Pancreatic lipase inhibitory activity of monogalactosyldiacylglycerols isolated from the freshwater microalga *Chlorella sorokiniana*

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Abstract Chemical investigation of the freshwater microalga *Chlorella sorokiniana* led to the isolation of a monogalactosyldiacylglycerol (MGDG)-rich fraction possessing dose-dependent inhibitory activity against pancreatic lipase activity. The MGDG-rich fraction contains 12 MGDGs identified by LC/HRMS analysis. Among them, three MGDGs were new compounds, namely, (2*S*)-1-*O*-(7*Z*,10*Z*-hexadecadienoyl)-2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-3-*O*-β-D-galactopyranosylglycerol (**1**), (2*S*)-1-*O*-linoleoyl-2-*O*-(7*Z*,10*Z*-hexadecadienoyl)-3-*O*-β-D-galactopyranosylglycerol (**6**), and (2*S*)-1-*O*-oleoyl-2-*O*-(7*Z*,10*Z*-hexadecadienoyl)-3-*O*-β-D-galactopyranosylglycerol (**8**). The major galactolipids were isolated by semipreparative HPLC and tested for their effect toward pancreatic lipase inhibitory activity. All the tested MGDGs showed significant reduction of pancreatic lipase activity indicating possible beneficial use for management of lipase-related disorders such as obesity.

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Introduction

Microalgae, unicellular organisms commonly found in freshwater and marine environments, are capable of performing photosynthesis. They exist both in prokaryotic and eukaryotic forms and are considered as a promising source of biofuel (Chisti 2007). They are also known for their ability to produce other commercially interesting products such as omega-3 fatty acids and carotenoids (Spolaore et al. 2006; Plaza et al. 2009). *Chlorella sorokiniana*, a freshwater microalga well-studied for lipid accumulation for biodiesel production (Xia et al. 2013; Vigeolas et al. 2012; Zheng et al. 2012), has also been investigated as a potential natural health product (Chacón-Lee and González-Marino 2010). In our previous study, we have reported the isolation and identification of monogalactosylmonoacylglycerols (MGMGs) from *C. sorokiniana* with their anti-inflammatory activity (Banskota et al. 2013c). Moreover, extracts from *C. sorokiniana* were reported to possess strong antioxidant activity (Matsukawa et al. 2000). Similarly, anti-invasion and apoptosis induction of *C. sorkiniana* in Hep G2 human hepatocellular carcinoma cells reported by Chung et al. (2012) demonstrate the health benefit of this unicellular microalga.

Obesity is a growing global health problem; more than 1.1 billion people worldwide are above their ideal

weight, and 312 million of them are obese (Hossain et al. 2007). Obesity is associated with many diseases, including diabetes, hypertension, and heart disease. Inhibition of pancreatic lipase activity is one of the promising targets for the development of new antiobesity nutraceutical/pharmaceutical products by reducing energy intake through gastrointestinal mechanisms. One of the few drugs that is currently available for the treatment of obesity is orlistat, which reduces intestinal fat absorption via inhibiting pancreatic lipase (Padwal and Majumdar 2007). In this regard, we have tested various microalgal extracts and fractions derived from microalgae against pancreatic lipase. Monogalactosyldiacylglycerol (MGDG)-rich fraction isolated from *C. sorokiana* possessed dose-dependent lipase inhibitory activity. In this paper, we describe the isolation and identification of galactolipids from *C. sorokiana* and their lipase inhibitory activity.

Material and methods

General

The NMR spectra were measured on a Bruker 700 MHz spectrometer with deuterated solvents. Both analytical and preparative HPLC were carried out on an Agilent 1200 Series HPLC equipped with a diode array detector and the HPLC coupled with a 6100B Series Single Quadrupole LC/MS system for LC/MS analysis. High-resolution mass spectra were recorded with a Thermo Fisher Scientific (USA) Exactive mass spectrometer. GC analysis was carried out on an Agilent Technologies 7890A GC spectrometer using an Omegawax 250 fused silica capillary column (30 m×0.25 mm×0.25 μm film thicknesses). Supelco 37 component FAME mix and PUFA-3 (Supelco, USA) were used as fatty acid methyl ester standards. HPLC grade solvents were used for the extraction and purification processes. Roar Lipoprotein Lipase (LPL) activity assay kit was obtained from Roar Biomedicals (New York, NY, USA). Orlistat, porcine pancreatic lipase (type VI-S), 4-methylumbelliferyl oleate (4-MU oleate), and all other reagent chemicals were purchased from Sigma (USA).

Chlorella sorokiniana culture

Chlorella sorokiniana UTEX 1230 was obtained from the UTEX Culture Collection of Algae at The University of Texas, Austin, Texas, USA and was maintained in flask culture in Proteose Medium containing 0.1 % proteose peptone (VWR, USA). Production of *C. sorokiniana* biomass was carried out in a proprietary, internally illuminated closed photobioreactor

maintaining 500 L of cultivation volume as described previously (Banskota et al. 2013c). Approximately 400 L of culture ($\sim 25 \times 10^6$ cells mL^{-1}) was harvested weekly by centrifugation, and the collected material was immediately dried by lyophilization resulting in a typical weekly yield of 200 g of dry biomass.

Extraction and isolation

The extraction process was reported previously (Banskota et al. 2013c). In brief, freeze-dried algal biomass (1.0 kg) was extracted with MeOH (6 L×2) stirring at room temperature for 2 h and filtered. The filtrate was evaporated and coated into Diaion HP-20 resin (2 kg, Supelco, USA) and subjected to silica gel column chromatography (10×12 cm, 70–230 mesh, Sigma-Aldrich, USA) eluting with hexane/EtOAc (2:1, 8 L) followed by EtOAc/MeOH (1:1, 4 L) yielding two fractions of 23.1 and 132.2 g, respectively. The EtOAc/MeOH fraction (30 g) was further subjected to silica gel column chromatography (10×33 cm) eluting with $CHCl_3$/MeOH gradient under gravity collecting 52 fractions (500 mL each). Fractions 18–32 eluted with 5–10 % MeOH in $CHCl_3$ contained mainly MGDGs (7.31 g, ^{1}H and ^{13}C NMR spectra; Fig. S1). The pooled fractions were dissolved in MeOH (500 mg/5.0 mL), and semipreparative HPLC was performed using a Synergi MAX-RP column (10×250 mm, 4 μm, Phenomenex, USA) with MeOH/H_2O (19:1) as the mobile phase under isocratic conditions for 60 min and post column UV detection at 205 nm. The major galactolipids, i.e., (2*S*)-1-*O*-α-linolenoyl-2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-3-*O*-β-D-galactopyranosylglycerol (**2**), (2*S*)-1-*O*-linoleoyl-2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-3-*O*-β-D-galactopyranosylglycerol (**4**), (2*S*)-1-*O*-linoleoyl-2-*O*-(7*Z*,10*Z*-hexadecadienoyl)-3-*O*-β-D-galactopyranosylglycerol (**6**), and (2*S*)-1-*O*-oleoyl-2-*O*-(7*Z*,10*Z*-hexadecadienoyl)-3-*O*-β-D-galactopyranosylglycerol (**8**), were eluted at 23.1, 29.6, 38.1, and 50.5 min, respectively. (2*S*)-1-*O*-(7*Z*,10*Z*-Hexadecadienoyl)-2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-3-*O*-β-D-galactopyranosylglycerol (**1**) was eluted at 20.7 min (Fig. S2).

1: UV (λ_{max}) 200 nm; MS (ESI in positive mode) *m/z* 743.5 (M + Na)$^+$; HRMS in Table 1. ^{1}H NMR (MeOH-d_4) δ 5.31–5.40 (10H, m, H-7′, 8′, 10′, 11′, 7″, 8″, 10″, 11″, 13″ and 14″), 5.26 (1H, m, H-2), 4.44 (1H, dd, *J*=12.0, 3.0 Hz, H_2-1a), 4.23 (1H, d, *J*=6.8 HZ, H-1‴), 4.21 (1H, dd, *J*=12.0, 6.7 Hz, H_2-1b), 3.99 (1H, dd, *J*=10.7, 5.2 Hz, H_2-3a), 3.82 (1H, brd, *J*=3.3 Hz, H-4‴), 3.75 (1H, dd, *J*=11.4, 6.9 Hz, H_2-6‴a), 3.72 (1H, dd, *J*= 10.7, 6.2 Hz, H_2-3b), 3.71 (1H, dd, *J*=11.4, 5.3 Hz, H_2-6‴b), 3.51 (2H, m, H-2‴ and 5‴), 3.45 (1H, dd, *J*=8.9, 3.1 Hz, H-3‴), 2.78–2.82 (6H, m, H_2-9′, 9″ and 12″), 2.33 (4H, m, H_2-2′ and 2″), 2.08 (8H, m, H_2-6′, 12′, 6″ and

Table 1 Na^+ and NH_4^+ adduct ions of MGDG present in MGDG-rich fraction isolated from *C. sorokiniana*

MGDG	RT (min)	Molecular formula	$[M + Na]^+$		$[M + NH_4]^+$		R1/R2
			Observed	Error (ppm)	Observed	Error (ppm)	
1	3.98	$C_{41}H_{68}O_{10}$	743.4696	-1.90	738.5147	−1.23	16:2/16:3
2	4.10	$C_{43}H_{70}O_{10}$	769.4851	-1.94	764.5302	−1.37	18:3/16:3
3	4.41	$C_{41}H_{70}O_{10}$	745.4855	-1.52	740.5305	−1.01	16:2/16:2
4	4.50	$C_{43}H_{72}O_{10}$	771.5012	-1.35	766.5456	−1.68	18:2/16:3
5	4.50	$C_{43}H_{72}O_{10}$	771.5012	-1.35	766.5456	−1.68	18:3/16:2
6	4.80	$C_{43}H_{74}O_{10}$	773.5163	-2.04	768.5615	−1.34	18:2/16:2
7	5.29	$C_{43}H_{76}O_{10}$	775.5321	-1.89	770.5771	−1.43	18:2/16:1
8	5.29	$C_{43}H_{76}O_{10}$	775.5321	-1.89	770.5771	−1.43	18:1/16:2
9	5.70	$C_{43}H_{78}O_{10}$	777.5480	-1.51	772.593	−1.16	18:2/16:0
10	5.76	$C_{43}H_{78}O_{10}$	777.5480	-1.51	772.5929	−1.23	18:1/16:1
11*	6.09	$C_{43}H_{80}O_{10}$	779.5639	-1.25	774.6088	−0.98	18:1/16:0
12*	6.60	$C_{43}H_{82}O_{10}$	781.5795	-1.36	776.624	−1.16	16:0/18:0

The position of fatty acids in *sn*-1 and *sn*-2 position was not known because of absence of appropriate fragment ions

15″), 1.62 (4H, m, H_2-3′ and 3″), 1.29–1.40 (14H, m, H_2-4′, 5′, 13′, 14′, 15′, 4″ and 5″), 0.97 (3H, t, *J*=8.0 Hz, H_3-16″), 0.91 (3H, t, *J*=7.0 Hz, H_3-16′).

6: UV (λ_{max}) 200 nm; MS (ESI in positive mode) *m/z* 751.6 $(M + H)^+$, 589.5 $(M + H - C_6H_{10}O_5)^+$; HRMS in Table 1. ^1H NMR (MeOH-d_4) δ 5.31–5.40 (8H, m, H-9′, 10′, 12′, 13′, 7″, 8″, 10″ and 11″), 5.26 (1H, m, H-2), 4.44 (1H, dd, *J*=12.0, 3.0 Hz, H_2-1a), 4.23 (1H, d, *J*=6.8 HZ, H-1‴), 4.21 (1H, dd, *J*=12.0, 6.7 Hz, H_2-1b), 3.99 (1H, dd, *J*=10.7, 5.2 Hz, H_2-3a), 3.82 (1H, brd, *J*=3.3 Hz, H-4‴), 3.75 (1H, dd, *J*=11.4, 6.9 Hz, H_2-6‴a), 3.72 (1H, dd, *J*=10.7, 6.2 Hz, H_2-3b), 3.71 (1H, dd, *J*=11.4, 5.3 Hz, H_2-6‴b), 3.51 (2H, m, H-2‴ and 5‴), 3.45 (1H, dd, *J*=8.9, 3.1 Hz, H-3‴), 2.78 (4H, m, H_2-11′ and 9″), 2.32 (4H, m, H_2-2′ and 2″), 2.07 (8H, m, H_2-8′, 14′, 6″ and 12″), 1.61 (4H, m, H_2-3′ and 3″), 1.29–1.40 (24H, m, H_2-4′, 5′, 6′, 7′, 15′, 16′, 17′, 4″, 5″, 13″, 14″ and 15″), 0.91 (6H, t, *J*=6.8 Hz, H_3-16″ and 18′).

8: UV (λ_{max}) 200 nm; MS (ESI in positive mode) *m/z* 753.5 $(M + H)^+$, 591.5 $(M + H - C_6H_{10}O_5)^+$; HRMS in Table 1. ^1H NMR (MeOH-d_4) δ 5.31–5.40 (6H, m, H-9′, 10′, 7″, 8″, 10″ and 11″), 5.26 (1H, m, H-2), 4.44 (1H, dd, *J*=12.0, 3.0 Hz, H_2-1a), 4.23 (1H, d, *J*=6.8 HZ, H-1‴), 4.21 (1H, dd, *J*=12.0, 6.7 Hz, H_2-1b), 3.99 (1H, dd, *J*=10.7, 5.2 Hz, H_2-3a), 3.82 (1H, brd, *J*=3.3 Hz, H-4‴), 3.75 (1H, dd, *J*=11.4, 6.9 Hz, H_2-6‴a), 3.72 (1H, dd, *J*=10.7, 6.2 Hz, H_2-3b), 3.71 (1H, dd, *J*=11.4, 5.3 Hz, H_2-6‴b), 3.51 (2H, m, H-2‴ and 5‴), 3.45 (1H, dd, *J*=8.9, 3.1 Hz, H-3‴), 2.78 (2H, m, H_2-9″), 2.32 (4H, m, H_2-2′ and 2″), 2.05 (8H, m, H_2-8′, 11′, 6″ and 12″), 1.61 (4H, m, H_2-3′ and 3″), 1.29–1.40 (28H, m, H_2-4′, 5′, 6′, 7′, 12′, 13′, 14′, 15′, 16′, 17′, 4″, 5″, 13″, 14″ and 15″), 0.91 (6H, m, H_3-16″ and 18′).

LC-HRMS conditions

LC-HRMS data was acquired on an Accela UHPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, USA), using conditions adapted from a previously reported method (MacDougall et al. 2011). The Accela system consisted of a quaternary pump and autosampler, using a ThermoHypersil Gold C8 column (100 × 2.1 mm, 1.9 μm) at 40 °C. The mobile phase consisted of A, 10 mM ammonium acetate in water (pH adjusted to 5.0 with acetic acid); B, acetonitrile; and C, isopropanol. A gradient with a flow rate of 500 μL min^{-1} was used: 30 % A/70 % B to 100 % B from 0 to 5 min, ramped to 95 % B/5 % C from 5 to 6 min, 20 % B/80 % C from 6 to 12 min, then held at 20 % B/80 % C from 12 to 14.5 min. The column was then re-equilibrated back to 30 % A/70 % B for 5 min. While isopropanol was not required to elute the MGDGs, it served to flush the column of any background triacylglycerols that accumulated on the column. Injection volume was 3.0 μL, and MGDG fraction was analyzed in triplicate.

The Exactive mass spectrometer was operated with an optional high-temperature ESI probe (HESI-II) and an HCD collision cell that allowed for "all-ion fragmentation" (AIF). In positive ion mode, the source parameters were set as follows: sheath gas, 60; auxiliary gas flow, 15; sweep gas, 0; spray voltage, 3.30 kV; capillary temperature, 380 °C; capillary voltage, 42.5 V; tube lens voltage, 125 V; skimmer voltage, 26 V; heater temperature, 350 °C. Alternating full spectrum scans and AIF scans at 50 eV were collected at 2 Hz using the "High" resolution setting (50,000 FWHM) over a mass range of 100–2000 *m/z*.

Alkaline hydrolysis of fraction containing MGDGs

The MGDG-rich fraction (650 mg in 10 mL MeOH) was treated with 6 % NaOMe solution (10 mL, Sigma-Aldrich, USA) at room temperature and stirred for 2 h. The reaction was quenched by adding water (20 mL) and extracted with hexane (50 mL×2). The aqueous/MeOH fraction was dried under reduced pressure, redissolved in water (5.0 mL), and applied onto an Amberlite MB-150 Mixed Bed Ion-exchange column (3.0×4.0 cm, Sigma-Aldrich, USA) and eluted with water (50 mL) yielding $2R$-1-O-β-D-galactopyranosylglycerol (120 mg) having identical spectral data with previous reported data (Oshima et al. 1994). The hexane fraction (188.9 mg) containing fatty acid methyl ester (FAME) was subjected to GC analysis. Methyl esters of hexadecadienoic acid, hexadecatrieonic acid, α-linolenic acid, linoleic acid, palmitic acid, oleic acid, palmitoleic acid, and stearic acid were detected in GC analysis (Fig. S3).

Pancreatic lipase inhibition assay

Extracts were screened for pancreatic lipase inhibitory activity in 96-well plates using LPL emulsion or 4-MU oleate as substrate. The reaction mixture consisted of 47-μL phosphate-buffered saline (pH 7.4), 2.0-μL extract solution in DMSO, and 1.0-μL porcine pancreatic lipase (PPL) solution (0.5 μg) which was incubated at 37 °C for 5 min. Following the incubation, 50 μL of substrate (100 μM 4-MU oleate or 200-fold dilution of LPL substrate) was added to give a final volume of 100 μL per well to start the reaction. Lipase activity was monitored for 30 min using a fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices) at an excitation wavelength of 327 nm and an emission wavelength of 450 nm for 4-MUoleate and excitation wavelength of 370 nm and an emission wavelength of 450 nm for LPL. MGDG-rich fraction was tested in μg mL^{-1}, and pure compounds were tested in molar concentrations. Orlistat, a known porcine pancreatic lipase (PPL) inhibitor, was used as a positive control. Inhibition effects are expressed as the mean value of two independent assays in duplicate for MGDG-rich fraction and pure MGDG with LPL substrate. Data for orlistat with both substrates and MGDG fraction with 4-MU oleate substrate were from single assay in duplicate.

Results and discussion

Galactolipids are reported to have a wide range of biological activities including antimicrobial, antitumor-promoting, anti-inflammatory, and anti-microfouling (Christensen 2009). In our earlier study, we have reported nitric oxide (NO) inhibitory activity of MGDGs and digalactosyldiacylglycerols (DGDG) isolated from both marine and freshwater microalgae

(Banskota et al. 2013a, b). In the present study, we have isolated a MGDG-rich fraction isolated from *C. sorokianina* and tested it for pancreatic lipase inhibitory activity. The MGDG-rich fraction showed dose-dependent pancreatic lipase inhibitory activity (Fig. 1a). At a 50-μg/mL concentration, 79.6 % of lipase inhibition was shown by the MGDG fraction when LPL was used as substrate. In a separate experiment, only 45.4 % lipase inhibition was observed using 4-MU oleate as substrate (Fig. S4a). To biologically compare 4-MU oleate substrate to the ROAR LPL emulsion substrate is not possible because Roar Biomedical cannot divulge the structure of their substrate. In the present study, MGDG-rich fraction gave a better dose-dependent response in LPL substrate compared to 4-MU oleate. Orlistat, a known lipase inhibitor used as a positive control, showed 83.9 % inhibition of lipase activity at 6.0 μM concentration with LPL as a substrate (Fig. 1b) and possessed stronger inhibition activity against pancreatic lipase when 4-MU oleate was used as a substrate (Fig. S4b). Even though the lipase inhibitory potency of the MGDG-rich fraction was weaker than orlistat, their ability to inhibit the lipase is itself significant because MGDGs are present in almost all green vegetables that we consume daily (Christensen 2009).
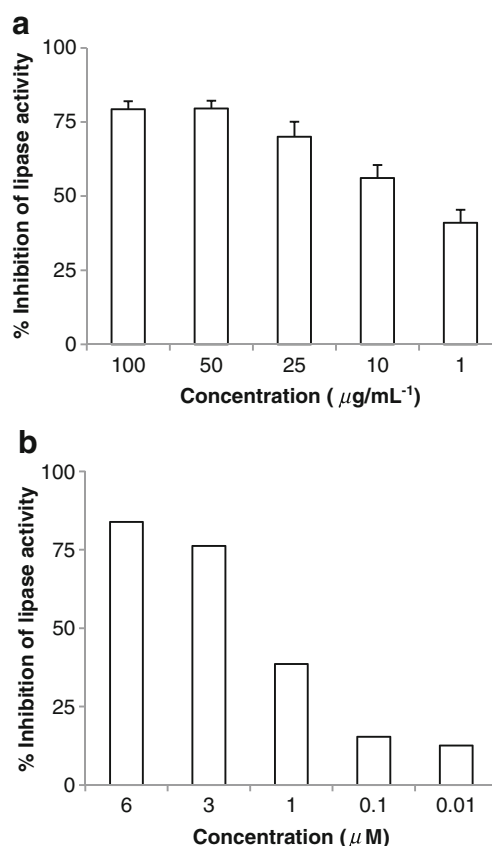

Fig. 1 Lipase inhibitory effect of monogalactosyldiacylglycerol (MGDG)-rich fraction of *C. sorokiniana* (**a**) and orlistat (**b**). Results are from two independent assays for MGDG fraction and a single assay for orlistat in LPL-substrate

For identification of the individual MGDGs, the glycolipid-rich fraction derived from *C. sorokianina* was subjected for LC/HRMS analysis. The LC/HRMS technique is well established for the structure determination of complex mixtures of polar lipids including galactolipids, sulfolipids, and phospholipids. Xu et al. (2010) described the UPLC-

Fig. 2 MS data of **1**. **a** Fragmentation pattern of MGDG following different pathways for ammonium or sodium adducts. **b** Positive-ion MS spectrum. **c** Positive-ion all-ion fragmentation (AIF) spectrum
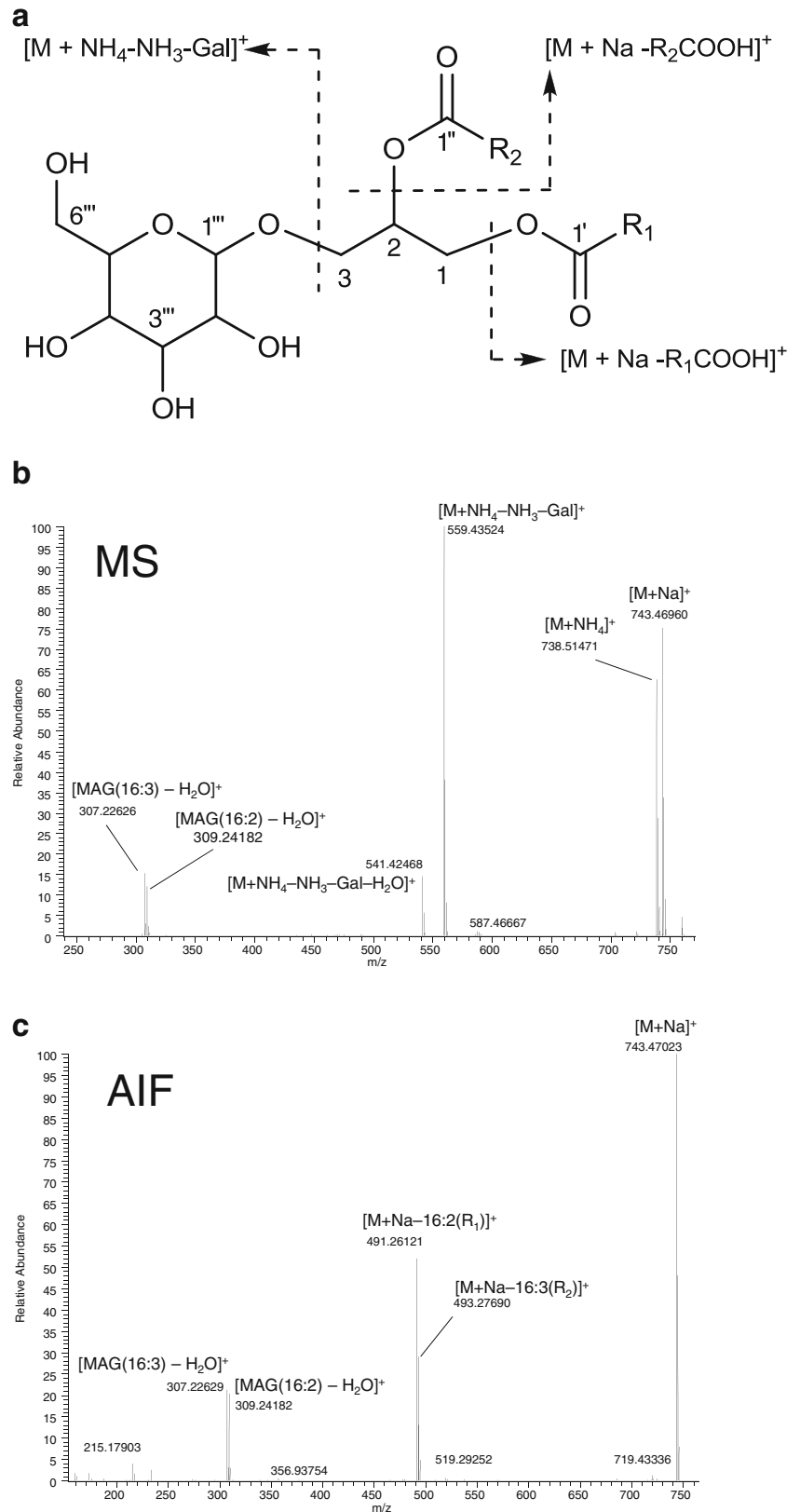
ESI-Q-TOF MS method for the identification of glycerolipids of the diatom *Stephanodiscus* sp., reporting that two strong fragment ions $[M + Na − R_xCOOH]^+$ resulting from the neutral loss of fatty acids from the precursor ion $[M + Na]^+$ and the mass difference between $[M + Na]^+$ and $[M + Na − R_xCOOH]^+$ were used for identification of the fatty acid acyl chain. Under different collision energy from 5 to 80 V, the abundances of $[M + Na − R_1COOH]^+$ were always higher than $[M + Na − R_2COOH]^+$ ion, indicating that the loss of acyl chain linking to *sn*-1 position of glycerol was more facile than the elimination of acyl chain connecting to the *sn*-2 position of MGDG (Li et al. 2008; Guella et al. 2003; Xu et al. 2010).

In the current study, LC-HRMS was performed using a Thermo Exactive bench-top mass spectrometer with Orbitrap technology. Data was acquired in a nontargeted fashion with alternating MS scans and "all-ion fragmentation (AIF)" scans using the optional HCD cell. It should be noted that AIF is performed without precursor ion isolation; so, these spectra should be considered pseudo-MS/MS spectra. A typical example of this data for MGDG **1** is shown in Fig. 2. Both NH_4^+ and Na^+ adduct ions were observed in the MS spectrum, along with fragment ions of the less stable NH_4^+ ion caused by in-source fragmentation. The ion at *m/z* 559.43524 represents the loss of NH_3 followed by loss of the galactosyl unit. The ions at *m/z* 307.22626 and 309.24182 are formed by losses of fatty acids 16:2 and 16:3, respectively, from the $[M + NH_4 − NH_3 − Gal]^+$ ion. For simplicity, these monoacylglycerol-type (MAG) ions are denoted by $[MAG(16:3) − H_2O]^+$ and $[MAG(16:2) − H_2O]^+$, respectively, in Fig. 2. In the AIF spectrum, the MGDG ammonium adduct and associated $[M + NH_4 − NH_3 − Gal]^+$ ion were completely eliminated, leaving only the monoacylglycerol-type ions remaining from this fragmentation pathway. By contrast, the highly stable MGDG sodium adduct dominated the AIF spectrum, along with its associated fatty acid neutral loss fragments at *m/z* 491.26121 and 493.27690. Based on the greater intensity of the *m/z* 491.26121 ion (16:2), the planer structure of **1** should be MGDG with the acyl group hexadecadienoyl in position *sn*-1 and hexadecatrienoyl in position *sn*-2, according to previous reports (Xu et al. 2010; Guella et al. 2003). Therefore, the MS and AIF scans provided complimentary data, where the MS scan confirmed the presence of MGDGs by loss of the galactosyl unit and associated fatty acid losses, and the AIF spectrum provided regiospecific information for the fatty acids. Similarly, 12 individual MGDGs were detected, and the regiospecificity of the two acyl side chains was thus identified (Table 1, Fig. 3, Fig. S5–S11), with the exception of compounds **11** and **12**, where sodium adduct ions were not observed, likely due to insufficient background sodium levels in the acetonitirile at the end of the LC gradient.

MGDGs **1–8** were further purified by semipreparative HPLC for structure confirmation, and major compounds were also tested for lipase inhibitory activity. The ^{1}H NMR data of **1** showed signal corresponding to 10 unsaturated protons, 6
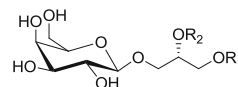
1 R₁ = 7Z,10Z-hexadecadienoyl, 16:2, R₂ = 7Z,10Z,14Z-hexadecatrinoyl
2 R₁ = α-linolenoyl, R₂ = 7Z,10Z,14Z-hexadecatrinoyl
3 R₁ = 7Z,10Z-hexadecadienoyl, R₂ = 7Z,10Z-hexadecadienoyl
4 R₁ = linoleoyl, R₂ = 7Z,10Z,14Z-hexadecatrinoyl
5 R₁ = α-linolenoyl, R₂ = 7Z,10Z-hexadecadienoyl
6 R₁ = linoleoyl, R₂ = 7Z,10Z-hexadecadienoyl
7 R₁ = linoleoyl, R₂ = palmitoleoyl
8 R₁ = oleoyl, R₂ = 7Z,10Z-hexadecadienoyl
9 R₁ = linoleoyl, R₂ = palmitoyl
10 R₁ = oleoyl, R₂ = palmitoleoyl

Fig. 3 Structure of MGDG (**1–10**) present in *C. sorokiniana*

oxygenated methine protons, 18 methylene groups including three oxygenated methylene, and 2 primary methyl groups (Fig. S12). Since all the olifenic proton signals of **1** were observed between 5.31 and 5.40 ppm as identical to the reported data of MGDG having polyunsaturated fatty acid acyl side chain with all *cis*-double bonds, it is likely that the double bonds of **1** should be all-*cis* (Banskota et al. 2013a, b). Moreover, NaOMe mediated hydrolysis the MGDG-rich fraction yielded 2R-1-O-β-D-galactopyranosylglycerol and mixture of FAME containing: hexadecadienoic acid, hexadecatrieonic acid, α-linolenic acid, linolenic acid, palmitoleic acid, oleic acid, palmitic acid, and stearic acid. Based on both LC-HRMS, NMR data, and hydrolyzed products, the stereostructure of **1** was elucidated as (2S)-1-O-(7Z,10Z-hexadecadienoyl)-2-O-(7Z,10Z,13Z-hexadecatrienoyl)-3-O-β-D-galactopyranosylglycerol. Similarly, stereostructures of **2–10** were also elucidated, and among the individual MGDGs, compounds **1**, **4**, and **8** were new natural products (Figs. S13 and S14). The spectral data of known compounds were identical to published data.

Because of the dose-dependent lipase inhibitory activity of the MGDG-rich fraction in LPL substrate, we further tested the major MGDGs, i.e, **2**, **4**, **6**, and **8** for their lipase inhibitory activity in LPL substrate, and the results are shown in Fig. 4.
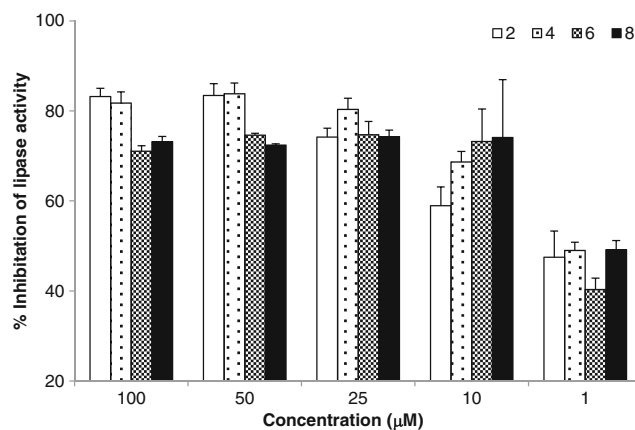
Fig. 4 Lipase inhibitory activity of major MGDGs (**2**, **4**, **6**, and **8**) isolated from *C. sorokiniana*

All the tested MGDGs showed strong lipase inhibitory activity of >70 % inhibition at 100- and 50-μM concentrations and around 50 % inhibition at 1.0-μM concentration. MGDGs **2** and **4** having higher degrees of unsaturation in their fatty acid acyl side chains gave dose-dependent activity. Murakami et al. (2003) used pancreatic lipase for selective hydrolysis of MGDG to MGMG, and the hydrolyzed products containing MGMGs inhibited the activities of all mammalian DNA polymerases. Similarly, Maeda et al. (2005) also used lipase to hydrolyze SQDG, MGDG, and DGDG obtained from spinach (*Spinacia oleracea*) to yield monoacyl derivatives having antitumor activity. This finding strongly suggests that MGDGs are directly hydrolyzed by pancreatic lipase, which is primarily responsible for hydrolysis of triglyceride (TAG) in the GI tract (Ros 2000). Thus, consumption of these MGDGs either from green vegetables or microalgae may reduce the absorption of unwanted fats by reducing pancreatic lipase activity. It is possible that MGDGs may directly compete with TAGs for hydrolysis by pancreatic lipase and help to reduce fat absorption; further study is needed to confirm such competition. In addition to the lipase inhibitory activity, six out of 12 MGDGs identified in the present study have at least one essential fatty acid in their side chain (*sn*-1 position), i.e., linoleic acid and α-linolenic acid, indicating that *C. sorokiniana* is a potential source of those essential fatty acids. Taken together, our results led us to conclude that *C. sorokianina* is an excellent source of MGDGs, which may have potential to fight against unwanted fat absorption within the GI track by reducing pancreatic lipase activity. Further in vivo study is warranted to confirm efficacy and potency of MGDG toward pancreatic lipase inhibitory activity.

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