# Glyceroglycolipids from *Citrus hystrix*, a Traditional Herb in Thailand, Potently Inhibit the Tumor-Promoting Activity of 12-*O*-Tetradecanoylphorbol 13-Acetate in Mouse Skin

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Two glyceroglycolipids were isolated from the leaves of *Citrus hystrix* (bitter orange), a traditional herb in Thailand. They were identified as 1,2-di-*O*-α-linolenoyl-3-*O*-β-galactopyranosyl-*sn*-glycerol (DLGG, **1**) and a mixture of two compounds, 1-*O*-α-linolenoyl-2-*O*-palmitoyl-3-*O*-β-galactopyranosyl-*sn*-glycerol (**2a**) and its counterpart (**2b**) (LPGG, **2**). Both lipids were potent inhibitors of tumor promoter-induced Epstein–Barr virus (EBV) activation. The $IC_{50}$ values of **1** and **2** were strikingly lower than those of representative cancer preventive agents such as α-linolenic acid, β-carotene, or (−)-epigallocatechin gallate. In a two-stage carcinogenesis experiment on ICR mouse skin with dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), compound **1** exhibited anti-tumor-promoting activity even at a dose 10 times lower than that of α-linolenic acid. As some synthetic detergents or saponins were entirely inactive in the EBV activation inhibition test, detergency was suggested not to play a major role in the mode of inhibitory action *in vivo*. The inhibition of the arachidonic acid cascade may be involved in anti-tumor promotion since **1** inhibited TPA-induced edema formation in the anti-inflammation test using ICR mouse ears.

**Keywords:** *Cancer chemoprevention; anti-tumor promoter; EB virus; Raji cells; glyceroglycolipid; Citrus hystrix; herb; spice*

## INTRODUCTION

Chemoprevention of cancer is regarded as one of the most promising avenues to cancer control (Greenwald et al., 1990; Weinstein, 1991; Wattenberg, 1992, 1993). To search for effective chemopreventive agents, we have been focusing on the inhibition of tumor promotion because promotion takes a long time and is the only reversible process during the multistages of carcinogenesis (Pitot et al., 1991; Hennings et al., 1993). Hitherto, we have conducted a convenient *in vitro* assay, the Epstein–Barr virus (EBV) activation test, for the detection of naturally occurring anti-tumor promoters (Ohigashi et al., 1986, 1992; Koshimizu et al., 1988; A. Murakami et al., 1992a, 1993; Kondo et al., 1993). Most inhibitors, identified by this assay, have further been proven to possess marked anti-tumor-promoting activities *in vivo* (Tokuda et al., 1986; A. Murakami et al., 1991, 1992b; Koshimizu et al., 1991; Ohigashi et al., 1992, 1994).

Cancer preventive agents, especially those from food items, are considered to be desirable for the general population, because of their relatively low toxicities. Recently we screened for EBV activation inhibitory activities of edible plants from Thailand used for flavors and/or condiments (A. Murakami et al., 1993). As a result, we found that the methanol extracts from such plants exhibited a significantly higher potential for anti-tumor promotion than those from common edible plants in Japan. In particular, an extract from *Citrus hystrix* (Rutaceae, bitter orange), the leaves of which are widely used as flavor for cooking in Thailand, was indicated to be a promising source for potent anti-tumor promoters. Here we describe isolation and identification of the active constituents of the plant, as well as their anti-tumor-promoting and anti-inflammatory activities.

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## MATERIALS AND METHODS

**General Procedure.** Fast atom bombartment mass spectra (FAB-MS) were obtained on a JEOL HX-100. Infrared (IR) spectra were taken on a Shimadzu Model 435. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX 400 (400 MHz) using tetramethylsilane (TMS) as an internal reference (δ 0.00). Optical rotation was measured by a JASCO DIP-4. Chromatographic materials used were as follows: Wako gel C-100 and C-200 from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), YMC I-40/64 gel from Yamamura Chemical Laboratory (Kyoto, Japan), Kieselgel 60 $F_{254}$ for TLC from Merck Co. Ltd. (Darmstadt, Germany), and ODS gel $KC_{18}F$ for TLC from Whatman (Clifton, NJ). Gas–liquid chromatography was performed on an FFS capillary column SS-10 (50 m × 0.24 mm i.d.) on a Shimadzu GC-7A with FID detection.

**Chemicals and Animals.** Teleocidin B-4 was isolated from *Streptoverticillium blastmyceticum* NA 34-17 as previously reported (Irie et al., 1984). TPA and *Rhizopus arrizus* lipase were purchased from Sigma Chemical Co. (St. Louis, MO). High-titer early antigen (EA)-positive sera from nasopharyngeal patients was a kind gift from Dr. T. Osato of Hokkaido University. Fluorescence isothiocyanate (FITC)-labeled anti-human IgG was obtained from Dako Co. Ltd. (Glostrup, Denmark). α-Linolenic acid, β-carotene, (−)-epigallocatechin gallate, Triton X-100, Excel 200, sorbitan monostearate, and saponins (soybean and green tea) were obtained from Wako Pure Chemical Industries. Female ICR mice (6 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan).

**Isolation and Identification of Active Constituents of *C. hystrix*.** Fresh leaves of *C. hystrix* (480 g) were extracted with 10 L of MeOH at room temperature for 2 weeks. After evaporation *in vacuo*, the aqueous concentrate (39 g) was extracted with ethyl acetate (EtOAc). The EtOAc layer (15 g)

0021-8561/95/1443-2779$09.00/0 

was chromatographed on Wako gel C-100, eluting stepwise with toluene containing increasing amounts of acetone to give active fractions (60–80% acetone eluates, 2.2 g). The fraction was further chromatographed on ODS gel ($MeOH/H_2O$ = 9:1) twice and then on ODS gel (MeOH/acetonitrile/$H_2O$ = 16:4:5). At this stage, two major compounds were detected as brown spots on ODS TLC (acetonitrile/$H_2O$ = 4:1) at $R_f$ values of 0.4 (**1**) and 0.3 (**2**) by spraying 5% sulfuric acid in EtOH followed by heating. Final purification was separately done by preparative TLC on silica gel ($CHCl_3$/MeOH = 9:1) to afford DLGG (**1**, 82 mg) and LPGG (**2**, 44 mg). DLGG (**1**): colorless oil, $[\alpha]^{26}_D$ −4.0° (*c* 0.48, $CHCl_3$); IR $\nu_{max}$ ($CH_2Cl_2$) 3460 (OH), 1730 (C=O) $cm^{-1}$; FAB-MS [*m*-nitrobenzyl alcohol (*m*NBA) as a matrix] *m*/*z* 797 $[M + Na]^+$; $^1H$ NMR ($CDCl_3$) δ 0.97 (t, 6H, *J* = 7.6 Hz, $[-CH_2CH_3] \times 2$), 1.2–1.4 (m, 24H, $-CH_2-$), 2.06 (m, 8H, $[-COCH_2-] \times 2$, $[=CHCH_2-] \times 2$), 2.32 (m, 4H, $[=CHCH_2CH_3] \times 2$), 2.81 (m, 8H, $[=CHCH_2CH=] \times 4$), 3.56 (m, 1H, H-5′), 3.60 (dd, 1H, *J* = 9.5, 3.4 Hz, H-*sn*-3a), 3.65 (dd, 1H, *J* = 9.5, 7.3 Hz, H-*sn*-3b), 3.75 (dd, 1H, *J* = 11.3, 6.4 Hz, H-6′a), 3.87 (dd, 1H, *J* = 10.4, 4.0 Hz, H-*sn*-1a), 3.91 (dd, 1H, *J* = 11.3, 5.5 Hz, H-6′b), 3.99 (dd, 1H, *J* = 11.3, 6.1 Hz, H-*sn*-1b), 4.02 (dd-like, 1H, H-4′), 4.21 (dd, 1H, *J* = 11.9, 7.3 Hz, H-2′), 4.28 (d, 1H, *J* = 7.3 Hz, H-1′), 4.39 (dd, 1H, *J* = 11.9, 3.4 Hz, H-3′), 5.28–5.43 (m, 13H, $[-CH=CH-] \times 6$, H-*sn*-2). LPGG (**2**): colorless oil, $[\alpha]^{26}_D$ −3.2° (*c* 0.48, $CHCl_3$); IR $\nu_{max}$ ($CH_2Cl_2$) 3460 (OH), 1730 (C=O) $cm^{-1}$; FAB-MS (*m*NBA as a matrix) *m*/*z* 775 $[M + Na]^+$; $^1H$ NMR ($CDCl_3$) δ 0.88 (br t, 3H, *J* = 7.0 Hz, $-CH_2CH_3$), 0.98 (t, 3H, *J* = 7.6 Hz, $-CH_2CH_3$), 1.2–1.4 (m, 24H, $-CH_2-$), 2.06 (m, 6H, $[-COCH_2-] \times 2$, $=CHCH_2-$), 2.31 (m, 2H, $=CHCH_2CH_3$), 2.80 (m, 4H, $[=CHCH_2CH=] \times 4$), 3.54 (m, 1H, H-5′), 3.59 (dd, 1H, *J* = 9.5, 3.4 Hz, H-*sn*-3a), 3.65 (dd, 1H, *J* = 9.5, 7.3 Hz, H-*sn*-3b), 3.7 (dd, 1H, *J* = 11.3, 6.4 Hz, H-6′a), 3.87 (dd, 1H, *J* = 10.4, 4.0 Hz, H-*sn*-1a), 3.91 (dd, 1H, *J* = 11.3, 5.5 Hz, H-6′b), 3.99 (dd, 1H, *J* = 11.3, 6.1 Hz, H-*sn*-1b), 4.01 (dd-like, 1H, H-4′), 4.21 (dd, 1H, *J* = 11.9, 7.3 Hz, H-2′), 4.26 (d, 1H, *J* = 7.3 Hz, H-1′), 4.39 (dd, 1H, *J* = 11.9, 3.4 Hz, H-3′), 5.28–5.41 (m, 7H, $[-CH=CH-] \times 6$, H-*sn*-2).

**Alkaline Treatment of DLGG (1) and LPGG (2).** DLGG (**1**, 10 mg) was treated with 1 mL of 0.2% NaOMe in MeOH solution at room temperature for 15 min. After neutralization with acetic acid (1 mL) on ice, the reaction mixture was partitioned between *n*-hexane and water. The water layer was purified by silica gel ($CHCl_3$/MeOH/$H_2O$ = 6:4:1) to afford a galactosylglycerol (**3**): colorless oil, $[\alpha]^{26}_D$ −8.0° (*c* 0.6, $H_2O$); IR $\nu_{max}$ (KBr) 3520 (OH) $cm^{-1}$; FAB-MS (*m*NBA as a matrix) *m*/*z* 277 $[M + Na]^+$; $^1H$ NMR (pyridine-$d_5$) δ 4.08 (dd, 1H, *J* = 5.3, 6.5 Hz, H-5′), 4.12 (d, 1H, *J* = 5.5 Hz, H-*sn*-1a), 4.15 (d, 1H, *J* = 4.9 Hz, H-*sn*-1b), 4.17 (dd, 1H, *J* = 3.3, 9.2 Hz, H-3′), 4.27 (dd, 1H, *J* = 3.8, 9.7 Hz, H-*sn*-3a), 4.45 (m, 4H, H-6′ab, H-*sn*-2, H-*sn*-3b), 4.52 (dd, 1H, *J* = 7.7, 9.2 Hz, H-2′), 4.57 (d, 1H, *J* = 3.3 Hz, H-4′), 4.91 (d, 1H, *J* = 7.7 Hz, H-1′). The *n*-hexane layer was subjected to GLC analysis, which was performed under the following conditions: FFS capillary column SS-10 (50 m × 0.24 mm i.d.), column temperature 200 °C; injector and detector temperature 240 °C; $N_2$ flow rate, 50 mL/min. A single peak at $t_R$ = 14.1 min corresponded with that of methyl α-linolenate. By the same treatment of LPGG (**2**), two peaks (1:1) corresponding with those of methyl palmitate and methyl α-linolenate were detected at $t_R$ = 5.4 and 14.1 min, respectively.

**Regioselective Hydrolysis of LPGG (2).** Regioselective hydrolysis of LPGG (**2**) was carried out according to the method of N. Murakami et al. (1994).

**Preparation of *sn*-1 Lysoglyceroglycolipid.** *R. arrizus* lipase (1800 units/0.63 mL in boric acid–borax buffer, pH 7.7) and Triton X-100 (2.5 μL) were added to dried LPGG (5 mg) in a brown vial. After sonication, the vial was incubated at 38 °C for 1 h. The reaction was stopped by the addition of acetic acid (0.1 mL) and EtOH (3 mL). The solvent was evaporated, and the residue was chromatographed on Wakogel C-200 ($CHCl_3$/MeOH = 7:1) to afford **4** (3.0 mg), α-linolenic acid (0.8 mg), and palmitic acid (0.8 mg). Compound **4**: colorless oil, $[\alpha]^{26}_D$ −5.6° (*c* 0.2, MeOH); IR $\nu_{max}$ (KBr) 3460 (OH), 1730 (C=O) $cm^{-1}$; FAB-MS (*m*NBA as a matrix) *m*/*z* 535 $[M + Na]^+$ (**4a**), 513 $[M + Na]^+$ (**4b**); $^1H$ NMR ($CD_3OD$) δ 0.90 (br t, 3H, *J* = 6.6 Hz, $CH_3$), 0.97 (t, 3H, *J* = 7.6 Hz, $CH_3$), 1.25–1.40 (br, 36H, $-(CH_2)_6-$), 2.07 (m, 6H, $COCH_2-$ and $=CHCH_2-$), 2.35 (m, 2H, $=CHCH_2CH_3$), 2.81 (m, 4H, $=CHCH_2CH=$), 3.45 (dd, 2H, *J* = 9.7, 3.3 Hz, H-3′), 3.51 (m, 4H, H-2′ and 5′), 3.66–3.79 (m, 5H, H-*sn*-1ab, H-*sn*-3a, and H-6′), 3.81 (d-like, 2H, *J* = 2.7 Hz, H-4′), 3.90 (dd, 2H, *J* = 10.5, 5.2 Hz, H-*sn*-3b), 4.22 (d, 2H, *J* = 7.6 Hz, H-1′), 5.03 (m, 2H, H-*sn*-2), 5.26–5.40 (m, 6H, $-CH=CH-$).

**Preparation of *sn*-2 Lysoglyceroglycolipid.** *R. arrizus* lipase (700 units/0.63 mL in Tris buffer, pH 7.7) and Triton X-100 (2.5 μL) were added to dried LPGG (5 mg) in a brown vial. After sonication, the vial was incubated at 38 °C for 17 h. The reaction was stopped with acetic acid (0.1 mL) and EtOH (3 mL). The solvent was evaporated and chromatographed on Wakogel C-200 ($CHCl_3$/MeOH = 7:1) to afford **5** (3.0 mg), α-linolenic acid (0.8 mg), and palmitic acid (0.8 mg). Compound **5**: colorless oil, $[\alpha]^{26}_D$ −5.9° (*c* 0.2, MeOH); IR $\nu_{max}$ (KBr) 3460 (OH), 1730 (C=O) $cm^{-1}$; FAB-MS (*m*NBA as a matrix) *m*/*z* 535 $[M + Na]^+$ (**5a**), 513 $[M + Na]^+$ (**5b**); $^1H$ NMR ($CD_3OD$) δ 0.90 (br t, 3H, *J* = 6.7 Hz, $CH_3$), 0.97 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.25–1.40 (br, 36H, $-(CH_2)_6-$), 2.07 (m, 6H, $COCH_2-$ and $=CHCH_2-$), 2.35 (m, 2H, $=CHCH_2CH_3$), 2.81 (m, 4H, $=CHCH_2CH=$), 3.45 (dd, 2H, *J* = 9.7, 3.3 Hz, H-3′), 3.51 (m, 4H, H-2′ and 5′), 3.64 (dd, 2H, *J* = 10.5, 4.6 Hz, H-*sn*-3a), 3.69–3.77 (m, 4H, H-6′), 3.81 (d-like, 2H, *J* = 2.7, H-4′), 3.90 (dd, 2H, *J* = 10.5, 5.2 Hz, H-*sn*-3b), 3.98 (m, 2H, H-*sn*-2), 4.14 (m, 4H, H-*sn*-1), 4.22 (d, 2H, *J* = 7.6 Hz, H-1′), 5.28–5.40 (m, 6H, $-CH=CH-$).

**Inhibitory Assay of EBV Activation.** The EBV activation inhibitory test was done as previously reported (A. Murakami et al., 1993). Human B-lymphoblastoid cells, Raji, were incubated in 1 mL of RPMI 1640 medium (supplemented with 10% fetal bovine serum) containing sodium *n*-butyrate (3 mM), teleocidin B-4 (50 nM), and the test compound at 37 °C under 5% $CO_2$ atmosphere for 48 h. After the cytotoxicity of each test sample was measured by staining the cells with trypan blue, smears were made from the cell suspension. Then, EA-induced cells were stained by a conventional indirect immunofluorescence technique with high-titer EA-positive sera from NPC patients followed by FITC-labeled IgG. The ratio of EA-induced cells was compared to that of a control experiment only with sodium *n*-butyric acid and teleocidin B-4, in which the ratio of EA-induced cells was ordinarily around 50%.

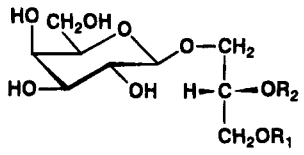
***In Vivo* Anti-tumor Promotion Test.** Anti-tumor promotion tests on mouse skin were performed as previously reported (A. Murakami et al., 1992). One group was composed of 15 female ICR mice, housed 5 mice per cage, and supplied with fresh water, and the diet was changed twice a week. The back of each mouse was shaved with surgical clippers. The mice at 7 weeks old were initiated with DMBA (0.19 μmol/0.1 mL in acetone). One week after initiation, the mice were promoted with TPA (1.6 nmol/ 0.2 mL in acetone) twice a week for 20 weeks. In the inhibitor-treated experiments, the mice were treated with DLGG (1.6, 16, or 160 nmol/0.1 mL in acetone) or α-linolenic acid (16 or 160 nmol/0.1 mL in acetone) 40 min before each TPA treatment. The anti-tumor-promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors (more than 1 mm in diameter) per mouse. Statistical analysis was done by the $\chi 2$-test on tumor-bearing mice and the Student *t*-test on the number of tumors per mouse.

**Anti-inflammatory Test on Mouse Ears.** Anti-inflammatory tests were done as previously reported (A. Murakami et al., 1991). Five mice at 7 weeks old were used in each experiment. The test compound (810 nmol/20 μL in 15% MeOH in $CHCl_3$) was applied to an inner part of an ICR mouse ear. After 20 min, a TPA solution (8.1 nmol/20 μL in 15% MeOH in $CHCl_3$) was applied to the same part of the ear. Only the TPA solution (8.1 nmol/20 μL in 15% MeOH in $CHCl_3$) was applied to the other ear of the same mouse as a positive control. After 6 h, a disk (6 mm in diameter) was obtained from both ears and weighed. The inhibitory effects (IE) were expressed by the increasing ratio of the weight of the treated disk to the control disk: IE (%) = [(TPA alone) − (test compound plus TPA)]/[(TPA) − (vehicle)] × 100. Statistical analysis was done by the Student *t*-test.

**Table 1. Inhibitory Activity of DLGG (1), LPGG (2), and Representative Anti-tumor Promoters toward EBV Activation[a]**

| compd | % inhibition (% cell viability) at concn of | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.005 μM | 0.05 μM | 0.5 μM | 5 μM | 25 μM | 50 μM | $IC_{50}$[b] μM |
| DLGG | 0 (86) | 10 (88) | 47 (86) | 93 (71) | 100 (58) | ND[c] | 0.63 |
| LPGG | 6 (88) | 25 (89) | 50 (82) | 94 (72) | 100 (55) | ND | 0.43 |
| α-linolenic acid | NT[d] | NT | NT | 0 (78) | 45 (73) | 87 (65) | 27 |
| β-carotene[e] | NT | NT | NT | 0 (88) | 44 (89) | 65 (95) | 30 |
| EGCG[f] | NT | NT | NT | 0 (87) | 12 (99) | 35 (80) | 68 |

[a] Data are the means of two experiments. [b] Fifty percent inhibition concentration. [c] Not detected due to cytotoxicity. [d] Not tested. [e] Dissolved in dioxane. [f] (−)-Epigallocatechin gallate.



1: $R_1$=$R_2$=α-linolenoyl
2a: $R_1$= α-linolenoyl; $R_2$= palmitoyl
2b: $R_1$= palmitoyl; $R_2$= α-linolenoyl
2: (2a: 2b =1: 1)
3: $R_1$=$R_2$=H
4a: $R_1$=H; $R_2$= α-linolenoyl
4b: $R_1$= H; $R_2$ = palmitoyl
4: (4a: 4b = 1: 1)
5a: $R_1$ = α-linolenoyl; $R_2$ =H
5b: $R_1$ = palmitoyl; $R_2$ = H
5: (5a: 5b = 1: 1)

**Figure 1.** Structures of DLGG (1), LPGG (2), and their derivatives.

## RESULTS AND DISCUSSION

**Isolation and Identification of Possible Anti-tumor Promoters from *C. hystrix*.** The active principles were traced by using the inhibitory assay of Epstein−Barr virus (EBV) activation induced by teleocidin B-4. Fresh leaves of *C. hystrix* were extracted with MeOH, and the extract was partitioned between ethyl acetate (EtOAc) and water. The active EtOAc layer was chromatographed on silica gel to afford active 60−80% acetone in toluene eluates. The combined fraction was further chromatographed on ODS gel ($MeOH/H_2O$ = 9:1) twice and then with MeOH/acetonitrile/$H_2O$ (16:4:5). Final purification was done by preparative TLC developing with $CHCl_3$/MeOH (9:1) to afford compounds **1** and **2**. On the basis of spectral data, compound **1** was identified as 1,2-di-*O*-α-linolenoyl-3-*O*-β-galactopyranosyl-*sn*-glycerol (DLGG, Figure 1) (Kitagawa et al., 1988; N. Murakami et al., 1990). The difference in the structures of **1** and **2** was suggested to be only the acyl residues by their $^1H$ NMR spectra. In a GLC analysis of the methanolysis products of **2**, two peaks, with the same intensities, were identified as methyl α-linolenate and methyl palmitate. This result was consistent with the data of the fast atom bombardment mass spectrum (FAB-MS) of **2** ($m/z$ 775 $[M + Na]^+$). The location of both acyl chains in **2** was determined by regioselective hydrolysis using *R. arrhizus* lipase (N. Murakami et al., 1994). Hydrolysis of **2** with the lipase at 700 units in a borate−borax buffer for 1 h gave an *sn*-1 lyso derivative (**4**). It was a mixture of **4a** and **4b** (1:1), bearing an α-linolenoyl and a palmitoyl group at the *sn*-2 position, respectively. Hydrolysis of **2** with the lipase at 1800 units in Tris buffer for 17 h gave an *sn*-2 lyso derivative (**5**). It was also a mixture of **5a** and **5b** (1:1), bearing the same acyl groups at the *sn*-1 position. These data revealed that LPGG (**2**) is a mixture of two glyceroglycolipids in a ratio of 1:1, i.e., 1-*O*-α-linolenoyl-2-*O*-palmitoyl-3-*O*-β-galactopyranosyl-*sn*-glycerol (**2a**) and its counterpart (**2b**) (N. Murakami *et al.*, 1990, 1991).

***In Vitro* Anti-tumor-Promoting Activity.** *In vitro* anti-tumor-promoting activity was examined by tumor promoter-induced Epstein−Barr virus (EBV) activation tests in Raji cells (A. Murakami et al., 1993). Teleocidin B-4 (50 nM), an indole alkaloid-type tumor promoter, was used as an EBV activator. DLGG (**1**) and LPGG (**2**) showed similar inhibitory effects on EBV activation in a dose−response manner (Table 1). Both compounds almost completely inhibited EBV activation at a concentration of 5 μM, and the 50% inhibition concentrations ($IC_{50}$) of **1** and **2** were 0.63 and 0.43 μM, respectively. These values are notably lower than those of representative, naturally occurring cancer preventive agents such as α-linolenic acid ($IC_{50}$ = 27 μM), β-carotene (30 μM), or (−)-epigallocatechin gallate (EGCG, 68 μM) (Table 1). Though there are some differences in the experimental conditions, **1** and **2** should be some of the most potent inhibitors among those glyceroglycolipids recently reported (Shirahashi et al., 1993; Nagatsu et al., 1994).

**Anti-tumor-Promoting Activity on Mouse Skin.** *In vivo* anti-tumor-promoting activity of DLGG (**1**) was evaluated by a two-stage carcinogenesis experiment on ICR mouse skin. α-Linolenic acid was used as a positive control because of its structural similarity with **1**. Test compounds were topically applied, 40 min prior to each TPA (1.6 nmol) treatment, on ICR mouse skin, which was initiated with DMBA (0.19 μmol) 1 week before promotion. The experiment was continued for 20 weeks after the start of promoting treatment. Anti-tumor-promoting activity was evaluated by the percentage of tumor-bearing mice and the numbers of tumors per mouse. As shown in Figure 2, pretreatment with **1**, at a dose of 160 nmol, markedly reduced tumor incidence by 39% ($P < 0.005$) and the number of tumors per mouse by 67% ($P < 0.001$) at 20 weeks. The anti-tumor-promoting potency of DLGG (**1**) was strikingly higher than that of α-linolenic acid because **1** significantly reduced the number of tumors by 50% ($P < 0.01$) at a dose of 16 nmol and α-linolenic acid was inactive at the same dose.

**Inhibitory Effects of Some Detergents on EBV Activation.** DLGG (**1**) possesses both hydrophilic sugar and hydrophobic acyl moieties and accordingly must show a detergent effect. It is important to clarify whether nonspecific interaction of **1** with cell membrane by the detergency is involved in the inhibitory action of **1** *in vivo*. Next, the inhibitory effects of some detergents on EBV activation were examined. As shown in Table 2, three synthetic detergents and two saponins were shown to be clearly inactive, up to a concentration of 20 μg/mL. Therefore, the detergent function of **1** was suggested not to play a critical role in anti-tumor-promoting action.

**Anti-inflammatory Activity on Mouse Ears.** Subsequently, anti-inflammatory effects of DLGG (**1**) and LPGG (**2**) were examined because tumor promotion is closely associated with inflammation and because anti-inflammatory activity of a glyceroglycolipid in a chorioallantoic membrane chick embryo test had been reported (Kikuchi et al., 1983). Anti-inflammatory activity

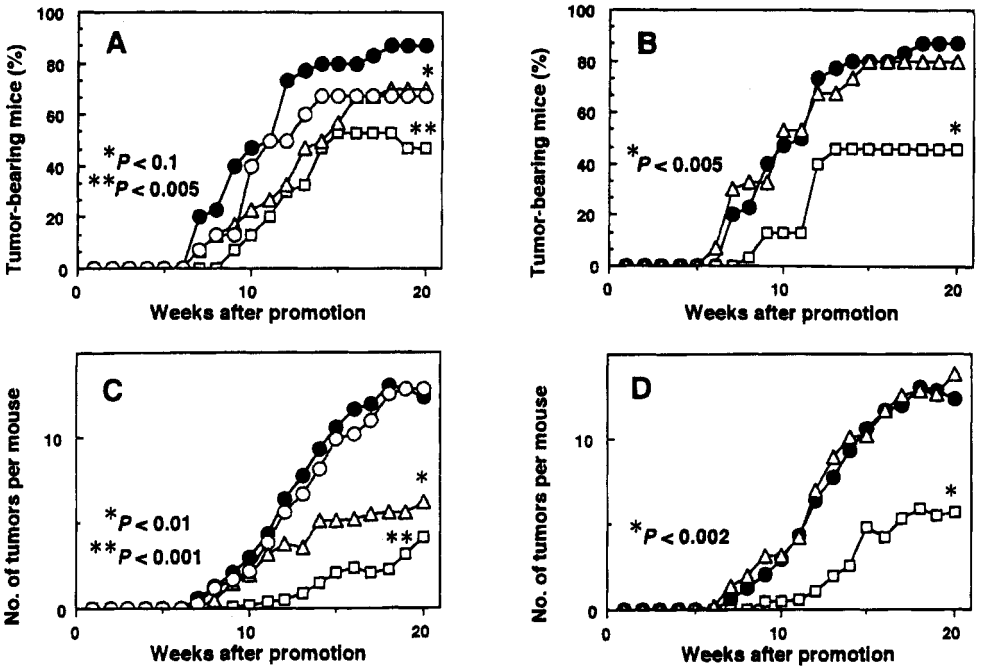



**Figure 2.** Anti-tumor-promoting activity of DLGG (**1**) and α-linolenic acid on mouse skin. One group was composed of 15 female ICR mice. The mice at 7 weeks old were initiated with DMBA (0.19 μmol). One week after initiation, the mice were promoted with TPA (1.6 nmol, ●) twice a week for 20 weeks. In the inhibitor-treated experiments, the mice were treated with DLGG [A and C: 1.6 (○), 16 (△), or 160 (□) nmol] or α-linolenic acid [B and D: 16 (△) or 160 nmol (□)] before each TPA treatment. The anti-tumor-promoting activities were evaluated by both the ratio of tumor-bearing mice (A and B) and the number of tumors per mouse (C and D). Statistical analysis was done by $\chi^2$-test on tumor-bearing mice and the Student $t$-test on the number of tumors per mouse.

**Table 2. Inhibitory Effect of Synthetic Detergents and Saponins on EBV Activation[a]**

| | % inhibition (% cell viability) at concn of | |
|---|---|---|
| detergent | 20 μg/mL | 4 μg/mL |
| Triton X-100[b] | 0 (90) | 0 (95) |
| Excel 200[b] | 0 (96) | 0 (88) |
| sorbitan monostearate | 0 (69) | 0 (80) |
| saponin (soybean)[b] | 0 (94) | 0 (92) |
| saponin (green tea)[b] | 0 (51) | 0 (84) |
| DLGG | ND[c] | 93 (72) |
| LPGG | ND[c] | 93 (72) |

[a] Data are the means of two experiments. [b] Mixtures with different molecular weights. [c] Not detected due to cytotoxicity.

**Table 3. Anti-inflammatory Activities of DLGG (1) and LPGG (2) on ICR Mouse Ears**

| compd | $m \pm$ SD,[a] mg | inhibition, % |
|---|---|---|
| DLGG | $3.3 \pm 0.7^b$ | 32 |
| LPGG | $4.1 \pm 0.6^c$ | 43 |
| indomethacin | $1.9 \pm 0.9^d$ | 19 |

[a] Mean differences in tissue weight between ear disks treated with TPA (8.1 nmol) and those treated with TPA (8.1 nmol) plus the test compounds. SD, Standard deviation. Data are the means of five experiments. The weights of mouse ear disks without any anti-inflammatory treatment and those only with TPA treatment were 8.3 ± 1.3 and 18.8 ± 0.7 mg, respectively. [b] $P < 0.001$ (vs control), $P < 0.05$ (vs indomethacin). [c] $P < 0.01$ (vs control), $P < 0.01$ (vs indomethacin). [d] $P < 0.05$ (vs control).

was measured by TPA-induced edema formation on mouse ears (Gschwendt et al., 1984). As shown in Table 3, both **1** and **2** exhibited statistically higher anti-inflammatory activity than did indomethacin, a well-known cyclooxygenase inhibitor. Tumor promoter-induced inflammation is triggered with the release of arachidonic acid by phospholipase $A_2$. Successively formed chemical mediators such as prostaglandins and leukotrienes are known to play critical roles in the inflammation process (Fischer et al., 1989). The inhibition of enzymes regulating such pathways may be responsible for anti-inflammatory effects of glyceroglycolipids.

**Conclusions.** This is the first report on anti-tumor-promoting activity of glyceroglycolipid in an animal model. The potential of DLGG (**1**) for cancer chemoprevention was evaluated to be conspicuous. As glyceroglycolipids are known to widely occur in chroloplast membranes in plant cells, its clinical application for cancer chemoprevention may be satisfactory. However, it is needless to say that assessment of toxicity as well as further mechanistic studies are indispensable for such application. Moreover, as **1** possesses a glycosidic linkage and two ester groups, the chemical stability of **1** in the human digestive system has not been confirmed yet. Cancer preventive effect by oral administration of **1** should be examined in the next study.

ACKNOWLEDGMENT

We thank Ms. Suratwadee Jiwajinda of Kasetsart University for sample collection.

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Received for review February 13, 1995. Accepted July 25, 1995.[⊗] This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan and the Japan Society for the Promotion of Science (A.M.) and by subsidies from Takeda Food Products, Ltd., and Yamazaki Spices Foundation.

JF950093H

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1995.