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Original article

# Anti-tumor-promoting activity of simple models of galactoglycerolipids with branched and unsaturated acyl chains

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**Abstract**

Six new galactoglycerolipid analogs, in which one or two 4-methylpentanoyl or *trans*-2-butenoyl groups are linked to the 2-*O*-β-D-galactosylglycerol skeleton, were tested for their anti-tumor-promoting activity using a short-term in vitro assay for Epstein–Barr virus early antigen (EBV-EA) activation. All these compounds were more active than their linear or saturated reference compounds in inhibiting the EBV activation promoted by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the diester 1-*O*-(4-methylpentanoyl)-2-*O*-[6-*O*-(4-methylpentanoyl)-β-D-galactopyranosyl]-*sn*-glycerol resulting the most active glycoglycerolipid analog till now tested. Four compounds (three butenoates and one 4-methylpentanoate), when tested in the in vivo two-stage carcinogenesis test, exhibited also inhibitory effects on mouse skin tumor promotion.


*Keywords:* Glycoglycerolipids; Cancer chemoprevention; Lipase transesterification; Epstein–Barr virus; Two-stage skin carcinogenesis test

## 1. Introduction

Chemoprevention is dedicated to identifying agents with potential preventive roles in cancer [1]. At present, the action mechanism of chemopreventive compounds is not known, however, it has been suggested that some of them could inhibit the tumor promotion stage by interacting with the protein kinase C [2], a family of serine/threonine kinases named PKC that play an essential role in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation and apoptosis [3].

PKC is activated by the potent tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that binds to it with very high affinity [4]. Recently, glycoglycerolipid analogs such as 2-*O*-glycosylglycerols linking short to medium length lipophilic chains, have shown anti-tumor-promoting activity in the TPA-promoted in vitro Epstein–Barr virus early antigen (EBV-EA) activation test [5,6] and also in the in vivo two-stage mouse skin carcinogenesis test [7]. The activity of such compounds (**1a**,**b**–**3a**,**b**) seems strictly related to the acyl chain length, six carbon atoms resulting in maximum effect. The shape of the lipophilic chain is also reported to influence the anti-tumor activity of some PKC ligands the structure of which is related to diacylglycerols [4]. In particular their activity was related to the presence of a 4-methyl-3-(methylethyl)pentanoyl chain, used to improve the hydrophobic contacts of these compounds with PKC. Moreover similar lipophilic residues such as *trans*-3,4-dimethylpent-2-enoyl or *cis*-2-butenoyl chains, seems to be a common motif that decorate the structure of some natural compounds exhibiting a potent inhibitory effect on EBV-EA activation induced by TPA [8,9].

On the basis of these evidences, which indicate the nature of the lipophilic chain as a crucial structural feature for the activity of many compounds, we decided to prepare simple branched and unsaturated glycoglycerolipid analogs in order to study the effect on the anti-tumor-promoting activity caused

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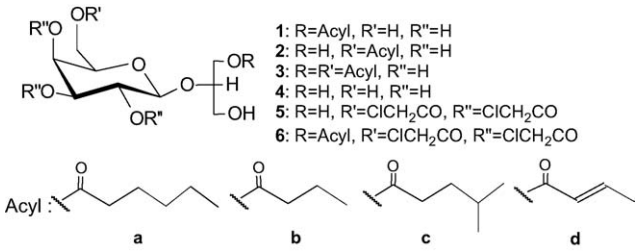
by introducing small modifications to the acyl chains of glycoglycerolipid analogs **1a**,**b**–**3a**,**b** and, eventually, to obtain more active compounds. So here we describe the anti-tumor-promoting activity of compounds **1c**,**d**–**3c**,**d** based on the short-term in vitro assay for the inhibition of EBV-EA activation induced by TPA. The inhibitory effect of compounds **1c** and **1d**–**3d** on mouse skin tumor promotion in an in vivo two-stage mouse skin carcinogenesis test will be also discussed.

## 2. Chemistry

Compounds **1c**–**3c** and **1d**–**3d** were prepared according to a well established chemo-enzymatic approach starting from 2-*O*-β-D-galactosylglycerol (**4**) [10]. In particular, *Pseudomonas cepacia* lipase (lipase PS) catalyzed transesterification of **4** with the proper vinyl ester as the acyl donor afforded compounds **2c**, **3c**, **1d** and **3d**, whereas compound **2d** was obtained using *Bacillus subtilis* protease (Proleather FG-F) as the catalyst. Compound **1c** was obtained through lipase PS catalyzed transesterification of the derivative **5** followed by removal of the chloroacetyl groups of **6c** with hydrazine acetate as reported in Ref. [11]. The assignment of the configuration of compounds **1c**–**d** and **3c**–**d** was determined as reported in Ref. [10], and the glycerol part in their structures is shown by Fischer projection formula [12] (Scheme 1).




Scheme 1.

## 3. Pharmacology

Epstein–Barr virus (EBV) is known to be activated by tumor promoters to produce viral early antigens (EA), and an evaluation of its inhibition is often used as a primary screening for in vitro anti-tumor-promoting activities [13]. The inhibitory effect of compounds **1c**–**3c** and **1d**–**3d** was assayed using a short-term in vitro assay for EBV-EA activation in Raji cells induced by the tumor promoter TPA, as described in Refs. [14,15].

Mouse skin tumor promotion inhibition of compounds **1c** and **1d**–**3d** was also evaluated in an in vivo two-stage mouse skin carcinogenesis test as reported in the experimental protocols.

## 4. Results and discussion

The three branched analogs **1c**–**3c** of hexanoates **1a**–**3a** and the three unsaturated analogs **1d**–**3d** of butyrates **1b**–**3b**, were prepared and tested for their anti-tumor-promoting activity using a short-term in vitro assay for EBV activation in Raji cells induced by TPA. Table 1 shows the in vitro tumor inhibitory activity of compounds **1c**,**d**–**3c**,**d** together with that of the already studied [5,6] esters **1a**,**b**–**3a**,**b**. Only weak cytotoxicity against Raji cells was observed for all compounds (70% viability at 1000 mol ratio/TPA and >80% at all the other mol ratio/TPA, Table 1) that showed potent inhibitory activity, as indicated by their percentage to control (7.9–17.5% at 500 mol ratio/TPA, Table 1).

In particular, comparing the new data with those previously published [5,6], both the branched 4-methylpentanoyl derivatives (**1c**–**3c**) and the unsaturated crotonyl derivatives (**1d**–**3d**) resulted more active than the corresponding linear hexanoates (**1a**–**3a**) and the saturated butanoates (**1b**–**3b**) respectively. In fact, going from linear to branched compounds, the averaged inhibitory activities increased of 22%, 14% and 36% at 500 mol ratio/TPA (see Table 1, **1c**–**3c** *vs*

Table 1
Inhibitory effects of **1a**–**d**, **2a**–**d** and **3a**–**d** on TPA-induced EBV-EA activation

| | Concentration (mol ratio/TPA) | | | | $IC_{50}$ |
|---|---|---|---|---|---|
| | % to control ± SE ($n$ = 3)[a] | | | | |
| | 1000 | 500 | 100 | 10 | |
| **1a** [5] | 0 ± 0 | 11.4 ± 0.3 | 32.1 ± 0.9 | 63.4 ± 1.3 | 25.0 |
| **2a** [5] | 0 ± 0 | 10.7 ± 0.1 | 30.1 ± 0.9 | 67.8 ± 1.2 | 28.3 |
| **3a** [6] | 0 ± 0 | 12.4 ± 0.2 | 32.3 ± 1.1 | 69.5 ± 1.8 | 32.2 |
| **1c** | 0 ± 0.4 | 8.9 ± 0.7 | 25.2 ± 1.2 | 65.0 ± 2.3 | 22.7 |
| **2c** | 0 ± 0.2 | 9.2 ± 0.9 | 28.9 ± 1.5 | 67.0 ± 2.1 | 26.5 |
| **3c** | 0 ± 0.2 | 7.9 ± 0.6 | 23.5 ± 1.1 | 62.0 ± 2.0 | 19.4 |
| **1b** [5] | 0 ± 0 | 22.4 ± 0.4 | 45.1 ± 1.1 | 80.2 ± 1.0 | 70.1 |
| **2b** [5] | 0 ± 0 | 20.4 ± 0.8 | 43.6 ± 1.0 | 78.2 ± 1.1 | 62.1 |
| **3b** [6] | 0 ± 0 | 21.1 ± 0.4 | 47.6 ± 1.2 | 80.5 ± 1.3 | 75.2 |
| **1d** | 0 ± 0.6 | 15.6 ± 1.1 | 34.5 ± 2.1 | 73.3 ± 1.3 | 39.8 |
| **2d** | 0 ± 0.5 | 17.5 ± 1.8 | 36.6 ± 2.0 | 75.7 ± 1.6 | 46.1 |
| **3d** | 0 ± 0.3 | 13.8 ± 1.4 | 31.5 ± 2.0 | 71.5 ± 1.5 | 34.2 |

[a] Values are EBV-EA activation (%) in the presence of the test compound relative to the control (100%). Activation was attained by treatment with TPA 32 pmol. $IC_{50}$ represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA.

**1a**-**3a**) and of 30%, 14% and 35% going from saturated to unsaturated compounds (Table 1, **1d–3d** *vs* **1b–3b** at 500 mol ratio/TPA). The comparison of the $IC_{50}$ values showed more enhanced activities for unsaturated vs. saturated compounds than for branched *vs* linear chain compounds (see Table 1). In the case of linear saturated acyl chains it has been shown that the introduction of a second acyl group does not significantly modify the activity of the monoesters [6]. On the contrary, the branched and unsaturated diesters **3c** and **3d** here studied represent the most active terms in the respective series (Table 1), the compound **3c** becoming the most active glycoglycerolipid analog till now prepared.

Compounds **1c** and **1d** were also submitted to an in vivo two-stage carcinogenesis test of mouse skin papillomas using dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter, in order to evaluate their inhibitory effect and compare it with that of **1a** that carries the linear lipophilic chain on the same glycosylglycerol position of **1c** and **1d**, and that is the most active glycoglycerolipid analog till now assayed in the in vivo test [7]. Also the butenoate **2d**, positional isomer of **1d**, and the diester **3d** were tested to evaluate, respectively, the influence of the position and of the number of the acyl chains on the in vivo activity. After 20 weeks of promotion, there was no statistically significant difference in body weights between control and any treated group. The activities, estimated by both the incidence (percentage of mice bearing papillomas) (Fig. 1A) and the multiplicity (average numbers of papillomas per mouse) (Fig. 1B), were compared with those of the control group. In the control group 100% of the mice bore papillomas at 10 weeks of promotion, and 5.4 and 9.3 papillomas per mouse were formed, respectively, after 10 and 20 weeks of promotion (Fig. 1). All the tested compounds were able to inhibit the tumor promotion in this in vivo assay showing a similar effect on reducing the percentage of mice bore papillomas. In fact, in all the groups about 27–33% of mice bore papillomas at 10 weeks of promotion (Fig. 1), and 87–93% of mice bearing papillomas resulted at 20 weeks of promotion (Fig. 1). In particular the differences in the incidence of papillomas in the treated cases with respect to control, analyzed by the $\chi^2$-test, appear significant after 10 weeks ($P < 0.005$) but not after 20 weeks. The decrease of the number of papillomas per mouse (papilloma multiplicity) was statistically significant ($P < 0.001$, Student's *t*-test for **1c** and **1d–3d**) in all the treated cases with respect to the control. The differences among the groups treated with the unsaturated esters **1d–3d** were not significant. However, a significantly more pronounced inhibitory effect was observed in the lowered number of papillomas per mouse produced by **1c** with respect to the unsaturated esters **1d** ($P < 0.02$) and **2d** ($P < 0.05$). In fact 1.6, 1.9 and 1.9 papillomas (30%, 35% and 35% with respect to control) were formed by treatment, respectively, with **1c**, **1d** and **2d** at 10 weeks of promotion, and 5.1, 6.1 and 5.9 (55%, 65% and 63% with respect to control) at 20 weeks of promotion (Fig. 1). The comparison of compounds **1a** and **1c** shows that despite of a quite similar in vitro activity (Table 1), compound **1c** (the branched hexanoate) is significantly ($P < 0.05$, papilloma incidence, and $P < 0.001$, multiplicity) less active than **1a** (the linear hexanoate) in the in vivo test (Fig. 1), in which the results might be also influenced by differences in the lipophilicity of the tested compounds. Glycoglycerolipid analogs could serve as non-specific physical barriers for TPA to get its target in the cells, so exerting their protective action simply by delaying the onset of papillomas induced by TPA on mouse skin. However, the hypothesis that non specific interaction of glycoglycerolipids with membranes due to detergency is involved in the anti-tumor-promoting action was ruled out by Murakami et al. [16]; moreover, the fact that **1a**, **1c** and **1d–3d** show significantly different activities (Fig. 1) despite very similar structures, could account for a possible specific interaction of glycoglycerolipid analogs with some biological target. Nevertheless, work is still required to clarify the action mechanism of tumor promotion inhibition by gly-

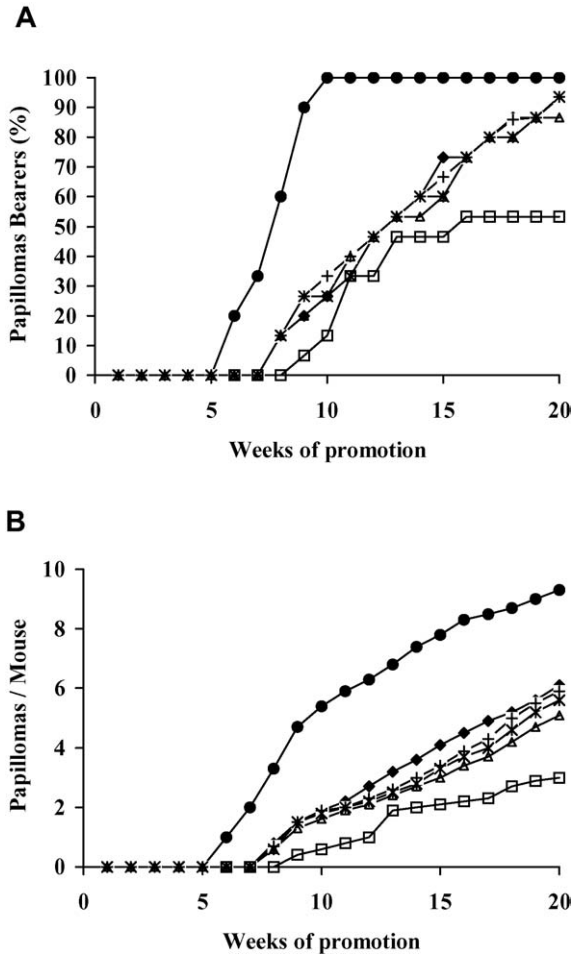



Fig. 1. Inhibitory effects of compounds **1c** and **1d–3d** (85 nmol) on DMBA-TPA mouse skin carcinogenesis and of **1a** (Ref. [7]). All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice a week starting 1 week after initiation. **A**: percentage of mice with papillomas; **B**: averaged number of papillomas per mouse (●, TPA alone; Δ, TPA + **1c**; ♦, TPA + **1d**; +, TPA + **2d**; *, TPA + **3d**; □, TPA + **1a**). At 20 weeks of promotion the averaged number of papillomas per mouse was reduced, with respect to the control group, by 45% ($P < 0.001$), 34% ($P < 0.001$), 37% ($P < 0.001$), 40% ($P < 0.001$) and 71% ($P < 0.001$) [7], respectively, for **1c**, **1d**, **2d**, **3d** and **1a**.

coglycerolipid analogs and, eventually, to identify their biological target (e.g. PKC).

In conclusion, six new potent anti-tumor-promoting compounds have been prepared, which structures are referred to simple branched and unsaturated galactoglycerolipid analogs, the branched diester **3c** resulting the most in vitro active glycoglycerolipid analog till now studied. The little changes made to the structures, with respect to the linear and saturated chains present in the previously studied galactoglycerolipid analogs, have exerted a positive effect on the anti-tumor-promoting activity of these compounds, suggesting that both unsaturated and branched acyl chains could be used to obtain new more potent cancer chemopreventing agents as anti-tumor-promoters. Besides the direct effects of their structural differences, the small variations in their chain lipophilicity might contribute to the modulation of their activity.

## 5. Experimental protocols

### 5.1. Chemistry

#### 5.1.1. Materials

*P. cepacia* lipase (lipase PS, specific activity 30.5 triacetin units/mg solid), a generous gift from Amano Pharmaceutical Co. (Mitsubishi Italia), was supported on celite [17] and kept overnight, under vacuum, prior to use. *B. subtilis* protease (Proleather FG-F, specific activity 10 units/mg solid) was purchased from Amano. Pyridine was distilled from calcium hydride. The acyl carriers were purchased from Aldrich (vinyl crotonate) or synthesized from 4-methylvaleric acid (Aldrich) and vinyl versatate (Aldrich) [18]. 2-*O*-β-D-(galactopyranosyl)glycerol (**4**) was synthesized according to literature procedures [19]. Evaporation under reduced pressure was always effected with a bath temperature below 40 °C. All the new compounds were characterized by $^1$H-NMR analysis at 500 MHz and chemical ionization mass spectrometry (CI-MS) [11]. The elemental analyses were consistent with the theoretical ones. Optical rotations were determined on a Perkin–Elmer 241 polarimeter in methanol solutions ($c$ = 1.0) in a 1 dm cell at 20 °C unless otherwise stated. Melting points (m.p.) were recorded on a Büchi 510 capillary m.p. apparatus and were uncorrected. Analyses of the new compounds, indicated by the symbols of the elements, were within ±0.4% of the theoretical values.

#### 5.1.2. General procedure for the enzymatic synthesis of compounds *2c, 3c, 1d–3d*

2-*O*-β-D-(galactopyranosyl)glycerol (**4**) (0.40 g, 1.57 mmol) was dissolved in pyridine (5 ml) and the appropriate vinyl ester (6 mmol) and lipase PS or Proleather FG-F (2.50 g) were added in the order. The mixture was stirred for 4 or 18 h (using vinyl crotonate) at 45 °C and the reaction was stopped by filtering off the enzyme and washing with pyridine and methanol. The solvent was removed under vacuum and the esters (pure **2c**, **3c**, **2d** and **3d** or diastereomerically enriched **1d**) could be obtained after repeated flash chromatographies (dichloromethane/methanol from 95:5 to 75:25, v/v).

*5.1.2.1. 2-*O*-[6-O-(4-methylpentanoyl)-β-D-galactopyranosyl]-*sn*-glycerol **(2c)**.* Yield 9%; oil; $[\alpha]_D^{20}$: +4.4. $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.75 (dd, 1H, $J_{6'a,5'}$ = 4.9 Hz, $J_{6'a,6'b}$ = 11.2 Hz, H-6′a); 4.89 (d, 1H, $J_{6'b,5'}$ = 7.7 Hz, H-6′b); 5.07 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); CI-MS $m/z$ 370 $[M + NH_4]^+$. Anal. $C_{15}H_{28}O_9$ (C, H, O).

*5.1.2.2. 1-*O*-(4-methylpentanoyl)-2-*O*-[6-O-(4-methylpentanoyl)-β-D-galactopyranosyl]-*sn*-glycerol **(3c)**.* Yield 40%; oil; $[\alpha]_D^{20}$ = –1.8. $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.63–4.70 (m, 2H, H-1a and H-1b); 4.74 (dd, 1H, $J_{6'a,5'}$ = 4.9 Hz, $J_{6'a,6'b}$ = 11.2 Hz, H-6′a); 4.88 (dd, 1H, $J_{6'b,5'}$ = 7.7 Hz, H-6′b); 4.98 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); CI-MS $m/z$ 468 $[M + NH_4]^+$. Anal. $C_{21}H_{38}O_{10}$ (C, H, O).

*5.1.2.3. 1-*O*-(trans-2-butenoyl)-2-*O*-(β-D-galactopyranosyl)-*sn*-glycerol **(1d)**.* Yield 10%; foam; 91% diastereomeric purity (by $^1$H-NMR); $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.65 (dd, 1H, $J_{1a,2}$ = 5.6 Hz, $J_{1a,1b}$ = 12.0 Hz, H-1a); 4.71 (dd, 1H, $J_{1b,2}$ = 4.9 Hz, H-1b); 5.01 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); CI-MS $m/z$ 340 $[M + NH_4]^+$. Anal. $C_{13}H_{22}O_9$ (C, H, O).

*5.1.2.4. 2-*O*-[6-O-(trans-2-butenoyl)-β-D-galactopyranosyl]-*sn*-glycerol **(2d)**.* Yield 5%; m.p. 120–121 °C (from $CH_2Cl_2$–$iPr_2O$); $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.83 (dd, 1H, $J_{6'b,5'}$ = 4.9 Hz, $J_{6'b,6'a}$ = 11.2 Hz, H-6′b); 4.87 (d, 1H, $J_{6'a,5'}$ = 7.7 Hz, H-6′a); 5.06 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); MS $m/z$ 340 $[M + NH_4]^+$. Anal. $C_{13}H_{22}O_9$ (C, H, O).

*5.1.2.5. 1-*O*-(trans-2-butenoyl)-2-*O*-[6-O-(trans-2-butenoyl)-β-D-galactopyranosyl]-*sn*-glycerol **(3d)**.* Yield 15%; foam; $[\alpha]_D^{20}$ = –1.1; $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.65 (dd, 1H, $J_{1a,2}$ = 5.6 Hz, $J_{1a,1b}$ = 12.0 Hz, H-1a); 4.73 (dd, 1H, $J_{1b,2}$ = 4.9 Hz, H-1b); 4.82 (dd, 1H, $J_{6'a,5'}$ = 5.6 Hz, $J_{6'b,6'a}$ = 11.2 Hz, H-6′a); 4.88 (dd, 1H, $J_{6'b,5'}$ = 7.0, H-6′b); 4.98 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); MS $m/z$ 408 $[M + NH_4]^+$. Anal. $C_{17}H_{26}O_{10}$ (C, H, O).

#### 5.1.3. Synthesis of compound *1c*

*5.1.3.1. 2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl-β-D-galactopyranosyl)glycerol **(5)**.* 1.2g (2.76 mmol) of 1,3-di-*O*-benzyl-2-*O*-(β-D-galactopyranosyl)glycerol [20] were dissolved in 36 ml of a dichlorometane/pyrydine 9:1 mixture and treated with chloroacetic anhydride (3.14 g, 18.4 mmol) at 0 °C for 15 min. Usual work-up and flash chromatography (petroleum ether/ethyl acetate 70:30, v/v) yielded the tetrachloroacetylated derivative (1,43 g, 1.93 mmol), oil, $[\alpha]_D^{20}$ = –1.24 ($c$ 1.0, $CHCl_3$). $^1$H-NMR selected signals ($CDCl_3$): $\delta$ 3.83, 3.95, 3.99 and 4.14 (4s, 8H, $4CH_2Cl$); 4.02 (m, 1H, H-2); 4.46–4.53 (m, 4H, $2CH_2Ph$); 4.73 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′). Anal. $C_{31}H_{34}Cl_4O_{12}$ (C, H, O). Following catalytic hydro-

genolysis (10% Pd/C in methanol) yielded 0.93 g (1.7 mmol) of 2-*O*-(2,3,4,6-*tetra*-*O*-chloroacetyl-β-D-galactopyranosyl) glycerol (**5**), amorphous solid, $[\alpha]_D^{20}$ = +10.8 (*c* 1.0, $CHCl_3$); m.p. 141–142 °C. $^1$H-NMR selected signals ($CDCl_3$): $\delta$ 3.79 (m, 1H, H-2); 3.97 and 4.17 (2s, 4H, $2CH_2Cl$); 4.01–4.13 (m, 5H, $2CH_2Cl$ and H-5′); 4.73 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′). Anal. $C_{17}H_{22}Cl_4O_{12}$ (C, H, O).

*5.1.3.2. 1*-O-*(4-methylpentanoyl)-2*-O-*(2,3,4,6-tetra*-O-*chloroacetyl-β-D-galactopyranosyl)*-sn-*glycerol* ***(6c)***. Compound **5** (0.93 g, 1.7 mmol) was dissolved in dry dichloromethane (20 ml) and vinyl 4-methylvalerate (15 mmol) and lipase PS (4.8 g) were added in the order. After stirring for 40 h at 45 °C the lipase was filtered off and the solvent removed under vacuum. Chromatographic purification (petroleum ether/ethyl acetate 1:1, v/v) yielded 0.76 g (1.15 mmol) of compound **6c**, 93% diastereomeric purity (by $^1$H-NMR), oil, $^1$H-NMR selected signals ($CDCl_3$): $\delta$ 0.89 (d, 6H, *J* = 6.3 Hz, $2CH_3$); 1.46–1.60 (m, 3H, $CH_2$ and CH); 2.30 (m, 2H, $CH_2CO$); 3.88 (m, 1H, H-2); 3.97 (s, 2H, $CH_2Cl$); 4.02–4.18 (m, 9H, $3CH_2Cl$, H1a, H1b and H-5′); 4.67 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′). Anal. $C_{23}H_{32}Cl_4O_{13}$ (C, H, O).

*5.1.3.3. 1*-O-*(4-methylpentanoyl)-2*-O-*(β-D-galactopyranosyl)*-sn-*glycerol* ***(1c)***. To a solution of **6c** (0.76 g, 1.15 mmol) in a 1:1 ethyl acetate/methanol mixture (22 ml) hydrazine acetate (1.6 g, 17.4 mmol) was added. After 4 h at room temperature the solvent was removed and the crude reaction mixture was submitted to repeated flash chromatografies (dichoromethane/methanol, 80:20, v/v) yielding 0.197 g (0.56 mmol) of compound **1c**, 93% diastereomeric purity (by $^1$H-NMR), oil, $^1$H-NMR selected signals (pyridine-$d_5$): $\delta$ 4.60–4.70 (m, 2H, H-1a and H-1b); 4.99 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); CI-MS *m/z* 370 $[M + NH_4]^+$. Anal. $C_{15}H_{28}O_9$ (C, H, O).

### *5.1.4. Configuration assignment at C-2 for **1c–d** and **3c–3d***

The 1-*O*-acyl-*sn*-glycerols obtained by treatment of **1c** and **1d** with β-galactosidase from *Aspergillus oryzae*, were reacted with acetone, 2,2-dimethoxypropane and *p*-toluenesulfonic acid. The obtained 1-*O*-acyl-2,3-*O*-isopropylidene-*sn*-glycerols were analyzed by chiral GLC and compared with authentic analytical standards [10]. The configuration assignments for the 1,6′-diesters **3c** and **3d** were obtained by selective LPS catalyzed transesterification of the 1-monoesters **1c** and **1d** with the proper vinyl ester [10].

### *5.2. Pharmacology*

#### *5.2.1. Short-term in vitro bioassay for anti-tumor promoters*

Inhibition was tested using a short-term in vitro assay for EBV activation in Raji cells cultivated in RPMI 1640 medium containing 10% fetal calf serum, and induced by TPA as described previously [14,15]. The assays were performed in triplicate for each compound. The average EBV-EA inhibitory activity of the test compounds was compared to that of control experiments (100%) with butyric acid (4 mM) and TPA (32 pM) in which EBV-EA induction was ordinarily around 30%. The viability of the cells was assayed against treated cells using the Trypan Blue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60% 2 days after treatment with the compounds for an accurate result.

#### *5.2.2. In vivo two-stage mouse skin carcinogenesis test*

Female ICR mice were obtained at 5–6 weeks of age from SLC Co. Ltd. (Shizuoka, Japan). Groups of animals (15 animals per group) were housed in bunches of five in polycarbonate cages. Mice were permitted free access to MP solid diet (Oriental Yeast Co. Ltd., Chiba, Japan) and drinking water at all times during the study. The back of each mouse was shaved with surgical clippers before the first day of initiation. Tumors on the back of the mice were initiated with DMBA (390 nmol) in acetone (0.1 ml). One week after initiation, they were promoted twice a week by application of TPA (1.7 nmol) in acetone (0.1 ml). For the animals in the test compound treated groups the mice were treated with the test compounds (85 nmol) in acetone (0.1 ml) 1 h before each TPA treatment. The incidence of papillomas was observed weekly for 20 weeks. The differences in mouse skin papillomas between control and experiments were analyzed by means of the Student's *t*-test at 20 weeks of promotion, whereas the differences in tumor bearing mice were analyzed by means of the $\chi^2$-test.

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