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2-*O*-β-D-Glucopyranosyl-*sn*-glycerol based analogues of sulfoquinovosyldiacylglycerols (SQDG) and their role in inhibiting Epstein-Barr virus early antigen activation

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ABSTRACT

New sulfoquinovosyldiacylglycerols derived from 2-*O*-β-D-glucopyranosyl-*sn*-glycerol, carrying acyl chains of various length on the glycerol moiety, were prepared through a convenient synthetic procedure in which a sulfonate is introduced at the C-6 position of glucose by oxidation of a thioacetate in the presence of the unprotected secondary hydroxyl groups, and tested for their anti-tumor-promoting activity using a short-term in vitro assay for Epstein-Barr virus early antigen (EBV-EA) activation. Our study has allowed to ascertain the role of the 6′-sulfonate group and the need of a free hydroxyl group on the glycerol moiety in inhibiting the EBV activation promoted by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

1. Introduction

Sulfoquinovosyldiacylglycerols (SQDG) are sulfo-glycolipids mainly associated with photosynthetic organisms discovered in microorganisms and higher plants by Benson in 1959[1] in which sulfoquinovose (6-deoxy-6-sulfo-glucose)[2] is α-linked to the *sn*-3 position of a diacylglycerol (Fig. 1). Even if their physiological functions are still under investigation, SQDG are thought to be involved in the maintenance of photosystem complexes function and to substitute phospholipids under phosphate-limiting growth conditions to maintain the total anionic lipids at a constant level in the thylakoid membranes of chloroplasts.[2,3] Concerning their behavior in experiments testing the potential therapeutic use of SQDG, recently reported[4,5] biological activities, including inhibitory effects on HIV-reverse transcriptase, mammalian DNA polymerase, proliferation of some cancer cell lines, angiogenesis (especially when coupled with tumor radiotherapy), and also apoptosis induction, make these compounds very attractive for their use in cancer therapy.

Also, Shirahshi et al. reported the in vitro anti-tumor-promoting activity of SQDG from Cyanobacterium *Phormidium tenue* together with that of mono- and digalactosyl diacylglycerols (MGDG and DGDG) isolated from the same micro-organism.[6] The inhibitory effect of MGDG and DGDG was higher than SQDG but a sure structure–activity relationship of SQDG was not possible[6] because they were tested as a mixture of compounds carrying acyl chains of different length, and because of the different nature of the sugars present in these natural compounds (i.e., β-galactopyranosides in the case of MGDG and DGDG, vs α-6-deoxy-glucopyranosides for SQDG). During our search for new glycoglycerolipids active in cancer chemoprevention, in recent years we have synthesized a number of esters of 2-*O*-β-D-glycosylglycerols in which the length, shape, number and position of the acyl chain, and the type of sugar (α and β glucose or galactose) were varied. These compounds were very active in inhibiting the tumor-promoting activity of TPA both

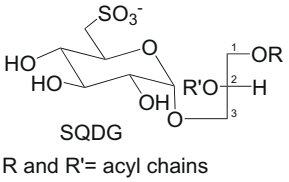

Figure 1.

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in in vitro and in in vivo tests, being such activities mainly influenced by the changes of the acyl chains length.[7] Thus, the above reported promising biological activities of SQDG prompted us to plan an easy synthesis of SQDG analogues based on the skeleton of 2-*O*-β-D-glucopyranosyl-*sn*-glycerol to which the previously tested glycoglycerolipid analogues were related and to test their anti-tumor-promoting activity.

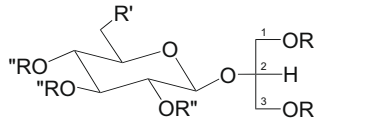
Starting from the known 2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (**2**),[8] SQDG analogues **1a–c** (Fig. 2), namely 1,3-di-*O*-octadecanoyl (**1a**), -dodecanoyl (**1b**), and -hexanoyl-2-*O*-β-D-sulfoquinovopyranosyl-*sn*-glycerol (**1c**) (in which acyl chains of different length were linked to the primary hydroxyls of 2-*O*-β-D-sulfoquinovopyranosyl-*sn*-glycerol), were prepared, exploiting chloroacetyls to temporary and selectively protect glucose hydroxyls with respect to glycerol. As a preliminary check-up of their biological behavior, in this paper the obtained compounds were tested as anti-tumor-promoters using a short-term in vitro assay for EBV-EA activation and, to get insight the influence exerted by the 6′-sulfonate, the corresponding 6′-hydroxy synthetic intermediates, 1,3-di-*O*-octadecanoyl (**4a**), -dodecanoyl (**4b**), and -hexanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (**4c**), were also tested.

2. Results and discussion

2.1. Chemistry

2-*O*-(2,3,4,6-Tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (**2**) was prepared from glucose pentaacetate and 1,3-dibenzylglycerol as previously reported.[8] Two acyl chains of the desired length were then linked to the glycerol hydroxyls of **2** by treatment with the proper acyl chloride (octadecanoyl, dodecanoyl or hexanoyl) in pyridine affording 1,3-diesters **3a–c** in good yields (see Section 4). Selective removal of the four chloroacetyls from the sugar was then efficiently performed by hydrazine acetate treatment[8] to yield the 1,3-di-*O*-acyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerols **4a–c**. The main task of introducing a sulfonate at C-6 position of glucose, was achieved by a strategy involving tosylation, substitution by thioacetate and 6′-SAc group oxidation.

In particular, zinc bromide catalyzed the selective tosylation[9] of the primary 6′-hydroxyl of **4a–c** in pyridine at −20 °C yielding 1,3-di-*O*-acyl-2-*O*-(6-*O*-tosyl-β-D-glucopyranosyl)-*sn*-glycerols **5a–c** in good yields, which further were converted into the corresponding 1,3-di-*O*-acyl-2-*O*-(6-deoxy-6-thioacetyl-β-D-glucopyranosyl)-*sn*-glycerols **6a–c** by potassium thioacetate treatment in DMF. The final oxidation step was carried out with OXONE® in acetic acid[10] directly on the unprotected compounds **6a–c**. The presence of free secondary hydroxyls on these intermediates made the oxidation step a challenging task. However, though the formation of 4′-O-acetylated sulfoquinovosides (diagnostic ^{1}H NMR signals: H-4′, dd, at 4.65 ppm and CH_3CO, s, at 2.08 ppm) occurred as a side reaction, and the amphiphilic nature of the final compounds made their work-up tedious (see Section 4) this procedure allowed us to obtain the target sulfoquinovosides **1a–c** in noteworthy 38–42% yields avoiding protecting group manipulation.



1: R= acyl, R'= SO_3^-, R"= H
2: R= H, R'= $ClCH_2COO$, R"= $ClCH_2CO$
3: R= acyl, R'= $ClCH_2COO$, R"= $ClCH_2CO$
4: R= acyl, R'= OH, R"= H
5: R= acyl, R'= OTs, R"= H
6: R= acyl, R'= $SCOCH_3$, R"= H
a: acyl= octadecanoyl **b**: acyl= dodecanoyl
c: acyl= hexanoyl

Figure 2.

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.[11] The inhibitory effects of sulfonated (**1a–c**) and de-sulfonated (**4a–c**) glucoglycerolipids were 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. 6 and 12 (see Section 4). Table 1 shows the in vitro tumor inhibitory activity of compounds **1a–c** and **4a–c**.

Only weak cytotoxicity against Raji cells was observed especially for compounds **1a–c** at high concentration (50% viability at 1000 mol ratio/TPA vs 60% for compounds **4a–c** Table 1) and, in general, all compounds were active as indicated by their percentage to control (46.8–65.1% at 500 mol ratio/TPA, Table 1), though previously tested glycoglycerolipids showed higher inhibitory effect on EBV activation.[7] In Table 1 the in vitro inhibitory effects of the already studied 1-*O*-hexanoyl-2-*O*-(6-*O*-hexanoyl-β-D-glucopyranosyl)-*sn*-glycerol (**7**)[13] and 1-*O*-hexanoyl-2-*O*-(β-D-galactopyranosyl)ethylene glycol (**8**)[14] are also reported for a comparison of the structural requirements. It is interesting to note that the 1,3-diester, 1,3-di-*O*-hexanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (**4c**), is significantly less active than the corresponding 1,6′-di-*O*-hexanoyl isomer (**7**)[13] in inhibiting the EBV-EA activation (IC_{50} 327 vs 37.3, respectively, Table 1) suggesting that the double acylation of glycerol plays a negative role on the activity in these compounds. These data in fact seem to support the reported observation that the anti-tumor-promoting activity of these 2-*O*-glycoglycerolipid analogues is closely related to the presence of a free hydroxymethylene group on the glycerol-like aglycone moiety.[14] Actually, 1-*O*-hexanoyl-2-*O*-(β-D-galactopyranosyl)ethylene glycol (**8**)[14] (in which glycerol is replaced by ethylene glycol as the aglycone) shows an activity very similar to that of the 1,3-diester **4c** (IC_{50} 350 vs 327, respectively, Table 1). Concerning the presence of the sulfonate group, it seems to exert an additional negative effect on the anti-tumor-promoting activity of glycoglycerolipids in this in vitro experimental model, as evidenced by the

Table 1
Inhibitory effects of **1a–c, 4a–c**, and on TPA-induced EBV-EA activation

	Concentration (mol ratio/TPA)				IC_{50}
	1000	500	100	10	
	% to control ± SE (n = 3)[a]				
1a	25.1 ± 1.6 (50)[b]	65.1 ± 2.4 (70)	87.4 ± 1.9 (80)	100 ± 0.2 (80)	632
1b	23.4 ± 1.6 (50)	62.6 ± 2.2 (70)	85.2 ± 1.7 (80)	100 ± 0.3 (80)	581
1c	20.3 ± 1.6 (50)	60.5 ± 2.1 (70)	84.3 ± 1.9 (80)	100 ± 0.3 (80)	539
4a	16.2 ± 1.5 (60)	57.8 ± 2.0 (70)	82.8 ± 1.8 (80)	100 ± 0.2 (80)	484
4b	13.0 ± 1.2 (60)	52.4 ± 2.0 (70)	80.5 ± 1.8 (80)	100 ± 0.4 (80)	402
4c	9.3 ± 0.7 (60)	46.8 ± 1.7 (70)	76.9 ± 1.8 (80)	100 ± 0.5 (80)	327
7[c]	0.0 ± 0.0 (70)	15.1 ± 0.5 (80)	34.6 ± 1.2 (80)	71.5 ± 1.9(80)	37.3
8[d]	6.7 ± 0.5 (70)	49.5 ± 1.4 (80)	78.5 ± 2.5 (80)	100 ± 0.2 (80)	350

[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] Values in parentheses are viability percentages of Raji cells.
[c] See Ref. 13.
[d] See Ref. 14.

comparison of the data reported in Table 1 for the 6′-sulfo-diesters **1a–c** (IC_{50} from 632 to 539, Table 1) and the 6′-hydroxy **4a–c** (IC_{50} from 484 to 327 Table 1). Nevertheless, the here reported 2-*O*-β-D-analogues **1a–c** show a significantly higher activity than the natural SQDG[6] (20.3–25.1%, Table 1, vs 91.7% at 1000 mol ratio/TPA, respectively). Finally, as further support to the data already reported,[7] also in this case shortening the acyl chains, from C18 (compounds of the **a** series) to C6 (compounds of the **c** series), resulted in the increasing of the activity both in the case of sulfoquinovosides **1** and glucosides **4** (see Table 1).

3. Conclusion

New sulfoquinovosyldiacylglycerols based on 2-*O*-β-D-glucopyranosyl-*sn*-glycerol and their 6′-hydroxy 'de-sulfonated' glucosyl analogues were prepared through an easy synthetic procedure and some interesting conclusions on their in vitro anti-tumor-promoting activity could be drawn from the obtained results. In fact, the new compounds have allowed to ascertain the influence of some till now unexplored structural features of these glycoglycerolipids (i.e., presence of a sulfonate group on the sugar moiety and double acylation of glycerol) on the tested activity. In particular, the sulfonate group at the C-6 position of glucose plays a negative role on the inhibition of EBV activation induced by the tumor promoter TPA, also increasing the cytotoxicity. In the same way, the double acylation of the glycerol moiety produces a negative effect, probably because of the masking of a primary hydroxyl group crucial for the activity. Basing on these observations, sulfoquinovosylmonoacylglycerols (SQMG) might be more active than the corresponding SQDG in the EBV-EA inhibition test. Due to the high interest related to bio-active natural sulfo-glycolipids, work is in progress to further characterize the biological potential of these new synthetically available sulfoquinovosylacylglycerol analogues.

4. Experimental

4.1. Chemical procedures

4.1.1. Materials

2-*O*-(2,3,4,6-Tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (**2**), was synthesized according to the literature procedure.[8] Optical rotations were determined on a Perkin–Elmer 241 polarimeter in 1% $CHCl_3$ or $CHCl_3$:CH_3OH:H_2O (65:25:4) solutions at 20 °C, in a 1 dm cell. Melting points were recorded on a Büchi 510 capillary melting point apparatus and were uncorrected. All reagents and solvents used were reagent grade and were purified before use by standard methods. Dry solvents and liquid reagents were distilled prior to use or dried on 4 Å molecular sieves. Column chromatography was carried out on flash silica gel (Merck 230–400 mesh). TLC analysis was carried out on silica gel plate (Merck $60F_{254}$) developing with 50% sulfuric acid or anisaldehyde based reagent. Evaporation under reduced pressure was always effected with a bath temperature below 40 °C. The structures of all the new synthesized compounds were confirmed through full ^1H and ^{13}C NMR characterization and mass spectroscopy. ^1H NMR analysis were performed at 500 MHz with a Bruker FT-NMR AVANCE™ DRX500 spectrometer using a 5 mm z-PFG (pulsed field gradient) broadband reverse probe at 298 K unless otherwise stated, and ^{13}C NMR spectra at 125.76 MHz were done of all the new compounds. The signals were unambiguously assigned by 2D COSY and HSQC experiments (standard Bruker pulse program). Chemical shifts are reported as δ (ppm) relative to residual $CHCl_3$ or CH_3OH (when $CDCl_3$:CD_3OD:D_2O solvent mixture was used) fixed at 7.24 and 3.30 ppm, respectively, for ^1H NMR spectra and relative to $CDCl_3$ fixed at 77.0 ppm (central line) or CD_3OD at 49.00 ppm (central line) for ^{13}C NMR spectra; scalar coupling constants are reported in hertz. Mass spectra were recorded in negative or positive-ion electrospray (ESI) mode on a Thermo Quest Finnigan LCQ™ DECA ion trap mass spectrometer; the mass spectrometer was equipped with a Finningan ESI interface; sample solutions were injected with a ionization spray voltage of 4.5 kV or 5.0 kV (positive and negative-ion mode, respectively), a capillary voltage of 32 V or −15 V (positive and negative-ion mode, respectively), and capillary temperature of 250 °C. Data were processed by Finnigan Xcalibur software system. TLC, ^1H NMR and MS confirmed purity of all synthesized compounds.

4.1.2. General procedure for the synthesis of diesters 3a–c

2-*O*-(2,3,4,6-Tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (**2**)[8] (2.0 g, 3.57 mmol) was dissolved in dry CH_2Cl_2 (30 mL) and cooled at −10 °C. The proper acyl chloride (8.8 mmol) as a 15% (v/v) CH_2Cl_2 solution and pyridine (22.0 mmol) as a 10% (v/v) CH_2Cl_2 solution, were added in the order and the mixture stirred at −10 °C under Ar atmosphere. The reaction was monitored by TLC (petroleum ether:EtOAc, 70:30 v/v and CH_2Cl_2:CH_3OH, 95:5 v/v) and stopped after 15–30 min diluting with dichloromethane (160 mL). The solution was washed with 1 M HCl (80 mL), water (80 mL), $NaHCO_3$ saturated solution (80 mL), and water (2 × 80 mL) in the order and the aqueous phases re-extracted with dichloromethane (2 × 160 mL). The collected organic layers were dried over Na_2SO_4, dried under reduced pressure and the crude residue submitted to silica gel flash chromatography (petroleum ether:EtOAc, from 75:25 to 80:20 v/v). In this way diesters **3a–c** were obtained.

4.1.2.1. 1,3-Di-*O*-octadecanoyl-2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (3a). Reaction time 15 min, yield 80%; mp = 78 °C; $[\alpha]_D^{20} = -2.5$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 6.7 Hz, $2CH_3$), 1.19–1.35 (m, 56H, $28CH_2$), 1.58 (m, 4H, $2CH_2$), 2.28 (m, 4H, $2CH_2$), 3.81 (m, 1H, H-5′), 3.96 (s, 2H, $ClCH_2$), 3.99 (m, 2H, $ClCH_2$), 4.02 (m, 2H, $ClCH_2$), 4.03–4.25 (m, 5H, 2H-1, H-2, and 2H-3), 4.11 (s, 2H, $ClCH_2$), 4.27 (dd, 1H, $J_{6'a,5'}$ = 2.3 Hz, $J_{6'a,6'b}$ = 12.4 Hz, H-6′a), 4.35 (dd, 1H, $J_{6'b,5'}$ = 5.1 Hz, H-6′b), 4.70 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 5.03 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, H-2′), 5.12 (dd, 1H, $J_{3',4'}$ = 9.5 Hz, H-4′), 5.30 (dd, 1H, H-3′); ^{13}C NMR ($CDCl_3$): δ 14.09 ($2CH_3$), 22.67 ($2CH_2$), 24.82 (CH_2), 24.85 (CH_2), 29.00–30.00 ($24CH_2$), 31.90 ($2CH_2$), 34.02 and 34.06 ($2CH_2CO$), 40.13, 40.22, 40.28 and 40.49 (4 CH_2Cl), 62.71 (C1 or C3), 62.74 (C1 or C3), 63.12 (C6′), 69.62 (C4′), 71.34 (C5′), 72.27 (C2′), 73.74 (C3′), 75.90 (C2), 100.17 (C1′), 165.90, 166.23, 166.90 and 167.01 ($4ClCH_2CO$), 173.36 (2CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 1115.6 $[M+Na]^+$, 100%. Calcd for $C_{53}H_{90}O_{14}Cl_4$, m/z 1092.5 [M].

4.1.2.2. 1,3-Di-*O*-dodecanoyl-2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (3b). Reaction time 15 min, yield 88%; mp = 57 °C; $[\alpha]_D^{20} = -2.3$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.84 (t, 6H, J = 6.7 Hz, $2CH_3$), 1.17–1.32 (m, 32H, $16CH_2$), 1.57 (m, 4H, $2CH_2$), 2.27 (m, 4H, $2CH_2$), 3.81 (m, 1H, H-5′), 3.95 (s, 2H, $ClCH_2$), 3.98 (m, 2H, $ClCH_2$), 4.01 (m, 2H, $ClCH_2$), 4.02–4.25 (m, 5H, 2H-1, H-2, and 2H-3), 4.11 (s, 2H, $ClCH_2$), 4.26 (dd, 1H, $J_{6'a,5'}$ = 2.3 Hz, $J_{6'a,6'b}$ = 12.4 Hz, H-6′a), 4.33 (dd, 1H, $J_{6'b,5'}$ = 5.1 Hz, H-6′b), 4.70 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 5.02 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, H-2′), 5.11 (dd, 1H, $J_{3',4'}$ = 9.5 Hz, H-4′), 5.30 (dd, 1H, H-3′); ^{13}C NMR ($CDCl_3$): δ 14.05 ($2CH_3$), 22.62 ($2CH_2$), 24.77 (CH_2), 24.81 (CH_2), 29.00–30.00 ($12CH_2$), 31.85 ($2CH_2$), 33.98 and 34.03 ($2CH_2CO$), 40.11, 40.20, 40.25 and 40.47 (4 CH_2Cl), 62.67 (C1 or C3), 62.70 (C1 or C3), 63.11 (C6′), 69.60 (C4′), 71.29 (C5′), 72.24 (C2′), 73.70 (C3′), 75.86 (C2), 100.12 (C1′), 165.87, 166.21, 166.88 and 167.00 ($4ClCH_2CO$), 173.33 (2CO). ESI-MS (CH_3OH, positive-ion

mode, relative intensity): m/z = 947.2 $[M+Na]^+$, 100%. Calcd for $C_{41}H_{66}O_{14}Cl_4$, m/z 924.3 [M].

4.1.2.3. 1,3-Di-*O*-hexanoyl-2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol (3c). Reaction time 30 min, yield 70%; oil; $[\alpha]_D^{20} = -2.1$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.85 (m, 6H, $2CH_3$), 1.18–1.35 (m, 8H, $4CH_2$), 1.57 (m, 4H, $2CH_2$), 2.27 (m, 4H, $2CH_2$), 3.81 (m, 1H, H-5′), 3.95 (s, 2H, $ClCH_2$), 3.98 (m, 2H, $ClCH_2$), 4.00 (m, 2H, $ClCH_2$), 4.02–4.25 (m, 5H, 2H-1, H-2, and 2H-3), 4.10 (s, 2H, $ClCH_2$), 4.26 (dd, 1H, $J_{6'a,5'}$ = 2.1 Hz, $J_{6'a,6'b}$ = 12.3 Hz, H-6′a), 4.33 (dd, 1H, $J_{6'b,5'}$ = 5.1 Hz, H-6′b), 4.70 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 5.02 (dd, 1H, $J_{2',3'}$ = 9.3 Hz, H-2′), 5.11 (dd, 1H, $J_{3',4'}$ = 9.4 Hz, H-4′), 5.29 (dd, 1H, H-3′); ^{13}C NMR ($CDCl_3$): δ 13.81 ($2CH_3$), 22.21 ($2CH_2$), 24.41 (CH_2), 24.45 (CH_2), 31.18 ($2CH_2$), 33.91 and 33.96 ($2CH_2CO$), 40.12, 40.20, 40.24 and 40.47 (4 CH_2Cl), 62.65 (C1 or C3), 62.69 (C1 or C3), 63.10 (C6′), 69.57 (C4′), 71.24 (C5′), 72.20 (C2′), 73.66 (C3′), 75.83 (C2), 100.07 (C1′), 165.85, 166.19, 166.85 and 166.96 ($4ClCH_2CO$), 173.29 (2CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 779.2 $[M+Na]^+$, 100%. Calcd for $C_{29}H_{42}O_{14}Cl_4$, m/z 756.1 [M].

4.1.3. General procedure for the synthesis of diacylglucosylglycerols 4a–c

Compound **3** (2.16 mmol) was dissolved in EtOAc:CH_3OH (56 mL, 1:1 v/v) and hydrazine acetate (3.0 g, 33 mmol) was added. The reaction was stirred under Ar atmosphere at room temperature for 18 h and monitored by TLC (CH_2Cl_2:CH_3OH, 95:5 and petroleum ether:EtOAc, 70:30 v/v). The solvent was evaporated under reduced pressure and the crude residue subjected to repeated flash column chromatography (CH_2Cl_2:CH_3OH, 95:5 v/v) followed by recrystallization from ethanol to remove hydrazine impurities yielding pure diacylglucosylglycerols **4a–c**.

4.1.3.1. 1,3-Di-*O*-octadecanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (4a). Reaction time 18 h; yield 68%; mp = 133 °C; $[\alpha]_D^{20} = -4.1$ ($CHCl_3$); ^1H NMR ($CDCl_3$:CD_3OD 95:5): δ 0.82 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.16–1.28 (m, 56H, $28CH_2$), 1.56 (m, 4H, $2CH_2$), 2.28 (m, 4H, $2CH_2$), 3.24 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 8.7 Hz, H-2′), 3.28 (m, 1H, H-5′), 3.36–3.45 (m, 2H, H-3′ and H-4′), 3.68 (dd, 1H, $J_{6'a,5'}$ = 5.2 Hz, $J_{6'a,6'b}$ = 12.1 Hz, H-6′a), 3.81 (dd, 1H, $J_{6'b,5'}$ = 3.1 Hz, H-6′b), 3.99 (m, 1H, H-2), 4.13–4.20 (m, 2H, H-1a and H-3a), 4.23 (dd, 1H, $J_{1b,2}$ = 4.9 Hz, $J_{1b,1a}$ = 12.5 Hz, H-1b or H-3b), 4.26 (dd, 1H, $J_{3b,2}$ = 4.0 Hz, $J_{3b,3a}$ = 12.0 Hz, H-3b or H-1b), 4.34 (d, 1H, H-1′); ^{13}C NMR ($CDCl_3$:CD_3OD 95:5): δ 14.02 ($2CH_3$), 22.62 ($2CH_2$), 24.79 ($2CH_2$), 29.00–30.00 ($24CH_2$), 31.86 ($2CH_2$), 34.09 and 34.11 ($2CH_2CO$), 62.12 (C6′), 63.10 (C1 or C3), 63.15 (C1 or C3), 70.01 (C3′), 73.41 (C2′), 75.65 (C2), 75.94 (C5′), 76.06 (C4′), 103.29 (C1′), 173.85 (CO), 174.10 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 809.7 $[M+Na]^+$, 100%. Calcd for $C_{45}H_{86}O_{10}$, m/z 786.6 [M].

4.1.3.2. 1,3-Di-*O*-dodecanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerols (4b). Reaction time 18 h; yield 60%; mp = 110 °C; $[\alpha]_D^{20} = -6.0$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.17–1.33 (m, 32H, $16CH_2$), 1.59 (m, 4H, $2CH_2$), 2.31 (m, 4H, $2CH_2$), 3.32 (m, 1H, H-2′), 3.39 (m, 1H, H-5′), 3.52 (m, 1H, H4′), 3.54 (m, 1H, H-3′), 3.75 (dd, 1H, $J_{6'a,5'}$ = 4.7 Hz, $J_{6'a,6'b}$ = 12.1 Hz, H-6′a), 3.89 (dd, 1H, $J_{6'b,5'}$ = 2.6 Hz, H-6′b), 4.02 (m, 1H, H-2), 4.10–4.20 (m, 2H, H-1a and H-3a), 4.25 (dd, 1H, $J_{1b,2}$ = 5.1 Hz, $J_{1b,1a}$ = 11.6 Hz, H-1b or H-3b), 4.36 (dd, 1H, $J_{3b,2}$ = 3.6 Hz, $J_{3b,3a}$ = 11.8 Hz, H-3b or H-1b), 4.38 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); ^{13}C NMR ($CDCl_3$): δ 14.11 ($2CH_3$), 22.67 ($2CH_2$), 24.84 (CH_2), 24.86 (CH_2), 28.9–29.8 (12 CH_2), 31.90 ($2CH_2$), 34.15 and 34.21 ($2CH_2CO$), 62.58 (C6′), 63.05 (C1 or C3), 63.20 (C1 or C3), 70.43 (C4′), 73.57 (C2′), 75.79 (C5′), 76.13 (C2), 76.27 (C3′), 103.35 (C1′), 173.65 (CO), 174.12 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 641.4 $[M+Na]^+$, 100%. Calcd for $C_{33}H_{62}O_{10}$, m/z 618.4 [M].

4.1.3.3. 1,3-Di-*O*-hexanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerols (4c). Reaction time 18 h; yield 60%; oil; $[\alpha]_D^{20} = -8.9$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.87 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.22–1.35 (m, 8H, $4CH_2$), 1.60 (m, 4H, $2CH_2$), 2.31 (m, 4H, $2CH_2$), 3.30–3.38 (m, 2H, H-2′ and H-5′), 3.47–3.55 (m, 2H, H-3′ and H-4′), 3.76 (dd, 1H, $J_{6'a,5'}$ = 5.0 Hz, $J_{6'a,6'b}$ = 12.0 Hz, H-6′a), 3.86 (dd, 1H, $J_{6'b,5'}$ = 2.8 Hz, H-6′b), 4.03 (m, 1H, H-2), 4.13–4.20 (m, 2H, H-1a and H-3a), 4.26 (dd, 1H, $J_{1b,2}$ = 5.0 Hz, $J_{1b,1a}$ = 11.8 Hz, H-1b or H-3b), 4.31 (dd, 1H, $J_{3b,2}$ = 4.0 Hz, $J_{3b,3a}$ = 11.8 Hz, H-3b or H-1b), 4.39 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H-1′); ^{13}C NMR ($CDCl_3$): δ 13.89 ($2CH_3$), 22.27 ($2CH_2$), 24.51 (CH_2), 24.50 (CH_2), 31.24 ($2CH_2$), 34.07 and 34.12 ($2CH_2CO$), 62.11 (C6′), 63.12 (C1 or C3), 63.14 (C1 or C3), 69.92 (C3′ or C4′), 73.44 (C2′), 75.83 (C2 and C5′), 76.25 (C3′ or C4′), 103.25 (C1′), 173.71 (CO), 174.07 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 473.2 $[M+Na]^+$, 100%. Calcd for $C_{21}H_{38}O_{10}$, m/z 450.2 [M].

4.1.4. General procedure for the synthesis of tosyl derivatives 5a–c

To a solution of compound **4** (1.10 mmol) in dry pyridine (33 mL) at −20 °C, zinc bromide (0.99 g, 4.4 mmol) was added resulting into the formation of white precipitate. After the addition of freshly recrystallized tosyl chloride (1.05 g, 5.5 mmol) reaction mixture turned green and precipitate went on diminishing. The reaction mixture was stirred at −20 °C for about 2 h (longer reaction times reduced the yields) and monitored by TLC (CH_2Cl_2:CH_3OH, 97:3 v/v). After the completion of reaction, CH_3OH (65 mL) was added to quench excess tosyl chloride and the solvent evaporated under vacuum at 35 °C. The residue was dissolved in EtOAc (110 mL) and the organic solution was washed with 1 M HCl (25 mL), H_2O (25 mL), $NaHCO_3$ saturated solution (25 mL), H_2O (2 × 25 mL), and the aqueous phases extracted again with EtOAc (2 × 50 mL). The collected organic layers were dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography using (CH_2Cl_2:CH_3OH, 97:3 or 95:5 v/v) to obtain the desired tosyl derivatives **5a–c**.

4.1.4.1. 1,3-Di-*O*-octadecanoyl-2-*O*-(6-deoxy-6-tosyl-β-D-glucopyranosyl)-*sn*-glycerol (5a). Reaction time 2 h; yield 73%; mp = 90 °C; $[\alpha]_D^{20} = -4.6$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.18–1.33 (m, 56H, $28CH_2$), 1.58 (m, 4H, $2CH_2$), 2.30 (m, 4H, $2CH_2$), 2.42 (s, 3H, CH_3), 3.29 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 9.0 Hz, H-2′), 3.42–3.53 (m, 3H, H-3′, H-4′, H-5′), 4.02 (m, 1H, H-2), 4.06–4.13 (m, 2H, H-1a and H-3a), 4.19 (dd, 1H, $J_{1b,2}$ = 5.3 Hz, $J_{1b,1a}$ = 11.5 Hz, H-1b or H-3b), 4.22 (dd, 1H, $J_{6'a,5'}$ = 4.0 Hz, $J_{6'a,6'b}$ = 10.9 Hz, H-6′a), 4.27 (dd, 1H, $J_{6'b,5'}$ < 1.0 Hz, H-6′b), 4.32 (d, 1H, H-1′), 4.36 (dd, 1H, $J_{3b,2}$ = 3.4 Hz, $J_{3b,3a}$ = 11.9 Hz, H-3b or H-1b), 7.33 (d, 2H, J = 8.3 Hz, Ph), 7.77 (d, 2H, Ph); ^{13}C NMR ($CDCl_3$): δ 14.09 ($2CH_3$), 21.64 ($PhCH_3$), 22.67 ($2CH_2$), 24.85 ($2CH_2$), 28.50–30.10 ($24CH_2$), 31.91 ($2CH_2$), 34.04 and 34.18 ($2CH_2CO$), 62.91 (C1 or C3), 63.00 (C1 or C3), 68.55 (C6′), 69.28 (C4′ or C5′), 73.27 (C2′), 73.66 (C4′ or C5′), 75.95 and 76.07 (C2 and C3′), 103.09 (C1′), 127.98 and 129.92 (4C, Ph), 132.70 and 145.02 (2C, Ph), 173.42 (CO), 174.15 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 963.6 $[M+Na]^+$, 100%. Calcd for $C_{52}H_{92}O_{12}S$, m/z 940.6 [M].

4.1.4.2. 1,3-Di-*O*-dodecanoyl-2-*O*-(6-deoxy-6-tosyl-β-D-glucopyranosyl)-*sn*-glycerol (5b). Reaction time 2 h, yield 70%; mp = 114 °C; $[\alpha]_D^{20} = -9.4$ ($CHCl_3$); ^1H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.18–1.32 (m, 32H, $16CH_2$), 1.58 (m, 4H, $2CH_2$), 2.30 (m, 4H, $2CH_2$), 2.43 (s, 3H, CH_3), 3.29 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 9.0 Hz, H-2′), 3.42–3.53 (m, 3H, H-3′, H-4′, H-5′), 4.02 (m,

1H, H-2), 4.05–4.13 (m, 2H, H-1a and H-3a), 4.19 (dd, 1H, $J_{1b,2}$ = 5.3 Hz, $J_{1b,1a}$ = 11.5 Hz, H-1b or H-3b), 4.22 (dd, 1H, $J_{6'a,5'}$ = 4.0 Hz, $J_{6'a,6'b}$ = 10.9 Hz, H-6′a), 4.27 (dd, 1H, $J_{6'b,5'}$<1.0 Hz, H-6′b), 4.32 (d, 1H, H-1′), 4.37 (dd, 1H, $J_{3b,2}$ = 3.4 Hz, $J_{3b,3a}$ = 11.9 Hz, H-3b or H-1b), 7.33 (d, 2H, J = 8.3 Hz, Ph), 7.77 (d, 2H, Ph); ^{13}C NMR ($CDCl_3$): δ 14.10 ($2CH_3$), 21.65 ($PhCH_3$), 22.67 ($2CH_2$), 24.84 ($2CH_2$), 28.77–29.85 ($12CH_2$), 31.89 ($2CH_2$), 34.04 and 34.18 ($2CH_2CO$), 62.91 (C1 or C3), 63.00 (C1 or C3), 68.53 (C6′), 69.25 (C4′ or C5′), 73.26 (C2′), 73.65 (C4′ or C5′), 75.93 and 76.10 (C2 and C3′), 103.11 (C1′), 127.98 and 129.93 (4C, Ph), 132.70 and 145.05 (2C, Ph), 173.44 (CO), 174.18 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 795.4 $[M+Na]^+$, 100%. Calcd for $C_{40}H_{68}O_{12}S$, m/z 772.4 [M].

4.1.4.3. 1,3-Di-*O*-hexanoyl-2-*O*-(6-deoxy-6-tosyl-β-D-glucopyranosyl)-*sn*-glycerol (5c).

Reaction time 2 h, yield 72%; mp = 137 °C; $[\alpha]_D^{20} = -12.5$ ($CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.20–1.34 (m, 8H, $4CH_2$), 1.58 (m, 4H, $2CH_2$), 2.30 (m, 4H, $2CH_2$), 2.42 (s, 3H, CH_3), 3.29 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 9.0 Hz, H-2′), 3.40–3.54 (m, 3H, H-3′, H-4′, H-5′), 4.02 (m, 1H, H-2), 4.06–4.14 (m, 2H, H-1a and H-3a), 4.19 (dd, 1H, $J_{1b,2}$ = 5.6 Hz, $J_{1b,1a}$ = 11.6 Hz, H-1b or H-3b), 4.21 (dd, 1H, $J_{6'a,5'}$ = 4.6 Hz, $J_{6'a,6'b}$ = 10.7 Hz, H-6′a), 4.26 (dd, 1H, $J_{6'b,5'}$ = 1.2 Hz, H-6′b), 4.36 (d, 1H, H-1′), 4.35 (dd, 1H, $J_{3b,2}$ = 3.7 Hz, $J_{3b,3a}$ = 11.9 Hz, H-3b or H-1b), 7.33 (d, 2H, J = 8.3 Hz, Ph), 7.77 (d, 2H, Ph); ^{13}C NMR ($CDCl_3$): δ 13.87 ($2CH_3$), 21.62 ($PhCH_3$), 22.24 (CH_2), 22.28 (CH_2), 24.49 ($2CH_2$), 31.20 (CH_2), 31.23 (CH_2), 33.98 and 34.11 ($2CH_2CO$), 62.87 (C1 or C3), 63.01 (C1 or C3), 68.67 (C6′), 69.28 (C4′ or C5′), 73.26 (C2′), 73.62 (C4′ or C5′), 75.92 (C3′ and C2), 102.97 (C1′), 127.96 and 129.92 (4C, Ph), 132.64 and 145.02 (2C, Ph), 173.45 (CO), 174.15 (CO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 627.3 $[M+Na]^+$, 100%. Calcd for $C_{28}H_{44}O_{12}S$, m/z 604.3 [M].

4.1.5. General procedure for the synthesis of thioacetates 6a–c

Compound **5** (0.74 mmol) was dissolved in dry DMF (14 mL) and potassium thioacetate (0.382 g, 3.35 mmol) was added. The mixture was stirred under Ar at room temperature and the changing of its color from blue to brown indicated the progress of reaction that was monitored by TLC (CH_2Cl_2:CH_3OH, 97:3 v/v). Owing to co-elution of the starting and target compound, the reaction was stopped after 24 h when the TLC spot color turned completely from green to brown (anisaldehyde based reagent). DMF was then co-evaporated with cyclohexane at reduced pressure at 45 °C and the following flash column chromatography (CH_2Cl_2:CH_3OH, 97:3 v/v) of the crude residue yielded the desired derivative **6**.

4.1.5.1. 1,3-Di-*O*-octadecanoyl-2-*O*-(6-deoxy-6-thioacetyl-β-D-glucopyranosyl)-*sn*-glycerol (6a).

Reaction time 24 h; yield 70%; brown amorphous solid; $[\alpha]_D^{20} = -27.5$ ($CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.18–1.33 (m, 56H, $28CH_2$), 1.59 (m, 4H, $2CH_2$), 2.31 (m, 4H, $2CH_2$), 2.37 (s, 3H, –$SCOCH_3$), 3.17 (dd, 1H, $J_{6'a,5'}$ = 3.4 Hz, $J_{6'a,6'b}$ = 14.5 Hz, H-6′a), 3.26 (dd, 1H, $J_{3',4'}$ = 9.0 Hz, $J_{4',5'}$ = 9.0 Hz, H-4′), 3.33 (dd, 1H, $J_{1',2'}$ = 7.6 Hz, $J_{2',3'}$ = 9.0 Hz, H-2′), 3.37 (dd, 1H, $J_{6'b,5'}$ = 4.4 Hz, H-6′b), 3.47 (m, 1H, H-5′), 3.54 (dd, 1H, H-3′), 4.02 (m, 1H, H-2), 4.07–4.17 (m, 2H, H-1a and H-3a), 4.21 (dd, 1H, $J_{1b,2}$ = 5.5 Hz, $J_{1b,1a}$ = 11.6 Hz, H-1b or H-3b), 4.36 (m, 2H, H-1′ and H-3b or H-1b); ^{13}C NMR ($CDCl_3$): δ 14.11 ($2CH_3$), 22.68 ($2CH_2$), 24.85 ($2CH_2$), 29.00–30.00 ($24CH_2$), 30.50 ($SCOCH_3$), 30.62 (C6′), 31.92 ($2CH_2$), 34.12 and 34.17 ($2CH_2CO$), 63.07 (C1 or C3), 63.14 (C1 or C3), 71.14 (C4′), 73.38 (C2′), 74.06 (C5′), 75.21 (C3′), 76.09 (C2), 103.15 (C1′), 173.40 (CO), 174.00 (CO), 198.56 (–SCO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 867.6 $[M+Na]^+$, 100%. Calcd for $C_{47}H_{88}O_{10}S$, m/z 844.6 [M].

4.1.5.2. 1,3-Di-*O*-dodecanoyl-2-*O*-(6-deoxy-6-thioacetyl-β-D-glucopyranosyl)-*sn*-glycerol (6b).

Reaction time 24 h; yield 71%; brown amorphous solid; $[\alpha]_D^{20} = -39.0$ ($CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.86 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.18–1.33 (m, 32H, $16CH_2$), 1.59 (m, 4H, $2CH_2$), 2.31 (m, 4H, $2CH_2$), 2.37 (s, 3H, –$SCOCH_3$), 3.16 (dd, 1H, $J_{6'a,5'}$ = 3.4 Hz, $J_{6'a,6'b}$ = 14.6 Hz, H-6′a), 3.26 (dd, 1H, $J_{3',4'}$ = 9.3 Hz, $J_{4',5'}$ = 9.3 Hz, H-4′), 3.33 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 9.3 Hz, H-2′), 3.37 (dd, 1H, $J_{6'b,5'}$ = 4.4 Hz, H-6′b), 3.47 (m, 1H, H-5′), 3.54 (dd, 1H, H-3′), 4.02 (m, 1H, H-2), 4.07–4.16 (m, 2H, H-1a and H-3a), 4.21 (dd, 1H, $J_{1b,2}$ = 5.5 Hz, $J_{1b,1a}$ = 11.6 Hz, H-1b or H-3b), 4.36 (d, 1H, H-1′), 4.37 (dd, 1H, $J_{3b,2}$ = 3.6 Hz, $J_{3b,3a}$ = 11.9 Hz, H-3b or H-1b); ^{13}C NMR ($CDCl_3$): δ 14.11 ($2CH_3$), 22.67 ($2CH_2$), 24.85 ($2CH_2$), 29.00–30.00 ($12CH_2$), 30.46 ($SCOCH_3$), 30.63 (C6′), 31.90 ($2CH_2$), 34.12 and 34.17 ($2CH_2CO$), 63.06 (C1 or C3), 63.14 (C1 or C3), 71.16 (C4′), 73.37 (C2′), 74.05 (C5′), 75.21 (C3′), 76.07 (C2), 103.12 (C1′), 173.40 (CO), 174.00 (CO), 198.52 (–SCO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 699.5 $[M+Na]^+$, 100%. Calcd for $C_{35}H_{64}O_{10}S$, m/z 676.4 [M].

4.1.5.3. 1,3-Di-*O*-hexanoyl-2-*O*-(6-deoxy-6-thioacetyl-β-D-glucopyranosyl)-*sn*-glycerol (6c).

Reaction time 24 h; yield 69%; brown oil; $[\alpha]_D^{20} = -34.3$ ($CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.22–1.35 (m, 8H, $4CH_2$), 1.60 (m, 4H, $2CH_2$), 2.31 (m, 4H, $2CH_2$), 2.36 (s, 3H, –$SCOCH_3$), 3.19 (dd, 1H, $J_{6'a,5'}$ = 3.3 Hz, $J_{6'a,6'b}$ = 14.5 Hz, H-6′a), 3.26 (dd, 1H, $J_{3',4'}$ = 9.1 Hz, $J_{4',5'}$ = 9.2 Hz, H-4′), 3.33 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 9.2 Hz, H-2′), 3.34 (dd, 1H, $J_{6'b,5'}$ = 4.7 Hz, H-6′b), 3.45 (m, 1H, H-5′), 3.53 (dd, 1H, H-3′), 4.02 (m, 1H, H-2), 4.08–4.16 (m, 2H, H-1a and H-3a), 4.22 (dd, 1H, $J_{1b,2}$ = 5.5 Hz, $J_{1b,1a}$ = 11.6 Hz, H-1b or H-3b), 4.35 (dd, 1H, $J_{3b,2}$ = 3.7 Hz, $J_{3b,3a}$ = 11.9 Hz, H-3b or H-1b), 4.36 (d, 1H, H-1′); ^{13}C NMR ($CDCl_3$): δ 13.87 (CH_3), 13.89 (CH_3), 22.26 (CH_2), 22.29 (CH_2), 24.51 ($2CH_2$), 30.45 ($SCOCH_3$), 30.65 (C6′), 31.22 (CH_2), 31.27 (CH_2), 34.07 and 34.11 ($2CH_2CO$), 63.06 (C1 or C3), 63.15 (C1 or C3), 71.30 (C4′), 73.39 (C2′), 74.10 (C5′), 75.28 (C3′), 76.00 (C2), 103.09 (C1′), 173.42 (CO), 174.00 (CO), 198.35 (–SCO). ESI-MS (CH_3OH, positive-ion mode, relative intensity): m/z = 531.2 $[M+Na]^+$, 100%. Calcd for $C_{23}H_{40}O_{10}S$, m/z 508.2 [M].

4.1.6. General procedure for the synthesis of sulfonates 1a–c

To a solution of compound **6** (0.37 mmol) in glacial acetic acid (18 mL), potassium monopersulfate triple salt (OXONE®) (0.68 g, 1.1 mmol) and potassium acetate (1.50 g, 15.3 mmol) were added in the order. The suspension was stirred at room temperature and the reaction was monitored by TLC ($CHCl_3$:CH_3OH:H_2O, 65:25:4). After disappearing of the starting, the solvent was evaporated under vacuum, the residue diluted with water (36 mL) and extracted with $CHCl_3$:CH_3OH, (80:20 v/v) (5 × 36 mL). The combined organic layers were washed with NaCl solution (2 × 70 mL). The water layers were then extracted several times following the same procedure checking by TLC the presence of residual target compound. All collected organic layers were reduced to a convenient volume and submitted to centrifugation at 4500 rpm for 10 min. Water layer was gently separated by sharp dropper, and the organic phase concentrated under reduced pressure. The obtained crude was purified by flash column chromatography (from $CHCl_3$:CH_3OH, 80:20 v/v to $CHCl_3$:CH_3OH:H_2O, 65:25:4 v/v) to yield the pure sulfonate **1**.

4.1.6.1. 1,3-Di-*O*-octadecanoyl-2-*O*-β-D-sulfoquinovopyranosyl-*sn*-glycerol sodium salt (1a).

Reaction time 6 h, yield 40%; white amorphous solid; $[\alpha]_D^{20} = +1.2$ ($CHCl_3$:CH_3OH:H_2O, 65:25:4). ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O, 65:25:4, 312 K): δ 0.84 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.15–1.32 (m, 56H, $28CH_2$), 1.56 (m, 4H, $2CH_2$), 2.28 (m, 4H, $2CH_2$), 3.03 (dd, 1H, $J_{6'a,5'}$ = 7.0 Hz, $J_{6'a,6'b}$ = 14.5 Hz, H-6′a), 3.18–3.25 (m, 2H, H-2′ and H-4′), 3.28 (dd, 1H, $J_{6'b,5'}$ = 3.5 Hz, H-6′b), 3.39 (dd, 1H, $J_{2',3'}$ = 9.1 Hz, $J_{3',4'}$ = 9.1 Hz, H-3′), 3.70 (m, 1H,

H-5′), 4.13–4.31 (m, 5H, 2H-1, 2H-3 and H-2), 4.41 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′); ^{13}C NMR ($CDCl_3$:CD_3OD: D_2O 65:25:4, 312 K): δ 14.26 ($2CH_3$), 23.00 ($2CH_2$), 25.22 (CH_2), 25.24 (CH_2), 29.40–30.20 ($24CH_2$), 32.28 ($2CH_2$), 34.47 and 34.50 ($2CH_2CO$), 53.85 (C6′), 62.96 (C1 or C3), 63.94 (C1 or C3), 72.75 (C5′), 73.72 (C2′ or C4′), 73.84 (C2′ or C4′), 75.32 (C2), 76.43 (C3′), 103.15 (C1′), 174.74 (CO), 174.86 (CO). ESI-MS (CH_3OH, negative-ion mode, relative intensity): m/z = 849.9 $[M]^-$, 100%. Calcd for $C_{45}H_{85}O_{12}S^-$, m/z 849.6 $[M]^-$.

4.1.6.2. 1,3-Di-*O*-dodecanoyl-2-*O*-β-D-sulfoquinovopyranosyl-*sn*-glycerol sodium salt (1b). Reaction time 7 h, yield 42%; white amorphous solid; $[\alpha]_D^{20} = +1.3$ ($CHCl_3$:CH_3OH:H_2O, 65:25:4). ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O 65:25:4, 312 K): δ 0.84 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.16–1.31 (m, 32H, $16CH_2$), 1.56 (m, 4H, $2CH_2$), 2.29 (m, 4H, $2CH_2$), 3.03 (dd, 1H, $J_{6'a,5'}$ = 7.0 Hz, $J_{6'a,6'b}$ = 14.5 Hz, H-6′a), 3.18–3.25 (m, 2H, H-2′ and H-4′), 3.27 (dd, 1H, $J_{6'b,5'}$ = 3.2 Hz, H-6′b), 3.39 (dd, 1H, $J_{2',3'}$ = 9.1 Hz, $J_{3',4'}$ = 9.1 Hz, H-3′), 3.69 (m, 1H, H-5′), 4.13–4.31 (m, 5H, 2H-1, 2H-3 and H-2), 4.41 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′); ^{13}C NMR ($CDCl_3$:CD_3OD: D_2O 65:25:4, 312 K): δ 14.33 ($2CH_3$), 23.04 ($2CH_2$), 25.23 ($2CH_2$), 29.20–30.20 ($12CH_2$), 32.29 ($2CH_2$), 34.44 and 34.49 ($2CH_2CO$), 53.59 (C6′), 62.86 (C1 or C3), 63.90 (C1 or C3), 72.61 (C5′), 73.47 (C2′ or C4′), 73.73 (C2′ or C4′), 75.36 (C2), 76.24 (C3′), 103.18 (C1′), 174.82 (CO), 174.90 (CO). ESI-MS (CH_3OH, negative-ion mode, relative intensity): m/z = 681.6 $[M]^-$, 100%. Calcd for $C_{33}H_{61}O_{12}S^-$, m/z 681.4 $[M]^-$.

4.1.6.3. 1,3-Di-*O*-hexanoyl-2-*O*-β-D-sulfoquinovopyranosyl-*sn*-glycerol sodium salt (1c). Reaction time 6 h, yield 38%; white amorphous solid; $[\alpha]_D^{20} = +3.7$ ($CHCl_3$:CH_3OH:H_2O, 65:25:4). ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O 65:25:4, 312 K): δ 0.85 (t, 6H, J = 7.0 Hz, $2CH_3$), 1.21–1.32 (m, 8H, $4CH_2$), 1.57 (m, 4H, $2CH_2$), 2.29 (m, 4H, $2CH_2$), 3.03 (dd, 1H, $J_{6'a,5'}$ = 7.1 Hz, $J_{6'a,6'b}$ = 14.5 Hz, H-6′a), 3.18–3.25 (m, 2H, H-2′ and H-4′), 3.28 (dd, 1H, $J_{6'b,5'}$ = 3.5 Hz, H-6′b), 3.39 (dd, 1H, $J_{2',3'}$ = 9.1 Hz, $J_{3',4'}$ = 9.1 Hz, H-3′), 3.70 (m, 1H, H-5′), 4.13–4.30 (m, 5H, 2H-1, 2H-3 and H-2), 4.41 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′); ^{13}C NMR ($CDCl_3$:CD_3OD:D_2O 65:25:4, 312 K): δ 13.98 ($2CH_3$), 22.56 ($2CH_2$), 24.82 ($2CH_2$), 31.58 ($2CH_2$), 34.39 and 34.41 ($2CH_2CO$), 53.74 (C6′), 63.01 (C1 or C3), 63.98 (C1 or C3), 72.71 (C5′), 73.62 (C2′ or C4′), 73.82 (C2′ or C4′), 75.34 (C2), 76.38 (C3′), 103.13 (C1′), 174.83 (CO), 174.92 (CO). ESI-MS (CH_3OH, negative-ion mode, relative intensity): m/z = 513.3 $[M]^-$, 100%. Calcd for $C_{21}H_{37}O_{12}S^-$, m/z 513.2 $[M]^-$.

4.2. Biological methods

4.2.1. 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 (obtained first from Professor G. Klein, Karolinska Institute, Stockholm, Sweden) cultivated in RPMI 1640 medium containing 10% fetal calf serum, and induced by TPA as described previously.[6,12] Raji cells (1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of a medium containing *n*-butyric acid (4 mM), 32 pmol of TPA in DMSO, and a known amount of the test compound in DMSO. The cells were stained by high titer EBV-positive sera from nasopharyngeal carcinoma patients and fluorescein-isothiocyanate-labeled anti-human IgG. After staining, they were detected by a conventional indirect immunofluorescence technique. The assays were performed in triplicate for each compound in which at least 500 cells were counted. 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 typically around 30%. The viability of the cells was assayed against treated cells using the Trypan Blue staining method. For an accurate determination of cytotoxicity, the cell viability was required to be more than 60% 2 days after treatment with the compounds.

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Supplementary data

Copies of ^{1}H NMR and ^{13}C NMR spectra of the target compounds **1a–1c** and **4a–4c** are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.06.064.

References and notes

1. Benson, A. A.; Daniel, H.; Wiser, R. *Proc. Natl. Acc. Sci.* **1959**, *45*, 1582.
2. Benning, C. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 53.
3. Sugimoto, K.; Midorikawa, T.; Tsuzuki, M.; Sato, N. *Biochem. Biophys. Res. Commun.* **2008**, *369*, 660.
4. Sakaguchi, K.; Sugawara, F. *Curr. Drug Ther.* **2008**, *3*, 44.
5. Mori, Y.; Sahara, H.; Matsumoto, K.; Takahashi, N.; Yamazaki, T.; Ohta, K.; Aoki, S.; Miura, M.; Sugawara, F.; Sakaguchi, K.; Sato, N. *Cancer Sci.* **2008**, *99*, 1063.
6. Shirahashi, H.; Murakami, N.; Watanabe, M.; Nagatsu, A.; Sakakibara, J.; Tokuda, H.; Nishino, H.; Iwashima, A. *Chem. Pharm. Bull.* **1993**, *41*, 1664.
7. Colombo, D.; Franchini, L.; Toma, L.; Tanaka, R.; Ronchetti, F.; Takayasu, J.; Nishino, H.; Tokuda, H. *Eur. J. Med. Chem.* **2006**, *41*, 1456. and references herein reported.
8. Colombo, D.; Compostella, F.; Ronchetti, F.; Scala, A.; Toma, L.; Tokuda, H.; Nishino, H. *Bioorg. Med. Chem.* **1999**, *7*, 1867.
9. Yamamura, H.; Kawasaki, J.; Saito, H.; Araki, S.; Kawai, M. *Chem. Lett.* **2001**, 706.
10. Hanashima, S.; Mizushina, Y.; Yamazaki, T.; Otha, K.; Takahashi, S.; Sahara, H.; Sakaguchi, K.; Sugawara, F. *Bioorg. Med. Chem* **2001**, *9*, 367.
11. Murakami, A.; Ohigashi, H.; Koshimizu, K. *Food Rev. Int.* **1999**, *15*, 335.
12. Tokuda, H.; Konoshima, T.; Kozuka, M.; Kimura, T. *Cancer Lett.* **1988**, *40*, 309.
13. Colombo, D.; Compostella, F.; Ronchetti, F.; Scala, A.; Tokuda, H.; Nishino, H. *Eur. J. Med. Chem.* **2001**, *36*, 691.
14. Colombo, D.; Ronchetti, F.; Scala, A.; Toma, L.; Tokuda, H.; Nishino, H. *Bioorg. Med. Chem.* **2003**, *11*, 909.