Antitumor-Promoting Activities of Various Synthetic 1-*O*-Acyl-3-*O*-(6′-*O*-Acyl-β-D-Galactopyranosyl)-*sn*-Glycerols Related to Natural Product from Freshwater Cyanobacterium *Anabaena flos-aquae f. flos-aquae*

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1-*O*-Acyl-3-*O*-(6′-*O*-acyl-β-D-galactopyranosyl)-*sn*-glycerol, which was isolated from a nitrogen-fixing freshwater cyanobacterium, *Anabaena flos-aquae f. flos-aquae*, was synthesized by utilizing lipase-catalyzed acylation. The antitumor-promoting activities of these galactolipids were evaluated using a short-term *in vitro* assay of Epstein–Barr virus activation in Raji cells induced by 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA). The glyceroglycolipids which have a palmitoleoyl residue at the 1-*O*-position exhibited more potent activities than the others in this assay.

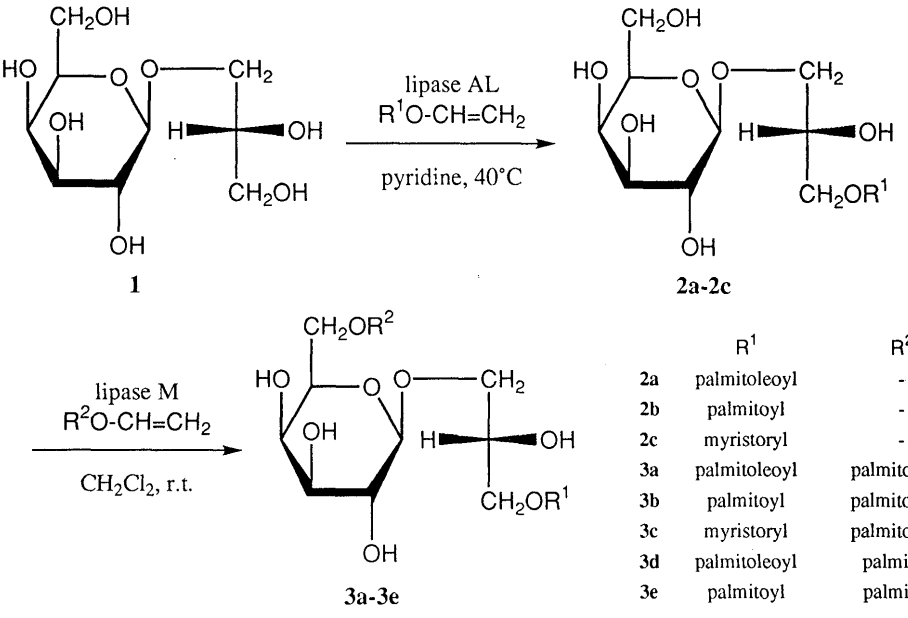
Key words glyceroglycolipid; antitumor-promoter; Epstein–Barr virus; *Anabaena flos-aquae f. flos-aquae*

Glyceroglycolipids are major components of the cell membrane in the plant kingdom. They have various biological activities and functions.[2)]

We previously isolated 1-*O*-acyl-3-*O*-(6′-*O*-acyl-β-D-galactopyranosyl)-*sn*-glycerol from nitrogen-fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae* as a mixture of several acyl pairs.[3)] Since this glyceroglycolipid was obtained in a small amount and it was difficult to isolate individual species, the biological activity was not examined. In order to obtain sufficient amounts of these glycolipids for testing, we planned to synthesize 1-*O*-acyl-3-*O*-(6′-*O*-acyl-β-D-galactopyranosyl)-*sn*-glycerols with various combinations of acyl residues.

In this paper, we describe the antitumor-promoting activities of various species of 1-*O*-acyl-3-*O*-(6′-*O*-acyl-β-D-galactopyranosyl)-*sn*-glycerol, which were synthesized by two-step regioselective enzymatic acylation.[4)]

Chemistry 1-*O*-Acyl-3-*O*-(6′-*O*-acyl-β-D-galactopyranosyl)-*sn*-glycerols (**3**) were prepared as described in our previous reports[4)] (Chart 1). The *sn*-1 position of 3-*O*-β-D-galactopyranosyl-*sn*-glycerol (**1**)[5)] was regioselectively acylated with vinyl palmitoleate by the use of *Achromobacter* sp. lipase (lipase AL). The product was purified by reversed-phase high-performance liquid chromatography (HPLC) to give **2a** (yield 26.6%, recovery 45.9%). In the ^{1}H-NMR spectrum of **2a**, the signal of the *sn*-1 position appeared at 4.43 ppm downfield relative to that of **1**. The FAB-MS of **2a** (*m/z*: 514 $(M+Na+1)^+$) also supported the structure. Then the 6′-*O*-position of **2a** was acylated in the presence of *Mucor javanics* lipase (lipase M) and vinyl palmitoleate to give **3a** (yield 49.9%, recovery 23.6%). The ^{1}H-NMR spectrum of **3a** showed the signals of 6′-H_2 at 4.83 ppm and 4.73 ppm downfield compared with those of **2a**. In the ^{13}C-NMR spectrum of **3a**, the signal due to 6′-C was also shifted down-field by 1.9 ppm.



1 2a-2c 3a-3e

	R^1	R^2
2a	palmitoleoyl	-
2b	palmitoyl	-
2c	myristoryl	-
3a	palmitoleoyl	palmitoleoyl
3b	palmitoyl	palmitoleoyl
3c	myristoryl	palmitoleoyl
3d	palmitoleoyl	palmitoyl
3e	palmitoyl	palmitoyl

Chart 1

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Table 1. Inhibitory Activities of Synthetic 1-*O*-Acyl-3-*O*-(6′-*O*-acyl-*β*-D-galactopyranosyl)-*sn*-glycerols (**3a—l**) and Natural Compounds on TPA-Induced EBV-EA[a] Activation TPA (32 pmol) = 100%

Compound	R^1	R^2	Concentration (molar ratio/TPA) 1000	500	100
			% to control (% viability)		
Natural	Mixture[b]	Mixture[c]	30.4 (70)	65.8	92.6
3a	Palmitoleoyl	Palmitoleoyl	0 (70)	42.1	73.7
3b	Palmitoyl	Palmitoleoyl	0 (70)	39.5	76.0
3c	Myristoyl	Palmitoleoyl	13.2 (70)	47.3	80.6
3d	Palmitoleoyl	Palmitoyl	18.5 (70)	55.8	77.2
3e	Palmitoyl	Palmitoyl	21.6 (70)	60.2	89.1
3f[d]	Myristoyl	Palmitoyl	7.2 (>60)	57.1	84.8
3g[d]	Oleoyl	Palmitoyl	60.5 (>60)	85.2	100
3h[d]	Oleoyl	Myristoyl	18.5 (>70)	68.7	100
3i[d]	Myristoyl	Linoleoyl	14.7 (>60)	66.2	90.5
3j[d]	Oleoyl	Linoleoyl	4.7 (>60)	46.2	75.9
3k[d]	Oleoyl	Oleoyl	17.2 (>70)	66.1	91.5
3l[d]	Myristoyl	Oleoyl	10.5 (>60)	55.8	76.8

a) Epstein–Barr virus early antigen (EBV-EA). *b*) Palmitoleoyl : palmitoyl : myristoyl = 5 : 94 : 1. *c*) Palmitoleoyl : palmitoyl = 14 : 86. *d*) T. Morimoto *et al.*, *Tetrahedron*, **51**, 6443 (1995).

Compounds **3b—e** were similarly obtained from **1** *via* the respective *sn*-1-*O*-acyl derivatives **2** by two-step enzymatic acylation.

Inhibitory Activity of EBV-EA and Discussion The antitumor-promoting activities of 1-*O*-acyl-3-*O*-(6′-*O*-acyl-*β*-D-galactopyranosyl)-*sn*-glycerols (**3a—e**), together with **3f—l** which were synthesized before,[4] were evaluated using a short-term *in vitro* assay of Epstein–Barr virus activation in Raji cells induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The results of this assay are summarized in Table 1. All samples exhibited low cytotoxicity at the concentration of 1000 mol excess over TPA. The galactolipids with unsaturated bonds on fatty acid residues inhibited tumor-promotion of activated Raji cells. Furthermore, the galactolipids with a palmitoleoyl group at the 6′-*O*-position showed more potent antitumor-promoting activities than the others. The EBV-EA inhibitory activities of **3a** and **3b** were comparable to those of potent anti-tumor-promoters.[6]

In conclusion, we have synthesized 12 kinds of 1-*O*-acyl-3-*O*-(6′-*O*-acyl-*β*-D-galactopyranosyl)-*sn*-glycerols, including six species having combinations of acyl residues that appear to occur in nature. Galactolipids that contained a palmitoleoyl group tended to exhibit potent antitumor-promoting activity.

Experimental

General Method Lipase AL was a gift from Meito Sangyo Co., Ltd. Lipase M was from Amano Pharmaceutical Co., Ltd. ^{1}H-NMR and ^{13}C-NMR spectra were obtained with JEOL GSX-400 and JEOL α-500 spectrometers using tetramethylsilane as an internal standard. FAB-MS were measured with JEOL DX-300 and JEOL DX-505 spectrometers. Infrared spectra were recorded with a Perkin-Elmer 1650QS Fourier-transform IR spectrometer. Optical rotations were obtained with a JASCO DIP-4 digital polarimeter. HPLC was performed using a JASCO 880-PU pump equipped with a JASCO 830-RI differential refractometer. Thin layer chromatography (TLC) was performed on Merck precoated Kieselgel 60F_{254}, and spots were detected by spraying 1% $Ce(SO_4)_2$–10% H_2SO_4, followed by heating. Column chromatography was carried out on silica gel BW-200 (Fuji Davison Chemicals Co., Ltd.).

General Procedure for Preparation of 1-*O*-Acyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol A mixture of 3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**1**, 81.2 mg), vinyl palmitoleate (260.0 mg) and lipase AL (243.6 mg) in pyridine (1.6 ml) was stirred at 40 °C. The reaction mixture was filtered and the solvent was removed under reduced pressure. The products were purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 10 : 3 : 1, lower layer) and HPLC (J′ sphere ODS-H80, MeOH : H_2O = 85 : 15) to give 1-*O*-palmitoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**2a**, 36.4 mg), 3-*O*-(6′-*O*-palmitoleoyl-*β*-D-galactopyranosyl)-*sn*-glycerol (7.0 mg) and 2-*O*-palmitoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (2.5 mg).

Compounds **2b, c** were obtained similarly. Vinyl palmitolate and vinyl myristate were used as acyl donors to give **2b** and **2c**, respectively.

2a: A colorless oil, $[\alpha]_D^{23}$ −5.7° (c = 0.1, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 3390, 1732. FAB-MS m/z: 514 $(M+Na+1)^+$. ^{1}H-NMR (500 MHz, pyridine-d_5 with one drop of D_2O) δ: 5.35 (2H, m), 4.71 (1H, d, J = 7.7 Hz, 1′-H), 4.43 (2H, d, J = 4.9 Hz, *sn*-1-H_2), 4.37 (1H, d, J = 3.7 Hz, 4′-H), 4.35 (1H, m, *sn*-2-H), 4.32 (1H, dd, J = 9.7, 7.7 Hz, 2′-H), 4.27 (1H, dd, J = 10.4, 5.5 Hz, *sn*-3-H), 4.23 (2H, m, 6′-H_2), 4.01 (1H, dd, J = 9.7, 3.7 Hz, 3′-H), 3.97 (1H, dd, J = 10.4, 4.9 Hz, *sn*-3-H), 3.91 (1H, dd, J = 6.4, 6.4 Hz, 5′-H), 2.22 (2H, t, J = 7.3 Hz), 1.93 (4H, quintet-like), 1.49 (2H, quintet, J = 7.3 Hz), 0.74 (3H, t, J = 7.0 Hz). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 175.4, 130.1, 130.0, 105.0 (1′-C), 76.7 (5′-C), 74.7 (3′-C), 72.5 (2′-C), 71.8 (*sn*-3-C), 70.2 (4′-C), 69.6 (*sn*-2-C), 66.5 (*sn*-1-C), 62.4 (6′-C).

2b: A white powder, $[\alpha]_D^{23}$ −20.0° (c = 0.2, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3330, 1743. FAB-MS m/z: 516 $(M+Na+1)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 4.89 (1H, d, J = 7.9 Hz, 1′-H), 4.57 (2H, m, *sn*-1-H_2), 4.53—4.47 (3H, m, 2′-H, 4′-H, *sn*-2-H), 4.45 (2H, d, J = 5.5 Hz, 6′-H_2), 4.40 (1H, dd, J = 10.4, 6.1 Hz, *sn*-3-H), 4.16 (1H, dd, J = 9.7, 3.5 Hz, 3′-H), 4.09 (1H, dd, J = 10.4, 4.9 Hz, *sn*-3-H), 4.08 (1H, d, J = 6.1 Hz, 5′-H), 2.32 (2H, quintet, J = 7.3 Hz), 0.87 (3H, t, J = 7.2 Hz).

2c: A white powder, $[\alpha]_D^{23}$ − 9.6° (c = 0.2, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3330, 1743. FAB-MS m/z: 487 $(M+Na)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 4.49 (1H, d, J = 4.9 Hz, 1′-H), 4.56 (2H, m, *sn*-1-H_2), 4.45—4.50 (3H, m, 2′-H, 4′-H, *sn*-2-H), 4.44 (2H, d, J = 5.5 Hz, 6′-H_2), 4.39 (1H, dd, J = 10.4, 6.1 Hz, *sn*-3-H), 4.15 (1H, dd, J = 8.9, 3.4 Hz, 3′-H), 4.09 (2H, m, 5′-H, *sn*-3-H), 2.32 (2H, t), 1.62 (2H, quintet, J = 7.3 Hz), 0.87 (3H, t, J = 7.0 Hz).

General Procedure for Preparation of 1-*O*-Acyl-3-*O*-(6′-*O*-acyl-*β*-D-galactopyranosyl)-*sn*-glycerol A mixture of 1-*O*-palmitoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**2a**, 19.9 mg), vinyl palmitoleate (33.1 mg) and lipase M (59.7 mg) in CH_2Cl_2 (1.0 ml) was stirred at room temperature. The reaction mixture was filtered to remove the enzyme and the filtrate was evaporated under reduced pressure. The residue was purified by SiO_2 column chromatography ($CHCl_3$: MeOH = 15 : 1) to furnish 1-*O*-palmitoleoyl-3-*O*-(6′-*O*-palmitoleoyl-*β*-D-galactopyranosyl)-*sn*-glycerol (**3a**, 14.7 mg). Compounds **3b—e** were obtained similarly.

3a: A white powder, $[\alpha]_D^{23}$ −1.5° (c = 0.3, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3489, 1736, 1720. FAB-MS m/z: 750 $(M+Na+1)^+$. ^{1}H-NMR (500 MHz, pyridine-d_5 with one drop of D_2O) δ: 5.42 (4H, m), 4.83 (1H, dd, J = 11.0, 7.3 Hz, 6-H′), 4.82 (1H, d, J = 7.7 Hz, 1′-H), 4.73 (1H, dd, J = 11.0, 5.1 Hz, 6′-H), 4.58 (1H, dd, J = 11.0, 4.8 Hz, *sn*-1-H), 4.54 (1H, dd, J = 11.0,

5.5 Hz, *sn*-1-H), 4.46 (1H, quintet-like, *sn*-2-H), 4.44 (1H, dd, $J=9.5$, 7.7 Hz, 2′-H), 4.39 (1H, dd, $J=10.3$, 5.3 Hz, *sn*-3-H), 4.35 (1H, d, $J=3.3$ Hz, 4′-H), 4.12 (1H, dd, $J=9.5$, 3.3 Hz, 3′-H), 4.11 (1H, t-like, 5′-H), 4.07 (1H, dd, $J=10.3$, 5.3 Hz, *sn*-3-H), 2.35 (2H, t, $J=7.3$ Hz), 2.28 (2H, t, $J=7.3$ Hz), 2.02 (8H, m), 1.61 (2H, quintet, $J=7.3$ Hz), 1.57 (2H, quintet, $J=7.3$ Hz), 0.80 (3H, t, $J=6.8$ Hz), 0.80 (3H, t, $J=6.8$ Hz). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 173.7, 173.7, 130.2, 130.2, 130.2, 130.2, 105.6 (1′-C), 74.8 (3′-C), 73.8 (5′-C), 72.1 (*sn*-3-C), 72.1 (2′-C), 69.9 (4′-C), 68.8 (*sn*-2-C), 66.5 (*sn*-1-C), 64.5 (6′-C).

3b: A white powder, $[\alpha]_D^{23}$ −1.1° ($c=0.2$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3489, 1735, 1719. FAB-MS *m/z*: 752 $(M+Na+1)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 5.48 (2H, t, $J=4.6$ Hz), 4.88 (1H, d, $J=11.0$, 7.3 Hz, 6′-H), 4.87 (1H, d, $J=7.7$ Hz, 1′-H), 4.79 (1H, dd, $J=11.0$, 5.1 Hz), 4.61 (2H, m, *sn*-1-H_2), 4.51 (1H, m, *sn*-2-H), 4.48 (1H, dd, $J=9.7$, 7.7 Hz, 2′-H), 4.44 (1H, dd, $J=10.3$, 5.5 Hz, *sn*-3-H), 4.40 (1H, d, $J=2.9$ Hz, 4′-H), 4.20—4.10 (3H, m, 3′-H, 5′-H, *sn*-3-H), 2.40 (2H, t, $J=7.3$ Hz), 2.35 (2H, t, $J=7.3$ Hz), 2.09 (4H, m), 1.65 (4H, m), 0.87 (6H, m).

3c: A white powder, $[\alpha]_D^{23}$ −0.5° ($c=0.4$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3489, 1736, 1724. FAB-MS *m/z*: 724 $(M+Na+1)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 5.46 (m)*, 4.86 (1H, d, $J=7.7$ Hz, 1′-H), 4.86 (1H, dd, $J=11.4$, 5.7 Hz, 6′-H), 4.76 (1H, dd, $J=11.4$, 4.8 Hz, 6′-H), 4.61 (2H, m, *sn*-1-H_2), 4.51 (1H, m, *sn*-2-H), 4.47 (1H, t-like, 2′-H), 4.43 (1H, dd, $J=10.3$, 5.5 Hz, *sn*-3-H), 4.39 (1H, d, $J=2.9$ Hz, 4′-H), 4.10—4.20 (3H, m, 3′-H, 5′-H, *sn*-3-H), 2.42 (2H, t, $J=7.0$), 2.37 (2H, t, $J=7.0$ Hz), 2.09 (4H, m), 1.66 (4H, m), 0.88 (6H, m). *) This peak overlapped with the HDO peak.

3d: A white powder, $[\alpha]_D^{23}$ −0.9° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3278, 1736, 1720. FAB-MS *m/z*: 752 $(M+Na+1)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 5.42 (2H, m), 4.85 (1H, dd, $J=11.4$, 7.7 Hz, 6′-H), 4.83 (1H, d, $J=7.7$ Hz, 1′-H), 4.75 (1H, dd, $J=11.4$, 5.1 Hz, 6′-H), 4.57 (1H, dd, $J=11.0$, 4.4 Hz, *sn*-1-H), 4.54 (1H, dd, $J=11.0$, 5.7 Hz, *sn*-1-H), 4.46 (1H, m, *sn*-2-H), 4.44 (1H, dd, $J=9.3$, 7.7 Hz, 2′-H), 4.39 (1H, dd, $J=10.3$, 5.7 Hz, *sn*-3-H), 4.36 (1H, d, $J=3.3$ Hz, 4′-H), 4.17—4.11 (2H, m, 3′-H, 5′-H), 4.08 (1H, dd, $J=10.3$, 5.1 Hz, *sn*-3-H), 2.36 (2H, t, $J=7.5$ Hz), 2.29 (2H, t, $J=7.5$ Hz), 2.02 (4H, m), 1.60 (4H, m), 0.83 (6H, m).

3e: A white powder, $[\alpha]_D^{23}$ −0.9° ($c=0.3$, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3373, 1734, 1720. FAB-MS *m/z*: 754 $(M+Na+1)^+$. ^{1}H-NMR (400 MHz, pyridine-d_5 with one drop of D_2O) δ: 4.86 (1H, d, $J=7.9$ Hz, 1′-H), 4.86 (1H, dd, $J=11.0$, 9.2 Hz, 6′-H), 4.75 (1H, dd, $J=11.0$, 4.6 Hz, 6′-H), 4.62 (1H, dd, $J=11.0$, 4.9 Hz, *sn*-1-H), 4.61 (1H, dd, $J=11.0$, 4.3 Hz, *sn*-1-H), 4.51 (1H, quintet-like, *sn*-2-H), 4.47 (1H, dd, $J=9.7$, 7.9 Hz, 2′-H), 4.43 (1H, dd, $J=10.7$, 5.2 Hz, *sn*-3-H), 4.40 (1H, d, $J=3.7$, 4′-H), 4.18 (1H, dd, $J=9.7$, 3.7 Hz, 3′-H), 4.16—4.11 (2H, m, 5′-H, *sn*-3-H), 2.45 (2H, t, $J=7.6$ Hz), 2.39 (2H, $J=7.6$ Hz), 1.70 (2H, quintet-like), 1.66 (2H, quintet-like), 0.88 (6H, t-like).

Bioassays for Antitumor–Promoting Activity The inhibition of EBV-EA activation was assayed using EBV genome-carrying human lymphoblastoid cells, Raji cells (nonproducer type), which were cultivated in 8% FBS RPMI 1640 medium (Nissui). The indicator cells (Raji) (1×10^6/ml) were incubated at 37 °C for 48 h in 1 mol of a medium containing *n*-butyric acid (4 mM),[7] 32 pmol of TPA in dimethyl sulfoxide (DMSO), and a known amount of the test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-positive sera from nasopharyngeal carcinoma (NPC) patients and fluorescein isothiocyanate-labeled anti-human IgG. After staining, they were detected by a conventional indirect immunofluorescence technique.[8,9] In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average EA induction was compared with that of the positive control experiments with *n*-butyric acid (4 mM) plus TPA (32 pmol), in which EA induction was ordinarily around 35%.

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