A New Digalactosyl Diacylglycerol from a Cultured Marine Dinoflagellate *Heterocapsa circularisquama*

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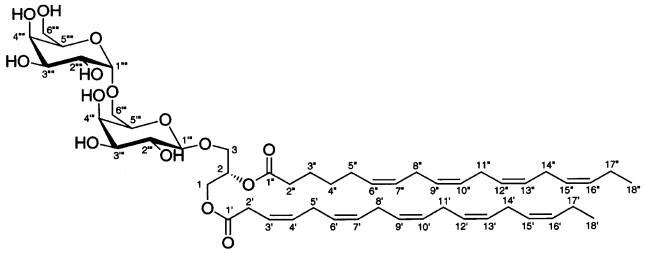
Received March 20, 2002

A new digalactosyl diacylglycerol (**1**) has been isolated from the cultured marine dinoflagellate *Heterocapsa circularisquama* together with known monogalactosyl diacylglycerols **2** and **3**, and their structures were elucidated by spectroscopic methods. The digalactosyl diacylglycerol **1** showed cytolytic activity toward heart cells of oysters.

Unicellular algae comprise an important group of phytoplankton and form the foundation of many marine food chains. However, harmful phytoplankton blooms have caused serious social and environmental problems and heavy damage to fisheries throughout the world.[1] In recent years, a novel dinoflagellate, *Heterocapsa circularisquama*, has caused mass mortalities of bivalves, e.g., oysters, pearl oysters, and mussels, in several inner bays of Japan.[2] A large number of studies have been reported on the toxic effects of this phytoplankton to the bivalves.[3,4] Recently, hemolytic activity has been found in the ethanol extracts of *H. circularisquama* cells.[3j,k]

In our search for the toxic substances in *H. circularisquama*, we have found that the EtOAc-soluble fraction of a 1-BuOH extract of the cultured *H. circularisquama* showed cytolytic activity toward heart cells of oysters. Bioassay-guided purification of the EtOAc-soluble fraction resulted in the isolation of a new digalactosyl diacylglycerol (**1**) together with two known monogalactosyl diacylglycerols, **2** and **3**. Previously, it has been reported that glycoglycerolipids derived from microalgae can be sources of biologically active substances including toxins.[5] Although a large number of studies have been made on the identification of galactolipids in some marine dinoflagellates,[6] little is known about the galactolipids in *H. circularisquama*. In this paper, we report on the structure of **1**, which has been deduced from its spectroscopic data, and on its biological activity.

The marine dinoflagellate *H. circularisquama* was cultured in seawater media (SW II)[7] in 10 L glass bottles at 22 °C. The cultivation was carried out applying a 16:8 h light to dark cycle using cool-white fluorescent lights for 2 weeks. The cells harvested by centrifugation (800 rpm, 5 min) from 90 L of cultured media were extracted with 1-BuOH, and the 1-BuOH extract (9.0 g) was partitioned between EtOAc and water. The EtOAc-soluble fraction (485 mg) was subjected to chromatography on silica gel using 2−100% methanol in chloroform as eluent to afford two bioactive fractions containing a mixture of glycolipids. Further separation by reversed-phase silica gel (ODS) column chromatography and HPLC (ODS) yielded digalactosyl diacylglycerol **1** from a latter fraction and monogalactosyl diacylglycerols **2** and **3** from a former fraction.



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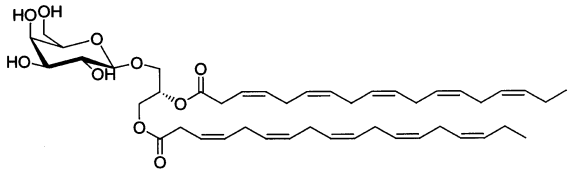

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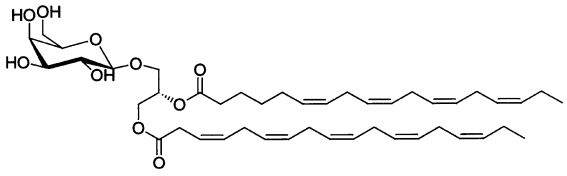

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The digalactosyl diacylglycerol **1**, $[\alpha]_D^{25}$ +54° (*c* 0.85, MeOH), was determined to have the molecular formula $C_{51}H_{78}O_{15}$ by positive mode high-resolution fast atom bombardment (HRFAB) mass spectrometry (*m*/*z* 953.5220, $[M + Na]^+$, Δ −1.8 mmu). Analysis of the ^1H and ^{13}C NMR, $^1H-^1H$ COSY, and HMQC spectra of **1** allowed the assignment of all of the ^{13}C NMR signals and the ^1H NMR signals for the sugar and the glycerol moieties as shown in Table 1. The anomeric H-1‴ proton of **1** showed HMBC cross-peaks with C-3 and C-2‴, and the anomeric H-1⁗ proton with C-6‴ and C-2⁗. The ^1H and ^{13}C NMR spectroscopic findings were consistent with **1** bearing a 1,2-di-*O*-acyl-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-*sn*-glycerol moiety. The ^{13}C NMR spectrum of **1** showed two carbonyl carbon signals arising from the acyl groups at δ_C 173.0 and 174.6, and their location in the molecule was confirmed by long-range coupling detected by HMBC. Thus, the carbonyl carbons at δ_C 174.6 (C-1″) and 173.0 (C-1′) were correlated with the proton signals of H-1 and H-2 of the glycerol part, respectively. Moreover, C-1″ was cor-

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10.1021/np0201265 CCC: $22.00
Published on Web 08/15/2002

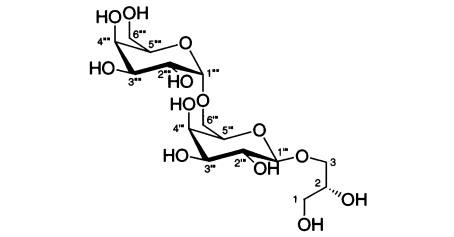
Table 1. ^{13}C and ^{1}H NMR Assignments for the Glycerol and Sugar Moieties of **1**

	^{13}C[a]	^{1}H [b](mult, *J* in Hz)
1	64.4	4.30 (dd, 6.6, 11.9)
		4.48 (dd, 3.0, 11.9)
2	71.7	5.30 (m)
3	68.7	3.78 (dd, 3.7, 10.8)
		3.98 (dd, 5.3, 10.8)
1‴	105.3	4.28 (d, 7.1)
2‴	72.4	3.56 (dd, 7.1, 9.6)
3‴	74.7	3.52 (dd, 3.2, 9.6)
4‴	70.0	3.92 (br d, 3.2)
5‴	74.6	3.78 (m)
6‴	67.8	3.71 (dd, 6.4, 9.9)
		3.94 (overlapped)[c]
1⁗	100.7	4.91 (d, 3.7)
2⁗	70.2	3.83 (dd, 3.7, 10.1)
3⁗	71.5	3.78 (dd, 3.7, 10.1)
4⁗	71.1	3.94 (br d, 3.7)
5⁗	72.6	3.89 (m)
6⁗	62.8	3.74 (dd, 5.5, 11.5)
		3.75 (dd, 6.4, 11.5)

[a] 125 MHz; CD_3OD (δ 49.0). [b] 500 MHz; CD_3OD (δ 3.35). [c] Signal pattern is unclear due to overlap.

related with proton signals at δ_H 2.40 (t, J = 7.3 Hz, H-2″) and 1.67 (tt, J = 7.3 and 7.8 Hz, H-3″), and C-1′ was correlated with proton signals at δ_H 3.18 (d, J = 5.0 Hz, H-2′) and 5.59 (m, H-3′ and H-4′). The connectivities observed in the $^{1}H-^{1}H$ COSY and HMBC measurements of the ^{1}H and ^{13}C signals of these ester chains were similar to those observed for compound **3**, thus indicating a 3,6,9,-12,15-octadecapentaenoate side chain and a 6,9,12,15-octadecatetraenoate being attached to 1-OH and 2-OH of the glycerol moiety, respectively.

To confirm the structure of the fatty acids, compound **1** was treated with NaOMe−MeOH according to a reported method,[6c] yielding a mixture of fatty acid methyl esters and digalactosyl glycerol **4**. The fatty acid composition was determined by GC and GC−MS analyses of the above methyl esters and found to be a 1:1 mixture of methyl 3,6,9,12,15-octadecapentaenoate (**5**) and methyl 6,9,12,15-octadecatetraenoate (**6**) by comparison with samples obtained by treatment of **3**.[6c]



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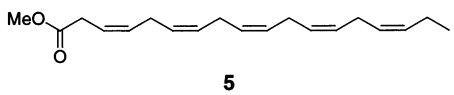

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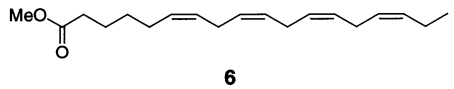

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Moreover, the glyceryl digalactoside **4**, $[\alpha]^{25}_D$ +86° (*c* 0.33, H_2O), was shown to be identical with (2*R*)-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-*sn*-glycerol on the basis of a comparison with the optical rotation and NMR data of previously reported data for this compound.[6c,8] Consequently, the chemical structure of **1** was determined as (2*S*)-1-*O*-3,6,9,12,15-octadecapentaenoyl-2-*O*-6,9,12,15-octadecatetraenoyl-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-*sn*-glycerol.

Compounds **2** and **3** were identified on the basis of their spectroscopical data as (2*S*)-1,2-di-*O*-3,6,9,12,15-octadecapentaenoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, which was previously isolated from the marine dinoflagellate *Scrippsiella trochoidea*,[6d] and (2*S*)-1-*O*-3,6,9,12,15-octadecapentaenoyl-2-*O*-6,9,12,15-octadecatetraenoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol (heterosigma-glycolipid III), which was obtained from the marine dinoflagellate *Heterosigma akashiwo*,[6c] respectively.

Compound **1** exhibited cytolytic activity toward the heart cells of oysters at a concentration of 0.5 μg/mL or greater. The activities of **2** and **3** were the same as that of **1** under the same concentrations. Thus, elucidation of the physiological functions of the galactosyl diacylglycerols **1**−**3** in *H. circularisquama* and their possible connection to the mass mortalities of bivalves would be an interesting subject for future investigation.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO FT/IR-7300 Fourier transform infrared spectrometer. ^{1}H and ^{13}C NMR spectra were measured and recorded on a JEOL ECP-500 spectrometer in CD_3OD. The resonances of CD_3OD at δ_H 3.35 ppm and δ_C 49.0 ppm were used as internal standards for NMR spectra. FABMS were recorded on a JEOL JMS-SX102A spectrometer. ESIMS were recorded on a Hitachi M-8000 mass spectrometer. GC−MS were measured and recorded on a Shimadzu QP-5050A mass spectrometer. HPLC was performed using a system composed of a JASCO PU-980 pump and a JASCO UV-970 or a JASCO RI-1530 detector.

Cultivation. The dinoflagellate *Heterocapsa circularisquama* was obtained from Hiroshima Fisheries Experimental Station. Cultures of *H. circularisquama* were grown in 10 L glass bottles filled with seawater medium (SW II)[7] at 22 °C for 2 weeks with air bubbling, applying a 16:8 h light:dark cycle provided by cool-white fluorescent lights.

Extraction and Isolation. The cultured cells were harvested by centrifugation (800 rpm, 5 min). The cells harvested from the culture were extracted with 1-BuOH (1 L × 3) to give a 1-BuOH extract (9.0 g). The 1-BuOH extract was partitioned between EtOAc and water. The EtOAc-soluble fraction (485 mg) was subjected to silica gel column chromatography, eluting with stepwise gradients of $CHCl_3$−MeOH (98:2, 95:5, 90:10, 80:20) and finally with MeOH alone, to give five fractions (I−V). Fraction III was further separated by an ODS silica gel column eluting with MeOH−H_2O (9:1) to give **1** in impure form. Final purification was carried out by repetitive separation on an analytical HPLC column [Inertsil ODS-3 (GL Science, 4.6 mm i.d. × 250 mm), flow rate 2.0 mL/min; solvent MeOH−H_2O (95:5); detection UV (215 nm)] to yield **1** (8 mg) in pure form. Using the same method as above, the pure forms of **2** (6 mg) and **3** (9 mg) were obtained from fraction II.

Digalactosyl diacylglycerol (1): $[\alpha]_D^{25}$ +54° (*c* 0.85, MeOH); IR (Nujol) ν_{max} 3338 (OH), 1736 cm^{-1} (C=O); ^{1}H NMR (500 MHz, CD_3OD) δ 5.59 (2H, m, H-3′, H-4′), 5.39 (16H, m, H-6′, H-7′, H-9′, H-10′, H-12′, H-13′, H-15′, H-16′, H-6″, H-7″, H-9″, H-10″, H-12″, H-13″, H-15″, H-16″), 3.18 (2H, d, J = 5.0 Hz, H-2′), 2.89 (14H, m, H-5′, H-8′, H-11′, H-14′, H-8″, H-11″, H-14″), 2.40 (2H, t, J = 7.3 Hz, H-2″), 2.15 (2H, m, H-5″), 2.13 (4H, m, H-17′, H-17″), 1.67 (2H, tt, J = 7.3, 7.8 Hz, H-3″), 1.45 (2H, quint, J = 7.8 Hz, H-4″), 1.02 (3H, t, J = 7.6 Hz), 1.01 (3H, t, J = 7.6 Hz) (H-18′, H-18″); ^{13}C NMR (125 MHz, CD_3OD) δ 174.6 (C-1″), 173.0 (C-1′), 132.8, 132.8 (C-16′, C-16″), 132.5 (C-4′), 130.7 (C-6″), 129.7, 129.5, 129.5, 129.3, 129.3, 129.3, 129.1, 129.0, 129.0, 128.9, 128.5 (C-6′, C-7′, C-9′, C-10′, C-12′, C-13′, C-7″, C-9″, C-10″, C-12″, C-13″), 128.2 (×2, C-15′, C-15″), 122.4 (C-3′), 35.0 (C-2″), 33.6 (C-2′), 30.1 (C-4″), 27.9 (C-5″), 26.7, 26.6 (×2), 26.6(×2), 26.5 (×2) (C-5′,

C-8′, C-11′, C-14′, C-8″, C-11″, C-14″), 25.6 (C-3″), 21.5 (×2, C-17′, C-17″), 14.7 (×2, C-18′, C-18″); ESIMS (negative mode) *m*/*z* 929 $[M - H]^-$; HRFABMS (positive mode) *m*/*z* 953.5220 $[M + Na]^+$ (calcd for $C_{51}H_{78}O_{15}Na$, 953.5238); ^{13}C NMR and ^1H NMR data for sugars and glycerol moieties, see Table 1.

Methanolysis of 1. A solution of **1** (6 mg) in methanol (1.0 mL) was treated with 2.8% NaOMe−MeOH (1.0 mL), and the mixture was stirred at room temperature for 30 min. The reaction mixture was neutralized with AG 50W-X8 (BIO-RAD, H^+ form). Evaporation of the solvent under reduced pressure yielded fatty acid methyl esters and digalactosyl glycerol **4**. The fatty acid methyl ester mixture was identified by GC and GC−MS [OV-1, HiCap-CPB1, Shimadzu, 0.32 mm × 50 m; column temperature, 100−300 °C, 3 °C/min] to be a 1:1 mixture of methyl 3,6,9,12,15-octadecapentaenoate (**5**) and methyl 6,9,12,15-octadecatetraenoate (**6**). The mixture was subjected to HPLC [Inertsil ODS-3, GL Science, MeOH−H_2O (93:7)] to furnish **4** (2 mg), **5** (2 mg), and **6** (2 mg). 1D and 2D NMR spectroscopic data and MS data for (2*R*)-3-*O*-[α-D-galactopyranosyl-(1‴→6‴)-*O*-*β*-D-galactopyranosyl]-*sn*-glycerol (**4**), methyl 3,6,9,12,15-octadecapentaenoate (**5**), and methyl 6,9,12,15-octadecatetraenoate (**6**) were identical with published data.[6c,8]

Bioassay. Hearts obtained from oysters were homogenized on 5 μm nylon mesh in Hank's balanced salt solution (HBSS) at room temperature.[9] Filtration through 5 μm nylon mesh gave a homogenate, which was treated with pronase for 10 min at 37 °C to afford small aggregates and single cells. After another filtration through 5 μm nylon mesh and centrifugation at 2000 rpm for 2 min at 4 °C, a homogeneous suspension of the harvested cells in HBSS was obtained for assay of the cytolytic activity. After incubation with a DMSO solution of the galactosyl diacylglycerol for 30 min at room temperature, the cells were stained with hematoxylin-eosin Y or fluorescein diacetate−propidium iodide. Imaging was performed with an Olympus CK40 microscope with a CK40-RFL fluorescence attachment.

Acknowledgment. The authors are grateful to Professor K. Ohkata and Dr. S. Kojima, Graduate School of Science, Hiroshima University, for their valuable advice and Dr. H. Takayama, Hiroshima Fisheries Experimental Station, for providing the dinoflagellate, *Heterocapsa circularisquama*. Spectral measurements were made at the Hiroshima Prefectural Research Institute for Science and Technology for NMR and ESIMS, and the Instrument Center for Chemical Analysis, Hiroshima University, for FABMS. This work was supported in part by a Grant-in-Aid for Scientific Research (Nos. 11558077 and 13024256) from the Japan Society for the Promotion of Science and by the Regional Science Promoter Program of Hiroshima Prefecture.

Supporting Information Available: ^1H, ^{13}C, COSY, HMQC, and HMBC spectra of **1**. ^1H and ^{13}C NMR and MS data of **2**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP0201265