A New Unsaturated Glycoglycerolipid from a Cultured Marine Dinoflagellate *Amphidinium carterae*

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From the cultured marine dinoflagellate *Amphidinium carterae*, a new unsaturated glycoglycerolipid (2*S*)-1,2-*O*-6,9,12,15-dioctadecatetraenoyl-3-*O*-[α-D-galactopyranosyl-(1''''→6''')-*O*-β-D-galactopyranosyl]-glycerol (1), has been isolated together with two known saturated ones, (2*S*)-1,2-distearoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (2) and (2*S*)-1-stearoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol (3). Their structures were elucidated on the basis of chemical and spectral data.

Key words dinoflagellate; *Amphidinium carterae*; glycoglycerolipid

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. In recent years, a large number of studies have been reported on the toxic effects of dinoflagellate to the bivalves.[1—6] In our search for the toxic substances from dinoflagellate *Amphidinium carterae*, we have found that the toluene extract of the cultured *A. carterae* showed toxic to pearl oysters. Bioassay-guided purification of the toluene soluble fraction resulted in the isolation of a new glycoglycerolipid (**1**), which is an unsaturated digalactosyl diacylglycerol, together with two saturated known ones (**2**, **3**). Previously, it has been reported that glycoglycerolipids derived from microalgae can be sources of biologically active substance including toxins.[7,8] Although a large number of studies have been made on the identification of glycoglycerolipids in some marine dinoflagellates,[9—13] little is known about the glycoglycerolipids in *A. carterae*. In this paper, we report the structure of a new unsaturated glycoglycerolipid (**1**), which has been identified from its chemical and spectral data.

The marine dinoflagellate *A. carterae* was cultured in seawater media (K) in 150 l pools at 25 °C. The cultivation was carried out applying a 14 : 10 h light to dark cycle using cool-white fluorescent lights for 4 weeks. The cells harvested by filtration and extracted with toluene/methanol (1 : 3, 2 l×4). The combined extract was partitioned between the toluene and 1 N NaCl solvent. The organic layer (26 g) was subjected to column chromatography on silica gel using 2—100% methanol in chloroform as eluent to afford a bioactive fraction containing a mixture of glycoglycerolipids. Further separation by reversed-phase silica gel (ODS) column chromatography and HPLC (ODS) yielded an unsaturated digalactosyl diacylglycerol (**1**) and two saturated glycoglycerolipids (**2**, **3**).

The digalactosyl diacylglycerol **1**, a pale yellow amorphous powder, was determined to have the molecular formula $C_{51}H_{80}O_{15}$ by positive mode high-resolution electrospray ionization (HR-ESI) mass spectrometry (*m*/*z*: 955.5390 $[M+Na]^+$, $C_{51}H_{80}O_{15}Na$ requires 955.5395). The ^{1}H- and ^{13}C-NMR spectra data of **1** indicated the presence of a sugar and long-chain unsaturated fatty acid ester moieties strongly suggesting a glycoglycerolipid. Analysis of the ^{1}H-, ^{13}C-NMR spectra of **1**, together with ^{1}H–^{1}H COSY, HMQC and HMBC experiments, led to the assignments of all the ^{1}H- and ^{13}C-NMR signals for the sugar and glycerol moieties as shown in Table 1. One group of signals at δ_C 105.1, 72.4, 74.6, 69.9, 74.4, 67.8 and δ_H 4.26 (d, *J*=7.6 Hz, diaxial, H-1'''), 3.52 (dd, *J*=3.2 Hz, axial–equatorial, 9.6 Hz, H-3''') indicated the presence of a 6-*O*-substituted β-D-galactopyranose unit. Another group of signals at δ_C 100.6, 70.2, 71.4, 71.2, 72.3, 62.8 and δ_H 4.90 (d, *J*=3.6 Hz, axial–equatorial, H-1''''), 3.76 (dd, *J*=3.6 Hz, axial–equatorial, 9.6 Hz, H-3'''') suggested a terminal α-D-galactopyranose unit. Moreover, the other group of signals at δ_C 64.1, 68.7, 71.7 and δ_H 4.24 (dd, *J*=6.5, 12.0 Hz), 4.48 (dd, *J*=3.0, 12.0 Hz), 5.30 (m), 3.78 (dd, *J*=3.5, 10.5 Hz), 3.98 (dd, *J*=5.3, 10.5 Hz) revealed a glycerol moiety. In the HMBC experiment, the anomeric H-

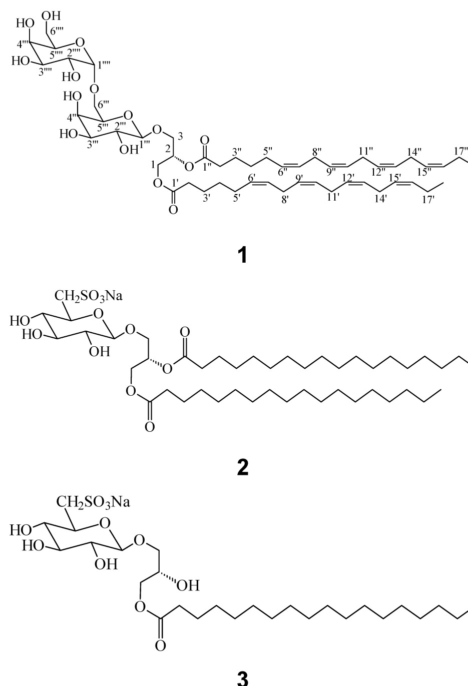

Fig. 1. Structures of Compounds **1**—**3**

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for the Glycerol and Sugar Moieties of **1** (500 MHz for ^{1}H and 125 MHz for ^{13}C, Methanol-d_4)

Carbon no.	^{1}H δ [mult., J (Hz)]	^{13}C δ
1	4.24 (dd, 6.5, 12.0)	64.1
	4.48 (dd, 3.0, 12.0)	
2	5.30 (m)	71.7
3	3.78 (dd, 3.5, 10.5)	68.7
	3.98 (dd, 5.3, 10.5)	
β-Gal		
1‴	4.26 (d, 7.6)	105.1
2‴	3.58 (dd, 7.6, 9.6)	72.4
3‴	3.52 (dd, 3.2, 9.6)	74.6
4‴	3.90 (br d, 3.2)	69.9
5‴	3.76 (m)	74.4
6‴	3.70 (dd, 6.0, 11.2)	67.8
	3.94 (dd, 5.4, 11.2)	
α-Gal		
1⁗	4.90 (d, 3.6)	100.6
2⁗	3.81 (dd, 3.6, 9.6)	70.2
3⁗	3.76 (dd, 3.6, 9.6)	71.4
4⁗	3.92 (br d, 3.6)	71.2
5⁗	3.88 (m)	72.3
6⁗	3.74 (dd, 5.4, 11.2)	62.8
	3.77 (dd, 6.0, 11.2)	

1‴ of the β-D-galactopyranosyl showed correlation to the C-3 of the glycerol moiety and the anomeric H-1⁗ of the α-D-galactopyranosyl to the C-6‴ of the β-D-galactopyranosyl. All the above findings were consistent with that **1** was a 1,2-di-*O*-acyl-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-glycerol. Furthermore, the ^{13}C-NMR spectrum of **1** showed two carbonyl carbon signals arising from the acyl groups at δ_C 174.7 and 174.5, and their locations in the molecule was similarly confirmed by the long-range coupling detected by the HMBC experiment. Thus the carbonyl carbons at δ_C 174.7 (C-1″) and 174.5 (C-1′) were correlated with the proton signals of H-1 and H-2 of the glycerol part, respectively.

To characterize the structure of the fatty acid moiety, compound **1** was treated with NaOMe–MeOH according to a reported method,[10] yielding a digalactosyl glycerol (**4**) and a fatty acid methyl ester (**5**). The digalactosyl glycerol **4**, $[\alpha]_D^{25}$ +83° (c=0.50, H_2O), was shown to be identical with (2*R*)-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-glycerol on the basis of a comparison with the optical rotation and NMR data of previously reported data for this compound.[10,14] And the fatty acid moiety **5** was determined by the GC-MS analyses of the above methyl ester and found to be a methyl 6,9,12,15-octadecatetraenoate by comparison with the standard sample. Consequently, the structure of **1** was determined as (2*S*)-1,2-*O*-6,9,12,15-dioctadecatetraenoyl-3-*O*-[α-D-galactopyranosyl-(1⁗→6‴)-*O*-β-D-galactopyranosyl]-glycerol.

By the same method, compounds **2** and **3** were identified as (2*S*)-1,2-distearoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol and (2*S*)-1-stearoyl-3-*O*-(6-sulpho-α-D-quinovopyranosyl)-glycerol, respectively.

Experimental

General Procedures NMR spectra were recorded in methanol-d_4 using a Bruker AV-500 spectrometer (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) with tetramethylsilane as internal standard. ESI-MS spectra were measured on a Bruker APEX II spectrometer in positive ion mode. Optical rotations were measured with an AA-10R digital polarimeter. Preparative HPLC was carried out on ODS columns (250×10 mm i.d., YMC) with a Waters 996 photodiode array detector. For CC, silica gel (200—300 mesh) (Qingdao Mar. Chem. Ind. Co. Ltd.), octadecylsilyl silica gel (80—100 mm) (Unicorn) and Sephadex LH-20 gel (Pharmacia) were used. The spray reagent used for TLC was 5% H_2SO_4 and 5% phosphomolybdic acid in 95% ethanol.

Material and Cultivation The dinoflagellate *Amphidinium carterae* strain was obtained from the Tropical Marine Biological Research Station in Hainan, Chinese Academy of Sciences. It was isolated from a shallow coastal reef in the Gulf of Sanya, Hainan Island, at the ambient seawater temperature of about 20 °C in March 2001. Its isolation and purification was done by the micropipette and serial-dilution technique, and in the agar petri dishes containing K medium until obtaining a uniagal culture. Liquid culture of *A. carterae* was grown in 150 l pools filled with seawater medium (K) at 25 °C for 3 weeks with air bubbling, applying a 14 : 10 h light : dark cycle provided by cool-white fluorescent lights.

Extraction and Isolation The cultured cells were harvested by filtration and extracted with toluene/methanol (1 : 3, 21×4). The combined extract was partitioned between the toluene and 1 N NaCl solvent. The organic layer (26 g) was subjected to column chromatography on silica gel using 2—100% methanol in chloroform as eluent to afford a bioactive fraction containing a mixture of glycolipids. This fraction was further separated by an ODS silica gel column chromatography (CC) eluted with MeOH–H_2O (9 : 1) to give in impure form. Final purification was carried out by repetitive separation on a preparative HPLC column (YMC-Pack ODS-5-A, 250×20 mm i.d.), flow rate 8.0 ml/min; solvent MeOH–H_2O (95 : 5); detected UV (215 nm) to yield an unsaturated glycoglycerolipid **1** (25 mg) in pure form. Using the same method as above, the other two saturated glycerolipids **2** (15 mg) and **3** (30 mg) were obtained from the CC of silica and ODS silica gel.

Digalactosyl Diacylglycerol (**1**): $[\alpha]_D^{25}$ +52° (c=0.80, MeOH); ^{1}H-NMR (500 MHz, CD_3OD) δ 5.36 (16H, m, H-6′, H-6″, H-7′, H-7″, H-9′, H-9″, H-10′, H-10″, H-12′, H-12″, H-13′, H-13″, H-15′, H-15″, H-16′, H-16″); 2.84 (12H, H_2-8′, H_2-8″, H_2-11′, H_2-11″, H_2-14′, H_2-14″), 2.34 (4H, t, J=7.0 Hz, H_2-2′, H_2-2″), 2.07 (8H, m, H_2-5′, H_2-5″, H_2-17′, H_2-17″), 1.67 (4H, m, H_2-3′, H_2-3″), 1.37 (4H, m, H_2-4′, H_2-4″), 0.96 (6H, t, J=7.6 Hz, H_3-18′, H_3-18″); ^{13}C-NMR (125 MHz, CD_3OD) δ 174.7 (C-1″), 174.5 (C-1′), 132.8 (×2, C-16′, C-16″), 130.6, 129.9, 129.9, 129.4, 129.3, 129.2, 129.2, 129.1, 129.1, 129.0, 128.9, 128.9 (C-6′, C-6″, C-7′, C-7″, C-9′, C-9″, C-10′, C-10″, C-12′, C-12″, C-13′, C-13″), 128.1 (×2, C-15′, C-15″), 35.0, 34.2 (C-2′, C-2″), 30.7, 30.0 (C-4′, C-4″), 27.8, 27.5 (C-5′, C-5″), 26.6 (×2), 26.6 (×2), 26.4 (×2) (C-8′, C-8″, C-11′, C-11″, C-14′, C-14″), 25.8, 25.6 (C-3′, C-3″), 21.5 (×2, C-17′, C-17″), 14.7 (×2, C-18′, C-18″); HR-ESI-MS m/z: $[M+Na]^+$ 955.5390 (Calcd for $C_{51}H_{80}O_{15}Na$: 955.5395).

Methanolysis of 1—3 A solution of **1** (10 mg) in methanol (1.5 ml) was treated with 3% NaOMe–MeOH (1.5 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was neutralized by with AG 50W-X8 (BIO-RAD, H^+ form) and partitioned between chloroform and water. Evaporation of the solvent under reduced pressure from the chloroform phase to yield a fatty acid methyl ester (**5**), which was identified by GC-MS to be a methyl 6,9,12,15-octadecatetraenoate. And the aqueous phase was evaporated under reduced pressure to give a residue, which was purified by CC of Sephadex LH-20 to furnish a digalactosyl glycerol (**4**).

By the same method, each of compounds **2** and **3** yielded stearic acid methyl ester and (6-sulpho-α-D-quinovopyranosyl)-glycerol, which were identified by GC-MS analysis and comparison of its spectral data with that of literature,[15] respectively.

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