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Time-of-Flight LC/MS Identification and Confirmation of a Kairomone in *Daphnia magna* Cultured Medium

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Daphnia kairomones induce morphological change to green alga. An active compound (8-methylnonyl sulfate), which was originally isolated and determined from *Daphnia pulex* body, was identified from a cultured medium of *Daphnia magna* by time-of-flight liquid chromatography/mass spectrometry with electrospray ionization after concentration by the Methylene Blue method.

A kairomone is a chemical substance released by an organism that affects other organisms in a food chain series. Hessen and Van Donk reported that a unicellular green alga achieved morphological change into 2-, 4-, and 8-colonies when the water was cultured with *Daphnia*.[1] However, neither isolation nor elucidation of active compounds has been completed due to the very low concentration of the compounds in the cultured medium.[2] A unique approach was tried: the active compounds were isolated from commercially available frozen *Daphnia pulex* body (10 kg) and the structure of the compounds determined with a combination of purification, chemical synthesis, and bioassay.[3,4] The synthesized aliphatic sulfates undoubtedly showed activity to induce morphological changes of phytoplankton at an optimum concentration of low ppb (10^{-6} g L^{-1}). Because the *Daphnia* kairomones are anion surfactants, they are quantitatively detected with the Methylene Blue method.[5] The concentration of total anion surfactants in *Daphnia* cultured medium was determined at 8.0×10^{-6} g L^{-1}. However, this method quantified only the total amount of the surfactants and each active compound was not chemically identified.[3,4] The absolute configuration of the aliphatic sulfates was elucidated by the same authors.[6]

The active compounds were isolated from *D. pulex*, but *D. magna* was used for the assay. The frozen *D. pulex* is commercially available, however, *D. magna* is larger in size than *D. pulex* and easy to assay albeit difficult to cultivate at kg-scale. It is possible for each species to release different compounds. Consequently, we report identification and confirmation of kairomones in *D. magna* cultured medium.

Electrospray ionization (ESI) in negative ion mode is best matched for these compounds because all target sulfates ($R–OSO_3^-M^+$) or amidosulfates ($R–NHSO_3^-M^+$) ionize well and can easily dissociate $R–OSO_3^-$ or $R–NHSO_3^-$ in aqueous solution respectively. Time-of-flight liquid chromatography/mass spectrometry (TOF LC/MS)[7] benefits from the increased identification capability of compounds in comparison to quadrupole analyzer due to its accurate mass measurement capability within 3 ppm mass error.[8]

Therefore, in this study, we chose TOF LC/MS with ESI to directly detect and identify the active compounds in the *D. magna* cultured medium.

Six sulfates (**1**, **2**, **3**, **5**, **6**, and **7**)[9,10] and one amidosulfate (**4**)[10,11] were separated by using the volatile buffered mobile phase (ammonium acetate and acetonitrile) for LC/MS, even though **2** and **3**, **5** and **6** were isomers. Analysis time was substantially reduced to less than 10 min by using a short column (50 mm) packed with small size particles (3.5×10^{-6} m). Automatic continuous mass-axis correction with the two known reference compounds gives extremely accurate mass. This provides fewer potential empirical formulas not only for the synthetic compounds but also the unknown in the *Daphnia* cultured medium.

To yield both deprotonated molecule and the m/z 97 fragment ($HOSO_3^-$), in source collision-induced dissociation (CID) was used. By setting the MS fragmentor to 250 V, familial fragments (m/z 97) in the mass spectra of all the aliphatic sulfates were observed together with each deprotonated molecule. Mass chromatogram of the m/z 97 fragment is selective indicator of the targets as shown in Fig. 1. The measured mass error of deprotonated molecule ($[M - H]^-$) in each standard compound is less than 0.4 mDa.[12]

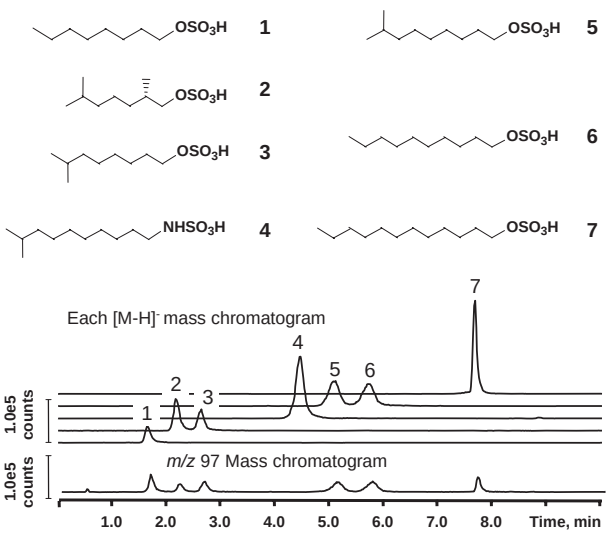

Fig. 1. Structures and mass chromatograms for the $[M - H]^-$ ions of authentic standards and the familial fragment ion for sulfate.

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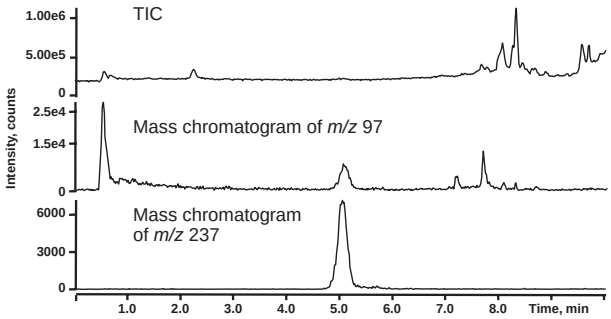

Fig. 2. Total ion chromatogram (TIC) and mass chromatogram of *Daphnia* cultured medium.

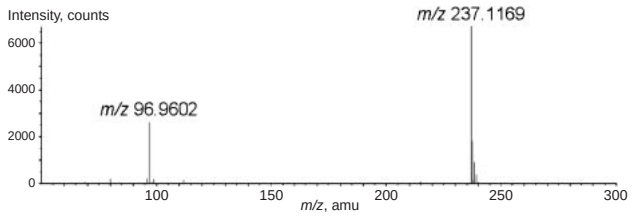

Fig. 3. Mass spectrum of the peak at 5.1 min in the *Daphnia* cultured medium in Fig. 2.

D. magna cultured medium was concentrated by the Methylene Blue method, and subsequently the Methylene Blue reagent was removed with cation-exchange resin. The method was modified to effect a 200× concentration of the Methylene Blue complex with anion in an organic layer. The concentrated cultured medium was analyzed by TOF LC/MS with the ESI source under the same condition.

The mass chromatogram of the m/z 97 fragment ion is a selective indicator of sulfate targets, especially useful for identification of compounds containing sulfate in complex matrices as in the cultured medium (Fig. 2). The retention time of the mass chromatogram of both m/z 97 and 237 in Fig. 2 matches that of standard **5** in Fig. 1.[12] This strongly suggests the cultured medium contains compound **5**.

Using the mass spectral data described below, **5** was identified and confirmed in the *D. magna* cultured medium. Accurate mass measurement of the deprotonated molecule ($[M - H]^-$ in negative ion mode) can give both the molecular weight of the compound and its empirical formula. Low level error has significant implications when trying to propose possible empirical formulas of unknowns. Actually, at 10 ppm accuracy, m/z 237.1169 (Fig. 3) provides only 3 possible empirical formulas with the elemental composition restricted to combinations of $C_{0–20}$, $H_{0–45}$, $N_{0–5}$, $O_{0–5}$, and $S_{0–5}$.[13] Accurate mass measurement of fragment ions also provides the atoms that those portions of the molecule contain (valuable structural information). At 10 ppm accuracy, m/z 96.9602 provides only one possible empirical formulas with the same condition described above.[14]

A calibration curve of the active compound **5** was drawn using the area of the mass chromatogram from m/z 237.10 to m/z 237.12 (Fig. 4).

The detected and confirmed 8-methylnonyl sulfate (**5**) is similar to one of the commonly used surfactants, sodium dodecyl sulfate (SDS, sodium salt of **7**). All other active kairomone compounds described here also behave as surfactants

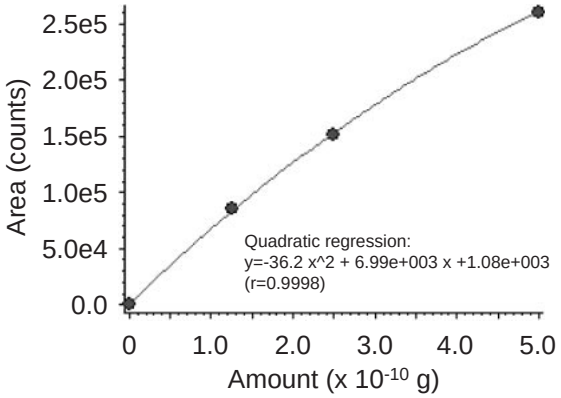

Fig. 4. Calibration curve of 8-methylnonyl sulfate (**5**).

due to both polar and non-polar sites in the molecule. Large amount of surfactants have been produced as detergents and partially released to the environment. Thus, it is a concern that environmentally released concentrations of surfactants acting as the kairomone would indirectly confuse the food chain in lakes and marshes and cause significant ecological disruption.

The kairomone is identified by the combination of TOF LC/MS with ESI with sample preparation by the simple Methylene Blue method. The identification is confirmed by comparison of the retention time and mass spectrum of the synthetic standard compound with those of the actual sample. The mass error is 0.3 mDa between actual sample result and calculated m/z. The method needs no derivatization and shows low background due to using ESI in negative ion mode. Target compounds including SO_4 were selectively screened by an MS instrument parameter (fragmentor voltage) adjustment. A fully automated introduction system of reference compounds for mass axis calibration gives very stable and reliable mass accuracy results.

Although the presence of other kairomone compounds may be assumed, this is the first direct chemical detection of the *Daphnia* kairomone from a cultured medium. Metamorphosis of *Scenedesmus obliquus* by artificial anion surfactants extractable from membrane filters was reported.[15] Phenomenon we found would be similar to a recent report of the aggregation phenomenon of the algal mono-cell for the Ulvales macro green algae.[16]

Experimental

LC/MS-grade acetonitrile and JIS (Japanese Industrial Standard) special grade ammonium acetate was obtained from WAKO Pure Chemical Industries (Osaka, Japan). A Milli-Q Gradient ultra-pure water system from Millipore (Billerica, MA, USA) was used throughout the study to obtain the HPLC-grade water used for the analyses.

The separation was carried out using an HPLC system consisting of a vacuum degasser, an autosampler and a binary pump (Agilent Series 1100, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed-phase C18 analytical column with dimensions of 50 mm × 2.1 mm and 3.5×10^{-6} m particle size (ZORBAX Eclipse® XDB-C18). Column temperature was maintained at 40 °C. Mobile phases A and B were 10 mM ammonium acetate aqueous solution and acetonitrile respectively. A binary gradient elution was made as follows: isocratic conditions for 3 min at 30% of solvent B, linear gradient from 30 to 95% of

solvent B from 3 to 8 min, then isocratic conditions for 4 min at 95% of solvent B. The flow rate used was kept at 0.3 mL min^{-1} and 1.0×10^{-5} L of sample was injected in each experiment of the study.

This HPLC system was interfaced to an Agilent 6210 LC/MS time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a dual electrospray interface operated in negative ion mode using the following operation parameters: capillary at 5000 V; nebulizer pressure at 3.5×10^5 Pa; drying gas at 10 L min^{-1}; gas temperature of 350 °C; fragmentor voltage (collision-induced dissociation voltage) at 250 V. The instrument performed the internal mass calibration automatically and constantly, using the second electrospray nebulizer with an automated calibrant delivery system that introduced a low flow of a calibrating solution containing the internal reference mass compounds (m/z 112.9856 and 1033.9881). The instrument software constantly corrects the measured masses of all the spectra using the known masses as reference. LC/MS accurate mass spectra were recorded across the range m/z 50–1100. The full-scan data recorded was processed with Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MS TOF software.

Five liter of *D. magna* cultured medium (250 adult bodies/L dechlorinated tap water, 1 week) was concentrated to 0.1 L, and was treated by the Methylene Blue method. Subsequently the Methylene Blue reagent was removed by cation-exchange resin (DOWEX 50WX8-100). The concentrated sample was dried and dissolved in 25 mL of Milli-Q water for LC/MS analysis. When treatment was complete, the cultured medium was concentrated to 200 times of the original volume. Authentic standards, shown in note 12 (each at 1.0×10^{-4} g L^{-1}, except for **4** because it was isolated from the cultured medium and no authentic standards was obtained), and the concentrated cultured medium sample were analyzed by TOF LC/MS with the ESI source under the same condition.

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9 Compounds **1**, **6**, and **7** were commercially available from Sigma-Aldrich Japan K.K. (Tokyo, Japan), Kanto Chemical, Co., INC. (Tokyo, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. Compounds **2**, **3**, and **5** were synthesized.[3,4,6]

10 The counter cation of natural sulfates and amidosulfate was not identified in aqueous solution and expressed as H.

11 Compound **4** was isolated from the cultured medium.[3,4]

12 Experimental data: Each retention time and $[M - H]^-$ Compound **1**: 1.7 min, m/z 209.0857 (calcd for $C_8H_{17}O_4S$: 209.0853), Compound **2**: 2.2 min, m/z 223.1011 (calcd for $C_9H_{19}O_4S$: 223.1009), Compound **3**: 2.7 min, m/z 223.1013 (calcd for $C_9H_{19}O_4S$: 223.1009), Compound **4**: 4.5 min, m/z 250.1484 (calcd for $C_{11}H_{24}NO_4S$: 250.1482), Compound **5**: 5.1 min, m/z 237.1167 (calcd for $C_{10}H_{21}O_4S$: 237.1166), Compound **6**: 5.7 min, m/z 237.1164 (calcd for $C_{10}H_{21}O_4S$: 237.1166), Compound **7**: 7.7 min, m/z 265.1481 (calcd for $C_{12}H_{25}O_4S$: 265.1479).

13 Accuracy of 10 ppm for m/z 237.1169 provides three possible empirical formulas with elemental composition restricted to combinations of $C_{0–20}$, $H_{0–45}$, $N_{0–5}$, $O_{0–5}$, and $S_{0–5}$: $C_{10}H_{21}O_4S$ (error 0.3 mDa), $C_{11}H_{17}N_4S$ (error −1.0 mDa) and $C_{14}H_{13}S$ (error 2.3 mDa).

14 Accuracy of 20 ppm for m/z 96.9602 provides three possible empirical formulas with elemental composition restricted to combinations same as note 13: HO_4S (error 0.1 mDa), C_4HOS (error −15 mDa), and HO_2S_2 (error 18 mDa).

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