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

Application

Natural Product Chemistry

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Abstract

***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 liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS)with electrospray ionization after concentration by the Methylene Blue method.**

Introduction

A pheromone is a chemical substance that triggers a variety of behavioral responses in another member of the same species. On the other hand, a kairomone is a chemical substance released by an organism that affects other organisms in a food chain series. It was reported that a unicellular green alga achieved morphological change into 2-, 4- and 8-colonies when the water was cultured with *Daphnia*. However, neither isolation nor elucidation of active compounds has been completed due to the very low concentration of the compounds in the cultured medium. 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. The synthesized aliphatic sulfates undoubtedly showed activity to induce morphological changes of phytoplankton at an optimum concentration of low ppb (10^{-6} g/L). Because the *Daphnia* kairomones are anion surfactants, they are quantitatively detected with the Methylene Blue method [1]. The concentration of total anion surfactants in *Daphnia* cultured medium was determined at 8.0 ppb. However, this method quantified only the total amount of the surfactants and each active compound was not chemically identified.

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 [2].

It is actually impossible to detect these aliphatic sulfates directly using HPLC with commonly used detectors such as ultraviolet absorption or fluorescence. As for LC/MS, electrospray ionization (ESI) in negative ion mode is best matched for these

compounds because all target sulfates ($R\text{-}OSO_3^-M^+$) or amidosulfates ($R\text{-}NHSO_3^-M^+$) ionize well and can easily dissociate to $R\text{-}OSO_3^-$ or $R\text{-}NHSO_3^-$ in aqueous solution, respectively. LC/TOF-MS benefits from the increased identification capability of compounds in comparison to a quadrupole analyzer due to its accurate mass measurement capability.

Therefore, in this study we chose LC/TOF-MS with ESI to directly detect and identify the active compounds in the *D. magna* cultured medium. Furthermore, the orthogonal spray position of this LC/MS ion source resists ion suppression from the sample even though it contains significant matrix components.

Experimental

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. Compound 4 was isolated from the cultured medium.

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.

Five liters of *D. magna* cultured medium (250 adult bodies per liter dechlorinated tap water, 1 week) was concentrated to 100 mL and 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 Table 1 (each at 100 ppb, except for 4, because it was isolated from the cultured medium and no authentic standard was obtained), and the concentrated cultured medium sample were analyzed by LC/ TOF-MS with the ESI source under the same conditions.

LC/MS Method Details

LC Conditions	
Instrument:	Agilent LC 1100
Column:	ZORBAX Eclipse XDB-C18 50 mm × 2.1 mm 3.5 µm (p/n 971700-902)
Column temp.:	40 °C
Mobile phase:	A: 10 mM ammonium acetate aqueous solution B: acetonitrile
Gradient and flow rate:	30% B at 0 min (0.3 mL/min) 30% B at 3 min (0.3 mL/min) 95% B at 8 min (0.3 mL/min) 95% B at 8.1 min (0.5 mL/min) 95% B at 12 min (0.5 mL/min) 30% B at 12.1 min (0.5 mL/min) 30% B at 17 min (0.5 mL/min)
Injection volume:	10 µL
MS Conditions	
Instrument:	Agilent 6210 TOF LC/MS
Source:	Negative ESI
Drying gas flow:	10 L/min
Nebulizer:	350 kPa
Drying gas temp.:	350 °C
V_{cap}:	5000 V
Fragmentor:	250 V
Scan mode:	*m/z* 50-1100, 10,000 transitents/scan, 0.89 scan/sec.
References mass:	*m/z* 112.9856 and 1033.9881

Results and Discussion

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

To yield both the 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. The extracted ion mass chromatogram of the *m/z* 97 fragment is a selective indicator of the targets, as shown in Figure 1. The measured mass error of the deprotonated molecule ($[M\text{-}H]^-$) in each standard compound is less than 0.4 mDa, as shown in Table 1.

Table 1. Measured Mass Accuracy of Authentic Standards

No.	$[M–H]^-$	Calcd. *m/z*	Measured *m/z*
1	$C_8H_{17}O_4S$	209.0853	209.0857
2	$C_9H_{19}O_4S$	223.1009	223.1011
3	$C_9H_{19}O_4S$	223.1009	223.1013
4	$C_{11}H_{24}NO_3S$	250.1482	250.1484
5	$C_{10}H_{21}O_4S$	237.1166	237.1167
6	$C_{10}H_{21}O_4S$	237.1166	237.1164
7	$C_{12}H_{25}O_4S$	265.1479	265.1481

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-fold concentration of the Methylene Blue complex with anion in an organic layer. The concentrated cultured medium was analyzed by LC/TOF-MS with the ESI source under the same conditions.

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

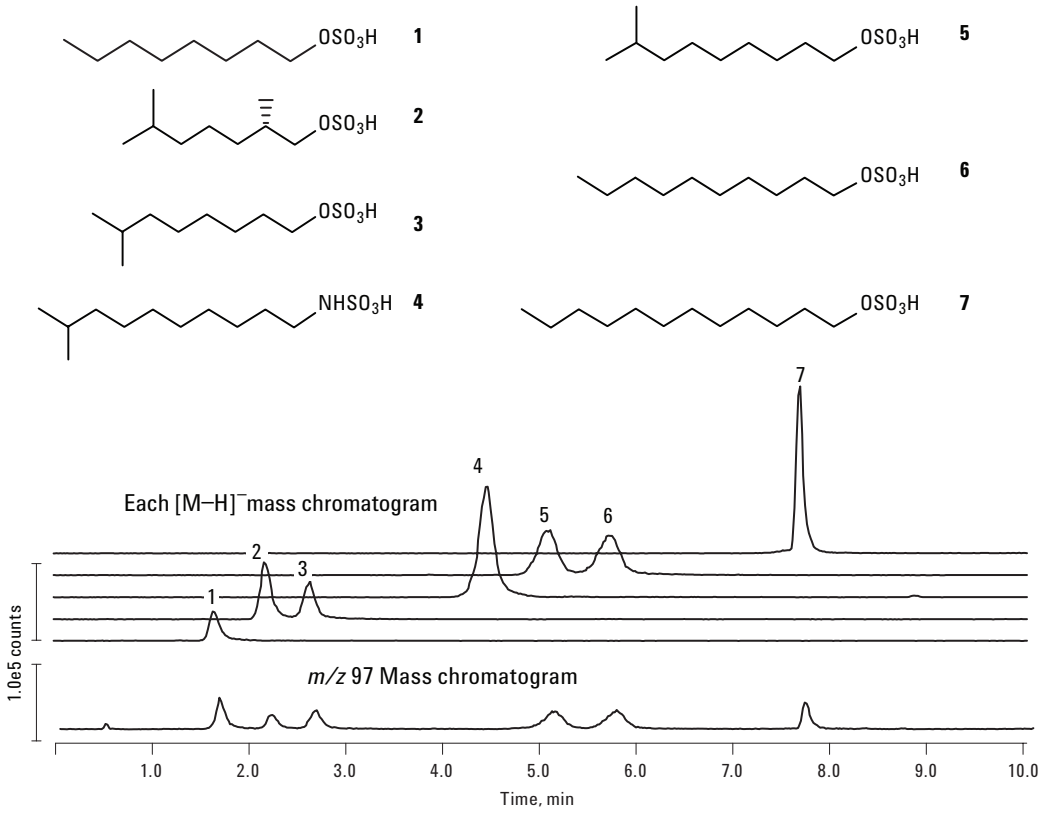

Figure 1. Structures and mass chromatograms for the $[M–H]^-$ ions of authentic standards and the familial fragment ion for sulfate.

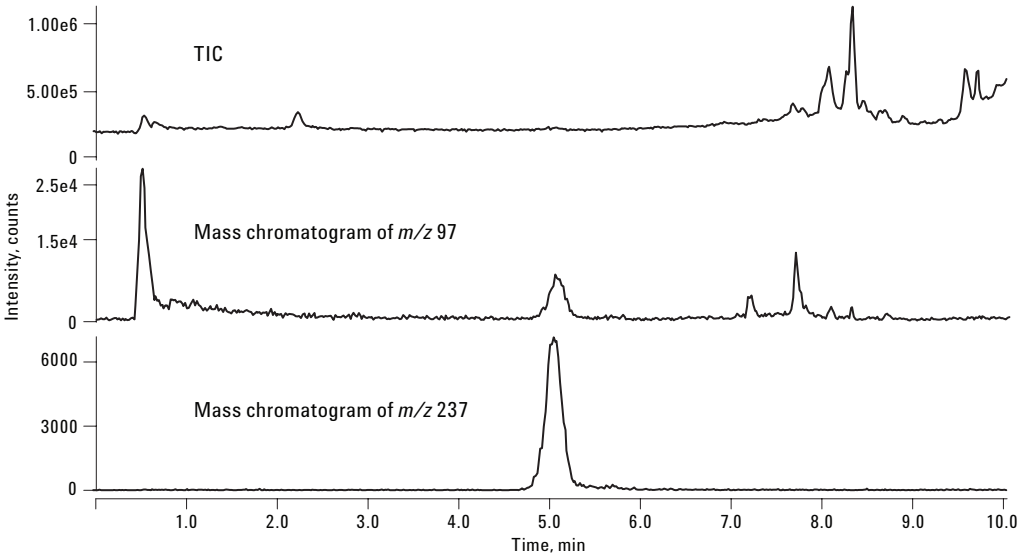

Figure 2. Total ion chromatogram (TIC) and mass chromatogram of *Daphnia* cultured medium.

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 (Figure 3) provides only three possible empirical formulas, with the elemental composition restricted to combinations of $C_{0\text{-}20}$, $H_{0\text{-}45}$, $N_{0\text{-}5}$, $O_{0\text{-}5}$, and $S_{0\text{-}5}$: $C_{10}H_{21}O_4S$ (error 0.3 mDa), $C_{11}H_{17}N_4S$ (error –1.0 mDa), and $C_{14}H_{13}S$ (error 2.3 mDa).

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 formula with the same condition described above: HO_4S (error 0.1 mDa). Accuracy of 20 ppm for *m/z* 96.9602 provides three possible empirical formulas with elemental composition restricted to the same combinations: HO_4S, C_4HOS (error –15 mDa) and HO_2S_2 (error 18 mDa).

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 due to both polar and nonpolar sites in the molecule. Large amounts 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.

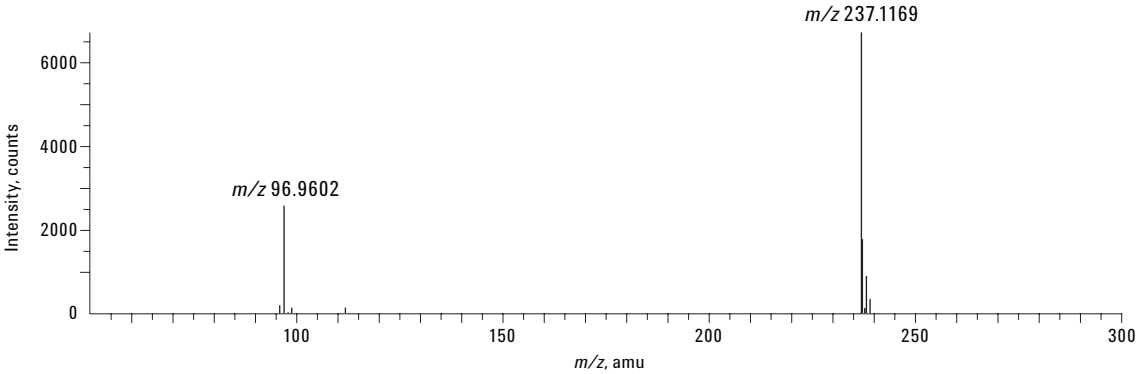

Figure 3. Mass spectrum of the peak at 5.1 min in the *Daphnia* cultured medium in Figure 2.

Conclusions

The kairomone is identified by the combination of LC/TOF-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 the actual sample result and the calculated *m/z*. The method needs no derivatization and shows low background due to using ESI in negative ion mode. Target compounds containing the SO_4 moiety 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.

Reference

1. K. Aomura, N. Ashidate, T. Aisawa, M. Fujita, K. Goto, K. Hasebe, S. Hikime, A. Ikehata, S. Kawamura, M. Kimura, et al., In Mizu no Bunseki ed., The Japan Society for Analytical Chemistry: Hokkaido, Kagaku-Dojin, Kyoto (1981), pp 374-378
2. Hideaki Uchida, Ko Yasumoto, Akinori Nishigami, Jerry A. Zweigenbaum, Takenori Kusumi, and Takashi Ooi. "Time-of-Flight LC/MS Identification and Confirmation of a Kairomone in *Daphnia magna* Cultured Medium," *Bull. Chem. Soc. Jpn.* Vol. 81, No. 2, 298-300, (2008)

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