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Original article

Cyclic and branched acyl chain galactoglycerolipids and their effect on anti-tumor-promoting activity

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Abstract

Fifteen new galactoglycerolipid analogues, in which one or two branched, alicyclic or aromatic acyl chains are linked to 2-*O*-β-D-galactosylglycerol (6′-position or 1,6′ positions), were prepared and tested for their anti-tumor-promoting activity using a short-term in vitro assay for Epstein–Barr virus early antigen (EBV-EA) activation. All compounds were active in inhibiting the EBV activation promoted by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the branched compounds resulting in the most active glycoglycerolipid analogues of the series. The branched 2-*O*-[6-*O*-(3-methylbutanoyl)-β-D-galactopyranosyl]-*sn*-glycerol (**1a**) and the structurally related alicyclic 2-*O*-[6-*O*-(2-cyclohexylethanoyl)-β-D-galactopyranosyl]-*sn*-glycerol (**1d**), when tested in an in vivo two-stage carcinogenesis test, exhibited inhibitory effects on mouse skin tumor promotion.

Keywords: Glycoglycerolipids; Cancer chemoprevention; Epstein–Barr virus

1. Introduction

Cancer chemoprevention consists of administration of synthetic or natural compounds to halt or inhibit the onset of cancer and is based on the conventional multistage carcinogenesis model. According to this model, tumor promotion is a long and reversible stage that can be efficiently suppressed [1]. Besides their structural and functional roles in the membranes of animals, plants and bacteria [2], glycoglycerolipids are known to inhibit the tumor-promoting activity of the potent tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) both in vitro and in vivo, their potential for cancer chemoprevention being considered conspicuous [3], and also to show in vitro anti-solid tumor activity [4]. During our search for new glycoglycerolipids active in cancer chemoprevention we have found that apparently minor structural changes of the lipophilic chain exert a positive effect on the anti-tumor-promoting activity of monoacyl- or diacylderivatives of 2-*O*-galactosylglycerol [5]. Therefore we have decided to synthesize a series of new analogues carrying different, but structurally related, acyl chains, focusing our attention on branched, alicyclic and aromatic structures. Here we describe the synthesis of galactoglycerolipid analogues **1a–j** and **2a**, **2c–f** and their anti-tumor-promoting activity based on the short-term in vitro assay for the inhibition of Epstein–Barr virus early antigen (EBV-EA) activation induced by TPA. The inhibitory effect of compounds **1a** and **1d** on mouse skin tumor promotion in an in vivo two-stage mouse skin carcinogenesis test will also be discussed.

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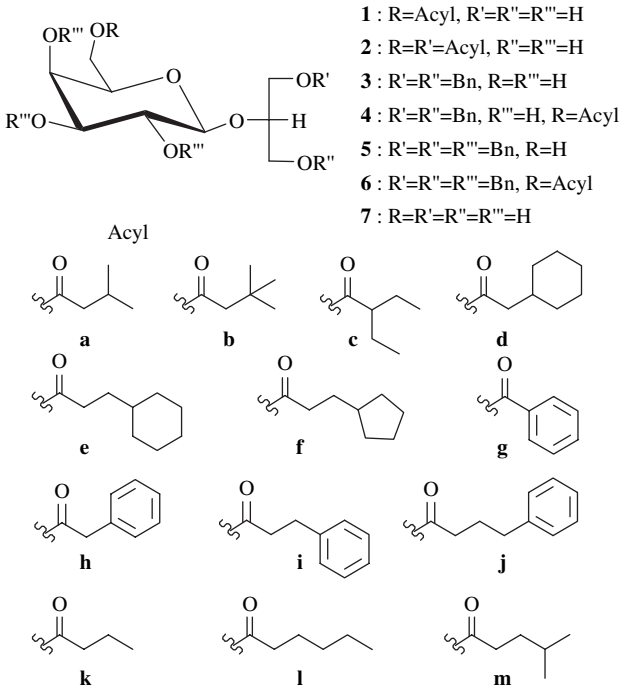
2. Results and discussion

2.1. Chemistry

Some of the desired compounds were prepared according to a well established chemo-enzymatic approach starting from 1,3-di-*O*-benzyl-2-*O*-β-D-(galactopyranosyl)-glycerol (**3**) or 2-*O*-β-D-galactopyranosylglycerol (**7**) [6]. In particular, *Pseudomonas cepacia* lipase (lipase PS) catalyzed transesterification of **3** yielded 45–90% of compounds **4a**, **4c–f** after 20–180 h depending on the acyl donor (see Section 3). Catalytic hydrogenation afforded the 6′-monoesters **1a**, **1c–f**. The same enzymatic procedure on **7** yielded, with lower yields (6–27%) and longer reactions times (20–330 h), the 1,6′-diesters **2a**, **2c–f** (for the assignment of the C-2 configuration see Section 3). Enzymatic reactions were very slow with the cyclic acyl donors and did not work in the case of the bulky 3,3-dimethyl-butanoyl acyl chain **b**, even if conducted with a different enzyme (*Candida antarctica* lipase) and solvents both on substrates **3** and **7**. Hence, only the monoesters **1b** and **1g–j** (and not the corresponding diesters **2b**, **2g–j**) were prepared by the treatment of the known 1,3-di-*O*-benzyl-2-*O*-(2,3,4-tri-*O*-benzyl-β-D-galactopyranosyl)-glycerol (**5**) [7] with the proper carboxylic acid to give the intermediates **6b** and **6g–j** followed by catalytic hydrogenation.



2.2. Biological evaluation

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 [1]. The inhibitory effect of branched (**1a–c**), alicyclic (**1d–f**) and aromatic (**1g–j**) monoesters and of diesters **2a**, **2c–f** 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. [8,9]. Mouse skin tumor promotion inhibition activity of compounds **1a** and **1d** was also evaluated in an in vivo two-stage mouse skin carcinogenesis test as reported in Section 3.

Table 1 shows the in vitro tumor inhibitory activity of compounds **1a–j** and **2a**, **2c–f** together with that of the previously studied [5] esters **1k–m** and **2k–m** for a comparison. 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 ratios/TPA, Table 1) that showed potent inhibitory activity, as indicated by their percentage to control (8.0–31.5% at 500 mol ratio/TPA, Table 1). Generally, the branched analogues **1a–c** and **2a**,**c** resulted in the most active of the tested compounds, IC_{50} ranging from 21.5 to 30.6 (Table 1), with both the alicyclic **1d–f**, **2d–f** and the aromatic derivatives **1g–j** being significantly less active (IC_{50} from 186 to 230, Table 1). In particular, a dramatic drop of activity could be observed both for adding an aliphatic or an aromatic ring to the acyl chain, e.g. **1k** vs **1j** (IC_{50} 62.1 and 205, respectively), and for cyclizing branched chains, e.g. **1a** vs **1d** or **1h** (IC_{50} 27.8, 205 or 223, respectively), **2a** vs **2d** (IC_{50} 21.5 and 133, respectively), **1c** vs **1g** (IC_{50} 30.6 and 230, respectively), **1m** vs **1e** or **1f** (IC_{50} 26.5, 217 or 186, respectively) and **2m** vs **2e** or **2f** (IC_{50} 19.4, 126 or 116, respectively). On the contrary no significant effect was observed when alicyclics

Table 1
Inhibitory effects of **1a–j** and **2a**, **2c–f** on TPA-induced EBV-EA activation

	Concentration (mol ratio/TPA)				IC_{50}
	1000	500	100	10	
	% To control ± SE ($n = 3$)[a]				
1a	0 ± 0.4 (70)	9.9 ± 0.8	29.6 ± 1.0	67.7 ± 1.5	27.8
1b	0 ± 0.3 (70)	9.2 ± 0.9	28.4 ± 1.1	66.3 ± 1.4	25.6
1c	0 ± 0.4 (70)	11.5 ± 0.8	31.4 ± 1.3	68.9 ± 1.4	30.6
1d	0 ± 0.6 (70)	28.3 ± 1.3	72.4 ± 2.2	95.3 ± 0.6	205
1e	0 ± 0.8 (70)	29.6 ± 1.4	73.9 ± 2.1	96.4 ± 0.7	217
1f	0 ± 0.6 (70)	24.9 ± 1.4	70.2 ± 1.9	94.3 ± 0.5	186
1g	0 ± 0.6 (70)	30.9 ± 1.4	75.7 ± 0.7	96.3 ± 1.7	230
1h	0 ± 0.3 (70)	30.5 ± 1.1	74.6 ± 1.3	95.9 ± 0.2	223
1i	0 ± 0.0 (70)	30.1 ± 0.1	73.1 ± 0.5	94.0 ± 0.8	213
1j	0 ± 0.5 (70)	30.0 ± 0.5	71.6 ± 1.0	92.8 ± 1.6	205
1k[b]	0 ± 0.0 (70)	20.4 ± 0.8	43.6 ± 1.0	78.2 ± 1.1	62.1
1l[b]	0 ± 0.0 (70)	10.7 ± 0.1	30.1 ± 0.9	67.8 ± 1.2	28.3
1m[b]	0 ± 0.2 (70)	9.2 ± 0.9	28.9 ± 1.5	67.0 ± 2.1	26.5
2a	0 ± 0.0 (70)	8.0 ± 0.9	25.5 ± 1.2	63.6 ± 2.0	21.5
2c	0 ± 0.0 (70)	8.6 ± 1.0	29.7 ± 1.4	70.1 ± 1.9	30.1
2d	0 ± 0.0 (70)	31.5 ± 1.8	54.2 ± 1.8	94.9 ± 0.5	133
2e	0 ± 0.0 (70)	30.4 ± 1.7	52.8 ± 1.9	94.6 ± 0.3	126
2f	0 ± 0.0 (70)	27.3 ± 1.8	51.6 ± 1.3	93.3 ± 0.4	116
2k[b]	0 ± 0.0 (70)	21.1 ± 0.4	47.6 ± 1.2	80.5 ± 1.3	75.2
2l[b]	0 ± 0.0 (70)	12.4 ± 0.2	32.3 ± 1.1	69.5 ± 1.8	32.2
2m[b]	0 ± 0.2 (70)	7.9 ± 0.6	23.5 ± 1.1	62.0 ± 2.0	19.4

[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.

[b] See Ref. [5].

were substituted with aromatic rings, e.g. **1d** vs **1h** (IC_{50} 205 and 223, respectively, Table 1) and **1e** vs **1i** (IC_{50} 217 and 213, respectively, Table 1). Also, differently from literature data for the gluco and galactoglycerolipids with linear acyl chains [10,11], all the tested cyclic chain compounds were almost equiactive regardless of their chain length (see Table 1 **1g–j** and **1d**,**e**). Concerning branching linear chains, this structural modification always afforded high activity of glycoglycerolipid analogues and the activity enhancement was especially marked, as already observed [5], for the diester series (the inhibitory effects of **2a** and **2c** were, respectively, 71% and 60% higher than **2k**, and **2m** and **2c** were, respectively, 40% and 6% more active than **2l**, see IC_{50} values, Table 1). In conclusion, the diester **2a** is, together with **2m** [5], the most potent glycoglycerolipid analogue synthesized to date, for the inhibition of TPA promoted EBV-EA activation.

For in vivo experiments monoesters **1a** and **1d** were chosen. They are representative members of the branched and cyclic series, respectively, and their structure is strictly related, the cyclohexylacetyl chain in **1d** resulting from the elongation and cyclization of the isopropylacetyl chain of **1a**. The diesters **2a** and **2d**, though appearing more active than the corresponding monoesters in the in vitro experiment, were not tested in vivo because 1-*O*-acyl and 1,6′-di-*O*-acylgalactosylglycerols are known to exhibit almost the same activity when assayed in the in vivo two-stage mouse skin carcinogenesis test [5]. Thus **1a** and **1d** were submitted to an in vivo two-stage carcinogenesis test of mouse skin papillomas using DMBA as an initiator and TPA as a promoter, in order to relate the inhibitory effect to their different structures. 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 number 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 (Fig. 1A), and 5 and 9 papillomas per mouse were formed, respectively, after 10 and 20 weeks of promotion (Fig. 1B). Both the tested compounds were able to inhibit the tumor promotion in this in vivo assay, reducing the percentage of mice bearing papillomas. In fact about 13% and 27% of mice bore papillomas, respectively, in the **1a** and **1d** treated groups at 10 weeks of promotion, and 80% and 93% of mice bearing papillomas resulted at 20 weeks of promotion (Fig. 1A). The differences in the incidence of papillomas in the treated cases with respect to control, analyzed by the χ^2-test, appear to be significant after 10 weeks both for **1a** ($P < 0.005$) and **1d** ($P < 0.005$) 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 **1a** and **1d**) with respect to the control, however, the differences among the two groups were not significant in the lowered number of papillomas per mouse, even if **1a** is significantly more active than **1d** in vitro (Table 1). In fact 1.1 and 1.2 papillomas (20% and 22% with respect to control) were formed by treatment, respectively, with **1a** and **1d** at 10 weeks of promotion, and 4.4 and 4.8 (47% and 52% with respect to control) at 20 weeks of promotion

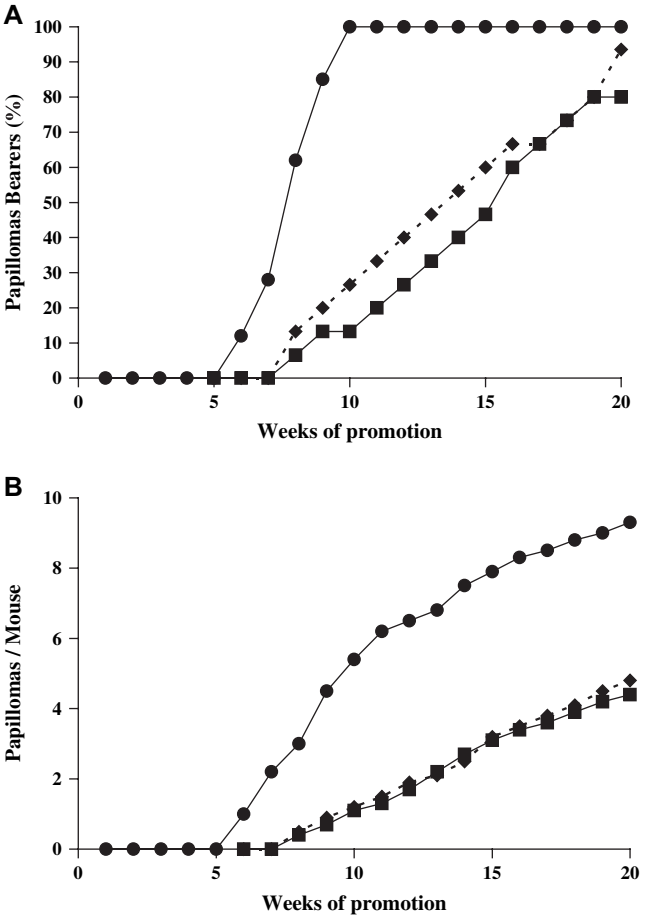


Fig. 1. Inhibitory effects of compounds **1a** and **1d** (85 nmol) on DMBA–TPA mouse skin carcinogenesis. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice a week starting one week after initiation. A: percentage of mice with papillomas; B: averaged number of papillomas per mouse (●, TPA alone; ■, TPA + **1a**; ◆, TPA + **1d**). At 20 weeks of promotion the averaged number of papillomas per mouse was reduced, with respect to the control group (9.3 ± 1.3), to 4.4 ± 0.6 for **1a** ($P < 0.001$) and to 4.8 ± 0.8 for **1d** ($P < 0.001$).

(Fig. 1B). Discrepancy in the structure–activity results between in vivo and in vitro experiments was already observed for glycoglycerolipid analogues and might be due to different lipophilicities of the tested compounds [5].

In conclusion, 15 new galactoglycerolipid analogues have been prepared which were active as anti-tumor-promoting compounds. Structure–activity relationship showed that while branching of the acyl chains enhances the in vitro anti-tumor-promoting activity, the presence of an aliphatic or aromatic ring exerts a negative effect. In particular the branched analogues are very potent, the diester **2a** being one of the most active glycoglycerolipids discovered by our in vitro assay.

3. Experimental protocols

3.1. Chemistry

3.1.1. Materials

P. cepacia lipase (LPS, specific activity: 30.5 triacetin units/mg solid), a generous gift from Amano Pharmaceutical Co

(Mitsubishi Italia), was supported on celite [12] and kept overnight, under vacuum, prior to use. Pyridine was distilled from calcium hydride. The trifluoroethyl esters were synthesized by reacting the proper acid with trifluoroethanol and dicyclohexylcarbodiimide, and the vinyl esters from the acid and vinyl neodecanoate (Aldrich) according to a literature procedure [13]. 1,3-Di-*O*-benzyl-2-*O*-β-D-(galactopyranosyl)-glycerol (**3**), 1,3-di-*O*-benzyl-2-*O*-(2,3,4-tri-*O*-benzyl-β-D-galactopyranosyl)-glycerol (**5**) and 2-*O*-β-D-galactopyranosylglycerol (**7**) were synthesized according to the literature procedures [14,7]. 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 with a Bruker FT-NMR AVANCE™ DRX500 spectrometer, and chemical ionization mass spectrometry (CI-MS) [15] using a Thermo Electron TRACE DSQ™ spectrometer through the rapid heating filament direct-exposure probe (DEP) insertion mode. ^{13}C NMR spectra at 125.76 MHz were recorded for all the target compounds, using 2D HSQC experiment (standard Bruker pulse program) for the full assignment of the carbon resonances. Chemical shifts are reported as δ (ppm) relative to $CHCl_3$, CH_3OH or pyridine, fixed at 7.24, 3.30 or 7.19 ppm, respectively, for ^{1}H NMR spectra and relative to CD_3OD fixed at 49.00 ppm (central line) for ^{13}C NMR spectra. The elemental analyses of all the new compounds were obtained with a Perkin–Elmer 2400 analyzer. Optical rotations were determined on a Perkin–Elmer 241 polarimeter in methanol or chloroform solutions ($c = 1.0$) in a 1 dm cell at 20 °C. Melting points were recorded on a Büchi 510 capillary melting point apparatus and were uncorrected.

3.1.2. General procedure for the synthesis of monoesters *1a, 1c–f*

*3.1.2.1. 1,3-Di-O-benzyl-2-O-(6-O-acyl-β-D-galactopyranosyl)-sn-glycerols (**4a, 4c–f**).* 1,3-Di-*O*-benzyl-2-*O*-β-D-(galactopyranosyl)-glycerol (**3**) (0.255 g, 0.59 mmol) was dissolved in dichloromethane (4.0 mL). The appropriate trifluoroethyl (**4d–f**) or vinyl (**4a**,**c**) ester (1.77 mmol) was added followed by LPS (0.75 g) and the suspension was stirred at 45 °C. The reaction was monitored by TLC (CH_2Cl_2–MeOH 90:10 v/v) and stopped after 20–180 h by filtering off the enzyme which was washed with dichloromethane. The solvent was removed under reduced pressure and the crude residue was submitted to silica gel flash chromatography (CH_2Cl_2–MeOH from 98:2 to 95:5 v/v). In this way 6′-*O*-acylderivatives were obtained.

*3.1.2.1.1. 1,3-Di-O-benzyl-2-O-[6-O-(3-methylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**4a**).* Reaction time 20 h, yield 90%; oil; $[\alpha]_D^{20} = +11.4$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.91 (d, 6H, $J = 6.5$ Hz, $2CH_3$), 2.06 (m, 1H, CH), 2.16 (d, 2H, $J = 6.5$ Hz, $COCH_2$), 3.44–3.72 (m, 7H, H-3′, H-5′, 2H-3, 2H-1 and H-2′), 3.75 (m, 1H, $J = 3.0$ Hz, H-4′), 4.05 (m, 1H, H-2), 4.26 (m, 2H, H-6′a and H-6′b), 4.35 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.44–4.54 (m, 4H, $2CH_2Ph$), 7.22–7.35 (m, 10H, Ph). CI-MS *m/z* 536 $[M + NH_4]^+$. $C_{28}H_{38}O_9$ calcd. C 64.85, H 7.39; found C 64.72, H 7.33.

*3.1.2.1.2. 1,3-Di-O-benzyl-2-O-[6-O-(2-ethylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**4c**).* Reaction time 144 h, yield 75%; oil; $[\alpha]_D^{20} = +10.6$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.92–0.96 (m, 6H, $2CH_3$), 1.43–1.53 (m, 4H, $2CH_2$), 2.10 (m, 1H, COCH), 3.52–3.70 (m, 7H, H-3′, H-5′, 2H-3, 2H-1 and H2′), 3.84 (d, 1H, $J = 3.0$ Hz, H-4′), 4.06 (m, 1H, H-2), 4.24 (dd, 1H, $J_{6'a,5'} = 7.0$ Hz, $J_{6'a,6'b} = 11.0$ Hz, H-6′a), 4.36 (dd, 1H, $J_{6'b,5'} = 6.0$ Hz, H-6′b), 4.39 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.46–4.56 (m, 4H, $2CH_2Ph$), 7.22–7.35 (m, 10H, Ph). CI-MS *m/z* 550 $[M + NH_4]^+$. $C_{29}H_{40}O_9$ calcd. C 64.40, H 7.57; found C 64.30, H 7.49.

*3.1.2.1.3. 1,3-Di-O-benzyl-2-O-[6-O-(2-cyclohexylethanoyl)-β-D-galactopyranosyl]-sn-glycerol (**4d**).* Reaction time 180 h, yield 71%; oil; $[\alpha]_D^{20} = +9.6$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.83–1.70 (m, 10H, $5CH_2$), 1.74 (m, 1H, CH), 2.16 (d, 2H, $J = 6.5$ Hz, $COCH_2$), 3.48–3.72 (m, 7H, H-3′, H-5′, 2H-3, 2H-1 and H-2′), 3.78 (d, 1H, $J = 3.0$ Hz, H-4′), 4.06 (m, 1H, H-2), 4.23 (dd, 1H, $J_{6'a,5'} = 7.0$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.29 (dd, 1H, $J_{6'b,5'} = 5.6$ Hz, H-6′b), 4.37 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.46–4.56 (m, 4H, $2CH_2Ph$), 7.23–7.36 (m, 10H, Ph). CI-MS *m/z* 576 $[M + NH_4]^+$. $C_{31}H_{42}O_9$ calcd. C 66.65, H 7.58; found C 66.50, H 7.64.

*3.1.2.1.4. 1,3-Di-O-benzyl-2-O-[6-O-(3-cyclohexylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**4e**).* Reaction time 140 h, yield 83%; oil; $[\alpha]_D^{20} = +8.3$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.80–1.74 (m, 13H, CH and $6CH_2$), 2.28 (m, 2H, $COCH_2$), 3.52–3.70 (m, 7H, H-3′, H-5′, 2H-3, 2H-1 and H-2′), 3.83 (d, 1H, $J = 3.0$ Hz, H-4′), 4.08 (m, 1H, H-2), 4.23 (dd, 1H, $J_{6'a,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.32 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, H-6′b), 4.39 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.46–4.56 (m, 4H, $2CH_2Ph$), 7.23–7.36 (m, 10H, Ph). CI-MS *m/z* 590 $[M + NH_4]^+$. $C_{32}H_{44}O_9$ calcd. C 67.11, H 7.74; found C 67.30, H 7.69.

*3.1.2.1.5. 1,3-Di-O-benzyl-2-O-[6-O-(3-cyclopentylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**4f**).* Reaction time 90 h, yield 85%; oil; $[\alpha]_D^{20} = +7.6$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.00–1.80 (m, 11H, CH and $5CH_2$), 2.30 (m, 2H, $COCH_2$), 3.50–3.70 (m, 7H, H-3′, H-5′, 2H-3, 2H-1 and H-2′), 3.81 (d, 1H, $J = 3.0$ Hz, H-4′), 4.07 (m, 1H, H-2), 4.23 (dd, 1H, $J_{6'a,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.30 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, H-6′b), 4.38 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.46–4.56 (m, 4H, $2CH_2Ph$), 7.23–7.36 (m, 10H, Ph). CI-MS *m/z* 576 $[M + NH_4]^+$. $C_{31}H_{42}O_9$ calcd. C 66.65, H 7.58; found C 66.49, H 7.68.

*3.1.2.2. 2-O-(6-O-Acyl-β-D-galactopyranosyl)-sn-glycerols (**1a, 1c–f**).* Compound **4** (0.40 mmol) was dissolved in methanol (20 mL) and 10% palladium on activated carbon (0.075 g) was added. The reaction mixture, monitored by TLC (CH_2Cl_2–MeOH 80:20), was shaken under hydrogen atmosphere for 2–3 h, and then filtered through a celite bed affording the debenzylated compounds.

*3.1.2.2.1. 2-O-[6-O-(3-Methylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**1a**).* Yield 92%; oil; $[\alpha]_D^{20} = +9.3$ (CH_3OH). ^{1}H NMR (CD_3OD): δ 0.88 (d, 6H, $J = 6.5$ Hz, $2CH_3$), 2.08 (m, 1H, CH), 2.22 (d, 2H, $J = 6.5$ Hz, $COCH_2$), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.60–3.70 (m, 4H, 2H-1 and 2H-3),

3.72 (m, 1H, H-2), 3.76 (m, 1H, H-5′), 3.80 (d, 1H, H-4′), 4.22 (dd, 1H, $J_{6'a,5'} = 4.4$ Hz, $J_{6'a,6'b} = 11.7$ Hz, H-6′a), 4.29 (dd, 1H, $J_{6'b,5'} = 8.0$ Hz, H-6′b), 4.37 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 22.69 ($2CH_3$), 26.77 (CH), 43.97 (CH_2), 62.73 (C1 or C3), 63.17 (C3 or C1), 64.62 (C6′), 70.16 (C4′), 72.48 (C2′), 74.03 (C5′), 74.43 (C3′), 83.45 (C2), 104.95 (C1′), 174.50 (C═O). CI-MS *m/z* 356 $[M + NH_4]^+$. $C_{14}H_{26}O_9$ calcd. C 49.70, H 7.75; found C 49.81, H 7.89.

*3.1.2.2.2. 2-O-[6-O-(2-Ethylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**1c**).* Yield 85%; oil; $[\alpha]_D^{20} = +9.4$ (CH_3OH). ^{1}H NMR (CD_3OD): δ 0.86–0.92 (m, 6H, $2CH_3$), 1.48–1.68 (m, 4H, $2CH_2$), 2.25 (m, 1H, COCH), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.62–3.70 (m, 4H, 2H-1 and 2H-3), 3.72 (m, 1H, H-2), 3.76 (m, 1H, H-5′), 3.80 (d, 1H, H-4′), 4.24–4.30 (m, 2H, H-6′a and H-6′b), 4.38 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 12.14 ($2CH_3$), 26.07 ($2CH_2$), 50.13 (CH), 62.71 (C1 or C3), 63.15 (C3 or C1), 64.71 (C6′), 70.24 (C4′), 72.51 (C2′), 74.09 (C5′), 74.47 (C3′), 83.26 (C2), 104.97 (C1′), 177.66 (C═O). CI-MS *m/z* 370 $[M + NH_4]^+$. $C_{15}H_{28}O_9$ calcd. C 51.13, H 8.01; found C 51.29, H 8.06.

*3.1.2.2.3. 2-O-[6-O-(2-Cyclohexylethanoyl)-β-D-galactopyranosyl]-sn-glycerol (**1d**).* Yield 88%; oil; $[\alpha]_D^{20} = +7.6$ (CH_3OH). ^{1}H NMR (CD_3OD): δ 0.86–1.82 (m, 11H, CH and $5CH_2$), 2.21 (d, 2H, $J = 6.5$ Hz, $COCH_2$), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.62–3.70 (m, 4H, 2H-1 and 2H-3), 3.72 (m, 1H, H-2), 3.76 (m, 1H, H-5′), 3.80 (d, 1H, H-4′), 4.21 (dd, 1H, $J_{6'a,5'} = 4.4$ Hz, $J_{6'a,6'b} = 11.0$ Hz, H-6′a), 4.28 (dd, 1H, $J_{6'b,5'} = 7.4$ Hz, H-6′b), 4.37 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 27.13 ($2CH_2$), 27.23 (CH_2), 34.06 ($2CH_2$), 36.12 (CH), 42.76 (CH_2CO), 62.77 (C1 or C3), 63.23 (C3 or C1), 64.68 (C6′), 70.23 (C4′), 72.53 (C2′), 74.11 (C5′), 74.50 (C3′), 83.50 (C2), 105.02 (C1′), 174.52 (C═O). CI-MS *m/z* 396 $[M + NH_4]^+$. $C_{17}H_{30}O_9$ calcd. C 53.96, H 7.99; found C 53.71, H 8.11.

*3.1.2.2.4. 2-O-[6-O-(3-Cyclohexylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**1e**).* Yield 88%; oil; $[\alpha]_D^{20} = +6.3$ (CH_3OH). ^{1}H NMR (CD_3OD): δ 0.80–1.80 (m, 13H, CH and $6CH_2$), 2.35 (m, 2H, $COCH_2$), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.57 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.62–3.70 (m, 4H, 2H-1′ and 2H-3′), 3.73 (m, 1H, H-2), 3.76 (m, 1H, H-5′), 3.80 (d, 1H, H-4′), 4.21 (dd, 1H, $J_{6'a,5'} = 5.1$ Hz, $J_{6'a,6'b} = 11.0$ Hz, H-6′a), 4.29 (dd, 1H, $J_{6'b,5'} = 7.4$ Hz, H-6′b), 4.37 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 27.34 ($2CH_2$), 27.63 (CH_2), 32.52 (CH_2CO), 33.42 (CH_2CH_2CO), 34.11 ($2CH_2$), 38.52 (CH), 62.81 (C1 or C3), 63.26 (C3 or C1), 64.69 (C6′), 70.21 (C4′), 72.55 (C2′), 74.10 (C5′), 74.52 (C3′), 83.64 (C2), 105.06 (C1′), 175.55 (C═O). CI-MS *m/z* 410 $[M + NH_4]^+$. $C_{18}H_{32}O_9$ calcd. C 55.09, H 8.22; found C 55.27, H 8.26.

*3.1.2.2.5. 2-O-[6-O-(3-Cyclopentylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**1f**).* Yield 86%; oil; $[\alpha]_D^{20} = +6.3$ (CH_3OH). ^{1}H NMR (CD_3OD): δ 1.04–1.84 (m, 11H, CH and $5CH_2$), 2.36 (m, 2H, $COCH_2$), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.61–3.70 (m, 4H, 2H-1 and 2H-3), 3.74 (m, 1H, H-2), 3.76 (m, 1H, H-5′), 3.80 (d, 1H, H-4′), 4.21 (dd, 1H, $J_{6'a,5'} = 5.1$ Hz, $J_{6'a,6'b} = 11.7$ Hz, H-6′a), 4.29 (dd, 1H, $J_{6'b,5'} = 8.0$ Hz, H-6′b), 4.37 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 26.05 ($2CH_2$), 32.21 (CH_2CH_2CO), 33.38 ($2CH_2$), 34.24 (CH_2CO), 40.90 (CH), 62.78 (C1 or C3), 63.23 (C3 or C1), 64.68 (C6′), 70.18 (C4′), 72.51 (C2′), 74.06 (C5′), 74.48 (C3′), 83.58 (C2), 105.00 (C1′), 175.35 (C═O). CI-MS *m/z* 396 $[M + NH_4]^+$. $C_{17}H_{30}O_9$ calcd. C 53.96, H 7.99; found C 53.89, H 8.04.

*3.1.3. General procedure for the synthesis of monoesters **1b**, **1g–j***

*3.1.3.1. 1,3-Di-O-benzyl-2-O-(2,3,4-tri-O-benzyl-6-O-acyl-β-D-galactopyranosyl)-sn-glycerols (**6b**, **6g–6j**).* To a solution of compound **5** (0.75 mmol) in dichloromethane (5.0 mL), the appropriate carboxylic acid (0.83 mmol), dicyclohexylcarbodiimide (0.83 mmol) and dimethylaminopyridine (0.08 mmol) were added in order. The reaction mixture was stirred at room temperature for 1–2 h and monitored by TLC (petroleum ether–ethyl acetate 70:30 v/v). After filtration of the formed dicyclohexylurea, dichloromethane (20 mL) was added and washed with 5% acetic acid (2 × 15 mL). The aqueous layers were extracted with dichloromethane (2 × 20 mL) and the organic layers were dried over sodium sulfate. The solvent was removed under reduced pressure, and the desired product was obtained by silica gel flash chromatography (petroleum ether–ethyl acetate 75:25 or 70:30 v/v) of the residue.

*3.1.3.1.1. 1,3-Di-O-benzyl-2-O-[2,3,4-tri-O-benzyl-6-O-(3,3-dimethylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**6b**).* Yield 90%; oil; $[\alpha]_D^{20} = +5.3$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.98 (s, 9H, $3CH_3$), 2.10 (s, 2H, CH_2), 3.45–3.52 (m, 2H, H-3′ and H-5′), 3.60–3.78 (m, 5H, 2H-1, 2H-3 and H-4′), 3.82 (dd, $J_{2',1'} = 7.7$ Hz, $J_{2',3'} = 10.0$ Hz, 1H, H-2′), 4.01–4.08 (m, 2H, H-2 and H-6′a), 4.18 (dd, 1H, $J_{6'b,5'} = 6.6$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′b), 4.57 (d, 1H, H-1′), 4.44–5.04 (m, 10H, $5CH_2Ph$), 7.14–7.40 (m, 25H, Ph). CI-MS *m/z* 820 $[M + NH_4]^+$. $C_{50}H_{58}O_9$ calcd. C 74.79, H 7.28; found C 74.66, H 7.42.

*3.1.3.1.2. 1,3-Di-O-benzyl-2-O-(2,3,4-tri-O-benzyl-6-O-benzoyl-β-D-galactopyranosyl-sn-glycerol (**6g**).* Yield 90%; oil; $[\alpha]_D^{20} = -10.6$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 3.53 (d, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H3′), 3.60–3.76 (m, 5H, 2H-1, 2H-3 and H-5′), 3.81 (d, 1H, H-4′), 3.86 (dd, $J_{2',1'} = 7.7$ Hz, 1H, H-2′), 4.06 (m, 1H, H-2), 4.28 (dd, 1H, $J_{6'a,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.42 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, H-6′b), 4.60 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.44–5.04 (m, 10H, $5CH_2Ph$), 7.16–7.90 (m, 30H, Ph). CI-MS *m/z* 826 $[M + NH_4]^+$. $C_{51}H_{52}O_9$ calcd. C 75.72, H 6.48; found C 75.58, H 6.43.

*3.1.3.1.3. 1,3-Di-O-benzyl-2-O-[2,3,4-tri-O-benzyl-6-O-(2-phenylethanoyl)-β-D-galactopyranosyl]-sn-glycerol (**6h**).* Yield 85%; oil; $[\alpha]_D^{20} = -2.6$ ($CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 3.42–3.46 (m, 2H, H-3′ and H-5′), 3.50 (s, 2H, $COCH_2Ph$), 3.60–3.74 (m, 5H, 2H-1, 2H-3 and H-4′), 3.77 (dd, $J_{2',1'} = 7.7$ Hz, $J_{2',3'} = 10.0$ Hz, 1H, H-2′), 4.00 (m, 1H, H-2), 4.05 (dd, 1H, $J_{6'a,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.18 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, H-6′b), 4.52 (d, 1H, H-1′), 4.42–5.00 (m,

10H, $5CH_2Ph$), 7.16–7.35 (m, 30H, Ph). CI-MS *m/z* $840 + NH_4]^+$. $C_{52}H_{54}O_9$ calcd. C 75.89, H 6.61; found C 75 [M, H 6.69.

3.1.3.1.4. 1,3-Di-O-benzyl-2-O-[2,3,4-tri-O-benzyl-6-O-(3-phenylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol ***(6i)***. Yield 87%; oil; $[\alpha]_D^{20} = -2.8$ ($CHCl_3$). ^1H NMR ($CDCl_3$): δ 2.50 (m, 2H, CH_2Ph), 2.86 (m, 2H, $COCH_2$), 3.40 (dd, 1H, $J_{5',6'a} = 6.0$ Hz, $J_{5',6'b} = 6.0$ Hz, H-5′), 3.46 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.60–3.76 (m, 5H, 2H-1, 2H-3 and H-4′), 3.80 (dd, $J_{2',1'} = 7.7$ Hz, 1H, H-2′), 4.01–4.06 (m, 2H, H-2 and H-6′a), 4.15 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′b), 4.54 (d, 1H, H-1′), 4.44–5.00 (m, 10H, $5CH_2Ph$), 7.10–7.36 (m, 30H, Ph). CI-MS *m/z* 854 $[M + NH_4]^+$. $C_{53}H_{56}O_9$ calcd. C 76.05, H 6.74; found C 76.30, H 6.69.

3.1.3.1.5. 1,3-Di-O-benzyl-2-O-[2,3,4-tri-O-benzyl-6-O-(4-phenylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol ***(6j)***. Yield 82%; oil; $[\alpha]_D^{20} = -5.9$ ($CHCl_3$). ^1H NMR ($CDCl_3$): δ 1.92 (m, 2H, CH_2), 2.23 (m, 2H, CH_2Ph), 2.64 (m, 2H, $COCH_2$), 3.51 (dd, 1H, $J_{5',6'a} = 6.0$ Hz, $J_{5',6'b} = 6.0$ Hz, H-5′), 3.54 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.64–3.82 (m, 5H, 2H-1, 2H-3 and H-4′), 3.86 (dd, $J_{2',1'} = 7.7$ Hz, 1H, H-2′), 4.05–4.12 (m, 2H, H-2 and H-6′a), 4.20 (dd, 1H, $J_{6'b,5'} = 6.3$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′b), 4.61 (d, 1H, H-1′), 4.48–5.08 (m, 10H, $5CH_2Ph$), 7.13–7.40 (m, 30H, Ph). CI-MS *m/z* 868 $[M + NH_4]^+$. $C_{54}H_{58}O_9$ calcd. C 76.21, H 6.87; found C 76.39, H 6.79.

*3.1.3.2. 2-O-(6-O-Acyl-β-D-galactopyranosyl)-sn-glycerol (****1b****, ****1g–j****).* Compound **6** (0.6 mmol) was dissolved in methanol (35 mL) and 10% palladium on activated carbon (0.09 g) was added. The reaction mixture, monitored by TLC (CH_2Cl_2–MeOH 80:20), was shaken under hydrogen atmosphere for 3–5 h, and then filtered through a celite bed affording the debenzylated compounds.

3.1.3.2.1. 2-O-[6-O-(3,3-Dimethylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol ***(1b)***. Yield 85%; oil; $[\alpha]_D^{20} = +10.3$ (CH_3OH). ^1H NMR (CD_3OD): δ 1.03 (s, 9H, $3CH_3$), 2.22 (s, 2H, CH_2), 3.50 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.62–3.70 (m, 4H, 2H-1 and 2H-3), 3.73 (m, 1H, H-2), 3.76 (dd, 1H, $J_{6'a,5'} = 4.4$ Hz, $J_{6'b,5'} = 7.4$ Hz, H-5′), 3.80 (d, 1H, H-4′), 4.22 (dd, 1H, $J_{6'a,6'b} = 11.0$ Hz, H-6′a), 4.26 (dd, 1H, H-6′b), 4.38 (d, 1H, H-1′). ^{13}C NMR (CD_3OD): δ 30.00 ($3CH_3$), 31.52 ($C(CH_3)_3$), 48.55 (CH_2), 62.73 (C1 or C3), 63.20 (C3 or C1), 64.58 (C6′), 70.28 (C4′), 72.55 (C2′), 74.16 (C5′), 74.51 (C3′), 83.30 (C2), 104.99 (C1′), 173.76 (C=O). CI-MS *m/z* 370 $[M + NH_4]^+$. $C_{15}H_{28}O_9$ calcd. C 51.13, H 8.01; found C 50.97, H 7.90.

3.1.3.2.2. 2-O-(6-O-Benzoyl-β-D-galactopyranosyl)-sn-glycerol ***(1g)***. Yield 83%; m.p. 152 °C (from $CHCl_3$–CH_3OH); $[\alpha]_D^{20} = +15.6$ (CH_3OH). ^1H NMR (CD_3OD): δ 3.54 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.60 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.60–3.71 (m, 4H, 2H1 and 2H-3), 3.75 (m, 1H, H-2), 3.90 (d, 1H, H-4′), 3.93 (m, 1H, $J_{5',6'a} = 6.6$ Hz, $J_{5',6'b} = 6.6$ Hz, H-5′), 4.44 (d, 1H, H-1′), 4.44–4.54 (m, 2H, H-6′a and H-6′b), 7.47 (dd, 2H, $J = 7.0$ Hz, $J = 7.0$ Hz, Ph), 7.60 (dd, 1H, $J = 7.0$ Hz, $J = 7.0$ Hz, Ph), 8.04 (d, 2H, $J = 7.0$ Hz, Ph). ^{13}C NMR (CD_3OD): δ 61.41 (C1 or C3), 61.76 (C3 or C1), 63.90 (C6′), 68.92 (C4′), 71.20 (C2′), 72.79 (C5′), 73.15 (C3′), 82.01 (C2), 103.66 (C1′), 128.20 (2CH, Ph), 129.25 (2CH, Ph), 129.83 (C, Ph), 132.95 (CH, Ph), 166.46 (C=O). CI-MS *m/z* 376 $[M + NH_4]^+$. $C_{16}H_{22}O_9$ calcd. C 53.63, H 6.19; found C 53.49, H 6.13.

*3.1.3.2.3. 2-O-[6-O-(2-Phenylethanoyl)-β-D-galactopyranosyl]-sn-glycerol (****1h****).* Yield 82%; oil; $[\alpha]_D^{20} = +6.2$ (CH_3OH). ^1H NMR (CD_3OD): δ 3.47 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.55 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.60–3.68 (m, 6H, $COCH_2$, 2H-1 and 2H-3), 3.69 (m, 1H, H-2), 3.74 (dd, 1H, $J_{5',6'a} = 4.4$ Hz, $J_{5',6'b} = 8.0$ Hz, H-5′), 3.76 (d, 1H, H-4′), 4.23 (dd, 1H, $J_{6'a,6'b} = 11.8$ Hz, H-6′a), 4.32 (dd, 1H, H-6′b), 4.34 (d, 1H, H-1′), 7.34–7.21 (m, 5H, Ph). ^{13}C NMR (CD_3OD): δ 41.79 (CH_2), 62.76 (C1 or C3), 63.23 (C3 or C1), 65.16 (C6′), 70.17 (C4′), 72.53 (C2′), 74.03 (C5′), 74.46 (C3′), 83.43 (C2), 104.96 (C1′), 128.07 (CH, Ph), 129.54 (2CH, Ph), 130.39 (2CH, Ph), 135.51 (C, Ph), 173.26 (C=O). CI-MS *m/z* 390 $[M + NH_4]^+$. $C_{17}H_{24}O_9$ calcd. C 54.83, H 6.50; found C 54.74, H 6.59.

*3.1.3.2.4. 2-O-[6-O-(3-Phenylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (****1i****).* Yield 80%; m.p. 125–126 °C (amorphous solid); $[\alpha]_D^{20} = +5.9$ (CH_3OH). ^1H NMR (CD_3OD): δ 2.66 (m, 2H, CH_2), 2.92 (m, 2H, CH_2), 3.48 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.61–3.74 (m, 6H, 2H-1, 2H-3, H-5′ and H-2), 3.76 (d, 1H, H-4′), 4.21 (dd, 1H, $J_{6'a,6'b} = 11.8$ Hz, $J_{6'a,5'} = 4.4$ Hz, H-6′a), 4.27 (dd, 1H, $J_{6'b,5'} = 8.1$ Hz, H-6′b), 4.34 (d, 1H, H-1′), 7.14–7.29 (m, 5H, Ph). ^{13}C NMR (CD_3OD): δ 30.49 (CH_2), 35.28 (CH_2), 61.42 (C1 or C3), 61.85 (C3 or C1), 63.32 (C6′), 68.75 (C4′), 71.14 (C2′), 72.68 (C5′), 73.09 (C3′), 82.24 (C2), 103.65 (C1′), 125.90 (CH, Ph), 127.98 (2CH, Ph), 128.10 (2CH, Ph), 140.54 (C, Ph), 172.97 (C=O). CI-MS *m/z* 404 $[M + NH_4]^+$. $C_{18}H_{26}O_9$ calcd. C 55.95, H 6.78; found C 55.78, H 6.81.

*3.1.3.2.5. 2-O-[6-O-(4-Phenylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (****1j****).* Yield 78%; wax; $[\alpha]_D^{20} = +5.6$ (CH_3OH). ^1H NMR (CD_3OD): δ 1.92 (m, 2H, CH_2), 2.35 (m, 2H, CH_2), 2.64 (m, 2H, CH_2), 3.49 (dd, 1H, $J_{3',2'} = 10.0$ Hz, $J_{3',4'} = 3.0$ Hz, H-3′), 3.56 (dd, 1H, $J_{2',1'} = 7.4$ Hz, H-2′), 3.60–3.70 (m, 4H, 2H-1 and 2H-3), 3.73 (m, 1H, H-2), 3.76 (dd, 1H, $J_{5',6'a} = 4.4$ Hz, $J_{5',6'b} = 8.0$ Hz, H-5′), 3.79 (d, 1H, H-4′), 4.20 (dd, 1H, $J_{6'a,6'b} = 11.8$ Hz, H-6′a), 4.29 (dd, 1H, H-6′b), 4.37 (d, 1H, H-1′), 7.13–7.29 (m, 5H, Ph). ^{13}C NMR (CD_3OD): δ 27.76 (CH_2), 34.24 (CH_2), 36.05 (CH_2), 62.79 (C1 or C3), 63.24 (C3 or C1), 64.72 (C6′), 70.21 (C4′), 72.55 (C2′), 74.10 (C5′), 74.51 (C3′), 83.54 (C2), 105.02 (C1′), 127.00 (CH, Ph), 129.41 (2CH, Ph), 129.52 (2CH, Ph), 142.82 (C, Ph), 174.98 (C=O). CI-MS *m/z* 418 $[M + NH_4]^+$. $C_{19}H_{28}O_9$ calcd. C 56.99, H 7.05; found C 57.12, H 7.19.

3.1.4. General procedure for the synthesis of diesters ***2a, 2c–f***

2-*O*-β-D-Galactopyranosylglycerol (0.2 g, 0.79 mmol) was dissolved in pyridine (3.0 mL); appropriate trifluoroethyl

(**2d–f**) or vinyl (**2a,c**) ester (3.95 mmol) and LPS (1.0 g) were added in order and the suspension was stirred at 45 °C. The reaction was monitored by TLC (CH_2Cl_2–MeOH 80:20) and stopped after 20–330 h by filtering off the enzyme which was washed with pyridine. The solvent was removed under reduced pressure and the crude residue was submitted to repeated flash chromatographies (CH_2Cl_2–MeOH from 95:5 to 80:20 v/v) to obtain pure (**2c–f**) or diastereomerically enriched (**2a**) 1,6′-di-*O*-acylderivatives.

*3.1.4.1. 1-O-(3-Methylbutanoyl)-2-O-[6-O-(3-methylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**2a**).* Reaction time 20 h, yield 27%; oil; 90% diastereomeric purity (by careful integration of the anomeric H-1′ NMR signals: δ 4.97 for the major 1-*O*-acyl isomer and δ 4.96 for the minor 3-*O*-acyl isomer). ^{1}H NMR (pyridine-d_5): δ 0.82–0.88 (m, 12H, $4CH_3$), 2.04–2.16 (m, 2H, 2CH), 2.18–2.27 (m, 4H, $2COCH_2$), 4.11–4.20 (m, 4H, 2H-3, H-3′ and H-5′), 4.36 (m, 1H, H-4′), 4.42 (m, 1H, H-2′), 4.45 (m, 1H, H-2), 4.66 (m, 2H, H-1a and H-1b), 4.73 (dd, 1H, $J_{6'a,5'} = 4.9$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.86 (dd, 1H, $J_{6'b,5'} = 7.7$ Hz, H-6′b), 4.97 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′). ^{13}C NMR (CD_3OD): δ 22.73 ($4CH_3$), 26.77 (CH), 26.85 (CH), 44.80 ($2CH_2$), 63.19 (C3), 64.50 (C1), 64.65 (C6′), 70.24 (C4′), 72.25 (C2′), 74.10 (C5′), 74.54 (C3′), 79.23 (C2), 104.67 (C1′), 174.51 (C=O), 174.65 (C=O). CI-MS *m/z* 440 $[M + NH_4]^+$. $C_{19}H_{34}O_{10}$ calcd. C 54.02, H 8.11; found C 54.30, H 8.28.

*3.1.4.2. 1-O-(2-Ethylbutanoyl)-2-O-[6-O-(2-ethylbutanoyl)-β-D-galactopyranosyl]-sn-glycerol (**2c**).* Reaction time 330 h, yield 12%; oil; $[\alpha]_D^{20} = +7.6$ ($CHCl_3$). ^{1}H NMR (pyridine-d_5): δ 0.86–0.92 (m, 12H, $4CH_3$), 1.40–1.72 (m, 8H, $4CH_2$), 2.24–2.34 (m, 2H, 2COCH), 4.11–4.20 (m, 4H, 2H-3, H-3′ and H-5′), 4.36 (m, 1H, H-4′), 4.41 (m, 1H, H-2′), 4.45 (m, 1H, H-2), 4.66 (dd, 1H, $J_{1a,1b} = 11.2$ Hz, $J_{1a,2} = 5.6$ Hz, H-1a), 4.76 (dd, 1H, $J_{1b,2} = 4.9$ Hz, H-1b), 4.78 (dd, 1H, $J_{6'a,5'} = 4.9$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.86 (dd, 1H, $J_{6'b,5'} = 7.7$ Hz, H-6′b), 4.98 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′). ^{13}C NMR (CD_3OD): δ 12.18 ($4CH_3$), 26.03 ($2CH_2$), 26.14 ($2CH_2$), 50.24 (2CH), 63.19 (C3), 64.39 (C1), 64.75 (C6′), 70.31 (C4′), 72.25 (C2′), 74.19 (C5′), 74.56 (C3′), 79.00 (C2), 104.58 (C1′), 177.66 (C=O), 177.73 (C=O). CI-MS *m/z* 468 $[M + NH_4]^+$. $C_{21}H_{38}O_{10}$ calcd. C 55.99, H 8.50; found C 55.70, H 8.43.

*3.1.4.3. 1-O-(2-Cyclohexylethanoyl)-2-O-[6-O-(2-cyclohexylethanoyl)-β-D-galactopyranosyl]-sn-glycerol (**2d**).* Reaction time 300 h, yield 6%; oil; $[\alpha]_D^{20} = +4.9$ ($CHCl_3$). ^{1}H NMR (pyridine-d_5): δ 0.84–1.84 (m, 22H, 2CH and $10CH_2$), 2.20–2.30 (m, 4H, $2COCH_2$), 4.10–4.20 (m, 4H, 2H-3, H-3′ and H-5′), 4.36 (m, 1H, H-4′), 4.42 (m, 1H, H-2′), 4.47 (m, 1H, H-2), 4.63–4.72 (m, 2H, H-1a and H-1b), 4.75 (dd, 1H, $J_{6'a,5'} = 4.2$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.87 (dd, 1H, $J_{6'b,5'} = 7.7$ Hz, H-6′b), 4.98 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′). ^{13}C NMR (CD_3OD): δ 27.18 ($4CH_2$), 27.28 ($2CH_2$), 34.11 ($4CH_2$), 36.13 (2CH), 42.85 ($2CH_2CO$), 63.23 (C3), 64.55 (C1), 64.72 (C6′), 70.28 (C4′), 72.28 (C2′), 74.16 (C5′), 74.57 (C3′), 79.21 (C2), 104.64 (C1′), 174.51 (C=O), 174.65 (C=O). CI-MS *m/z* 520 $[M + NH_4]^+$. $C_{25}H_{42}O_{10}$ calcd. C 59.74, H 8.42; found C 59.60, H 8.57.

*3.1.4.4. 1-O-(3-Cyclohexylpropanoyl)-2-O-[6-O-(3-cyclohexylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**2e**).* Reaction time 260 h, yield 20%; oil; $[\alpha]_D^{20} = +4.0$ ($CHCl_3$). ^{1}H NMR (pyridine-d_5): δ 0.68–1.64 (m, 26H, 2CH and $12CH_2$), 2.31–2.43 (m, 4H, $2COCH_2$), 4.12–4.20 (m, 4H, 2H-3, H-3′ and H-5′), 4.37 (m, 1H, H-4′), 4.43 (m, 1H, H-2′), 4.47 (m, 1H, H-2), 4.64–4.70 (m, 2H, H-1a and H-1b), 4.76 (dd, 1H, $J_{6'a,5'} = 4.9$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.89 (dd, 1H, $J_{6'b,5'} = 7.7$ Hz, H-6′b), 4.99 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′). ^{13}C NMR (CD_3OD): δ 27.36 ($4CH_2$), 27.65 ($2CH_2$), 32.58 ($2CH_2CO$), 33.48 ($2CH_2CH_2CO$), 34.14 ($4CH_2$), 38.54 (2CH), 63.24 (C3), 64.68 (C1 and C6′), 70.23 (C4′), 72.28 (C2′), 74.14 (C5′), 74.58 (C3′), 79.37 (C2), 104.70 (C1′), 175.54 (C=O), 175.64 (C=O). CI-MS *m/z* 548 $[M + NH_4]^+$. $C_{27}H_{46}O_{10}$ calcd. C 61.11, H 8.74; found C 61.02, H 8.69.

*3.1.4.5. 1-O-(3-Cyclopentylpropanoyl)-2-O-[6-O-(3-cyclopentylpropanoyl)-β-D-galactopyranosyl]-sn-glycerol (**2f**).* Reaction time 260 h, yield 27%; wax; $[\alpha]_D^{20} = +3.9$ ($CHCl_3$). ^{1}H NMR (pyridine-d_5): δ 0.88–1.72 (m, 22H, 2CH and $10CH_2$), 2.36 (m, 2H, $COCH_2$), 4.12–4.20 (m, 4H, 2H-3, H-3′ and H-5′), 4.37 (m, 1H, H-4′), 4.43 (m, 1H, H-2′), 4.47 (m, 1H, H-2), 4.64–4.72 (m, 2H, H-1a and H-1b), 4.76 (dd, 1H, $J_{6'a,5'} = 4.9$ Hz, $J_{6'a,6'b} = 11.2$ Hz, H-6′a), 4.90 (dd, 1H, $J_{6'b,5'} = 7.7$ Hz, H-6′b), 4.99 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′). ^{13}C NMR (CD_3OD): δ 26.07 ($4CH_2$), 32.24 (CH_2CH_2CO), 32.29 (CH_2CH_2CO), 33.42 ($4CH_2$), 34.31 ($2CH_2CO$), 40.93 (2CH), 63.21 (C3), 64.65 (C1 and C6′), 70.22 (C4′), 72.26 (C2′), 74.11 (C5′), 74.56 (C3′), 79.34 (C2), 104.67 (C1′), 175.35 (C=O), 175.45 (C=O). CI-MS *m/z* 520 $[M + NH_4]^+$. $C_{25}H_{42}O_{10}$ calcd. C 59.74, H 8.42; found C 59.88, H 8.39.

*3.1.5. Configuration assignment at C-2 for **2a**, **2c–f***

The 1-*O*-acyl-*sn*-glycerols obtained by treatment of **2a**, **2c–f** 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 on a chiral GLC-capillary column (dimethylpentyl-β-cyclodextrin, 25 m, ID 0.25 mm, from MEGA – Italy) and compared with authentic analytical standards [6] under the following conditions: oven temperature, from 120 to 200 °C, 0.2 °C/min; injector temperature, 250 °C; detector temperature, 295 °C; helium flow, 1 mL/min; split ratio 80:1. In the chromatogram, the lower retention time peak corresponded to the 1-*O*-acyl-2,3-*O*-isopropylidene-*sn*-glycerols and the higher retention time peak to the 3-*O*-acyl-isomers. The following retention times were found for the 3-methylbutanoyl, 2-ethylbutanoyl, cyclohexylethanoyl, cyclopentylpropanoyl, and cyclohexylpropanoyl derivatives: 12.08/13.07, 17.22/17.43, 81.33/82.13, 86.06/87.07 and 119.71/120.56 min.

3.2. Biological evaluation

3.2.1. 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 [8,9]. 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.

3.2.2. In vivo two-stage mouse skin carcinogenesis test

Female SENECAR 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 dimethylbenz[*a*]anthracene (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 χ^2-test.

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