Isolation and Identification of Anti-tumor-Promoting Principles from the Fresh-Water Cyanobacterium *Phormidium tenue*[1)]

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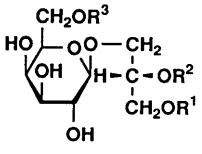
Bioassay-directed fractionation of the extract of the cyanobacterium *P. tenue* led to the isolation of the three classes of glycolipids, *viz.*, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG) as anti-tumor-promoters. In comparing the anti-tumor-promoting effect of the three classes of glycolipids with various acyl residues, MGDG and DGDG inhibited tumor promotion more intensely than SQDG. With respect to MGDG and DGDG, the inhibitory activity of each glycolipid possessing a single pair of acyl residues was also investigated after HPLC separation. Among the 17 tested MGDGs (1a—h) and DGDGs (2a—i), three of the DGDGs (2a, b, e) showed more potent inhibitory activity toward tumor promotion than the others.

Keywords *Phormidium tenue*; glyceroglycolipid; anti-tumor-promoter; monogalactosyl diacylglycerol (MGDG); digalactosyl diacylglycerol (DGDG); Epstein–Barr virus

In the last decade, microalgae have drawn much attention as prospective and excellent sources of biologically active constituents.[3)] Furthermore, *Chlorella* and *Spirulina* species have been utilized as commercially available health foods. Thus, the establishment of a procedure for the mass culture of these microalgae would allow them to be available as an inexhaustible resource of biologically active principles, similarly to microorganisms. On this basis, we have been engaged in a search for biologically active constituents in microalgae. After preliminary investigations of several biological activities of the extract of the fresh-water cyanobacterium *P. tenue*, the 70% EtOH extract of the alga was found to inhibit the tumor promoting stage of Epstein–Barr virus associated early antigen (EBV-EA) examination.[4)] Bioassay-directed separation of the extract led to the isolation of three classes of glycolipids as anti-tumor-promoters. In this paper, we present the anti-tumor-promoting activities of the glycolipids, *viz.*, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG), isolated from the fresh-water cyanobacterium *P. tenue*.

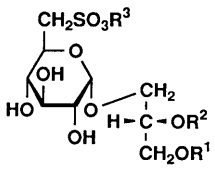
Results and Discussion

Isolation and Identification of Anti-tumor-Promoting Principles The 70% EtOH extract of *P. tenue* was partitioned between *n*-hexane and H_2O. The H_2O layer was successively extracted with the EtOAc and *n*-BuOH. Of the three extracts, the *n*-BuOH extract was separated by silica gel column chromatography [$CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)→65 : 35 : 10 (lower phase)] in monitoring the inhibition of EBV-activation to afford the three active fractions. The first and second active fractions were composed almost exclusively of MGDG (**1**) and DGDG (**2**), respectively. Further separation of both fractions revealed **1** and **2** to be responsible for the activity. The third fraction was purified by a silica gel column by elution with $CHCl_3$: MeOH : 28% aq. NH_3 = 13 : 4 : 1 to give **3** as an anti-tumor-promoting principle. MGDG (**1**) and DGDG (**2**) were identified in comparison with the physicochemical data of the authentic materials previously isolated from the alga.[5)] The fast arom bombardment mass spectrum (FAB-MS) of **3** showed two comparatively intense quasimolecular ion peaks at *m/z*: 863 [$(M+Na)^+$, acyl residue: $C_{18:2}$, $C_{16:0}$] and *m/z*: 835 [$(M+Na)^+$, acyl residue: $C_{18:2}$, $C_{14:0}$]. The molecular formula of the former peak was determined to be $C_{43}H_{77}Na_2O_{12}S$, and that of the latter one to be $C_{41}H_{73}Na_2O_{12}S$ by high resolution (HR) FAB-MS. The infrared (IR) spectrum of **3** indicated the presence of a sulfonic group (1121, 1028 cm^{-1}), while proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **3** showed a signal-splitting triplet due to some methyl protons in the range of 0.86 to 0.99 ppm, as well as a broad signal ascribable to a number of methylene protons at 1.29 ppm. It also exhibited 12H proton signals ranging from 2.91 to 5.33 ppm as well as two methylene proton signals (δ 2.07, 4H, m) attached to a carbonyl function. Detailed analysis of homonuclear decoupling spectra revealed that the coupling constants and splitting patterns of the protons in the sugar moiety of **3** were very similar to those of α-glucopyranosides, but the chemical shifts were in some ways different. Namely, observation of the C6-methylene proton at 3.34 and 2.91 ppm indicated the attachment of a sulfonyl group on the C6 carbon, so the sugar moiety proved to be sulfoquinovose. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **3**, in which the C6



1: MGDG
R^1=R^2=acyl residue
R^3=H

2: DGDG
R^1=R^2=acyl residue
R^3=α-D-galactopyranosyl



3: SQDG
R^1=R^2=acyl residue
R^3=Na, K, NH_4, 1/2Ca, 1/2Mg

3a: R^1=H, R^2=acyl residue

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

Fig. 1

carbon signal appeared at 54.2 ppm, also substantiated this conclusion. The stereochemistry of *sn*-2 in the glycerol portion was determined to be *S* by comparing the specific rotation of 3-α-sulfoquinovosyl-*sn*-glycerol obtained by alkaline treatment of **3** with that reported previously.[6,7] Therefore, **3** was determined to be SQDGs.

Counter cations of the SQDGs were elucidated to be Na^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+} by positive ion chromatography. The SQDGs from *P. tenue* had too many acyl pairs to be separated into a compound possessing a single acyl pair by the HPLC method.[1] Fatty acid distribution of **3** was determined by enzymatic hydrolysis using *Rhizopus arrhizus* lipase. Lipase-catalyzed regioselective deacylation at the *sn*-1 position quantitatively afforded 1-lysosulfoquinovosyl diacylglycerol (**3a**) and a mixture of fatty acids.[1] The alkaline trreatment (NaOMe–MeOH) of **3a** gave a mixture of the methyl esters derived from the fatty acids. Gas-liquid chromatography (GLC) analysis of both mixtures as methyl esters provided the fatty acid distribution of **3**. The sulfoquinovosyl diacylglycerol isolated from *P. tenue* contained unsaturated fatty acids to a great extent at the *sn*-1 position. This feature was fairly similar to that of MGDG (**1**) and DGDG (**2**) from the alga.[5]

Anti-tumor-Promoting Effects of the Glyceroglycolipids The anti-tumor-promoting activities of the glycolipids were determined using a short-term *in vitro* assay of Epstein–Barr virus activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The inhibitory effect on the activation and the viabilities of Raji cells are summarized in Table I. The inhibitory effect of MGDG (**1**) and DGDG (**2**) was more potent than SQDG (**3**) with respect to those glycolipids which included several acyl pairs.

In the case of MGDG (**1**) and DGDG (**2**), the anti-tumor-promoting activity of each component possessing a pair of fatty acid residues was also examined. In general, DGDGs (**2a—i**) showed more potent anti-tumor-promotion activities than MGDGs (**1a—h**) at the dose of 5×10^3 mol ratio toward TPA. No apparent relationship was observed between the anti-tumor-promoting effect and acyl pairs in the glycolipids, but the three MGDGs (**1a**, **b**, **e**) and the three DGDGs (**2a**, **b**, **e**) possessing a common pair of acyl residues inhibited tumor promotion more intensely than the other glycolipids, as shown in Table I. However, the inhibiting activity toward tumor promotion of the three MGDGs (**1a**, **b**, **e**) was presumed to be attributed to cytotoxicity. On the other hand, the three DGDGs (**2a**, **b**, **e**) inhibited tumor-promotion of activated Raji cells involving very little cytotoxicity. This finging suggests that the linkage of another galactose unit to the three MGDGs decreases cytotoxicity without eliminating the anti-tumor-promoting effect. Since no one has yet been able to completely separate a single constituent of SQDG, the structure–activity relationship of SQDGs are still uncertain at present.

In conclusion, we have found the three classes of membrane glycolipids (**1—3**) from the cyanobacterium *P. tenue* to inhibit the tumor-promoting stage. It may be of interest from the standpoint of structure–activity relationship that the three MGDGs (**1a**, **b**, **e**) and three DGDGs (**2a**, **b**, **e**) which showed greater anti-tumor-promoting activity than the other glycolipids, were composed of common fatty acid pairs similarly distributed. The three DGDGs (**2a**, **b**, **e**) are promising because of their combination of a high anti-tumor-promoting effect with good viability (60—80%) at a 5×10^3 mol ratio toward TPA. Detailed investigation of their pharmacological activities *in vivo* is now in progress.

TABLE I. Inhibitory Effects of Glyceroglycolipids on TPA Induced EVB-EA Activation

Compound	R^1	R^2	Concentration (mol ratio/TPA) 5000	2500	1000
MGDG (**1**)[a]			0[c] (10)[d]	29.1 (60)	62.3
DGDG (**2**)[a]			0 (10)	17.8 (60)	71.6
SQDG (**3**)[a]			24.9 (60)	62.1	91.7
1a	$C_{18:3}$	$C_{18:3}$	0 (0)	17.6 (20)	87.7 (70)
1b	$C_{18:3}$	$C_{14:0}$	0 (0)	13.9 (20)	67.8 (70)
1c	$C_{18:3}$	$C_{16:1}$ (9-*t*)[b]	30.8 (70)	43.2	60.7
1d	$C_{16:1}$	$C_{14:0}$	21.7 (70)	39.4	56.3
1e	$C_{18:2}$	$C_{14:0}$	0 (10)	33.7	51.4
1f	$C_{18:1}$	$C_{14:0}$	38.7 (70)	54.7	62.2
1g	$C_{16:0}$	$C_{14:0}$	33.2 (70)	41.2	57.8
1h	$C_{18:2}$	$C_{16:0}$	10.3 (70)	55.4	69.4
2a	$C_{18:3}$	$C_{18:3}$	0 (70)	22.6	43.2
2b	$C_{18:3}$	$C_{14:0}$	0 (80)	50.4	73.1
2c	$C_{18:3}$	$C_{18:2}$	12.6 (60)	23.3	65.8
2d	$C_{16:1}$	$C_{14:0}$	8.4 (70)	20.5	60.1
2e	$C_{18:2}$	$C_{14:0}$	0 (60)	35.7	70.4
2f	$C_{18:2}$	$C_{18:2}$	9.6 (70)	23.2	57.5
2g	$C_{18:1}$	$C_{14:0}$	18.7 (70)	32.6	47.9
2h	$C_{16:0}$	$C_{14:0}$	7.7 (70)	37.7	58.3
2i	$C_{18:2}$	$C_{16:0}$	11.8 (70)	22.5	50.9

a) The mol ratio was determined using the average molecular weight evaluated on the basis of the fatty acid distribution. *b*) Palmitelaidic acid. *c*) Values represent percentages relative to the positive control value (100%). *d*) Values in parentheses are the viability percentages of Raji cells.

Experimental

The instruments used to obtain the physicochemical data and experimental conditions for cultivation of the alga and chromatography were the same as reported in our previous paper.[1]

Extraction and Isolation The lyophilized alga (7.85 g) was homogenized in 70% EtOH (300 ml) and extracted three times during a 12 h period. Removal of the solvent gave a residue which was partitioned between *n*-hexane and water. The water layer was successively extracted with EtOAc and *n*-BuOH. The *n*-BuOH extract was fractionated by silica gel column chromatography with $CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase) followed by $CHCl_3$: MeOH : H_2O = 65 : 35 : 10 (lower phase), by monitoring the inhibition of EBV-EA activation,[4] to afford three active fractions. Further purification of the first and second fractions to isolate MGDGs (**1a—h**) and DGDGs (**2a—i**) was carried out as described previously.[5] The third fraction was purified by silica gel column chromatography using $CHCl_3$: MeOH : 28% aq. NH_3 = 13 : 4 : 1 as the eluent to furnish pure solfoquinovosyl diacylglycerol (**3**, 46.0 mg).

Sulfoquinovosyl Diacylglycerol (3) Colorless oil. IR ν_{max}^{film} cm^{-1}: 3420, 1736, 1121, 1028. Positive-FAB-MS *m/z*: 863 $[(M+Na)^+, R^1=C_{18:2}, R^2=C_{16:0}]$, 835 $[(M+Na)^+, R^1=C_{18:2}, R^2=C_{14:0}]$. Negative-FAB-MS *m/z*: 817 $(M-Na)^-$, 789 $(M-Na)^-$. HRFAB-MS: Calcd for $C_{43}H_{77}Na_2O_{12}S$ 863.4931, Found 863.4960; Calcd for $C_{41}H_{73}Na_2O_{12}S$ 835.4618, Found 835.4664. ^{1}H-NMR (CD_3OD, 400 MHz (assignable common signals of the mixture)) δ: 2.35 (4H, m, $COCH_2$), 2.91 (1H, dd, *J*=9.2, 14.3 Hz, 6-H), 3.08 (1H, dd, *J*=9.0, 9.5 Hz, 3-H), 3.34 (1H, dd, *J*=2.2, 14.3 Hz, 6-H), 3.40 (1H, dd, *J*=3.7, 9.5 Hz, 2-H), 3.57 (1H, dd, *J*=6.4, 10.8 Hz, *sn*-3-H), 3.64 (1H, dd, *J*=9.0, 9.5, 4-H), 4.07 (1H, ddd, *J*=2.2, 9.2, 9.5 Hz, 5-H), 4.10 (1H, dd, *J*=5.3, 10.8 Hz, *sn*-3-H), 4.18 (1H, dd, *J*=7.0, 12.1 Hz, *sn*-1-H), 4.50 (1H, dd, *J*=2.9, 12.1 Hz, *sn*-1-H), 4.76 (1H, d, *J*=3.7 Hz, 1-H), 5.33 (m, 2-H, overlapping with the olefinic proton signal). ^{13}C-NMR (OD_3OD, 100 MHz (assignable common signals of the mixture)) δ_C: 100.1 (1-C), 73.5 (2-C), 75.0 (3-C), 74.9 (4-C), 69.9 (5-C), 54.2 (6-C), 64.3 (*sn*-1-C), 71.7 (*sn*-2-C), 67.1 (*sn*-3-C).

Fatty Acid Distribution of 3 A solution of **3** (5 mg) and the lipase in the presence of Triton X-100 (2.5 mg) in a boric acid–borax buffer (0.63 ml, pH 7.7) was stirred at 38 °C for 5 h. 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 SiO_2 using $CHCl_3$: MeOH : H_2O = 65 : 35 : 10 (lower phase) as an eluent to yield **3a** (3.3 mg, quant.) and a mixture of fatty acids (1.7 mg). The fatty acids, obtained by the previous enzymatic hydrolysis of **3**, were converted to the corresponding methyl esters by treatment with ethereal diazomethane. Alkaline treatment (5% NaOMe–MeOH) of **3a** afforded 3-α-D-sulfoquinovosyl-*sn*-glycerol (**3b**) and a mixture of fatty acid methyl esters. The distribution of fatty acid in **3** was determined by GLC analysis of both methyl esters.

3a: Colorless oil. IR ν_{max}^{film} cm^{-1}: 3384, 1736, 1121, 1029. Positive-FAB-MS *m/z*: 601 ($[M+Na]^+$, $R^2 = C_{16:0}$), 573 ($[M+Na]^+$, $R^2 = C_{14:0}$). ^{1}H-NMR (CD_3OD, 400 MHz (assignable common signals of the mixture)) δ: 2.08 (2H, m, $COCH_2$), 2.91 (1H, dd, J = 9.2, 14.5 Hz, 6-H), 3.08 (1H, dd, J = 9.0, 9.9 Hz, 3-H), 3.35 (1H, dd, J = 2.2, 14.5 Hz, 6-H), 3.40 (1H, dd, J = 3.7, 9.5 Hz, 2-H), 3.53 (1H, dd, J = 6.2, 10.6 Hz, *sn*-3-H), 3.62 (1H, dd, J = 9.5, 9.5 Hz, 4-H), 3.71 (1H, dd, J = 5.3, 12.3 Hz, *sn*-1-H), 3.76 (1H, dd, J = 4.2, 12.3 Hz, *sn*-1-H), 4.08 (1H, ddd, J = 2.2, 9.2, 9.5 Hz, 5-H), 4.09 (1H, dd, J = 5.3, 10.6 Hz, *sn*-3-H), 4.75 (1H, d, J = 3.7 Hz, 1-H), 5.09 (1H, m, *sn*-2-H). Fatty acid distribution of **3** is as follows, *sn*-1: $C_{14:0}$: $C_{16:0}$: $C_{16:1}$: $C_{18:0}$: $C_{18:1}$: $C_{18:2}$: $C_{18:3}$ = 10 : 15 : 5 : 16 : 14 : 32 : 8, *sn*-2: $C_{14:0}$: $C_{16:0}$ = 36 : 64.

Bioassays for Anti-tumor-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 ml of a medium containing *n*-butyric acid (4 mM),[8] 32 pmol of TPA in 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-labelled anti-human IgG. After staining, they were detected by a conventional indirect immunofluorescence technique.[9,10] 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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References and Notes

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