Studies on Glycolipids. VI.[1] New Acyl-Distributed Glyceroglycolipids from the Nitrogen-Fixing Cyanobacterium *Anabaena flos-aquae f. flos-aquae*

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Two unprecedented acyl-distributed glyceroglycolipids have been isolated from the nitrogen-fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae*. The structures were determined as (2′*S*)-3′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (1) and (2′*R*)-2′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (2) on the basis of physicochemical evidence. The positional distribution of fatty acid residues was elucidated by enzymatic hydrolysis using *Rhizopus arrhizus* lipase.

Keywords glyceroglycolipid; nitrogen-fixing algae; *Anabaena flos-aquae f. flos-aquae*; cyanobacterium

Galactolipids are major constituents of the chloroplast membrane in the plant kingdom. The biological function as well as occurrence and distribution of galactolipids has been an area of intense interest and investigation.[3] Furthermore, glycolipids possessing pharmacological activity or unique structural features have been isolated from various organisms other than higher plants.[4] Cyanobacteria belong to the category of photosynthetic microalgae and are gram-negative bacteria. Some of them conduct nitrogen-fixation in a heterocyst possessing a thicker cell wall than a usual vegetate cell.[5] Since glycolipids are involved in several membrane functions such as photosynthesis, a specific glycolipid with unique structure and function may be located in heterocyst cell walls. Nevertheless, only limited investigations have been undertaken on glycolipids of nitrogen-fixing cyanobacteria. Thus, we have examined glycolipids of the nitrogen-fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae* as a part of our studies on glycolipids in microalgae.[6] In a previous paper, we characterized a new acyl-distributed glyceroglycolipid, (2′*S*)-3′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (**1**), from this nitrogen-fixing cyanobacterium.[7] Further investigation of glycolipid constituents in the alga led us to isolate another unprecedented acyl-distributed glyceroglycolipid, (2′*R*)-2′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (**2**). We present here the full details of the structural elucidation of these glyceroglycolipids.

Cultures were grown for 3 weeks in Closterium-Bicine (CB)-medium in 5 l Erlenmeyer flasks at 25 °C illuminated continuously with cool-white fluorescent lights (1500 lux) and aerated vigorously with sterilized air through a 0.2 μm membrane filter at the rate of 0.5 l per minute. The alga was harvested by centrifugation at 20000 × *g* and lyophilized to give 8.02 g of lyophilized cells from the combined 40-l culture. Extraction of the alga (8.02 g) with $CHCl_3$: MeOH (1 : 1) at room temperature gave 1.16 g of an extract. Thin layer chromatographic (TLC) analysis of the extract disclosed a spot characteristic of this nitrogen-fixing cyanobacterium and showing a positive coloration to anthrone reagent.[8] Repeated silica gel chromatography ($CHCl_3$: MeOH = 20 : 1 → 10 : 1) of the extract and subsequent normal-phase high-performance liquid chromatography (HPLC) ($CHCl_3$: MeOH = 96 : 4) afforded two glycolipids **1** (6.0 mg) and **2** (3.1 mg).

Compound **1** was obtained as a white amorphous powder and showed a hydroxyl and two ester carbonyl absorption bands in the infrared (IR) spectrum. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** exhibited features of a glyceroglycolipid: it showed the presence of terminal methyls (δ 0.88, t, 3H × 2) and a mass of methylene groups (δ 1.28) as a 12H signal covering the range from 4.14 to 4.90 ppm. Detailed analysis of the homonuclear decoupling spectra defined β-D-galactopyranoside. In addition, observation of carbon signals due to two ester carbonyl at 173.6 ppm (C = O × 2) and two terminal methyl groups at 14.3 ppm (CH_3 × 2) in the 13-carbon nuclear magnetic resonance (^{13}C-NMR) spectrum indicated the presence of two acyl groups in **1**. Although the glycolipid contained both a β-galactosyl-glycerol moiety and two acyl residues, it was apparently different from a monogalactosyl diacylglycerol distributed widely in the plant membrane.[6a)]

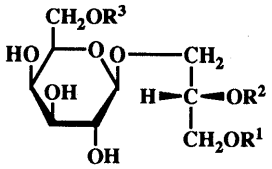
Intensive comparison of the ^{1}H-NMR spectra of both glyceroglycolipids disclosed the apparent difference that the proton signal due to the C-6 methylene group in **1** was observed at lower field and the signal assignable to glycerol 2′-H in **1** appeared at higher field than those of monogalactosyl diacylglycerol. In the ^{13}C-NMR spectrum, the C-6 signal in **1** was displaced downfield by 2.2 ppm and the neighboring C-5 signal was shifted upfield by 3.0 ppm. With respect to the carbon signals of glycerol, those of C-1′, C-2′, and C-3′ were also shifted by +4.0, −2.2, and +3.2 ppm. These acylation shifts suggested the two acyl residues to be present at C-6 and C-3′.[7] This presumption was confirmed by the heteronuclear multiple bond correlation (HMBC) spectrum of **1** in which two carbonyl carbon signals showed cross peaks due to long-range coupling with the proton signals of the C-6 and C-3′ methylene groups.

Compound **2**[9] was obtained as a white amorphous powder and showed a hydroxyl and two ester absorption bands in the IR spectrum. The ^{1}H- and ^{13}C-NMR spectra were fairly similar to those of **1** except for the glycerol moiety, which indicated **2** to be a glyceroglycolipid with a different acyl distribution from that of **1** (Table I). Differences in spectral properties between the two glycolipids were as follows. In the ^{1}H-NMR, the signal ascribable to the C-2′ methine proton was moved downfield and the signal due to the C-3′ methylene proton was displaced upfield. Thus, the two acyl residues in **2** were presumed to

TABLE I. ^{1}H- and ^{13}C-NMR Data for **1**, **2**, **1a**, and **2a**[a]

	1[b]		**2**[b]		**1a**	**2a**
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{1}H
1	4.89 (1H, d, J=7.9)	105.5	4.85 (1H, d, J=8.1)	105.7	4.91 (1H, d, J=7.9)	4.88 (1H, d, J=7.7)
2	4.50 (1H, dd, J=7.9, 9.6)	72.0	4.44 (1H, dd, J=8.1, 9.6)	72.1	4.51 (1H, dd, J=7.9, 9.8)	4.48 (1H, dd, J=7.7, 9.5)
3	4.18 (1H, dd, J=3.4, 9.6)	74.7	4.07—4.22 (m)[c]	74.7	4.15—4.20 (m)[d]	4.16 (1H, dd, J=3.4, 9.5)
4	4.41 (1H, d, J=3.4)	69.9	4.37 (1H, d, J=3.3)	69.9	4.40 (1H, d, J=3.3)	4.56 (1H, d, J=3.4)
5	4.17 (1H, dd, J=5.3, 7.5)	73.8	4.07—4.22 (m)[c]	73.8	4.15—4.20 (m)[d]	4.07 (1H, dd, J=6.4, 6.4)
6	4.90 (1H, dd, J=7.5, 11.2)	64.5	4.89 (1H, dd, J=7.9, 10.4)	64.5	4.88 (1H, dd, J=5.0, 10.9)	4.45 (2H, d, J=6.4)
	4.80 (1H, dd, J=5.3, 11.2)		4.80 (1H, dd, J=5.0, 10.4)		4.80 (1H, dd, J=5.0, 10.9)	
1′	4.45 (1H, dd, J=5.3, 10.4)	72.0	4.51 (1H, dd, J=5.0, 10.4)	68.8	4.51 (1H, dd, J=5.3, 9.7)	4.52 (1H, dd, J=5.6, 10.7)
	4.14 (1H, dd, J=5.3, 10.4)		4.29 (1H, dd, J=5.0, 10.9)		4.29 (1H, dd, J=4.5, 9.7)	4.26 (1H, dd, J=5.1, 10.7)
2′	4.52 (1H, m)	68.8	5.65 (1H, quintet-like)	74.6	4.45 (1H, quintet-like)	5.65 (1H, quintet-like)
3′	4.62 (2H, m)	66.5	4.07—4.22 (m)[c]	61.2	4.15—4.20 (m)[d]	4.21 (1H, dd, J=4.8, 11.6)
						4.15 (1H, dd, J=5.1, 11.6)

a) The ^{1}H-NMR spectra were measured in pyridine-d_5 containing one drop of D_2O at 400 MHz, while the ^{13}C-NMR spectrum was recorded in pyridine-d_5 at 100 MHz. *b*) Assignments were made with the aid of the homonuclear decoupling and the C–H correlation spectroscopy spectra. *c*, *d*) The signals overlapped.



a: $CO(CH_2)_{12}CH_3$
b: $CO(CH_2)_{14}CH_3$
c: $CO(CH_2)_7CH{=}CH(CH_2)_5CH_3$

1 : R^1 = a:b:c = 1 : 94 : 5
R^2 = H
R^3 = b:c = 86 : 14

1a : $R^1 = R^2$ = H
R^3 = b:c = 86 : 14

1b : $R^1 = R^2 = R^3$ = H

2 : R^1 = H
R^2 = b:c = 88 : 12
R^3 = a:b:c = 2 : 87 : 11

2a : $R^1 = R^3$ = H
R^2 = b:c = 88 : 12

Chart 1

be linked to C-6 and C-2′. The ^{13}C-NMR spectrum supported this presumption: the C-2′ signal was observed at lower field, while the C-1′ and C-3′ signals appeared at higher field than those of **1**.

On alkaline treatment, both glycolipids quantitatively gave (2′*R*)-glyceryl β-D-galactopyranoside[10] and a mixture of methyl esters of fatty acids. On the basis of the above spectral and chemical evidence, the structures of **1** and **2** were established as (2′*S*)-3′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (**1**) and (2′*R*)-2′,6-*O*-diacyl-glyceryl β-D-galactopyranoside (**2**), respectively.

Alkaline treatment of **1** and **2** revealed that both glycolipids contained several fatty acid residues,[11] so the distribution of fatty acids was determined by enzymatic hydrolysis using *Rhizopus arrhizus* lipase. The lipase-catalyzed hydrolysis of **1** gave (2′*R*)-6-*O*-acyl-glyceryl β-D-galactopyranoside (**1a**) and the fatty acids linked to C-3′ in 30% yield, with recovery of **1** (52%).[12] On the other hand, the lipase predominantly hydrolyzed the acyl substituent on C-6 in **2** to yield **2a**, together with recovery of **2** (56.3%).[12] The structures of **1a** and **2a** were confirmed by the following spectral evidence. In the ^{1}H-NMR spectra, the proton signal ascribable to 3′-H_2 in **1a** was observed in the range of 4.15—4.20 ppm and the proton signal due to 6-H_2 in **2a** was apparently shifted to lower field as compared with those of **1** and **2a**.

The distribution of the fatty acid residues was determined to be as shown in Chart 1 by gas-liquid chromatographic (GLC) analysis of the methyl esters of the fatty acids obtained by enzymatic hydrolysis, and NaOMe treatment of **1a** and **2a**. Even though they are plant glycolipids, the two glycolipids (**1** and **2**) contained unsaturated fatty acids in low proportions. This unique feature stimulated us to compare fatty acid composition between the two glycolipids and monogalactosyl diacylglycerol, a major membrane diacylglyceryl β-D-galactoside in microalgae. Determination of the fatty acid distribution of the monogalactosyl diacylglycerol was carried out by the same procedure as reported previously.[6b] Its fatty acid composition was as follows: *sn*-1, $C_{14:0}$: $C_{16:0}$: $C_{16:1}$: $C_{18:0}$: $C_{18:1}$: $C_{18:2}$: $C_{18:3}$ = 4 : 33 : 13 : 8 : 33 : 4 : 5; *sn*-2, $C_{16:0}$: $C_{16:1}$ = 98 : 2. Comparative analysis of the fatty acid distributions of the three glycolipids reveals that the content in the algae and the proportion of unsaturated fatty acids of two glycolipids are both smaller than those of the monogalactosyl diacylglycerol (see Experimental). Further, it also shows reversal of fatty acid groups between **1** and **2**.

In conclusion, we have characterized two new glycero-glycolipids with unprecedented acyl distributions from the nitrogen-fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae*. The two glycolipids are unique in both their low content in the alga and the low proportion of unsaturated fatty acids as compared with other glycero-glycolipids from microalgae. Therefore, their physicochemical properties and biological functions may be of interest.

Experimental

IR spectra were recorded on a Perkin-Elmer 1650QS FTIR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained with a JEOL GSX-400 (400 MHz) spectrometer using tetramethylsilane as an internal standard. FAB-MS were determined with a JMS SX-102 spectrometer. Optical rotations were measured on a JASCO DIP-4 digital polarimeter. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-13A. The conditions for identification of methyl esters of fatty acids were as follows: column, ULBON HR-SS-10 (0.25 mm i.d. × 50 m, Shinwa Kako Co., Ltd.); column temperature, 150—220 °C, 3 °C/min; injection temperature, 250 °C; carrier gas, N_2, 2.2 kg/cm^2. HPLC was performed using a JASCO 880-PU pump equipped with a Shodex RI, SE-11 differential refractometer. Thin layer chromatography (TLC) was performed on Merck precoated Kieselgel 60F_{254}, and spots were detected by illumination with an ultraviolet lamp, or by spraying 1% $Ce(SO_4)_2$–10% H_2SO_4 followed by heating. Column chromatography was performed on Silica gel BW-200 (Fuji Davison Chemicals Co., Ltd.).

Rhizopus arrhizus lipase (lipase type XI) was purchased from Sigma Co., Ltd.

Culture Conditions The strain of *Anabaena flos-aquae f. flos-aquae* (NIES-74) was purchased from the National Institute for Environment Agency. The alga was maintained in CB medium adjusted to pH 9.0 at 25 °C with cool-white fluorescent illumination of 1000 lux. It was cultured in 5-l Erlenmeyer flasks containing CB medium, *viz.* $Ca(NO_3)_2 \cdot 4H_2O$ 0.15; KNO_3 0.1; β-Na_2 glycerophosphate 0.05; $MgSO_4 \cdot 7H_2O$ 0.04; Bicine 0.5; minor elements solution 3.0 ml/l; trace elements solution 0.1 ml/l. The minor elements solution was composed of, in g/l $FeCl_3 \cdot 6H_2O$ 0.196; $MnCl_2 \cdot 4H_2O$ 0.036; $ZnSO_4 \cdot 7H_2O$ 0.022; $CoCl_2 \cdot 6H_2O$ 0.004; $Na_2MnO_4 \cdot 2H_2O$ 0.0025; $Na_2EDTA \cdot 2H_2O$ 1.0. The trace elements solution consisted of, in mg/l, vitamin B_{12} 0.1; biotin 0.1; thiamine·HCl 10.0. The pH of the medium was adjusted to 9.0 with sodium hydroxide prior to autoclaving. Cultures were illuminated continuously at an incident intensity of 1500 lux with cool-white fluorescent lamps and vigorously aerated with sterilized air passed through a 0.2 μm membrane filter (Millipore, Mirex FG-50) at the rate of 0.5 l/min. After three weeks, the alga was harvested by centrifugation at 20000 × *g* from the combined 40-l culture and lyophilized. Yields of lyophilized cells were typically in the range of 0.2—0.25 g/l of culture.

Isolation The lyophilized alga (8.02 g) was homogenized in $CHCl_3$: MeOH = 1 : 1 and extracted at room temperature for 6 h three times. The extract was submitted to silica gel column chromatography using $CHCl_3$: MeOH = 20 : 1 → 10 : 1 as the eluent to give a mixture of **1** and **2**, as a crude fraction that contained monogalactosyl diacylglycerols. The mixture was subjected to HPLC (column: YMC-A-024, solvent: $CHCl_3$: MeOH = 96 : 4) to furnish **1** (6.0 mg) and **2** (3.1 mg). The monogalactosyl diacylglycerol (MGDG) fraction was purified on a column of Sephadex LH-20 using $CHCl_3$ as the eluent to furnish pure MGDGs (87.4 mg).

1: A white amorphous powder. $[\alpha]_D^{25}$ −2.1° (c = 1.2, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3364, 1733, 1721. FAB-MS *m/z*: 737 $(M+Li)^+$. ^{1}H-NMR: Table I. ^{13}C-NMR: Table I. **2**: A white amorphous powder. $[\alpha]_D^{25}$ +0.9° (c = 0.5, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3448, 1735. FAB-MS *m/z*: 753 $(M+Na)^+$. ^{1}H-NMR: Table I. ^{13}C-NMR: Table I.

Alkaline Treatment of 1 and 2 A solution of **1** (3.0 mg) in dry MeOH (0.5 ml) was treated with 5% NaOMe–MeOH (0.5 ml) at room temperature for 10 min. The reaction mixture was neutralized by using ion-exchange resin (Dowex 50W × 8) and the resin was removed by filtration. The filtrate was extracted with hexane and the hexane layer was concentrated under reduced pressure to yield a mixture of methyl myristate, methyl palmitate and methyl palmitoleate (2.1 mg). The mixture of methyl esters was identified by GLC comparison with authentic samples. Removal of the MeOH layer under reduced pressure gave a residue, which was purified by silica gel column chromatography ($CHCl_3$: MeOH : H_2O = 6 : 4 : 1) to furnish **1b** (0.9 mg). Treatment of **2** (2.0 mg) with NaOMe–MeOH was similarly carried out to give **1b** (0.5 mg). The physicochemical properties of **1b** were identical with those of **1a** in the literature.

***Rhizopus arrhizus* Lipase-Catalyzed Hydrolysis of 1 and 2** A solution of **1** (5.0 mg) and *Rhizopus arrhizus* lipase (500 unit) in the presence of Triton X-100 (2.5 mg) in boric acid–borax buffer (625 μl, pH 7.7) was stirred at 38 °C for 20 min. The reaction was quenched with acetic acid (0.1 ml), then EtOH was added to the reaction mixture. The solvent was removed under reduced pressure and the resulting residue was chromatographed on silica gel with $CHCl_3$: MeOH = 10 : 1 as the eluent to yield **1a** (1.0 mg) together with recovered **1** (2.4 mg). In the same procedure, **2a** was prepared from **2** (1.3 mg) with recovered of **2** (2.7 mg).

1a: A white amorphous powder. $[\alpha]_D^{25}$ +6.0° (c = 0.4, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3406, 1737. FAB-MS *m/z*: 499 $(M+Li)^+$ ($R^3 = C_{16:0}$). ^{1}H-NMR: Table I. **2a**: A white amorphous powder. $[\alpha]_D^{25}$ −7.0° (c = 0.3, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3425, 1730. FAB-MS *m/z*: 499 $(M+Li)^+$ ($R^2 = C_{16:0}$). ^{1}H-NMR: Table I.

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7) Presented in part in our previous communication. N. Murakami, H. Shirahashi, J. Sakakibara, and Y. Tsuchida, *Chem. Pharm. Bull.*, **40**, 285 (1992).
8) This spot was not observed in TLC analysis of some other cyanobacteria and green algae maintained in our laboratory.
9) The FAB-MS of **1** and **2** showed the most intense peak at *m/z* 737 $(M+Li)^+$, corresponding to a molecular formula of $C_{41}H_{78}O_{10}Li$ (**1**: *m/z* 737.5693, −6.2 mmu error, **2**: *m/z* 737.5755, +4.3 mmu error).
10) B. Wickberg, *Acta Chem. Scand.*, **12**, 1187 (1958).
11) Since low solubility of both glycolipids in CH_3CN and MeOH caused difficulty in applying reversed-phase HPLC, further separation to obtain compounds with a single acyl composition has not been achieved yet.
12) In the lipase catalyzed hydrolysis, deacylation proceeded regioselectively without generating other monoacyl derivatives and monogalactosyl glycerol at this stage.