# A NOVEL GLYCEROGLYCOLIPID FROM THE NITROGEN-FIXING CYANOBACTERIUM *ANABAENA FLOS-AQUAE F. FLOS-AQUAE*

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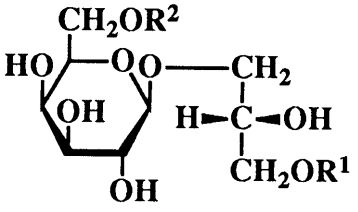
A novel glyceroglycolipid, (2'*S*)-3',6-*O*-diacyl-glyceryl β-D-galactopyranoside (**1**), was isolated from the nitrogen-fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae*. The chemical structure was elucidated on the basis of spectral properties, and the distribution of fatty acid residues was determined by enzymatic hydrolysis of **1** using lipase (from *Rhizopus arrhizus*).

**KEYWORDS** cyanobacterium; *Anabaena flos-aquae f. flos-aquae*; nitrogen-fixation; glycolipid

Some cyanobacteria are known to carry out nitrogen-fixation in a heterocyst possessing a thicker cell wall than that of the usual vegetative cell.[1] Since glycolipids are shown to take part in several membrane functions in plants,[2] the unique functions and structural features of heterocyst cell walls may be related to a specific glycolipid. This assumption prompted us to investigate glycolipids in the nitrogen-fixing cyanobacteria *Anabaena flos-aquae f. flos-aquae*.[3] Here we describe the isolation and characterization of a novel glyceroglycolipid (**1**).

Cultures were grown for three weeks in CB-medium[4] 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 collected by centrifugation at 20,000 × g and lyophilized to afford 8.02 g of the lyophilized cells from the 35-L culture medium. Extraction of the alga (8.02 g) with $CHCl_3$-MeOH (1:1) gave a residue (1.16 g), which was subjected to successive $SiO_2$ column chromatography ($CHCl_3$-MeOH=20:1→10:1) and normal phase HPLC ($CHCl_3$:MeOH=96:4) to furnish **1** (6.0mg).[5]

Compound **1**, a white amorphous powder, $[\alpha]_D^{25}$ -2.1° (c=1.2, $CHCl_3$), exhibited a hydroxyl (3364 $cm^{-1}$) and two ester carbonyl absorption bands (1733, 1721 $cm^{-1}$) in its IR spectrum. The $^1H$ NMR spectrum of **1** indicated the presence of terminal methyl (δ0.88, t, 3H×2) and a number of methylene groups (δ1.28). It also showed a 12H signal covering the range from 4.14 to 4.90 ppm. The physicochemical features outlined above suggested that **1** was a glycolipid. Detailed analysis of the homonuclear decoupling spectra defined a sugar component as a galactose. In addition, observation of carbon signals



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
$R^2$ = b : c = 86 : 14

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

Table I. $^{1}H$ and $^{13}C$ NMR Data for **1** and **2** a)

| | **1** b) | | **2** |
|---|---|---|---|
| | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 4.89 (1H, d, J=7.9) | 105.5 | 4.91 (1H, d, J=7.9) |
| 2 | 4.50 (1H, dd, J=7.9, 9.6) | 72.0 | 4.51 (1H, dd, J=7.9, 9.8) |
| 3 | 4.18 (1H, dd, J=3.4, 9.6) | 74.7 | 4.15-4.20 (m)c) |
| 4 | 4.41 (1H, d, J=3.4) | 69.9 | 4.40 (1H, d, J=3.3) |
| 5 | 4.17 (1H, dd, J=5.3, 7.5) | 73.8 | 4.15-4.20 (m)c) |
| 6 | 4.90 (1H, dd, J=7.5, 11.2) | 64.5 | 4.88 (1H, dd, J=5.0, 10.9) |
| | 4.80 (1H, dd, J=5.3, 11.2) | | 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=5.3, 9.7) |
| | 4.14 (1H, dd, J=5.3, 10.4) | | 4.29 (1H, dd, J=4.5, 9.7) |
| 2' | 4.52 (1H, m) | 68.8 | 4.45 (1H, quintet-like) |
| 3' | 4.62 (2H, m) | 66.5 | 4.15-4.20 (m)c) |

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 COSY spectra. c)These signals overlapped.

from two carbonyl (δc173.6, C=O×2) and two terminal methyl groups (δc14.3, $CH_3$×2) in the $^{13}C$ NMR spectrum revealed the presence of two acyl residues. The glycolipid (**1**) is, however, different from a monogalactosyl diacylglycerol distributed widely in the plant membrane.

Intensive comparison of their $^{1}H$ NMR spectra disclosed the apparent difference that the proton signal due to C-6 methylene group in **1** was shown in a lower field and the signal assignable to 2'-H of glycerol portion in **1** appeared in a higher field than those of monogalactosyl diacylglycerol.[6] In the $^{13}C$ NMR spectrum, the C-6 signal in **1** was observed downfield by 2.2 ppm and the neighboring C-5 signal was shifted upfield by 3.0 ppm. Each $^{13}C$ signal of C-1', C-2', and C-3' were also shifted by +4.0, -2.2, and +3.2 ppm with respect to the glycerol moiety. These acylation shifts probably showed the two acyl residues to be present at both C-6 and C-3'.[7]

The presumption was confirmed by the HMBC spectrum of **1** : two carbonyl carbon signals showed the cross peak due to long-range coupling with the proton signals of C-6 and C-3' methylene groups. On treatment of **1** with NaOMe-MeOH, (2'*R*)-glyceryl β-D-galactopyranoside, $[\alpha]_D^{24}$ -8.4° (c=0.5, $H_2O$),[8] was quantitatively prepared along with a mixture of methyl esters of fatty acids. On the basis of the above findings, the structure of **1** was established as (2'*S*)-6,3-*O*-diacylglyceryl β-D-galactopyranoside.

Since the glycolipid (**1**) contained several fatty acid residues,[9] the distributions of fatty acids were determined by enzymatic hydrolysis using lipase (from *Rhizopus arrhizus*).[10] The lipase catalyzed hydrolysis of **1** afforded (2'R)-6-*O*-acylglyceryl β-D-galactopyranoside (**2**), [11] and the fatty acid attached at C-3' in 30% yield, involving the recovery of **1** (52%).[12] The structure of **2** was confirmed by the physicochemical property that the proton signal ascribable to 3'-$H_2$ was observed in the range of 4.15-4.20 ppm. Treatment of **2** with NaOMe-MeOH gave a mixture of methyl esters of fatty acids liberated from C-6. Gas-liquid chromatography analysis of both fatty acid mixtures as their methyl esters showed the composition of two acyl residues linked to C-6 and C-3'.[13]

In conclusion, we have isolated and characterized the novel glycolipid attaching two acyl groups to C-6 and C-3' in (2'*R*)-glyceryl β-D-galactopyranoside from the nitrogen-fixing cyanobacterium. The physicochemical properties and biological activities of **1** may be of interest because of the low content of unsaturated fatty acids and the unprecedented positions of acyl groups.

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3) The strain of *Anabaena flos-aquae f. flos-aquae* (NIES-74) used for the present investigation was purchased from the National Institute for Environmental Studies of the Environment Agency.

4) CB-medium is composed of $Ca(NO_3)_2 \cdot 4H_2O$ 0.15; $KNO_3$ 0.1; β-$Na_2$ glycerophosphate 0.05; $MgSO_4$ $7H_2O$ 0.04; Bicine $FeCl_3 \cdot 6H_2O$ 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; in g/L.The trace elements solution is composed of vitamin $B_{12}$ 0.1; biotin 0.1; thiamine-HCl 10.0; in mg/L. The pH of the medium was adjusted to 9.0.

5) The algal extract was separated on the constituents, positive coloration to anthrone reagent in order to obtain glycolipids. The FAB-MS spectrum of **1** showed the most intense peak at m/z737, corresponding to a molecular formula of $C_{41}H_{78}O_{10}Li$ (m/z737.5693, -6.2 mmu error).

6) N. Murakami, H. Imamura, J. Sakakibara, and N. Yamada, *Chem. Pharm. Bull.*, **38**, 3497(1990).

7) K. Yamasaki, R. Kasai, Y. Masaki, M. Okihara, and O. Tanaka, *Tetrahedron Lett.*, **1977**, 1231.

8) lit. $[\alpha]_D^{15}$ -9° (c=0.6, $H_2O$). H. Kikuchi, Y. Tsukitani, T. Manda, T. Fujii, H. Nakanishi, M. Kobayashi, and I. Kitagawa, *Chem. Pharm. Bull.*, **30**, 3544(1982).

9) Since low solubility of the glycolipid (**1**) in $CH_3CN$ and MeOH caused difficulty in applying the reversed phase HPLC, further separation of **1** to a compound having a single acyl composition has not been achieved yet.

10) N. Murakami, H. Imamura, T. Morimoto, T. Ueda, S. Nagai, J. Sakakibara, and N. Yamada, *Tetrahedron Lett.*, **32**, 1331(1991).

11) A white amorphous powder. $[\alpha]_D^{25}$ +6.0° (c=0.4, $CHCl_3$). IR (KBr, $cm^{-1}$) : 3406, 1737. FAB-MS(m/z) : 499$(M+Li)^+$.

12) In the hydrolysis of **1** using the lipase, deacylation of C-3' acyl group predominated without generation of a 3-monoacyl derivative and monogalactosyl glycerol at this stage.

13) The conditions for identification of fatty acid methyl esters: column, ULBON HR-SS-10 (0.25mm i.d. × 50m, Shinwa kako Co.Ltd.); column temperature, 150-220°C, 3°C/min; injection temperature, 250°C; carrier gas, $N_2$, 2.2kg/$cm^2$.

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