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ANTI-TUMOUR-PROMOTING GLYCEROGLYCOLIPIDS FROM THE GREEN ALGA, *CHLORELLA VULGARIS**

TAKASHI MORIMOTO, AKITO NAGATSU, NOBUTOSHI MURAKAMI,† JINSAKU SAKAKIBARA,‡ HARUKUNI TOKUDA,§ HOYOKU NISHINO§ and AKIO IWASHIMA§

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan; §Department of Biochemistry, Kyoto Prefectural University of Medicine, Kawaramachi-dori, Hirokoji, Kamigyo-ku, Kyoto 602, Japan

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Key Word Index—*Chlorella vulgaris*; Chlorophyceae; monogalactosyl diacylglycerols; digalactosyl diacylglycerols; anti-tumour-promoter.

Abstract—Two new monogalactosyl diacylglycerols were isolated from the freshwater green alga, *Chlorella vulgaris*, as anti-tumour promoters, together with three monogalactosyl diacylglycerols and two digalactosyl diacylglycerols. The new monogalactosyl diacylglycerol containing (7*Z*,10*Z*)-hexadecadienoic acid showed a more potent inhibitory effect toward tumour promotion than the other glycerolipids isolated.

INTRODUCTION

In the last decade, microalgae have drawn much attention as prospective and rich sources of biologically active constituents [1–3]. Furthermore, several microalgae (e.g. species of *Chlorella*, *Spirulina* and *Scenedeamus*) have been considered as a supplemental food or for biomass [4–6]. We have reported that monogalactosyl diacylglycerols (MGDG) and digalactosyl diacylglycerols (DGDG) isolated from a cyanobacterium, *Phormidium tenue*, showed an inhibitory effect on the Epstein–Barr virus-associated early antigen (EBV-EA) activation on Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [7]. In addition, we have synthesized MGDG possessing several pairs of fatty acid residues to clarify the relationship between acyl pairs and this inhibitory effect [8]. It was found that the activities of glycero-glycolipids were influenced by fatty acid residue and distribution. As a part of our continuing search for galactolipids as anti-tumour promoters, we found that glycero-glycolipids from the freshwater green alga, *C. vulgaris*, inhibited tumor promotion more effectively than those from *P. tenue*. In this paper, we present the isolation and structural elucidation of the anti-tumour-promoting glyceroglycolipids from *C. vulgaris*.

RESULTS AND DISCUSSION

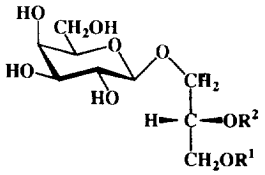
The alga was cultured in modified Detmer (MD) medium for three weeks. The alga was harvested by centrifugation and lyophilized, and the lyophilized alga (6.88 g) extracted with $CHCl_3$–MeOH (1:2). The extract (1.2 g) was successively subjected to silica gel column chromatography ($CHCl_3$–MeOH–H_2O (10:3:1, lower layer) and (13:7:2 lower layer)) to afford the crude MGDG and DGDG fractions. The crude MGDG fraction was further purified on LH-20 eluting with $CHCl_3$. Then, the purified MGDG fraction was separated by reverse-phase HPLC with monitoring of the inhibition of EBV-activation to furnish compounds **1**–**5**. Separation of the crude DGDG fraction was performed by silica gel column chromatography ($CHCl_3$–MeOH–28% aq. NH_3, 13:5:1). HPLC purification of the DGDG fraction provided compounds **6** and **7** as the major active components.

Compound **1** gave a $[M + Na]^+$ at *m/z* 747 in its FAB mass spectrum and the IR spectrum showed the presence of hydroxyl and ester groups. The ^1H NMR spectrum exhibited two terminal methyl signals (δ0.88 and 0.96, each 3H, both *t*), a broad methylene signal at δ1.26 and a signal (δ2.41, 4H, *m*) due to two methylene protons linked to carbonyl functions. In addition, coupling analysis in the homonulcear decoupling spectra suggested the presence of a sugar component, such as a galactose. Treatment of **1** with NaOMe–MeOH liberated a glycerol galactoside (**8**) and a mixture of fatty acid methyl esters. The spectral and physicochemical data of **8** were in complete agreement with those of (2*R*)-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, which was obtained by NaOMe treatment of MGDG isolated from *P. tenue* [9]. The fatty

*Part 9 in the series 'Studies on Glycolipids'. For part 8 see Ref. [7].

†Present address: Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan.

‡Author to whom correspondence should be addressed.



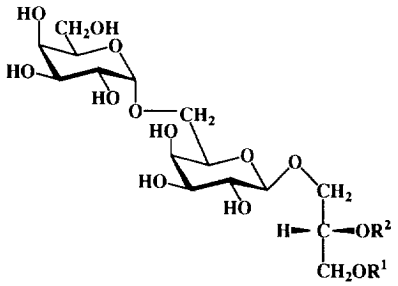
1 : R^1= (7Z,10Z,13Z)-hexadecatrienoyl, R^2= (7Z,10Z)-hexadecadienoyl
R^2= (7Z,10Z)-hexadecadienoyl

2 : R^1, R^2= (7Z,10Z)-hexadecadienoyl

3 : R^1= linolenoyl
R^2= (7Z,10Z,13Z)-hexadecatrienoyl

4 : R^1= linoeoyl
R^2= (7Z,10Z)-hexadecadienoyl

5 : R^1=R^2=linoleoyl



6 : R^1=linoleoyl
R^2= (7Z,10Z)-hexadecadienoyl

7 : R^1= linolenoyl
R^2= (7Z,10Z,13Z)-hexadecatrienoyl

acid composition was determined by GC-MS as a 1:1 mixture of methyl hexadecadienoate and methyl hexadecatrienoate. To determine the location of the two fatty acid residues in **1**, we attempted regioselective deacylation by lipase. Enzymatic hydrolysis of **1** using Lipase type XI (from *Rhizopus arrhizus*) [10] gave 2-*O*-monoacyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**1a**) and a fatty acid, which was isolated as the methyl ester after CH_2N_2 treatment; this was identified as methyl hexadecatrienoate containing a 1,4,7-*cis*-triene system by its mass, ^1H and ^{13}C NMR spectra [11]. In the heteronuclear multiple bond coherence (HMBC) spectrum, the C-14 olefinic carbon (δ_C 131.9) was coupled with the terminal methyl protons (δ_H 0.98) of C-16 and the methylene protons (δ_H 2.31) of C-15. Thus, the fatty acid was established as (7Z,10Z,13Z)-hexadecatrienoic acid. Furthermore, treatment of **1a** with NaOMe–MeOH liberated galactosyl glycerol (**8**) and methyl hexadecadienoate, which was identified by its mass, ^1H and ^{13}C NMR spectra. Then, the picolinyl ester was prepared in order to clarify the position of its double bonds [12]. The methyl ester was hydrolyzed followed by treatment with thionyl chloride and the resulting acid chloride converted to the picolinyl ester using 3-pyridylcarbinol in MeCN. The GC-MS fragmentation pattern of the picolinyl ester revealed that the double bonds were present at C-7 and C-10 (see Experimental). On the basis of above finding, the chemical structure of compound **1** was determined as (2*S*)-1-*O*-(7Z,10Z,13Z)-hexadecatrienoyl-2-*O*-(7Z,10Z)-hexadecadienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol. The structures of the other MGDG (**2**–**5**) were elucidated in the same manner.

Compound **6** showed a $[M + Na]^+$ at *m/z* 935 in its FAB mass spectrum and its IR resembled those of MGDG. ^1H and ^{13}C NMR spectra of compound **6**, however, suggested that it was a DGDG by comparison with the spectral data of galactolipids obtained from *P. tenue* [9]. The α-1,6 linkage of the glycosidic bond between the two galactose residues was determined on the basis of the anomeric carbon (C-1″) signal at δ 100.7 and the downfield-shifted C-6′ carbon signal. Deacylation of **6** with *Rhizopus arrhizus* lipase gave a 1-*O*-deacylated galactolipid (**6a**) and linoleic acid, which was identified by GC analysis and GC-MS after CH_2N_2 treatment. Treatment of **6a** with NaOMe–MeOH liberated (2*R*)-3-*O*-(α-D-galactopyranosyl(1-6)-*β*-D-galactopyranosyl)-*sn*-glycerol (**9**) [13] and methyl (7Z,10Z)-hexadecadienoate. Consequently, the structure of **6** was deduced to be (2*S*)-1-*O*-(9Z,12Z)-octadecadienoyl-2-*O*-(7Z,10Z)-hexadecadienoyl-3-*O*-(α-D-galactopyranosyl(1-6)-*β*-D-galactopyranosyl)-*sn*-glycerol. The chemical structure of the other DGDG (**7**) was elucidated in a similar manner.

The anti-tumour-prompting activities of the glyceroglycolipids from *C. vulgaris* were determined using a short-term *in vitro* assay of EBV-EA activation on Raji cells induced by TPA [14]. The inhibitory effect on the activation and viabilities of Raji cells are summarized in Table 1. The effect of the MGDG fraction was 2.5 times as great as that from *P. tenue*. In the case of the DGDG fraction, the effect was four-fold greater than that from *P. tenue*. These fractions and compounds showed high anti-tumour-promotion activity at ratio of 1×10^3 M toward TPA without cytotoxity. Compound **2**, which contained (7Z,10Z)-hexadecadienoic acid, was the most potent anti-tumour-promoting constituent at 1×10^3 or 5×10^2 mol ratio toward TPA. On the other hand, compound **5**, which contained linoleic acid, exhibited lower activity. The DGDG (**6** and **7**) were more potent than the MGDG (**3** and **4**) possessing the same acyl pairs.

In conclusion, we isolated five MGDG (**1**–**5**) and two DGDG (**6** and **7**) from *C. vulgaris*. In particular, (2*S*)-1-*O*-(7Z,10Z,13Z)-hexadecadienoyl-2-*O*-(7Z,10Z)-hexadecadienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**1**) and (2*S*)-1,2-di-*O*-(7Z,10Z)-hexadecadienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**2**) were new glyceroglycolipids. These MGDG and DGDG showed high anti-tumour promoting effect *in vitro* without cytotoxity. We are now synthesizing various glyceroglycolipids to establish structure–activity relationships.

EXPERIMENTAL

General. ^1H and ^{13}C NMR spectra were obtained with a JEOL GSX-400 spectrometer using TMS as int. standard. GC identification of Me esters of fatty acids was carried cut using a ULBON HR-SS-10 column (0.25 mm i.d. × 50 m, Shinwa Kako); column temp.,

Table 1. Inhibitory effects of galactolipids on TPA-induced EBV-EA* activation

	Acyl group		Concentration (mol ratio/TPA)				
			2500		1000	500	100
	sn-1	*sn*-2	% to positive control ± s.e.† (% viability)				
MGDG mixt.‡			0 ± 1.2	(60)	26.3 ± 2.4	57.5 ± 2.5	82.8 ± 2.0
1	16:3	16:2	0 ± 1.2	(70)	22.8 ± 2.5	47.3 ± 3.7	61.5 ± 2.9
2	16:2	16:2	0 ± 0	(70)	4.2 ± 2.0	36.9 ± 2.5	77.5 ± 2.1
3	18:3	16:3	0 ± 1.0	(70)	27.3 ± 2.8	63.7 ± 3.4	88.4 ± 2.0
4	18:2	16:2	0 ± 1.5	(70)	39.6 ± 2.5	68.4 ± 3.2	91.5 ± 1.8
5	18:2	18:2	0 ± 0.4	(70)	23.6 ± 3.1	69.5 ± 3.2	87.8 ± 1.8
DGDG mixt.‡			0 ± 0.3	(60)	16.4 ± 1.9	49.7 ± 2.3	90.4 ± 1.6
6	18:3	16:3	0 ± 0	(60)	13.7 ± 1.3	50.6 ± 2.1	69.4 ± 2.0
7	18:2	16:2	0 ± 0	(60)	20.4 ± 1.6	48.5 ± 2.2	76.0 ± 2.4
Glycyrrhizin			...		26.4 ± 2.3 (70)	63.5 ± 3.1	82.3 ± 2.0

*Epstein–Barr virus early antigen.

†Standard error ($n = 3$).

‡The mol ratio was determined using the average M_r evaluated on the basis of fatty acid distribution.

150–220°, 3° min^{-1}; inj. temp. 250°; carrier gas, N_2, 2.2 kg cm^{-2}. Conditions for GC-MS measurement were as follows: column, SE-30 (3.0 mm i.d. × 2.0 m); inj. temp. 250°; column temp. 150–220°, 10° mm^{-1}; carrier gas, N_2, 1 ml min^{-1}; ionizing energy 70 eV; ion source temp. 230°. TLC was performed on Merck precoated Kieselgel $60F_{254}$ and spots were detected by UV or by spraying with 5% vanillin–70% $HClO_4$ or 1% $Ce(SO_4)_2$–10% H_2SO_4 followed by heating. CC was performed on silica gel BW-200 or BW-300 (Fuji Davison Chemicals).

Culture conditions. Chlorella vulgaris was purchased from the National Institute for Environmental Studies of Japan. The alga was cultured in 5 l Erlenmeyer flasks containing MD medium. The pH of the medium was adjusted to 8 at 25° with 1 M NaOH prior to autoclaving. Cultures were illuminated continuously at an incident intensity of 3000 lux with cool-white fluorescent lamps and vigorously aerated with sterilized air passed through a 0.2 mm membrane filter (Millipore, Mirex FG-50) at a rate of 0.5 l min^{-1}. After 3 weeks, algae were harvested by centrifugation at 20000 *g* from the comb. 36 l culture and lyophilized. Yields of lyophilized cells were typically in the range of 0.17–0.19 g l^{-1} of culture. MD medium (g l^{-1}): KNO_3 1, $MgSO_4 \cdot 7H_2O$ 0.25, K_2HPO_4 0.25 NaCl 0.1, $CaCl_2 \cdot 2H_2O$ 0.1, Fe soln ($FeSO_4 \cdot 7H_2O$ 2, conc H_2SO_4 4 drops) 1 ml l^{-1}, A_5 soln (H_3BO_4 2.86, $CuSO_4 \cdot 5H_2O$ 79 mg l^{-1}, $NaMoO_4 \cdot 2H_2O$ 21 mg l^{-1}) 1 ml.

Isolation. The lyophilized algae (6.88 g) were homogenized in $CHCl_3$–MeOH (1:2) (300 ml) and extracted ×3 during 12 hr. The $CHCl_3$–MeOH sol. portion was evapd under red. pres. to give 1.2 g of extract. This was subjected to silica gel CC using $CHCl_3$–MeOH–H_2O (10:3:1, lower phase) to afford a crude MGDG fr. Then, the column was eluted with $CHCl_3$–MeOH–H_2O (65:35:10, lower phase) to yield a crude DGDG fr. The crude MGDG fr. was applied to a column of Sephadex LH-20 using $CHCl_3$ as eluent to furnish the pure MGDG fr. (101 mg). The MGDG fr. was purified by HPLC (Develosil ODS-5, 10 mm i.d. × 250 mm, Nomura Chemical Co. MeOH–Me_2CO–H_2O, 50:40:10), to furnish **1** (5.5 mg), **2** (9.4 mg), **3** (15.1 mg), **4** (130.5 mg) and **5** (20.9 mg). The crude DGDG fr. was subjected to silica gel CC using $CHCl_3$–MeOH–28% aq. NH_3, 13:4:1) to afford the pure DGDG fr. (70 mg). The fraction was purified further by HPLC (Develosil ODS-5, 10 mm i.d. × 250 mm, MeOH–Me_2CO–H_2O, 13:1:2) to furnish **6** (13.4 mg) and **7** (27.2 mg).

Compound **1**. Colourless oil. $[\alpha]_D^{26} - 2.1°$ ($CHCl_3$; *c* 0.1). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1735 (C=O). FAB-MS *m/z*: 743 $[M + Na]^+$. ^1H NMR (d_5-pyridine, 400 MHz): δ0.88 (3H, *t*, *J* = 6.8 Hz), 0.96 (3H, *t*, *J* = 7.3 Hz), 2.37 (4H, *m*), 2.90 (6H, *m*), 4.09 (*dd*, *J* = 5.0 and 5.5 Hz, H-5′), 4.10 (*dd*, *J* = 5.2 and 11.0 Hz, *sn*-3-H), 4.16 (*dd*, *J* = 3.4 and 9.3 Hz, H-3′), 4.40 (*dd*, *J* = 5.3 and 11.0 Hz, *sn*-3-H), 4.42 (2H, *m*, H-6′), 4.46 (1H, *m*, H-2′), 4.54 (*dd*, *J* = 6.2 and 10.8 Hz, *sn*-1-H), 4.57 (*d*, *J* = 3.4 Hz, H-4′), 4.74 (*dd*, *J* = 10.8 and 3.1 Hz, *sn*-1-H), 4.84 (*d*, *J* = 7.6 Hz, H-1′), 5.44 (4H, *m*), 5.71 (1H, *m*, *sn*-2-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ105.4 (C-1′), 72.5 (C-2′), 75.0 (C-3′) 70.1 (C-4′), 76.8 (C-5′), 62.5 (C-6′), 64.0 (*sn*-1-C), 72.0 (*sn*-2-C), 68.7 (*sn*-3-C).

Compound **2**. Colourless oil. $[\alpha]_D^{26} - 2.1°$ ($CHCl_3$; *c* 0.2). IR ν_{max}^{KBr} cm^{-1}: 3435 (OH), 1735 (C=O). FAB-MS *m/z*: 745 $[M + Na]^+$. ^1H NMR (d_5-pyridine, 400 MHz): δ0.89 (3H, *t*, *J* = 7.0 Hz), 0.96 (3H, *t*, *J* = 7.5 Hz), 2.37 (4H, *m*), 2.95 (4H, *m*), 4.08 (*dd*, *J* = 5.7 and 5.9 Hz, 5-H), 4.09 (*dd*, *J* = 5.0 and 10.8 Hz, *sn*-3-H), 4.16 (*dd*, *J* = 3.4 and 9.3 Hz, H-3′), 4.40 (*dd*, *J* = 5.3 and 10.8 Hz, *sn*-3-H), 4.42 (2H, *m*, H-6′), 4.46 (1H, *m*, H-2′), 4.54 (*dd*, *J* = 6.2 and 10.8 Hz, *sn*-1-H), 4.57 (*d*, *J* = 3.4 Hz, H-4′), 4.72 (*dd*, *J* = 10.8 and 3.1 Hz, *sn*-1-H), 4.84 (*d*, *J* = 7.6 Hz, H-1′), 5.44 (4H, *m*), 5.71 (1H, *m*, *sn*-2-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ105.4 (C-1′), 72.4 (C-2′), 74.9 (C-3′) 70.1

(C-4'), 76.8 (C-5'), 62.5 (C-6'), 64.0 (*sn*-1-C), 72.0 (*sn*-2-C), 68.7 (*sn*-3-C).

Compound **3**. Oil. $[\alpha]_D^{26}$ − 2.8 ($CHCl_3$; *c* 0.1). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 (C=O). FAB-MS *m/z*: 769 $[M + Na]^+$. ^1H NMR (d_5-pyridine, 400 MHz): δ0.88 (3H, *J* = 6.8 Hz), 0.96 (3H, *t*, *J* = 7.5 Hz), 2.36 (4H, *m*), 2.94 (8H, *m*), 4.09 (*dd*, *J* = 5.7 and 5.7 Hz, H-5'), 4.10 (*dd*, *J* = 5.4 and 10.9 Hz, *sn*-3-H), 4.16 (*dd*, *J* = 3.3 and 9.3 Hz, H-3'), 4.40 (*dd*, *J* = 4.8 and 10.9 Hz, *sn*-3-H), 4.42 (2H, *m*, H_2-6'), 4.46 (1H, *m*, H-2'), 4.54 (*dd*, *J* = 3.3 and 11.9 Hz, *sn*-1-H), 4.57 (*d*, *J* = 3.3 Hz, H-4'), 4.70 (*dd*, *J* = 11.9 and 3.3 Hz, *sn*-1-H), 4.84 (*d*, *J* = 7.7 Hz, H-1'), 5.42–5.56 (12H, *m*), 5.70 (1H, *m*, *sn*-2-H). Neither spectral nor physicochemical data are described in Ref. [15].

Compound **4**. Oil. $[\alpha]_D^{26}$ − 3.1° ($CHCl_3$; *c* 0.4). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 (C=O). FAB-MS *m/z*: 773 $[M + Na]^+$. ^1H NMR (d_5-pyridine, 400 MHz): δ0.88 (6H, *m*), 2.39 (4H, *m*), 2.93 (4H, *m*), 4.06–4.14 (2H, *m*, H-5', *sn*-3-H), 4.17 (*dd*, *J* = 3.5 and 9.4 Hz, H-3'), 4.40 (*dd*, *J* = 5.4 and 10.9 Hz, *sn*-3-H), 4.43–4.50 (3H, *m*, H-2', H_2-6'), 4.52–4.59 (2H, *m*, H-4', *sn*-1-H), 4.72 (*dd*, *J* = 11.9 and 3.3 Hz, *sn*-1-H), 4.85 (*d*, *J* = 7.7 Hz, H-1'), 5.52 (8H, *m*), 5.70 (1H, *m*, *sn*-2-H). Neither spectral nor physicochemical data are described in Ref. [15].

Compound **5**. Oil. $[\alpha]_D^{26}$ − 2.8° ($CHCl_3$; *c* 0.4). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 (C=O). FAB-MS *m/z*: 801 $[M + Na]^+$. ^1H NMR (d_5-pyridine, 400 MHz): δ0.89 (6H, *m*), 2.40 (4H, *m*), 2.93 (4H, *m*), 4.05–4.12 (2H, *m*, H-5', *sn*-3-H), 4.17 (*dd*, *J* = 3.3 and 9.5 Hz, H-3'), 4.40 (*dd*, *J* = 5.4 and 10.8 Hz, *sn*-3-H), 4.42–4.50 (3H, *m*, H-2', H_2-6'), 4.52–4.60 (2H, *m*, H-4', *sn*-1-H), 4.73 (*dd*, *J* = 11.9 and 3.1 Hz, *sn*-1-H), 4.85 (*d*, *J* = 7.7 Hz, H-1'), 5.50 (4H, *m*), 5.71 (1H, *m*, *sn*-2-H). Neither NMR spectral nor physicochemical data are described in Ref. [16].

Compound **6**. Oil. $[\alpha]_D^{26}$ + 43.9° ($MeOH_3$; *c* 0.8). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (C=O). FAB-MS *m/z*: 935 $[M + Na]^+$. ^1H NMR (CD_3OD, 400 MHz): δ0.90 (3H, *t*, *J* = 7.1 Hz), 0.97 (3H, *t*, *J* = 7.5 Hz), 2.09 (4H, *m*), 2.32 (4H, *m*), 2.81 (4H, *m*), 3.92–3.83 (4H, *m*), 3.80–3.63 (8H, *m*), 3.49 (*dd*, *J* = 4.1 and 9.7 Hz, H-3'), 3.51 (*dd*, *J* = 6.8 and 9.7 Hz, H-2'), 3.94 (*dd*, *J* = 11.0 and 5.5 Hz, *sn*-3-H), 4.22 (*dd*, *J* = 12.1 and 6.8 Hz, *sn*-1-H), 4.24 (*d*, *J* = 6.8 Hz, H-1'), 4.43 (*dd*, *J* = 12.1 and 2.9 Hz, *sn*-1-H), 5.25 (1H, *m*, *sn*-2-H), 5.30 (6H, *m*). ^{13}C NMR (CD_3OD, 100 MHz): δ175.1, 174.8, 105.3 (C-1'), 72.6 (C-2'), 74.7 (C-3') 70.1 (C-4'), 74.6 (C-5'), 67.9 (C-6'), 100.7 (C-1''), 70.3 (C-2''), 71.5 (C-3''), 71.2 (C-4''), 72.5 (C-5''), 62.9 (C-6''), 64.0 (*sn*-1-C), 71.8 (*sn*-1-C), 68.8 (*sn*-3-C). Neither spectral nor physicochemical data are described in Ref. [15].

Compound **7**. Oil. $[\alpha]_D^{26}$ + 44.6° (MeOH; *c* 0.9). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1735 (C=O). FAB-MS *m/z*: 931 $[M + Na]^+$. ^1H NMR (CD_3OD, 400 MHz): δ0.90 (3H, *t*, *J* = 7.0 Hz), 0.91 (3H, *t*, *J* = 7.0 Hz), 2.06 (4H, *m*), 2.32 (4H, *m*), 2.80 (8H, *m*), 3.92–3.83 (4H, *m*), 3.80–3.63 (8H, *m*), 3.48 (*dd*, *J* = 3.4 and 9.8 Hz, H-3'), 3.51 (*dd*, *J* = 6.6 and 9.8 Hz, H-2'), 3.93 (*dd*, *J* = 10.8 and 5.4 Hz, *sn*-3-H), 4.22 (*dd*, *J* = 12.0 and 6.6 Hz, *sn*-1-H), 4.24 (*d*, *J* = 6.6 Hz, H-1'), 4.43 (*dd*, *J* = 12.1 and 2.9 Hz, *sn*-1-H), 5.24 (1H, *m*, *sn*-2-H), 5.33 (4H, *m*). ^{13}C NMR (CD_3OD, 100 MHz): δ175.0, 174.8, 105.3 (C-1'), 72.6 (C-2'), 74.7 (C-3'), 70.1 (C-4'), 74.6 (C-5'), 67.9 (C-6'), 100.7 (C-1''), 70.3 (C-2''), 71.5 (C-3''), 71.2 (C-4''), 72.4 (C-5''), 62.9 (C-6''), 64.0 (*sn*-1-C), 71.8 (*sn*-1-C), 68.8 (*sn*-3-C). Neither spectral nor physicochemical data are described in Ref. [15].

Alkaline methanolysis of **1** *and* **6**. A soln of **1** (5 mg) in dry MeOH (1 ml) was treated with 5% NaOMe–MeOH (0.2 ml) at room temp. for 10 min. The reaction mixt. was neutralized using an ion-exchange resin (Dowex 50W × 8) and the resin was removed by filtration. The filtrate was extracted with hexane and the hexane layer concd under red. pres. to yield a mixt. of Me (7*Z*,10*Z*)-hexadecadienoate and Me (7*Z*,10*Z*,13*Z*)-hexadecadienoate (3.5 mg). The mixt. of Me esters was subjected to GC and R_ts values compared with those of authentic samples. Removal of solvent from the MeOH layer under red. pres. gave a residue, which was purified by silica gel CC ($CHCl_3$–MeOH–H_2O, 6:4:1) to furnish **8** (1.7 mg). In the case of the methanolysis of **6** (5 mg), **9** (0.9 mg) was purified by silica gel CC ($CHCl_3$–MeOH–H_2O, 6:4:1).

Compound **8**. Oil. $[\alpha]_D^{26}$ − 8° (MeOH; *c* 0.8). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH). ^1H NMR (d_5-pyridine; 400 MHz): δ4.08 (*dd*, *J* = 5.3 and 6.6 Hz, H-5'), 4.13 (*d*, *J* = 5.5 Hz, *sn*-1-H), 4.14 (*d*, *J* = 4.9 Hz, *sn*-1-H), 4.17 (*dd*, *J* = 3.3 and 9.3 Hz, H-3'), 4.27 (*dd*, *J* = 3.8 and 9.7 Hz, *sn*-3-H), 4.45 (3H, *m*, H_2-6', *sn*-1-H, *sn*-2-H), 4.52 (*dd*, *J* = 7.7 and 9.3 Hz), 4.56 (*d*, *J* = 3.3 Hz, H-4'), 4.91 (*d*, *J* = 7.7 Hz, H-1'). ^{13}C NMR (CD_3OD, 100 MHz): δ105.3 (C-1'), 72.6 (C-2'), 74.9 (C-3'), 70.4 (C-4'), 76.8 (C-5'), 62.6 (C-6'), 64.1 (*sn*-1-C), 72.2 (*sn*-2-C), 72.1 (*sn*-3-C).

Compound **9**. Oil. $[\alpha]_D^{26}$ + 81° (H_2O; *c* 0.6). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH). ^1H NMR (d_5-pyridine; 400 MHz): δ4.10 (6H, *m*, H_2-6', H_2-6'', *sn*-3-H), 4.14 (1H, *m*, H-2'), 4.53 (2H, *m*, H-4', *sn*-1-H), 4.61 (1H, *dd*, *J* = 10.1 and 3.7 Hz, H-3''), 4.68 (1H, *dd*, *J* = 10.1 and 3.7 Hz, H-2''), 4.82 (1H, *d*, *J* = 7.6 Hz, H-1'), 5.50 (1H, *d*, *J* = 3.7 Hz, H-1''). ^{13}C NMR (CD_3OD, 100 MHz): δ105.6 (C-1'), 72.7 (C-2'), 74.7 (C-3') 70.4 (C-4'), 74.6 (C-5'), 68.0 (C-6'), 101.0 (C-1''), 70.5 (C-2''), 71.5 (C-3''), 71.2 (C-4''), 72.5 (C-5''), 62.3 (C-6''), 64.4 (*sn*-1-C), 71.4 (*sn*-2-C), 70.0 (*sn*-3-C).

Enzymatic hydrolysis of **1** *and* **6**. A soln of **1** (5 mg) and Lipase type XI (700 unit, Sigma) in the presence of Triton X-100 (2.5 mg) in boric acid–borax buffer (0.63 ml, pH 7.7) was stirred at 38° for 1 hr. The reaction was quenched with HOAc (0.1 ml), then EtOH was added to the reaction mixt. The solvent was removed under red. pres. and the resulting residue purified on silica gel using $CHCl_3$–MeOH (7:1) to yield **1a** (3.2 mg) and hexadecatrienoic acid (1.8 mg). (7*Z*,10*Z*,13*Z*)-Hexadecatrienoic acid was converted to the Me ester by treatment with CH_2N_2. Treatment of **1a** with NaOMe–MeOH gave compound **8** (1.7 mg) and Me (7*Z*,10*Z*)-hexadecadienoate (1.4 mg). In the case of lipase-catalysed hydrolysis of **6** (5.0 mg), **6a** (3.6 mg) was isolated by silica gel CC ($CHCl_3$–MeOH–H_2O, 7:3:1, lower phase). Enzymatic hydrolysis of the other MGDG (**2**–**5**) and DGDG (**7**) was carried out in the same manner.

Compound **1a**. Oil. $[\alpha]_D^{26}$ − 1.2° (MeOH; *c* 0.2). FAB-MS *m*: 511 $[M + Na]^+$. IR ν_{max}^{KBr} cm^{-1}: 3410, 1730. ^1H NMR (CD_3OD, 400 MHz): δ0.90 (3H, *t*, *J* = 7.3 Hz), 2.36 (2H, *t*, *J* = 7.3 Hz), 3.45 (1H, *dd*, *J* = 3.7 and 9.8 Hz, H-3″), 3.50 (1H, *m*, H-5′), 3.51 (1H, *dd*, *J* = 7.3 and 9.8 Hz, H-2′), 3.66–3.79 (5H, *m*, H_2-6′, *sn*-1-H_2, *sn*-3-H), 3.82 (1H, *dd*, *J* = 1.2 and 3.7 Hz, H-4′), 3.96 (1H, *dd*, *J* = 5.5 and 10.4 Hz, *sn*-3-H), 4.23 (1H, *d*, *J* = 7.3 Hz, H-1′), 5.04 (1H, *m*, *sn*-2-H), 5.32 (4H, *m*, olefinic H). ^{13}C NMR (CD_3OD, 100 MHz): δ175.3 (C=O), 105.3 (C-1′), 72.5 (C-2′), 74.9 (C-3′), 70.3 (C-4′), 76.8 (C-5′), 62.5 (C-6′), 61.8 (*sn*-1-C), 74.6 (*sn*-2-C), 68.8 (*sn*-3-C).

Compound **6a**. Oil. $[\alpha]_D^{26}$ + 17.0° (MeOH; *c* 0.2). IR ν_{max}^{KBr} cm^{-1}: 3400, 1740. FAB-MS *m/z*: 649 $[M + Na]^+$. ^1H NMR (CD_3OD, 400 MHz): δ0.90 (3H, *t*, *J* = 7.0 Hz), 2.35 (2H, *m*), 3.49 (2H, *m*, H-2′, H-3′), 3.63–3.80 (10H, *m*), 3.84–3.95 (5H, *m*), 4.24 (1H, *d*, *J* = 7.3 Hz, 1H-1′), 5.04 (1H, *m*, *sn*-2-H). ^{13}C NMR (CD_3OD, 100 MHz): δ175.3, 105.2 (C-1′), 72.5 (C-2′), 74.7 (C-3′) 70.2 (C-4′), 74.6 (C-5′), 67.9 (C-6′), 100.6 (C-1″), 70.2 (C-2″), 71.5 (C-3″), 71.1 (C-4″), 72.5 (C-5″), 62.8 (C-6″), 61.8 (*sn*-1-C), 74.6 (*sn*-2-C), 68.9 (*sn*-3-C).

Methyl (7Z,10Z)-hexadecadienoate. Oil. EI-MS *m/z*: 226 $[M]^+$. ^1H NMR ($CDCl_3$, 400 MHz): δ5.40 (4H, *m*, olefinic H), 3.70 (3H, *s*, OMe), 2.84 (2H, *m*, allylic H), 2.35 (2H, *t*, *J* = 7.5 Hz, H_2-2), 2.10 (4H, *m*, H_2-6, H_2-12), 0.98 (3H, *t*, *J* = 7.5, H_3-16). ^{13}C NMR ($CDCl_3$, 100 MHz): δ174.3, (C-1′), 51.6 (OMe), 34.1 (C-2), *27.2 (C-12), *27.0 (C-6), 25.6 (C-8), 24.9 (C-3), 14.5 (C-16); *interchangeable.

Methyl (7Z,10Z,13Z)-hexadecatrienoate. Oil. EI-MS *m/z*: 264 $[M]^+$. ^1H NMR ($CDCl_3$, 400 MHz): δ5.36 (6H, *m*, olefinic H), 3.66 (3H, *s*, OMe), 2.80 (4H, *m*, allylic H), 2.31 (2H, *t*, *J* = 7.6 Hz, H_2-2), 2.07 (4H, *m*, H_2-6, H_2-15), 0.98 (3H, *t*, *J* = 7.5 Hz, H_3-16). ^{13}C NMR ($CDCl_3$, 100 MHz): δ174.3, (C-1′), 131.9 (C-14), 130.0 (C-7), *128.3 (C-10), *128.2 (C-11), 128.0 (C-8), 127.1 (C-13), 51.7 (OMe), 34.1 (C-2), 29.3 (C-5) 28.8 (C-4), 27.0 (C-6), †25.6 (C-9), †25.5 (C-12), 24.9 (C-3), 20.6 (C-15), 14.3 (C-16); * and † interchangeable.

Identification of hexadecadienoic acid. A soln of compound **1a** in dry MeOH (1.0 ml) was treated with 10% KOH–MeOH (1 ml) at room temp. for 10 min. The reaction mixt. was extracted with *n*-hexane and the extract concd *in vacuo*. The remaining extract was treated with $SOCl_2$ (1 ml) at room temp. for 5 min. Excess $SOCl_2$ was removed and the corresponding acid chloride in dry MeCN (0.1 ml) was treated with a 10% soln of 3-pyridylcarbinol in MeCN (0.1 ml) at room temp. for 10 min. Removal of solvent in a stream of N_2 gave the picolinyl ester, which was subjected to GC-MS analysis. EIMS *m/z*: 343 $[M]^+$, 314 $[M - CH_2H_5]^+$, 300 $[M - C_3H_7]^+$, 286 $[M - C_4H_9]^+$, 272 $[M - C_5H_{11}]^+$, 259 $[M - C_6H_{12}]^+$, 246 $[M - C_7H_{13}]^+$, 232 $[M - C_8H_{15}]^+$, 206 $[M - C_{10}H_{17}]^+$, 192 $[M - C_{11}H_{19}]^+$.

Bioassay for anti-tumour-promoting activity. Inhibition of EBV-EA activation was assayed using EBV genome-carrying human lymphoblastoid cells, Raji cells (non-producer type), which were cultivated in 8% FBS RPMI 1640 medium (Nissui). The indicator cells (Raji, $\times 10^6$ ml^{-1}) were incubated at 37° for 48 hr in 1 ml of a medium containing *n*-butyric acid (4 mM), 32 pmol of TPA in DMSO and a known amount of the test compound in DMSO. Smears were made from the cell suspension. Activated cells were stained by high titre EBV-positive sera from nasopharyngeal carcinoma (NPC) patients and fluorescein isothiocyanate-labelled anti-human Ig G. After staining, they were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted; the expts were repeated twice. The average EA induction was compared with that of positive control expts with *n*-butyric acid (4 mM) plus TPA (32 pmol) in which EA indication was ordinarily *ca* 35%.

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