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Citation	Journal of Oleo Science, 65(9), 723-732 https://doi.org/10.5650/jos.ess16026
Issue Date	2016-09
Doc URL	https://hdl.handle.net/2115/64896
Type	journal article
File Information	J. Oleo Sci. (2016, Honda).pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Journal of Oleo Science
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doi : 10.5650/jos.ess16026
J. Oleo Sci. **65**, *(9) 723-732 (2016)*

JOURNAL OF OLEO SCIENCE
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Lipid Classes, Fatty Acid Composition, and Glycerolipid Molecular Species of the Red Alga *Gracilaria vermiculophylla*, a Prostaglandin-Producing Seaweed

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Abstract: The red alga *Gracilaria vermiculophylla* is a well-known producer of prostaglandins, such as PGE_2 and $PGF_{2\alpha}$. In this study, the characteristics of glycerolipids as substrates of prostaglandin production were clarified, and the lipid classes, fatty acid composition, and glycerolipid molecular species were investigated in detail. The major lipid classes were monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), as well as phosphatidylcholine (PC), which accounted for 43.0% of the total lipid profile. Arachidonic acid (20:4n-6), a prostaglandin precursor, and palmitic acid (16:0) were the predominant fatty acids in the total lipid profile. The 20:4n-6 content was significantly high in MGDG and PC (more than 60%), and the 16:0 content was significantly high in DGDG and SQDG (more than 50%). Chiral-phase high-performance liquid chromatography determined that fatty acids were esterified at the *sn*-1 and *sn*-2 positions of those lipids. The main glycerolipid molecular species were 20:4n-6/20:4n-6 (*sn*-1/*sn*-2) for MGDG (56.5%) and PC (40.0%), and 20:4n-6/16:0 for DGDG (75.4%) and SQDG (58.4%). Thus, it was considered that the glycerolipid molecular species containing one or two 20:4n-6 were the major substrates for prostaglandin production in *G. vermiculophylla*.

Key words: *Gracilaria vermiculophylla*, lipid class, fatty acid, glycerolipid, molecular species

1 INTRODUCTION

The red alga *Gracilaria vermiculophylla*, found mostly in the Northwest Pacific, is an important marine resource in the production of agar[1, 2]. This alga is a well-known producer of prostaglandins, such as PGE_2 and $PGF_{2\alpha}$[3–6]. Although this alga is known to have a cyclooxygenase (COX) gene, the metabolic pathway and biological significance of prostaglandins have not been fully elucidated to date[7, 8]. Prostaglandins are important compounds that are widely used in the pharmaceutical field because they inhibit platelet aggregation[9], and have oxytocic[10] and gastric antisecretory actions[11]. *G. vermiculophylla* is a promising source of naturally occurring prostaglandins[4, 12].

Algal prostaglandins are produced from glyceroglycolipid (GL) and phospholipid (PL) substrates, which are hydrolyzed by acyl-hydrolases that are activated by physical wounding[4, 6, 7, 13]. Wounding releases arachidonic acid (20:4n-6) or eicosapentaenoic acid (20:5n-3), which are then transformed to prostaglandins by the action of COXs or other related enzymes[7, 8]. Several studies have reported on the 20:4n-6 metabolites of *G. vermiculophylla*[3–6], but few studies have focused on the substrate glycerolipids (GL and PL).

In the present study, to elucidate the metabolic pathway of prostaglandins in *G. vermiculophylla*, characteristics of the substrates were investigated in detail. Namely, lipid

Abbreviations: ASG, acyl steryl glycoside; CID, collision-induced dissociation; COX, cyclooxygenase; DGDG, digalactosyldiacylglycerol; 3,5-DNPU, 3,5-dinitrophenylurethane; 2D-TLC, two-dimensional thin-layer chromatography; ELSD, evaporative light scattering detection; FFA, free fatty acid; GC, gas chromatography; GL, glyceroglycolipid; HPLC, high-performance liquid chromatography; MGDG, monogalactosyldiacylglycerol; MS, mass spectrometry; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; St, Sterol; SQDG, sulfoquinovosyldiacylglycerol; TIC, total ion chromatogram.

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Accepted April 20, 2016 (received for review February 2, 2016)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

classes, fatty acid composition, and glycerolipid molecular species of the alga were clarified using two-dimensional thin-layer chromatography (2D-TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS).

2 EXPERIMENTAL PROCEDURES

2.1 Materials

G. vermiculophylla was collected from Shinori Beach (Hakodate, Japan) in late October 2008. The algal sample was immediately immersed in a boiling water bath for 10 min to inactivate hydrolytic enzymes before lipid extraction and then processed within 24 h.

Analytical- and HPLC-grade solvents, and 1,1-dimethylhydrazine were purchased from Kanto Chemical (Tokyo, Japan). Standard samples of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) from plant leaves were obtained from Lipid Products (Redhill, United Kingdom). Soybean phosphatidylcholine (PC) was also used as a standard, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,5-Dinitrophenyl isocyanate was obtained from Sumika Chemical Analysis Service (Osaka, Japan).

2.2 Lipid extraction

Lipids were extracted from *G. vermiculophylla* by the Bligh-Dyer method[14] with some modifications. In brief, the algal sample (100 g) was cut into small pieces (3–5 mm), which were homogenized in 200 mL methanol and 100 mL chloroform. After filtration, 100 mL chloroform and 100 mL water were added to the algal residue, homogenized, and then filtered. Next, 200 mL chloroform was added to the residue; the solvent was separated in the same manner, and all the obtained solvent fractions were mixed. After standing for 12 h in the dark at room temperature, the organic solvent layer (lower layer) was recovered, the solvent was evaporated until dry under reduced pressure, and the resultant lipids were obtained.

2.3 Lipid class analysis

2D-TLC analysis was performed on silica gel 60F_{254} aluminum sheets (10 × 10 cm, 0.25 mm thick; Merck, Darmstadt, Germany) according to the method described previously[15–17]. The solvent systems were a mixture of chloroform/methanol/water (75:25:2.5, by vol) for the first separation and chloroform/methanol/acetic acid/water (80:9:12:2, by vol) for the second separation. Spots were visualized by heating at 130℃ after spraying with 50% sulfonic acid. Lipid components were identified by comparing the *Rf* values of standards and the chromatograms shown in previous reports[16, 17].

Quantitative analysis of the major glycerolipids (MGDG, DGDG, SQDG, and PC) was performed by HPLC with evaporative light-scattering detection (ELSD)[18, 19]. In brief, glycerolipids were separated on a LiChrospher 100 DIOL column (250 × 4.0 mm i.d., 5 μm particles; Merck) using two solvent systems consisting of (A) chloroform and (B) methanol/acetone/water/acetic acid (30:60:9:1, by vol) as the mobile phase. The gradient profile was as follows: 0 min 0% B, 1 min 0% B, 2 min 30% B, 6 min 30% B, 8 min 50% B, 13 min 50% B, 15 min 100% B, and 16 min 100% B. The flow rate was 0.9 mL/min and the column temperature was 35℃. The detector (SEDEX model 55; SEDERE, Alfortville, France) temperature and nebulizer pressure (air) were set at 50℃ and 250 kPa, respectively. Peaks were identified by comparison of retention times with those of standard lipids. MGDG, SQDG, PC, and DGDG eluted with retention times of 7.5–8.2, 10.1–11.0, 13.2–14.0, and 14.1–14.6 min, respectively. Standard curves were prepared for MGDG, SQDG, PC, and DGDG.

2.4 Isolation of glycerolipids

MGDG, DGDG, SQDG, and PC were isolated from the total lipids of *G. vermiculophylla* by TLC on silica gel 60 F_{254} glass sheets (20 × 20 cm, 0.25 mm thick; Merck), using chloroform/methanol/water/ethyl acetate/2-propanol (5:2:1:5:5, by vol) as the developing solvent[20]. SQDG was further purified by TLC, with a solvent system of chloroform/acetone/methanol/water/acetic acid (10:6:2:1:2, by vol).

2.5 Fatty acid analysis

Individual glycerolipids were converted to fatty acid methyl esters by heating at 90℃ for 1 h in 5% (w/v) HCl in methanol[21]. GC analysis of the methyl esters was performed using a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an Omegawax 320 column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA, USA)[13]. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The split ratio was 1:50. The column temperature was elevated from 170 to 230℃ at 1℃/min. The sample injection port and flame-ionization detector (FID) temperatures were maintained at 230℃. Peaks were monitored on a Shimadzu Chromatopac C-R6A integrator and identified by comparing the retention data of authentic standards and known fatty acids from marine algae[22].

2.6 Release of diacylglycerols from glycerolipids

MGDG, DGDG, SQDG, and PC were converted into 1,2-diacylglycerols as described by Heinze *et al.*[23]. Briefly, 2 mg glycoglycerolipid and 52 mg $HIO_4 \cdot 4H_2O$ were dissolved in 1 mL methanol and kept at room temperature for 90 min in the dark. Then, 4 mL chloroform and 2.5 mL 0.45% NaCl solution were added and shaken vigorously. After brief centrifugation, the lower phase was evaporated under nitrogen gas. The residue was dissolved with 5 μL

1,1-dimethylhydrazine in chloroform/water/2-propanol/acetic acid (6:7:2:3, by vol) and kept in the dark at 25℃ for 4 h for DGDG, and 20 h for MGDG, SQDG and PC. After adding 3 mL hexane, the mixture was washed twice with 2 mL 50 mM KH_2PO_4 solution and dried over anhydrous Na_2SO_4. The residue obtained after removal of the solvent contained the diacylglycerols released from glycerolipids.

2.7 Preparation of derivatives

The 1,2-diacylglycerols released were immediately converted into their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives without prior isolation from the reaction products to minimize acyl migration as described previously[20, 24]. Namely, the reaction products and 3,5-dinitrophenyl isocyanate (5 mg) were dissolved in dry toluene (5 mL) in the presence of dry pyridine (30 μL), and the solution was stirred at 30℃ for 3 h. After removal of the solvent, the crude urethane derivatives were purified by TLC on silica gel 60 F_{254} glass sheets (Merck), using hexane/dichloromethane/ethanol (40:10:3, by vol) as the developing solvent. Bands were visualized under UV irradiation (*Rf* 0.6–0.7), and the adsorbent containing the derivatives was scraped off and extracted with diethyl ether.

2.8 Chiral-phase HPLC

Chiral-phase HPLC of the diacylglycerols as 3,5-DNPU derivatives was performed as described previously[20, 24–26]. The analysis was done on a Shimadzu LC-6A instrument equipped with a chiral column (YMC-Pack A-K03, 250 × 4.6 mm i.d., 5 μm particles; YMC, Kyoto, Japan), using a mixture of hexane/dichloromethane/ethanol (40:10:1, by vol) as the mobile phase at a constant flow rate of 1 mL/min. The column temperature was kept at 10℃. Peaks were monitored with a Shimadzu SPD-6A variable wavelength detector set at 254 nm, and chromatograms were recorded on a Shimadzu Chromatopac C-R3A. The chirality of the diacylglycerols released from glycerolipids was determined by comparing the retention times of the 3,5-DNPU derivatives with those of standard *sn*-1,2- and *sn*-2,3-diacylglycerols generated from fish oil triacylglycerols by partial Grignard degradation[20, 24, 25].

2.9 Reversed-phase HPLC

Molecular species analysis of diacylglycerols as 3,5-DNPU derivatives was performed by reversed-phase HPLC, using a system comprised of an L-7100 pump (Hitachi, Tokyo, Japan) equipped with a Superspher 100 RP-18 column (250 × 4.0 mm i.d., 4 μm particles; Merck) and an L-7455 diode array detector (Hitachi). The analysis was done isocratically at 20℃ using acetonitrile as the mobile phase at a constant flow rate of 0.5 mL/min. Peaks were monitored at 254 nm.

2.10 Reversed-phase HPLC-ESI-MS

Reversed-phase HPLC-electrospray ionization (ESI)-MS was performed by admitting the entire HPLC column effluent to a LCQ ion trap mass spectrometer (Thermo Separation Products, San Jose, CA, USA). Separations of the glycerolipid molecular species were performed under the same conditions as the HPLC analysis with UV detection, as described above. Mass spectra were taken in a scan range of 150–1200 atomic mass units (amu). The capillary temperature was 270℃. The tube lens offset and capillary voltages were −60 V and −28 V, respectively. Flow rates of the nitrogen sheath and auxiliary gases were set to 80 arbitrary units (arb) and 30 arb, respectively. The deprotonated molecules ($[M-H]^-$) and carboxylate anions ($[RCOO]^-$) obtained in negative ESI mode were used to identify individual molecular species of glycerolipids. The relative intensities of two carboxylate anions ($[R^1COO]^-$ and $[R^2COO]^-$) produced from glycerolipids by collision-induced dissociation (CID) was used for determining the *sn*-position (*sn*-1 or *sn*-2) of the acyl groups in the molecules[24, 27].

3 RESULTS AND DISCUSSION

3.1 Lipid class composition

Three GLs (MGDG, DGDG, and SQDG) and four PLs (PC; phosphatidylethanolamine, PE; phosphatidylglycerol, PG; phosphatidylinositol, PI) were clearly recognizable as dark violet spots on the 2D-TLC plate (Fig. 1). Previous reports have described that glycerolipids are present in *Gracilaria* species[12, 21, 28], including some minor compounds, possibly

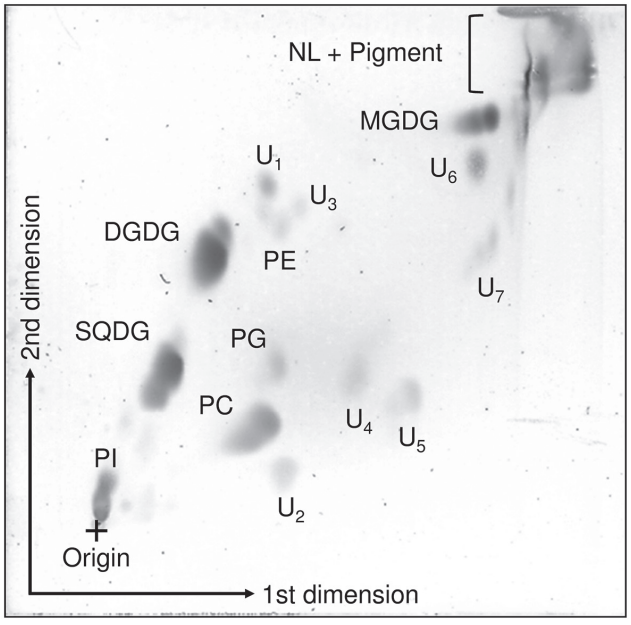


Fig. 1 2D-TLC separation of total lipids from *Gracilaria vermiculophylla*.

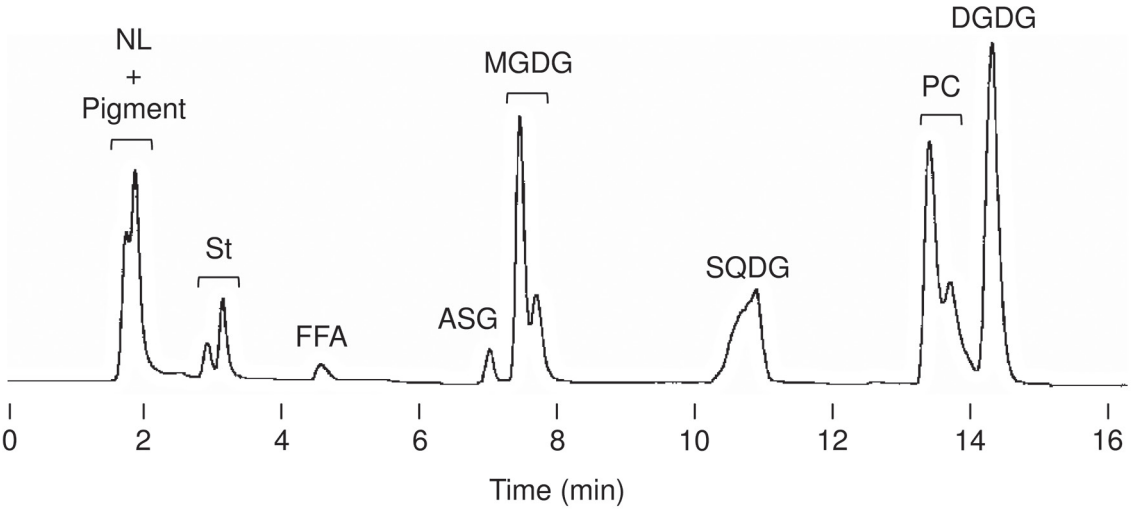


Fig. 2 HPLC-ELSD chromatogram of total lipids from *Gracilaria vermiculophylla*.

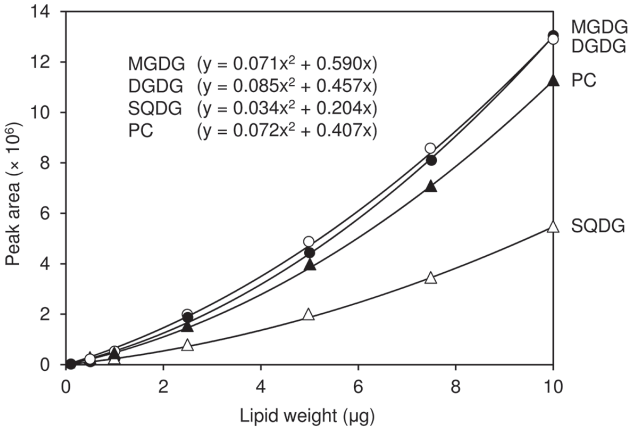


Fig. 3 Calibration curves of MGDG, DGDG, SQDG, and PC in HPLC-ELSD.

sphingophospholipids and inositolphosphoceramides (U_1–U_7 in Fig. 1), that have also been identified in *G. verrucosa* (=*G. vermiculophylla*)[28]. The major glycerolipid components (MGDG, DGDG, SQGD, and PC) were also detected by HPLC-ELSD, but no clear peaks of minor PLs (PE, PG, and PI) were detected (Fig. 2), probably due to the limited sensitivity and dynamic range of ELSD[29] when compared to TLC detection. Quantitative estimations of the major glycerolipids were performed by HPLC-ELSD, using a calibration curve for each glycerolipid (Fig. 3). The sum of the major four glycerolipids of *G. vermiculophylla* accounted for 43.0% of the total lipid profile, the contents of which (mg/g of total lipids ± SD of three analyses) were in the order of DGDG (123.7 ± 2.9) > PC (110.7 ± 8.9) > SQDG (107.8 ± 5.8) > MGDG (88.1 ± 1.2). A similar order (DGDG > PC > MGDG > SQDG) has been observed in *G. verrucosa*[28]. On the other hand, some other *Gracilaria* species have different glycerolipid compositions, e.g., in *G. bursa-pastoris* and *G. chorda*, MGDG is the most predominant constituent, while in *G. textorii* and *G. gigas*, PC is the richest one[30]. The results obtained in this study, in which MGDG, DGDG, SQGD, and PC comprised the majority of the total glycerolipids in *G. vermiculophylla*, suggest that these glycerolipids are the main substrates of prostaglandin production.

3.2 Fatty acid composition of glycerolipids

Previous studies have reported that 16:0 and 20:4n-6 are rich in lipids from *G. vermiculophylla*[5, 22, 28, 30]. The main fatty acids of the total lipids in *G. vermiculophylla* examined in this study were 16:0 (31.6%) and 20:4n-6 (44.6%) (Table 1). Other *Gracilaria* species, such as *G. bursa-pastoris*, *G. textorii*, and *G. chorda*, also have similar fatty acid compositions[30], whereas the most predominant fatty acid in *G. gigas* and *G. indica* is 20:5n-3[30, 31], and *G. edulis* and *G. folifera* contain almost no polyunsaturated fatty acid (PUFA)[32]. The four major lipid classes, MGDG, DGDG, SQDG, and PC, had a characteristic composition of fatty acids: the 20:4n-6 content was especially high (> 60%) in MGDG and PC, and the 16:0 content was especially high (>50%) in DGDG and SQDG. PC was particularly rich in PUFAs (74.6%), whereas SQDG contained a large amount of saturated fatty acid (SFA) (79.1%). A similar composition was also observed in *G. chorda*[30]. These results suggest that MGDG and PC, which are abundant in 20:4n-6, are the main substrates of prostaglandin production.

3.3 Positional isomers of glycerolipids

Figure 4 shows the chiral-phase HPLC profiles of 3,5-DNPU derivatives of the diacylglycerols released from MGDG, DGDG, SQDG, and PC of *G. vermiculophylla*, and of standard *sn*-1,2(2,3)-diacylglycerols generated from tuna orbital oil triacylglycerols by partial Grignard degradation. The standard was clearly resolved into two groups,

Table 1 Fatty acid composition of the red alga *Gracilaria vermiculophylla* (wt%).

Fatty acid	Total lipid	Lipid class			
		MGDG	DGDG	SQDG	PC
12:0	0.3	0.1	0.2	1.1	tr
14:0	5.3	1.7	1.8	14.7	1.2
15:0	0.6	1.1	0.8	1.0	0.2
16:0	31.6	21.7	54.0	61.7	15.3
iso 17:0	0.6	nd	nd	nd	nd
17:0	0.4	1.1	0.3	0.2	0.2
18:0	0.7	0.5	0.4	0.4	0.7
22:0	0.1	nd	nd	nd	nd
Σ saturated	**39.6**	**26.2**	**57.5**	**79.1**	**17.6**
16:1n-9	3.7	1.5	0.7	1.3	tr
16:1n-7	tr	nd	nd	nd	0.7
18:1n-9	3.3	3.2	3.8	0.8	2.1
18:1n-7	1.7	0.5	0.2	0.3	3.2
20:1n-9	0.2	0.2	tr	tr	nd
24:1n-9	tr	nd	nd	nd	nd
Σ monounsaturated	**8.9**	**5.3**	**4.7**	**2.4**	**6.0**
16:3n-3	0.2	0.9	tr	nd	nd
16:4n-3	0.2	0.7	nd	nd	tr
18:2n-6	0.8	0.4	0.5	0.2	0.9
18:3n-6	0.4	0.1	tr	tr	1.1
18:3n-3	0.3	0.4	0.2	0.1	0.1
18:4n-3	0.4	1.0	0.2	nd	0.2
20:2n-6	0.2	0.2	0.2	nd	0.2
20:3n-6	2.0	0.8	0.6	0.3	5.5
20:4n-6	44.6	63.4	35.2	17.2	63.8
20:5n-3	1.7	1.6	0.5	tr	2.3
22:4n-6	0.2	tr	nd	nd	0.5
Σ polyunsaturated	**51.0**	**68.1**	**37.4**	**17.8**	**74.6**
Others	0.5	0.4	0.4	0.7	1.8

tr: Trace (<0.1%). nd: Not detected.

representing the *sn*-1,2- and *sn*-2,3-enantiomers (Fig. 4A): the faster elution consisted of *sn*-1,2-enantiomers, and the subsequent elution consisted of *sn*-2,3-enantiomers on the A-K03 column[20, 24, 25, 33]. Each enantiomeric group was split because of the presence of various molecular species. All diacylglycerols released from MGDG, DGDG, SQDG, and PC of *G. vermiculophylla*, which were eluted within 20 min of injection with a partial resolution of molecular species, were *sn*-1,2-enantiomers (Fig. 4B–E). These results confirm that the glycerol moieties of all MGDG, DGDG, SQDG, and PC of *G. vermiculophylla* have the *S*-configuration, a finding consistent with higher plants and other algae[20, 24, 34].

3.4 Molecular species of glycerolipids

Figure 5 shows the reversed-phase HPLC chromatograms of 3,5-DNPU derivatives of the diacylglycerols released from MGDG, DGDG, SQDG, and PC of *G. vermiculophylla*. All major molecular species were eluted from the C18 column with clear resolution. Each peak was identified by MS with CID energy as described previously[26, 27]. Figure 6 shows the reversed-phase HPLC-ESI-MS profiles of DNPU derivatives of *sn*-1,2-diacylglycerols released from MGDG of *G. vermiculophylla*. Prominent $[M-H]^-$ mole-

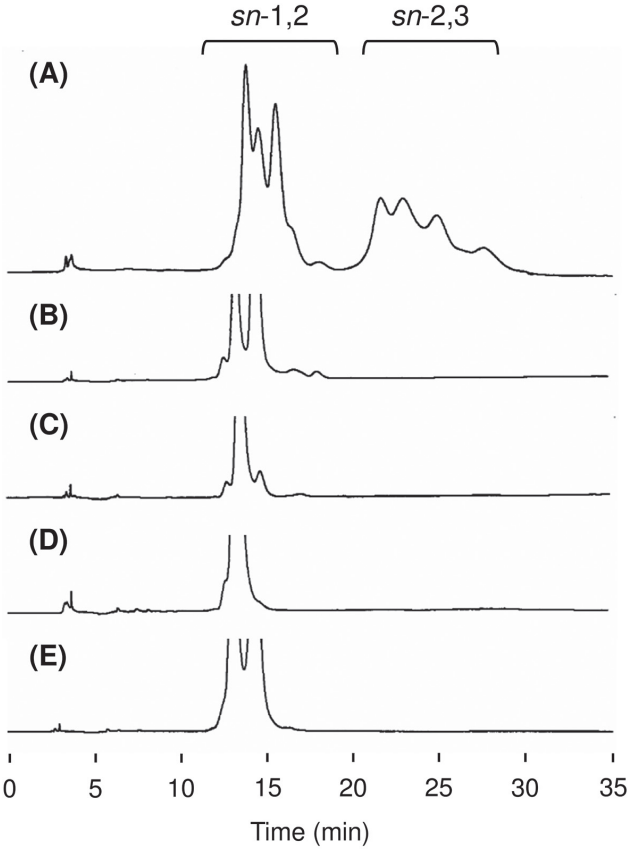


Fig. 4 Chiral-phase HPLC profiles of the 3,5-DNPU derivatives of (A) standard *sn*-1,2 (2,3)-diacylglycerols generated from tuna orbital oil triacylglycerols by partial Grignard degradation, and of the diacylglycerols released from (B) MGDG, (C) DGDG, (D) SQDG, and (E) PC of *Gracilaria vermiculophylla*.

cules were detected without CID energy (Fig. 6B), whereas when 40% CID energy was applied, $[RCOO]^-$ ions were produced (Fig. 6C). The *sn*-positions of the acyl groups were determined from $[RCOO]^-$ ion intensities (Fig. 6C). When CID energy was added, the intensities of $[R^2COO]^-$ (carboxylate anions generated from the *sn*-2 position) were higher than those of $[R^1COO]^-$ (carboxylate anions from the *sn*-1 position). The same observation was made in ESI-MS and FAB-MS analyses of phospholipids[35, 36]. The preference of the formation of the carboxylate anion from the *sn*-2 position is most likely due to the stability of the five-membered ring transition state, compared to the six-membered ring transition state, which is generated by attack of the anionic site of the phosphate group either at the glycerol *sn*-2 or *sn*-1 carbon, respectively[35, 36]. With the 3,5-DNPU derivatives of diacylglycerols, the anionic site ($[M-H]^-$) would be generated by deprotonation of the amide bond[26, 27].

Table 2 shows the molecular species composition of the glycerolipids from *G. vermiculophylla* examined in this study. In MGDG, DGDG, SQDG, and PC, 15, 14, 12, and 7 molecular species were identified, respectively. The 20:4n-6/20:4n-6 (*sn*-1/*sn*-2) species accounted for the largest proportion of MGDG and PC: 56.5% and 40.0%, respectively. On the other hand, the 20:4n-6/16:0 species was the predominant species in DGDG and SQDG: 75.4% and 58.4%, respectively. MGDG also contained a high proportion of 20:4n-6/16:0 (17.7%), and PC contained an abundance of 16:0/20:4n-6 (26.3%). The *sn*-position of 16:0 and 20:4n-6 in MGDG, DGDG, and SQDG molecules was opposite that of the main molecular species of PC. In SQDG, a saturated 14:0/16:0 species was also abundant (29.4%). In addition to the major molecular species, 20:4n-6/16:0, MGDG, DGDG, and SQDG contained a small amount of its reverse isomer, 16:0/20:4n-6, whereas PC did not contain 20:4n-6/16:0. The 16:0/20:4n-6 isomer was also found in MGDG of three species of red algae (e.g., *Porphyra yezoensis*, *Corallina pilulifera*, and *G. vermiculophylla*), but 16:0/20:4n-6 was not found in DGDG and SQDG, nor was 20:4n-6/16:0 found in PC of these algal species[37]. The coexistence of both isomers indicates that *G. vermiculophylla* biosynthesizes glycerolipids from both the prokaryotic (biosynthesized in the chloroplast envelope) and the eukaryotic pathways (biosynthesized in the chloroplast envelope after passing through the endoplasmic reticulum), as with spinach, tobacco, and *Arabidopsis*[38–41]. When glycerolipids are biosynthesized in the prokaryotic pathway, they predominantly have PUFAs at the *sn*-1 position and SFAs at the *sn*-2 position. In contrast, when biosynthesized through the eukaryotic pathway, glycerolipids predominantly have SFAs at the *sn*-1 position and PUFAs at the *sn*-2 position[41]. The first stage reaction of prostaglandin production from *G. vermiculophylla* is the hydrolysis of membrane glycerolipids by acyl-hydrolases, which is triggered by physical wounding[4, 13, 20]. From the above results, 20:4n-6 would mainly be released from the 20:4n-6/20:4n-6 species of MGDG and PC, the 20:4n-6/16:0 species of MGDG, DGDG, and SQDG, and the 16:0/20:4n-6 species of PC.

4 CONCLUSIONS

This study investigated the lipid classes, fatty acids, and molecular species of the red alga *G. vermiculophylla* to elucidate the metabolic pathway of algal prostaglandins. The main glycerolipids were MGDG, DGDG, SQDG, and PC, which comprised 43.0% of the total lipid profile, and 20:4n-6 and 16:0 were the predominant fatty acids in these glycerolipids. The 20:4n-6/20:4n-6 species was predominant in MGDG (56.5%) and PC (40.0%), and the 20:4n-6/16:0 species was predominant in DGDG (75.4%) and SQDG (58.4%). Thus, the glycerolipid molecular species

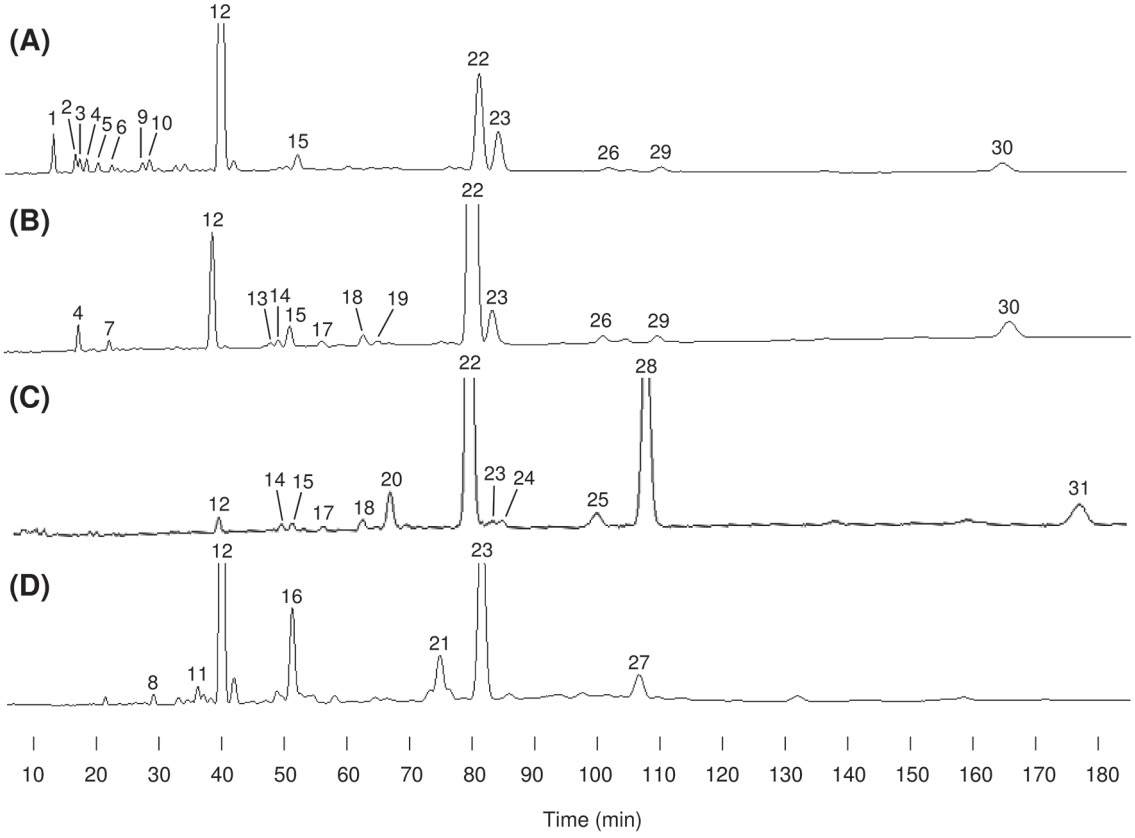


Fig. 5 Reversed-phase HPLC chromatograms of the 3,5-DNPU derivatives of diacylglycerols released from (A) MGDG, (B) DGDG, (C) SQDG, and (D) PC of *Gracilaria vermiculophylla*. Peak numbers correspond to those in Table 2.

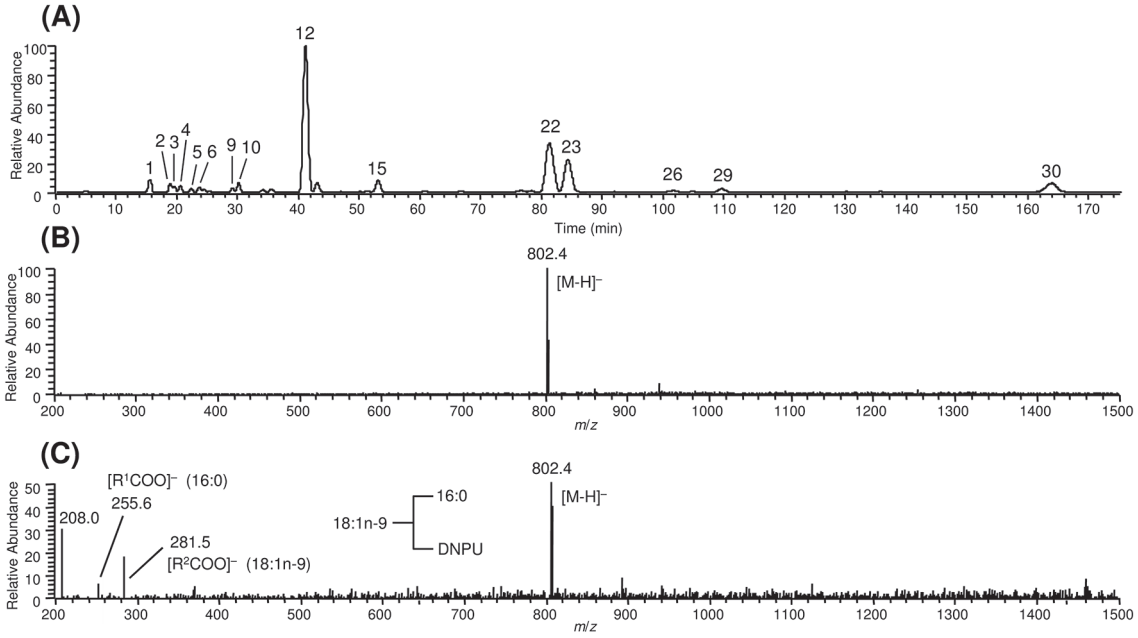


Fig. 6 Reversed-phase HPLC-ESI-MS profiles of the 3,5-DNPU derivatives of *sn*-1,2-diacylglycerols released from MGDG of *Gracilaria vermiculophylla*. (A): TIC; (B) and (C): mass spectra averaged over the peak 30 between 162 and 166 min on the TIC. Source CID: (B) 0%; (C) 40%. Peak numbers on TIC (A) correspond to those in Fig. 5 and Table 2.

Table 2 Molecular species composition of the red alga *Gracilaria vermiculophylla* (mol%).

Peak no.[a]	ECN[b]	Molecular species	Lipid class			
		(*sn*-1/*sn*-2)	MGDG	DGDG	SQDG	PC
1	18	20:5n-3/16:4n-3	2.0	nd	nd	nd
2	20	18:4n-3/18:4n-3	1.0	nd	nd	nd
3	20	20:5n-3/16:3n-3	0.8	nd	nd	nd
4	20	20:5n-3/18:4n-3	0.9	1.1	nd	nd
5	22	16:2n-6/16:3n-3	0.8	nd	nd	nd
6	22	20:4n-6/20:5n-3	1.0	nd	nd	nd
7	22	20:5n-3/16:2n-6	nd	0.5	nd	nd
8	22	20:5n-3/20:2n-6	nd	nd	nd	0.9
9	24	14:0/18:4n-3	0.5	nd	nd	nd
10	24	16:2n-6/16:2n-6	0.8	nd	nd	nd
11	24	20:4n-6/18:3n-6	nd	nd	nd	1.2
12	24	20:4n-6/20:4n-6	56.5	7.8	0.8	40.0
13	26	18:2n-6/20:4n-6	nd	0.2	nd	nd
14	26	20:4n-6/14:0	nd	0.4	0.3	nd
15	26	14:0/20:4n-6	1.8	1.7	0.2	nd
16	26	20:4n-6/20:3n-6	nd	nd	nd	10.2
17	26	20:5n-3/16:0	nd	0.5	0.2	nd
18	27	20:4n-6/15:0	nd	1.0	0.7	nd
19	27	15:0/20:4n-6	nd	0.3	nd	nd
20	28	12:0/16:0	nd	nd	3.2	nd
21	28	18:1n-9/20:4n-6	nd	nd	nd	7.7
22	28	20:4n-6/16:0	17.7	75.4	58.4	nd
23	28	16:0/20:4n-6	7.3	4.3	0.2	26.3
24	29	15:0/14:0	nd	nd	0.7	nd
25	30	16:1n-9/16:0	nd	nd	1.2	nd
26	30	14:0/18:1n-9	0.3	0.8	nd	nd
27	30	16:0/20:3n-6	nd	nd	nd	3.8
28	30	14:0/16:0	nd	nd	29.4	nd
29	30	16:0/20:3n-6	0.3	0.6	nd	nd
30	32	16:0/18:1n-9	2.5	3.5	nd	nd
31	32	16:0/16:0	nd	nd	3.6	nd
		Others	5.8	1.9	1.1	9.9

[a] Peak numbers correspond to those given in Fig. 5.
[b] Equivalent carbon number (total number of carbon atoms in the two constituent fatty acids – 2 x total number of their double bonds).
nd: Not detected.

containing one or two 20:4n-6 would be the major substrates for prostaglandin production in this alga.

ACKNOWLEDGMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 21658074) and Regional Innovation

Cluster Program (Global Type), Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT). The authors thank Prof. Hajime Yasui (Hokkaido University) for identification of the alga.

REFERENCES

1) Bellorin, A. M.; Oliveira, M. C.; Oliveira, E. C. *Gracilaria vermiculophylla*: A western Pacific species of Gracilariaceae (Rhodophyta) first recorded from the eastern Pacific. *Phycol. Res.* **52**, 69-79 (2004).
2) Daugherty, B. K.; Bird, K. T. Salinity and temperature effects on agar production from *Gracilaria verrucosa* Strain G-16. *Aquaculture* **75**, 105-113 (1988).
3) Fusetani, N.; Hashimoto, K. Prostaglandin E_2: a candidate for causative agent of "ogonori" poisoning. *Nippon Suisan Gakkaishi* **50**, 465-469 (1984).
4) Nakajima, I.; Suzaki, K.; Oba, K. Production of Prostaglandins by the marine alga, *Gracilaria verrucosa. J. Jpn. Oil Chem. Soc.* **47**, 753-758 (1998).
5) Imbs, A. B.; Vologodskaya, A. V.; Nevshupova, N. V.; Khotimchenko, S. V.; Titlyanov, E. A. Response of prostaglandin content in the red alga *Gracilaria verrucosa* to season and solar irradiance. *Phytochemistry* **58**, 1067-1072 (2001).
6) Rempt, M.; Weinberger, F.; Grosser, K.; Pohnert, G. Conserved and species-specific oxylipin pathways in the wound-activated chemical defense of the noninvasive red alga *Gracilaria chilensis* and the invasive *Gracilaria vermiculophylla. Beilstein J. Org. Chem.* **8**, 283-289 (2012).
7) Nakajima, I.; Suzaki, K.; Oba, K. Mechanism for prostaglandin synthesis in the marine alga, *Gracilaria verrucosa. J. Jpn. Oil Chem. Soc.* **47**, 759-763 (1998).
8) Kanamoto, H.; Takemura, M.; Ohyama, K. Identification of a cyclooxygenase gene from the red alga *Gracilaria vermiculophylla* and bioconversion of arachidonic acid to $PGF_{2\alpha}$ in engineered *Escherichia coli. Appl. Microbiol. Biotechnol.* **91**, 1121-1129 (2011).
9) Sim, A. K.; McCraw, A. P.; Cleland, M. E.; Aihara, H.; Otomo, S.; Hosoda, K. The effect of prostaglandin E_1 incorporated in lipid microspheres on thrombus formation and thrombus disaggregation and its potential to target to the site of vascular lesions. *Arzneimittel-Forschung* **36**, 1206-1209 (1986).
10) Sugimoto, Y.; Yamasaki, A.; Segi, E.; Tsuboi, K.; Aze, Y.; Nishimura, T.; Oida, H.; Yoshida, N.; Tanaka, T.; Katsuyama, M.; Hasumoto, K.; Murata, T.; Hirata, M.; Ushikubi, F.; Negishi, M.; Ichikawa, A.; Narumiya, S. Failure of parturition in mice lacking the prostaglandin F receptor. *Science* **277**, 681-683 (1997).
11) Takeuchi, K.; Ueki, S.; Tanaka, H. Endogenous prostaglandins in gastric alkaline response in the rat stomach after damage. *Am. J. Physiol.* **250**, 842-849 (1986).
12) Imbs, A. B.; Latyshev, N. A.; Svetashev, V. I.; Skriptsova, A. V.; Le, T. T.; Pham, M. Q.; Pham, L. Q. Distribution of polyunsaturated fatty acids in red algae of the genus *Gracilaria*, a promising source of prostaglandins. *Russ. J. Mar. Biol.* **38**, 339-345 (2012).
13) Illijas, M. I.; Terasaki, M.; Nakamura, R.; Iijima, N.; Hara, A.; Fusetani, N.; Itabashi, Y. Purification and characterization of glycerolipid acyl-hydrolase from the red alga *Gracilaria vermiculophylla. Fish. Sci.* **74**, 670-676 (2008).
14) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917 (1959).
15) Bigogno, C.; Khozin-Goldberg, I.; Boussiba, S.; Vonshak, A.; Cohen, Z. Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid. *Phytochemistry* **60**, 497-503 (2002).
16) Bisanz, C.; Bastien, O.; Grando, D.; Jouhet, J.; Maréchal, E.; Cesbron-Delauw, M-F. *Toxoplasma gondii* acyl-lipid metabolism: de novo synthesis from apicoplast-generated fatty acids versus scavenging of host cell precursors. *Biochem. J.* **394**, 197-205 (2006).
17) Christie, W. W. Thin-layer chromatography of lipids. *AOCS Lipid Library.* (2011). http://lipidlibrary.aocs.org/Analysis/content.cfm?ItemNumber=40388.
18) Sugawara, T.; Miyazawa, T. Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **34**, 1231-1237 (1999).
19) Yunoki, K.; Sato, M.; Seki, K.; Ohkubo, T.; Tanaka, Y.; Ohnishi, M. Simultaneous quantification of plant glyceroglycolipids including sulfoquinovosyldiacylglycerol by HPLC-ELSD with binary gradient elution. *Lipids* **44**, 77-83 (2009).
20) Takahashi, Y.; Itabashi, Y.; Suzuki, M.; Kuksis, A. Determination of stereochemical configuration of the glycerol moieties in glycoglycerolipids by chiral phase high-performance liquid chromatography. *Lipids* **36**, 741-748 (2001).
21) Christie, W. W.; Han, X. Preparation of derivatives of fatty acids. in *Lipid Analysis*. 4th ed. The Oily Press, Bridgwater, England. pp. 145-158 (2010).
22) Takagi, T.; Asahi, M.; Itabashi, Y. Fatty acid composition of twelve algae from Japanese waters. *J. Jpn. Oil Chem. Soc.* **34**, 1008-1012 (1985).
23) Heinze, F. J.; Linscheid, M.; Heinz, E. Release of diacylglycerol moieties from various glycosyl diacylglycerols. *Anal. Biochem.* **139**, 126-133 (1984).
24) Honda, M.; Kashima, A.; Takahashi, K.; Itabashi, Y. Molecular species of digalactosyldiacylglycerols in the

brown alga *Sargassum yezoense*. *Nippon Suisan Gakkaishi* **75**, 1061-1069 (2009).
25) Itabashi, Y.; Kuksis, A.; Marai, L.; Takagi, T. HPLC resolution of diacylglycerol moieties of natural triacylglycerols on a chiral phase consisting of bonded (R)-(+)-1-(1-naphthyl) ethylamine. *J. Lipid Res.* **31**, 1711-1717 (1990).
26) Itabashi, Y. Development and application of chromatographic methods for glycerolipid analysis. *Chromatography* **32**, 59-72 (2011).
27) Hashidate, T.; Itabashi, Y. Electrospray ionization mass spectrometry of regioisomeric 1,2-diacylglycerols. *Bunseki Kagaku* **54**, 807-816 (2005).
28) Khotimchenko, S. V. Lipids from the marine alga *Gracilaria verrucosa*. *Chem. Nat. Compd.* **41**, 285-288 (2005).
29) Cleaver, G.; Bullock, S. Development in ELSD technology to improve sensitivity and linearity of response over a wider dynamic range. *Chromatogr. Today* **8**, 38-41 (2015).
30) Araki, S.; Sakurai, T.; Oohusa, T.; Kayama, M.; Nisizawa, K. Content of arachidonic and eicosapentaenoic acids in polar lipids from *Gracilaria* (Gracilariales, Rhodophyta). *Hydrobiologia* **204/205**, 513-519 (1990).
31) Kumari, P.; Kumar, M.; Gupta, V.; Reddy, C. R. K.; Jha, B. Tropical marine macroalgae as potential sources of nutritionally important PUFAs. *Food Chem.* **120**, 749-757 (2010).
32) Bhaskar, N.; Kinami, T.; Miyashita, K.; Park, S. B.; Endo, Y.; Fujimoto, K. Occurrence of conjugated polyenoic fatty acids in seaweeds from the Indian Ocean. *Z. Naturforsch. C: Biosci.* **59**, 310-314 (2004).
33) Itabashi, Y. Chiral-phase HPLC of glycerolipids. in *HPLC in Acyl Lipids* (Lin, J.-T; McKeon, T. A. eds.). HNB Publishing, New York, pp. 167-198 (2005).
34) Ishizuka, I.; Yamakawa, T. Glycoglycerolipids. *New Compr. Biochem.* **10**, 101-197 (1985).
35) Zirrolli, J. A.; Clay, K. L.; Murphy, R. C. Tandem mass spectrometry of negative ions from choline phospholipid molecular species related to platelet activating factor. *Lipids* **26**, 1112-1116 (1991).
36) Vernooij, E. A. A. M.; Brouwers, J. F. H. M.; Kettenes-von den Bosch, J. J.; Crommelin, D. J. A. RP-HPLC/ESI MS determination of acyl chain positions in phospholipids. *J. Sep. Sci.* **25**, 285-289 (2002).
37) Itabashi, Y. Reverse isomers of glycoglycerolipids in marine red Algae. in *Proceedings of the 3rd Japan-Korea Joint Seminar on Fisheries Sciences*. Jinju-Tongyeong, pp. 161-163 (2003).
38) Cho, S. H.; Thompson, G. A. On the metabolic relationships between monogalactosyldiacylglycerol and digalactosyldiacylglycerol molecular species in *Dunaliella salina*. *J. Biol. Chem.* **262**, 7586-7593 (1987).
39) Ohta, H.; Awai, K.; Takamiya, K. Glyceroglycolipids of photosynthetic organisms-Their biosynthesis and evolutionary origin. *Trends Glycosci. Glycotech.* **12**, 241-253 (2000).
40) Somerville, C.; Browse, J. Plant lipids: metabolism, mutants, and membranes. *Science* **252**, 80-87 (1991).
41) Ohlrogge, J.; Browse, J. Lipid biosynthesis. *Plant Cell* **7**, 957-970 (1995).