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Aliphatic sulfates released from *Daphnia* induce morphological defense of phytoplankton: isolation and synthesis of kairomones

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Abstract—Six aliphatic sulfates, kairomones released from a crustacean *Daphnia pulex* induce morphological changes of phytoplankton *Scenedesmus gutwinskii* at ppb (10^{-9} g/mL) concentrations.

In a series of food chain in aquatic world, phytoplankton is the bottom creature that supports the lives of animals. It has been soundly believed that phytoplankton is docile and never resists against its fate. This belief has been disputed by Hessen and van Donk, who reported that a unicellular green alga, *Scenedesmus subspicatus*, achieved morphological change into 2-, 4-, and 8-coenobia (colonies) when the water in which a crustacean *Daphnia magna*, a grazer of the alga, had been cultured (*Daphnia* water) was added to the cultivation medium.[1] They also found that the grazing rate of the coenobium morph was lower than that of the unicellular morph, owing to the increased size of the former. This metamorphosis was supposed to be a self-defense mechanism acquired by the green alga and triggered by a kairomone secreted from *D. magna*. Their report has aroused the interest of many scientists to attempt to identify the kairomone,[2–10] although the chemical structure has not been disclosed thus far.

Here we report identification of the *Daphnia* kairomones that cause the morphological change in a unicellular green alga *Scenedesmus gutwinskii* var. *heterospina* (NIES-802) at 10^{-1}–10^{3} ng/mL concentrations (Fig. 1).

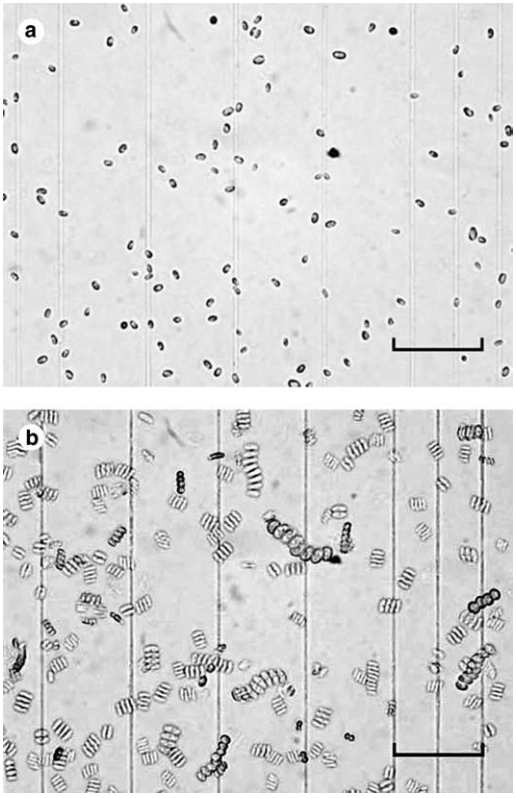

Figure 1. Microscopic (×200) pictures of *S. gutwinskii*. (a) Control (10 days cultivation without active sample). (b) Ten days after the addition of active compound **4** (1 ng/mL). The scales are 50 μm.

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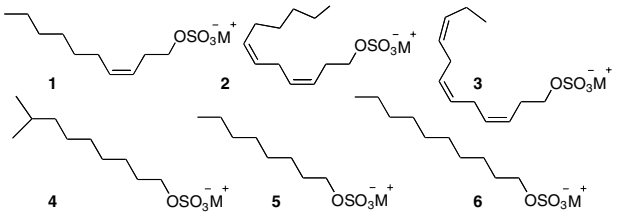
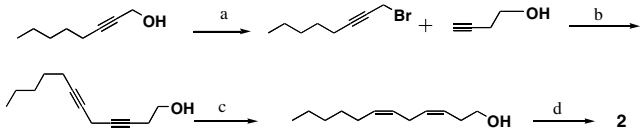


In our experiment, we selected commercially available frozen *Daphnia* (*Daphnia pulex*) rather than *Daphnia* water itself as a starting material, because the concentration of the kairomone in the latter seemed too low to allow elucidation of its chemical structure. Frozen *Daphnia* (10 kg; Aso Tropical Fish Co. Ltd, Osaka) was soaked with methanol (20 L × 3), and the methanol solution was evaporated, the residue being treated with water (9 L). The mixture was successively extracted with hexane, dichloromethane, and butanol, and the most active butanol extract was separated by HPLC monitoring the activity using a newly introduced bioassay system (see Bioassay). The activity (A) was expressed by the equation $A = N_t/N_c$, where N_t and N_c correspond with total number of cells (e.g., 1-cell; 1, 8-cell; 8) and number of coenobium (e.g., 1-cell; 1, 8-cell; 1), respectively. The active substances isolated at first were **1**,[11,17] **2**,[17] and **6**.[12] While *Daphnia* water (10% dilution) showed A = 3.5–3.8, the activities of **1**, **2**, and **6** were A = 4.2, 4.2, and 3.0 at 10 ng/mL, respectively. To rule out the possibility that the isolated substances are inactive and they might still be contaminated with a minute amount of 'super active compound', compounds **1** and **2** were synthesized. [Compound **1**: catalytic hydrogenation of 3-decynol (Lindler catalyst) followed by sulfation (pyridine–SO_3). Compound **2**: see Scheme 1.]

The activities of synthetic **1** and **2** decreased to A = 2.8–3.1 at 10 ng/mL, indicating that these substances contained more active components. Further separation of the fraction composed of **1** and **2** afforded triene and alkyl sulfates, **3**,[17] **4**,[17] and **5**,[12] the activities of which were A = 2.2 at 100 ng/mL, 4.2 at 1 ng/mL, and 4.3 at 10 ng/mL, respectively. Synthetic **4** [LAH reduction of methyl 8-methylnonanoate, followed by sulfation (SO_3-pyr)] exhibited the same activity (A = 4.2 at 1 ng/mL, A = 3.4 at 0.1 ng/mL) (Fig. 2).

Figure 1 exhibits the effect of compound **4** on the morphology of *S. gutwinskii*. Figure 1a shows the control (cultivated for 10 days without active sample), in which every alga exists as a 1-cell type. When **4** is added to the cultivation medium (1 ng/mL) and the alga is cultured for



Scheme 1. Reagents: (a) CBr_4, Ph_3P, quant; (b) K_2CO_3, NaI, CuI, DMF,[13] 20%; (c) H_2,quinoline, Pd–$BaSO_4$, MeOH, quant; (d) SO_3–pyr, THF, 47%.

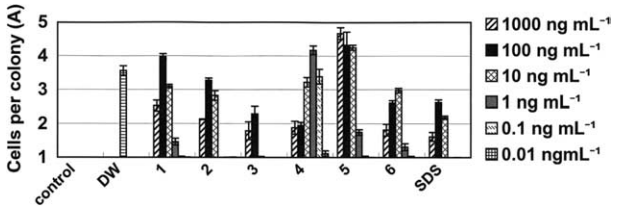


Figure 2. Activities ($A = N_t/N_c$) of sulfates **1**–**6**. DW indicates *Daphnia* water (diluted; 10%, v/v). SDS = sodium dodecyl sulfate. Sulfates **1**, **2**, and **4** are synthetic materials (Na salts), and **5** and **6** (Na salts) are commercial products. All results are expressed as means ± s.d. for triplicate determinations.

10 days, dramatic change of the algal form is observed as shown in Figure 1b, in which most of the alga exist as 2-, 4-, and 8-cell types.

It is notable that the aliphatic (unsaturated and saturated) sulfates are produced by a crustacean *Daphnia* in relatively large amounts, **1**; 6.4 mg, **2**; 0.4 mg, **3**; 0.7 mg, **4**; 0.2 mg, **5**; 0.2 mg, **6**; 3.2 mg per 100 g of the frozen *Daphnia*. It is unlikely that **5** and **6** (common surfactants) are contaminants from the environment, because (1) their contents are so high for environmental pollutants accumulated in the *Daphnia* body, and (2) the concomitant **1**, **2**, **3**, and **4** must be the genuine natural products, obvious from their unusual structures.

Attempts to detect the active substances (transparent above 210 nm) in *Daphnia* water were futile because of their extremely low concentrations and existence of huge amounts of contaminants. The 'methylene blue method',[14] a quantitative analysis of environmental anion surfactants, was therefore applied to the *Daphnia* water. The concentration of total anion surfactants was determined to be 8 ng/mL, which was large enough to cause the morphological changes of the microalga.

The active substances obtained in this study are closely related to commonly used anion surfactants. Therefore, activity of sodium dodecyl sulfate (SDS) and sodium dodecylbenzenesulfonate (LAS), the representative detergents, was tested. Although LAS was inactive (1000–0.01 ng/mL), SDS showed moderate activity (A = 2.2 at 10 ng/mL) (Fig. 2). The fact that SDS is active at extremely low concentration may stimulate ecological controversy on the usage of this famous detergent.

Lürling and Beekman reported that *artificial* anion surfactants extractable from membrane filters caused metamorphosis of *Scenedesmus obliquus*.[15]

The present findings seem to be closely related to the aggregation phenomenon of the algal mono-cell recently reported for the Ulvales macro green algae.[16]

Bioassay: Each 200 μL of C medium of *S. gutwinskii* (5.0×10^2 cells/mL) is delivered into the central 30–50 wells of 96- well polystyrene tissue culture plate (CELLSTAR, Greiner Bio-one Co., Ltd) containing the test samples (1000–0.01 ng/mL), and the outer wells are filled

with distilled water to avoid dehydration of the system. The plate is covered with a plastic lid, and incubated at 20 °C (12 light/12 dark) for 10 days. A drop of the medium is placed on a Thoma's hemacytometer, and the numbers of 1-, 2-, 4-, and 8-cell types were counted under a microscope (×200).

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17. *Experimental data:* (The structures of the compounds were established by COSY, HSQC, HMBC, and NOESY spectra. The countercation of natural sulfates was not identified and expressed as M^+.)
Compound **1**: ^{1}H NMR (400 MHz, CD_3OD) δ 5.53 (1H, dt, J = 11, 7 Hz; H-4), 5.44 (1H, dt, J = 11, 7 Hz; H-3), 4.00 (2H, t, J = 7 Hz; H-1), 2.46 (2H, q, J = 7 Hz; H-2), 2.11 (2H, q, J = 7 Hz; H-5), 1.38 (2H, overlapped, H-6), 1.34 (6H, envelop, H-7, 8, 9), 0.94 (3H, t, J = 7 Hz; H-10). NOESY cross-peaks were observed between H-2/H-5 and H-3/H-4. (Underlined coupling constants and NOEs support the *Z*-configuration of the olefin.) ^{13}C NMR (100 MHz, CD_3OD): δ 133.4 (C-4), 125.6 (C-3), 68.6 (C-1), 32.9 (C-8), 30.7 (C-6), 30.0 (C-7), 28.5 (C-2), 28.2 (C-5), 23.7 (C-9), 14.4 (C-10). (Underlined chemical shifts of allylic carbons support the *Z*-configurations of the olefins.) FABHRMS(−): *m/z* 235.1017 (calcd for $C_{10}H_{19}O_4S$: 235.1004).
Compound **2**: ^{1}H NMR (400 MHz, CD_3OD): δ 5.47 (1H, dt, J = 11, 7 Hz; H-4), 5.45 (1H, dt, J = 11, 7 Hz; H-3), 5.42 (1H, dt, J = 11, 7 Hz; H-7), 5.36 (1H, dt, J = 11, 7 Hz; H-6), 4.02 (2H, t, J = 7 Hz; H-1), 2.86 (2H, t, J = 7 Hz; H-5), 2.49 (2H, q, J = 7 Hz; H-2), 2.12 (2H, q, J = 7 Hz; H-8), 1.40 (2H, overlapped; H-9), 1.37 (4H, envelop; H-10, 11), 0.96 (3H, t, J = 7 Hz; H-12). The underlined coupling constants support the *Z*-configurations of the two olefins. NOESY cross-peaks between H-2/H-5 and H-5/H-8 are consistent with the *Z*-configurations. ^{13}C NMR (100 MHz, CD_3OD): δ 131.7 (C-4), 131.3 (C-7), 128.7 (C-6), 125.9 (C-3), 68.4 (C-1), 32.6 (C-10), 30.5 (C-9), 28.6 (C-2), 28.1 (C-8), 26.6 (C-5), 23.6 (C-11), 14.4 (C-12). (Underlined chemical shifts of allylic carbons support the *Z*-configurations of the olefins.) FABHRMS(−): *m/z* 261.1150 (calcd for $C_{12}H_{21}O_4S$: 261.1161).
Compound **3**: ^{1}H NMR (400 MHz, CD_3OD): δ 5.45–5.54 (2H, m; H-3,4), 5.30–5.45 (4H, m; H-6,7,9,10), 4.03 (2H, t, J = 7 Hz; H-1), 2.90 (2H, t, J = 5 Hz; H-5), 2.86 (2H, t, J = 5 Hz: H-8), 2.50 (2H, q, J = 7 Hz; H-2), 1.01 (2H, t, J = 7 Hz; H-12). NOESY cross-peaks between H-2/H-5 and H-8/H-11 lead to the *Z*-configurations of 3- and 9-olefins. ^{13}C NMR (100 MHz, CD_3OD): δ 132.8 (C-10), 131.4 (C-4), 129.5 (C-7), 128.8 (C-6), 128.2 (C-9), 126.2 (C-3), 68.4 (C-1), 28.6 (C-2), 26.6 (C-5), 26.4 (C-8), 21.4 (C-11), 14.6 (C-12). The underlined chemical shifts of the allylic methylene carbons support the *Z*-configurations of the three olefins. HMBC cross-peaks to/from; C-3/H-1,2,5, C-4/H-2,5, C-6/H-4, C-9/H-8,11, C-10/H-8,11,12. FABHRMS(−): *m/z* 259.0096 (calcd for $C_{12}H_{19}O_4S$: 259.1004).
Compound **4**: ^{1}H NMR (400 MHz, CD_3OD): δ 4.03 (2H, t, J = 7 Hz; H-1), 1.70 (2H, quint, J = 7 Hz; H-2), 1.57 (1H, nonet, J = 7 Hz; H-8), 1.50–1.40 (2H, m; H-3), 1.47–1.30 (6H, envelop; H-4,5,6), 1.22 (2H, m; H-7), 0.92 (6H, d, J = 7 Hz; H-9,10). ^{13}C NMR (100 MHz, CD_3OD): δ 69.1 (C-1), 40.2 (C-7), 31.0 (C-5), 30.5 (C-2), 30.4 (C-4), 29.2 (C-8), 28.5 (C-6), 26.9 (C-3), 23.1 (C-9,10). FABHRMS(−): *m/z* 237.1157 (calcd for $C_{10}H_{21}O_4S$: 237.1166).