.0 ± 1.0	—	16.4 ± 0.9	—	39.7 ± 5.5	7.3 ± 2.6
Enteromorpha intestinalis	24.8 ± 0.8	3.1 ± 0.6	2.6 ± 1.5	—	11.2 ± 1.4	—	63.1 ± 2.0	16.5 ± 5.8
Enteromorpha sp.	8.5 ± 1.2	1.3 ± 0.5	2.3 ± 1.5	—	12.5 ± 2.1	—	34.1 ± 0.2	12.0 ± 2.1
Ulva conglobota	7.8 ± 3.4	—	—	—	10.0 ± 0.9	—	15.0 ± 2.0	2.8 ± 0.9
Ulva fasciata	93.3 ± 7.6	14.9 ± 3.6	75.2 ± 3.3	—	71.3 ± 1.6	—	100.0 ± 0.0	6.7 ± 5.8
Ulva pertusa	32.5 ± 3.5	1.9 ± 0.9	11.4 ± 1.1	—	10.1 ± 2.3	—	44.2 ± 5.5	2.1 ± 0.1
Urospora pencilliformis	—	—	—	—	16.9 ± 2.6	—	45.8 ± 2.8	—
Phaeophyta								
Colpomenia sinuosa	15.5 ± 1.5	1.6 ± 0.6	1.3 ± 0.6	—	12.2 ± 3.1	—	13.9 ± 1.5	9.2 ± 4.6
Cutleria cylindrica	10.9 ± 2.7	—	7.8 ± 1.0	—	—	—	11.6 ± 3.5	—
Dictyopteris prolifera	4.1 ± 0.6	—	3.4 ± 0.9	1.5 ± 0.4	1.3 ± 0.6	—	41.7 ± 2.9	—
Dictyota dichotoma	14.3 ± 5.9	—	8.0 ± 1.7	3.3 ± 0.5	55.8 ± 4.7	1.3 ± 0.3	87.4 ± 5.8	—
Dictyota linearis	—	—	2.5 ± 1.1	—	—	—	3.8 ± 1.2	—
Ishige foliacea	7.9 ± 2.5	—	—	—	11.4 ± 1.9	—	20.4 ± 3.0	12.7 ± 3.2
Laminaria japonica	—	—	—	—	—	—	24.0 ± 1.6	—
Padina crassa	16.8 ± 1.6	—	6.0 ± 2.4	—	17.7 ± 2.8	—	46.3 ± 3.9	—
Sargassum micracanthum	10.6 ± 2.8	—	1.6 ± 0.6	—	10.7 ± 1.5	—	22.7 ± 2.3	2.0 ± 1.0
Sargassum muticum	6.4 ± 3.1	—	58.3 ± 2.2	—	27.3 ± 3.0	—	53.8 ± 1.7	—
Sargassum siliquatrum	9.1 ± 1.3	2.1 ± 1.0	88.0 ± 8.1	—	19.6 ± 2.7	—	38.4 ± 2.0	—
Sargassum thunbergii	3.9 ± 1.0	—	—	—	7.5 ± 2.6	—	—	—
Undaria pinnatifida	1.8 ± 0.2	—	6.7 ± 1.4	1.3 ± 0.6	5.2 ± 1.4	—	33.1 ± 2.1	—
Rhodophyta								
Asparagopsis taxiformis	—	—	2.5 ± 1.0	—	—	—	46.1 ± 1.8	—
Bangia atropurpurea	4.2 ± 2.4	—	—	—	—	—	—	—
Corallina pilulifera	3.2 ± 1.5	1.5 ± 0.5	1.6 ± 0.6	—	9.5 ± 2.0	—	11.0 ± 1.0	2.6 ± 1.1
Demonema pulvinatum	8.6 ± 2.3	—	—	—	—	—	—	—
Gelidium japonicum	7.0 ± 3.7	—	—	—	—	—	—	—
Gracilaria chorda	7.1 ± 1.9	1.7 ± 0.9	3.7 ± 0.8	—	12.1 ± 1.8	—	46.6 ± 3.8	—
Gracilaria gigas	—	—	3.9 ± 0.3	—	—	—	—	—
Gracilaria vermiculophylla	7.9 ± 2.7	—	9.0 ± 1.0	—	19.8 ± 1.7	—	51.4 ± 6.0	1.3 ± 1.2
Grateloupia filicina	6.5 ± 1.5	—	—	—	12.7 ± 2.6	—	2.6 ± 1.4	—
Hypnea pannosa	2.0 ± 1.3	—	—	—	—	—	—	—
Plocamium telfairiae	6.4 ± 1.9	—	3.8 ± 1.7	0.9 ± 0.1	6.4 ± 1.4	—	33.0 ± 2.7	—
Porphyra yezoensis	4.8 ± 1.1	—	—	—	8.0 ± 1.0	—	9.5 ± 3.0	—
Pterocladia capillacea	8.7 ± 3.8	1.3 ± 0.6	1.0 ± 0.0	—	7.5 ± 2.9	—	2.4 ± 1.7	—
Symphyocladia marchantioides	—	—	3.4 ± 1.4	—	11.5 ± 1.3	—	11.1 ± 2.2	—

—, no activity.

m/z (M^+): Calcd. for $C_{18}H_{28}O_2$: 276.2089, Found: 276.2082.

Compound ***3*** *(α-linolenic acid).* NMR δ_H (400 MHz, $CDCl_3$): 0.98 (t, 3H, $J = 7.6$ Hz, $-CH_2CH_3$), 1.27–1.37 (m, 8H, $-CH_2- \times 4$), 1.59–1.67 (m, 2H, $-CH_2-CH_2-$COOH), 2.01–2.11 (m, 4H, CH=CH–$CH_2- \times 2$), 2.35 (t, 2H, $J = 7.2$ Hz, $-CH_2$–COOH), 2.77–2.85 (m, 4H, CH=CH–CH_2–CH=CH × 2), 5.28–5.43 (m, 6H, –CH=CH– × 3), 7.26 (br. s, 1H, –COOH). NMR δ_c (100 MHz, $CDCl_3$): 14.3 ($-CH_3$), 20.6 ($-CH_2-CH_3$), 24.8 (CH=CH–CH_2–CH=CH), 25.6 (CH=CH–CH_2–CH=CH), 27.2 ($-CH_2$–CH=CH), 29.1 ($-CH_2-CH_2$COOH), 29.1 ($-CH_2-$), 29.2 ($-CH_2-$), 29.6 ($-CH_2-$), 29.7 ($-CH_2-$), 34.0 ($-CH_2$–COOH), 127.0 (–CH=CH–), 127.6 (–CH=CH–), 128.1 (–CH=CH–), 128.2 (–CH=CH–), 130.1 (–CH=CH–), 131.9 (–CH=CH–), 180.6 (COOH). EIMS m/z: 278 (M^+, base), 228, 222, 108, 95, 79, 75, 67, 55. HRMS m/z (M^+): Calcd. for $C_{18}H_{30}O_2$: 278.2246, Found: 278.2229.

Results

Screening of seaweed

Thirty-seven species of seaweed distributed among the three major divisions of macro algae (Chlorophyta, Phaeophyta, and Rhodophyta) were collected in the coastal region of Nagasaki Prefecture, Japan, and their methanol and some water extracts were screened for algicidal activity against red-tide phytoplankton, *H. akashiwo*, *O. luteus*, *G. mikimotoi*, and *F. japonica* (Table 1). Ten species had activity over 10% on

H. akashiwo at a dose of 10 µl of extract/ml of medium, almost all of which converged in Chlorophyta and Phaeophyta. The activity of *U. fasciata* was remarkable among the 10 active seaweeds (significance, $p < 0.05$).

Isolation and characterization of algicidal principles of U. fasciata

The methanol extract of *U. fasciata* was partitioned into hexane-, 50% methanol-, and water-soluble fractions. The active hexane-soluble fraction was separated by silica gel chromatography, followed by reversed-phase (ODS) chromatography and finally reversed-phase HPLC monitoring the algicidal activity against *H. akashiwo* to give three active compounds, compounds **1** (13.1 mg), **2** (21.4 mg) and **3** (22.1 mg).

Compound **1**, obtained as a colorless oil, had the molecular formula $C_{16}H_{24}O_2$ determined by HREIMS at 248.1768 [M^+] (calcd. 248.1776), requiring 5 degrees of unsaturation. The characteristic IR absorptions showed the presence of a carbonyl group (1,713 cm^{-1}) and a hydroxyl group (3,200–3,400 cm^{-1}). The ^{13}C NMR spectrum, including the DEPT experiment, revealed the presence of eight sp^2 methines, six sp^3 methylenes, one methyl (δ 14.25), and one carbonyl carbon (δ 178.26), and the 5 degrees of unsaturation was fully accounted for by one carbonyl group and four olefins. These spectral data indicated the tetra-unsaturated fatty acid nature of the compound. The ^1H NMR spectrum showed an eight-proton olefinic multiplet at δ 5.322–5.426, which was coupled with a six-proton multiplet located at δ 2.797–2.853 due to three bis-allylic methylenes, indicating the presence of a skipped tetraene substructure of –CH=CH–CH_2–CH=CH–CH_2–CH=CH–CH_2–CH=CH–. In the ^1H–^1H COSY spectrum, the olefinic signal was additionally coupled with a two-proton multiplet δ 2.075, which in turn coupled with a three-proton triplet at δ 0.974 ($J = 7.6$ Hz), indicating that an ethyl group was attached at one end of the tetraene substructure. The remaining two methylenes appeared as overlapping multiplets at δ 2.410, which corresponds to the methylenes α to the carbonyl and α to the double bond. The two-methylene multiplets coupled with the olefin multiplet at δ 2.797–2.853 in the ^1H–^1H COSY spectrum, indicating that the other terminal was –CH_2–CH_2–COOH. From this spectroscopic evidence, compound **1** was determined to be hexadeca-4,7,10,13-tetraenoic acid. The geometry of the four double bonds was assigned on the basis of the ^{13}C chemical shifts of the bis-allylic methylene carbons. The chemical shift values of the three bis-allylic methylene carbons (δ 25.53, 25.58, and 25.60) assigned by HETCOR experiment were in the range characteristic of the methylene carbon between two *cis*-substituted olefins.[16–18] Thus the stereochemistry of the four double bonds was assigned to be all *cis*. This assignment was partially supported by the NOE experiment. In the NOESY spectrum, the overlapping bisallylic methylenes (δ 2.797–2.853) showed NOE correlation with the methylene of the terminal ethyl group (δ 2.075) or with the C-3 methylene (δ 2.410), indicating that the Δ4,5 and Δ13,14 double bonds had *cis*-geometry, but no information about the stereochemistry of the inner two double bonds was obtained from the NOESY spectrum due to overlapping of the three bisallylic methylene signals. The selected COSY and NOESY correlations are depicted in Fig. 2.

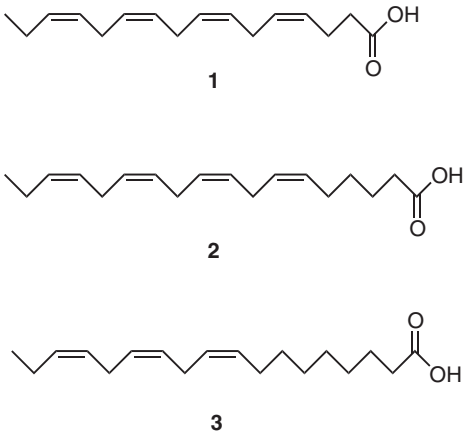

Fig. 1. Algicidal Unsaturated Fatty Acids Isolated from *Ulva fasciata.*

Compound **2**, obtained as a colorless oil, had the molecular formula $C_{18}H_{28}O_2$ determined by HREIMS at 276.2802 [M^+] (calcd. 276.2089), requiring five degrees of unsaturation. By comparison of its ^1H and ^{13}C NMR spectra with those of compound **1**, it was evident that compound **2** was a two-carbon homolog of compound **1**. The ^{13}C NMR spectrum showed two extra alkyl methylene signals at δ_c 24.35 and 29.04 ppm, which appeared as a four-proton multiplet at δ_H 2.03–2.12 ppm in its ^1H NMR spectrum. Thus compound **2** was identified as octadeca-6,9,12,15-tetraenoic acid (ODTA), which had been isolated as an allelopathic principle of the brown alga, *Cladisiphon okamuranus*. The spectroscopic data involving ^1H NMR and ^{13}C NMR were completely identical with the reported values.[11]

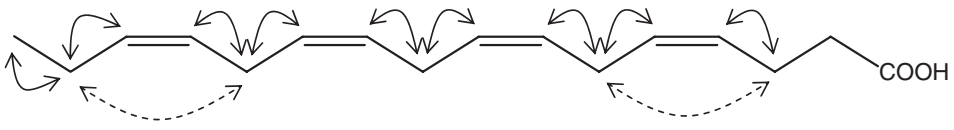

Fig. 2. Selected ^1H–^1H COSY (⟷) and NOESY (⇠⇢) Correlations of Compound **1**.

Compound **3**, obtained as a colorless oil, had the molecular formula $C_{18}H_{30}O_2$ determined by HREIMS at 278.2229 [M^+] (calcd. 278.2246). Regarding its degrees of unsaturation (four) and comparing its NMR spectra with those of compound **2**, compound **3** was immediately deduced to be α-linolenic acid. In fact, the ^{1}H NMR spectrum of compound **3** was completely superimposed with that of commercial α-linolenic acid.

Discussion

In the present study, the algicidal principles of *U. fasciata* were found to be polyunsaturated fatty acids (PUFAs) such as octadeca-6,9,12,15-tetraenoic acid (ODTA), α-linolenic acid, and hexadeca-4,7,10,13-tetraenoic acid (HDTA), one of which (ODTA) was the same compound that had been isolated as the allelopathic substance of *Cladosiphon okamuranus*. These PUFAs are commonly found in seaweeds, especially green algae.[19,20] Other research has also reported that the green algae of *Chlorophyta*, such as *U. pertusa*, *Monostroma lattisium*, and *Enteromorpha* sp. obtained in Japanese waters, contain large amounts (63% to 72% of total lipids) of PUFAs composed mainly of 16:4(n-3), 18:3(n-3), and 18:4(n-3).[21] Dose-response curves of isolated HDTA, ODTA, and α-linolenic acid as well as commercial ODTA and α-linolenic acid are shown in Fig. 3. Commercial ODTA and α-linolenic acid showed the same levels of activity as corresponding natural compounds. The LC_{50} values of ODTA, α-linolenic acid, and HDTA on *H. akashiwo* calculated by regression analysis were 0.83, 1.13, and 1.35 µg/ml respectively. *H. akashiwo* cells treated with these PUFAs become swollen with time and finally caused rupture, as observed for the allelopathic PUFAs of *Cladosiphon okamuranus* against *H. akashiwo*.[11] Although the detailed mechanism of action is unknown, it is supposed that these amphiphatic molecules interact with the cell membrane of *H. akashiwo* and disrupt osmolarity regulation. Interestingly, however, the saturated fatty acid, palmitic acid, isolated from an active fraction during the isolation process, showed quite low algicidal activity (2.5% at 20 µg/ml). This indicates that the polyunsaturated nature of the active compounds plays an important role in the algicidal activity. Marine microalgae have also been reported to be a rich source of PUFAs. *H. akashiwo* itself was reported to contain the PUFAs, predominantly n-3 PUFA, up to 59.1% of total fatty acids.[22] Recently, other researchers have isolated three hemolytic PUFAs, ODTA, 5,8,11,14,17-eicosapentanoic acid (EPA), and 5,8,11,14-eicosatetraenoic acid (AA), from the ichthyotoxic microalga *F. japonica*.[23] The researchers assumed that the PUFAs are part of a defense strategy that rapidly converts an essential cell constituent into a highly toxic grazer toxin. In the marine plant kingdom, the PUFAs might be commonly used as allelochemicals to inhibit the growth of competing microalgae.

The main factors generally considered when selecting harmful algal bloom control methods are effectiveness, toxicity, cost, and practicability. The effect of algicidal PUFAs of *U. fasciata* appeared rather immediately: all these compounds killed all *H. akashiwo* cells in less than 1 h at 2.0 µg/ml (Fig. 4). Thus, this method has an

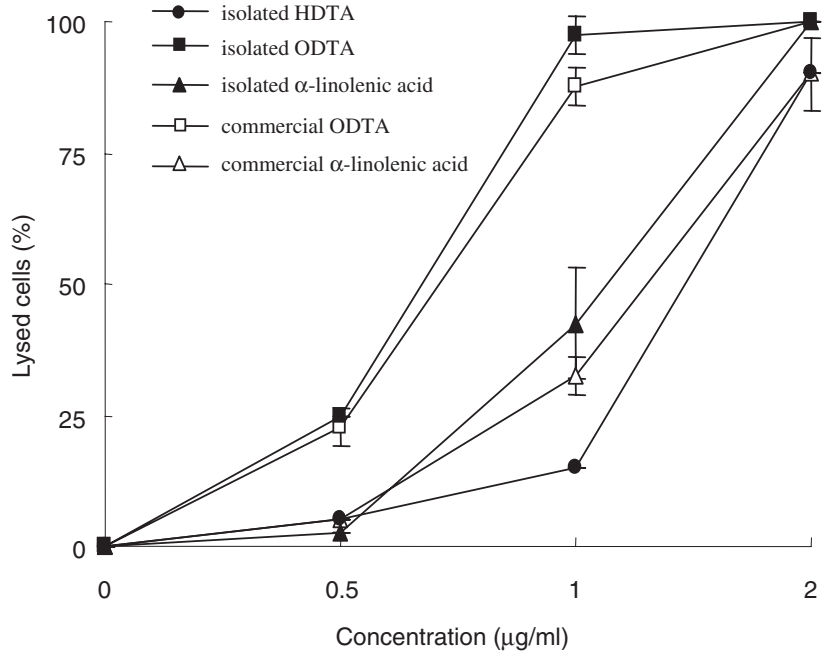

Fig. 3. Lysis of *Heterosigma akashiwo* Cells by Natural HDTA (●), ODTA (■) and α-Linolenic Acid (▲), and Commercial ODTA (□) and α-Linolenic Acid (△), Determined at 4 h after Treatment.
Bars indicate standard errors (n = 3).

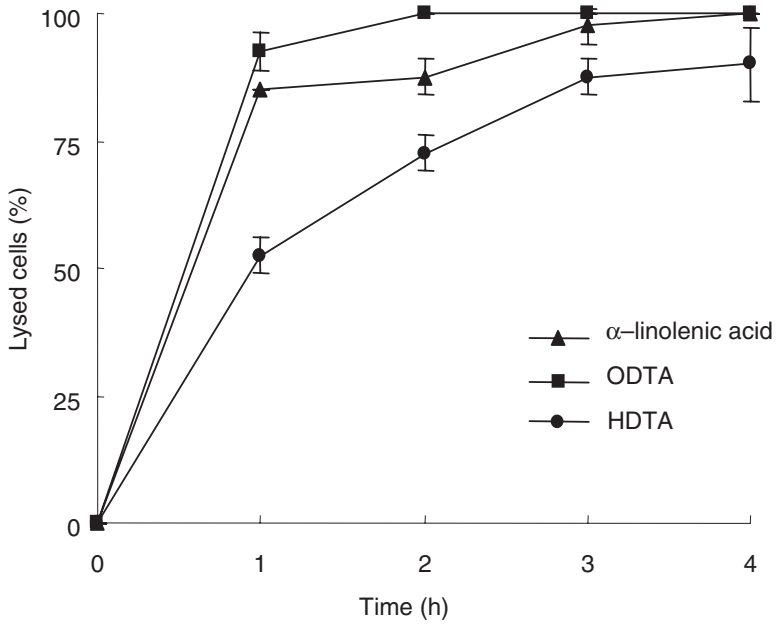

Fig. 4. Time Course of Algicidal Activity of HDTA, ODTA, and α-Linolenic Acid on *Heterosigma akashiwo* at 2 µg/ml. Bars indicate standard errors (n = 3).

advantage over bacterial methods, which need at least several days to kill or inhibit the growth of microalgae. Taking into account the characteristics described so far, the algicidal compounds of *U. fasciata* are promising for controlling HABs because (1) they have a high inhibition effect on the harmful algae tested, especially *H. akashiwo*, without recovery at a relatively low concentration, (2) they have high biodegradability, ecological acceptability, and a relatively environmentally benign nature, and (3) their source, *U. fasciata*, is distributed widely in nature and is readily collectable. Study toward development of a practical method for controlling HAB using the extract or the whole body of *U. fasciata* is ongoing in our laboratory.

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