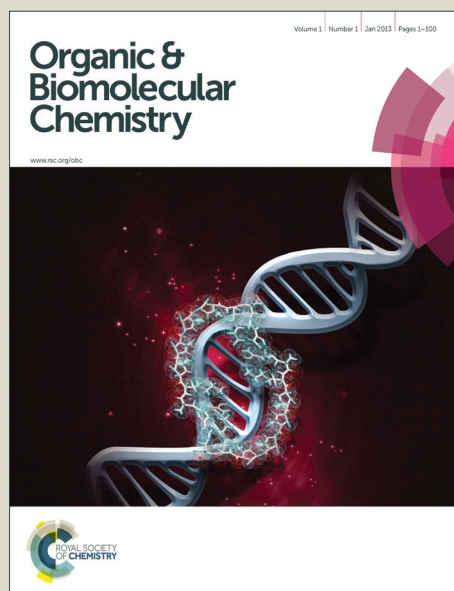


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ARTICLE TYPE

Anionic glycolipids related to glucuronosyldiacylglycerol inhibit protein kinase Akt

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New glucuronosyldiacylglycerol (GlcADG) analogues based on a 2-*O*-β-D-glucopyranosyl-*sn*-glycerol scaffold and carrying one or two acyl chains of different lengths have been synthesized as phosphatidylinositol 3-phosphate (PI3P) mimics targeting the protein kinase Akt. Akt inhibitory effect of prepared compounds, was assayed using an *in vitro* kinase assay. The antiproliferative activity of the compounds was tested in the human ovarian carcinoma IGROV-1 cell line in which we found that two of them could inhibit proliferation, in keeping with the target inhibitory effect.

Introduction

Glucuronosyldiacylglycerols (GlcADGs, figure 1) are unusual acidic glycolipids found in bacteria,¹ fungi,² algae³ and in some higher plants such as *Arabidopsis thaliana* and rice,⁴ and only very recently their first synthesis has been reported.⁵ Their presence in these photosynthetic organisms seems related to conditions of reduced phosphorous availability in which they replace phospholipids for their biological functions.^{4,6} GlcADGs participate to this lipid remodelling as the better-known anionic sulfolipids, sulfoquinovosyldiacylglycerols (SQDGs, figure 1),^{7,8} sharing also the same biosynthetic pathway, which requires a common SQDG synthase.⁴ Differently from GlcADGs, in the last years both natural and synthetic sulfoquinovosyldiacylglycerols have been tested for their antitumor, antiviral, anti-inflammatory, immunosuppressive and other bioactivities.⁹⁻¹³ This prompted us to prepare some SQDG analogues (figure 1) based on 2-*O*-β-D-glucosyldiacylglycerol, which were tested as inhibitors of tumor-promoters in cancer prevention studies.^{14,15} These unnatural sulfolipids, similarly to other glucose-based compounds,¹⁶ display a structure which can be related to that of 3-phosphorylated phosphatidylinositol (PI3P, figure 1), one of the natural phospholipids involved in the regulation of specific lipid-binding domains (e.g. PH, FYVE or PX) in phosphatidylinositol-3-kinase (PI3K) effector proteins.^{17a-d} So they are currently investigated as potential inhibitors of protein kinase B (PKB or Akt),^{18a,b} a kinase involved in sustaining multiple aggressiveness features of tumor cells such as invasion capability and reduced sensitivity to antitumor agents.¹⁹ PI3P and the other 3-phosphorylated phosphatidylinositols *i.e.* PI(3,4,5)P₃ and PI(3,4)P₂, are generated by PI3K and, at the plasma membrane, can bind the pleckstrin homology (PH) domain promoting kinase activation.^{17a,20} Many proteins with PH domain are known, but only few of them are regulated by the direct binding of 3-phosphoinositides.^{17a} For this reason, even if different types of small molecules have been synthesized as Akt inhibitors (e.g. ATP and allosteric inhibitors),

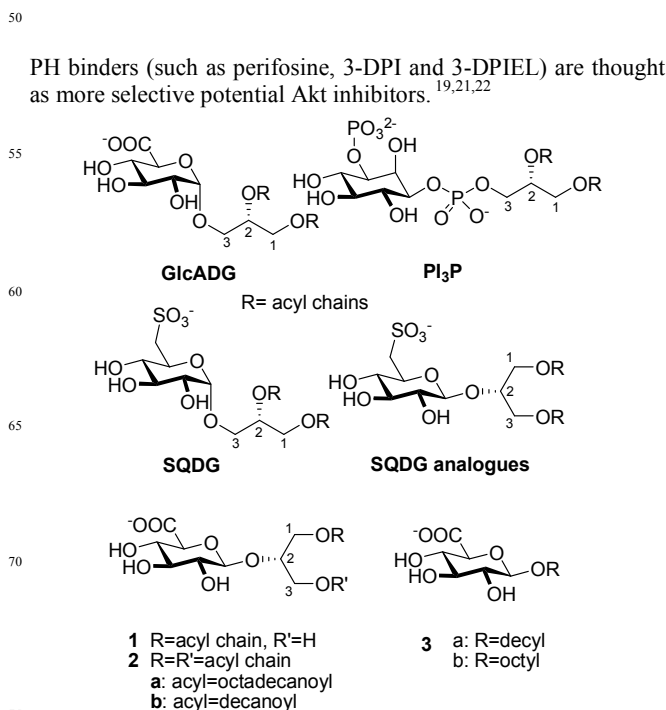


Fig. 1 Structures of PI3P, related natural anionic glycolipids and synthetic analogues.

Here we describe the synthesis of the new glucuronosyldiacylglycerol (GlcADG) analogues **1a,b** and **2a,b**, carrying acyl chains which differ in lengths and number (figure 1) as PI3P analogues that could target the Akt pleckstrin homology domain. They maintain the same 2-*O*-β-D-glucopyranosyl-*sn*-glycerol scaffold of SQDG analogues but a diverse anionic group is installed at position 6 of the sugar moiety (COO⁻ vs SO₃⁻) for the interaction with the PI3P binding pocket of PH domain,

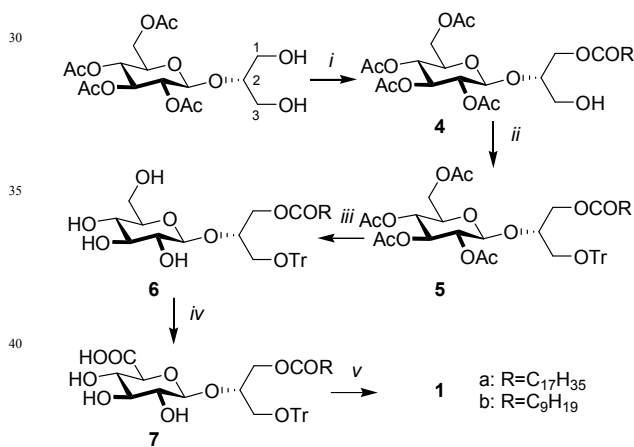
which is characterized by positively charged residues.²² At the same time the glucuronides **3a,b** (Fig. 1) have been prepared as simplified anionic models for binding the PH domain. The biological activity of selected compounds is also presented as inhibitory activity against Akt, both in an isolated enzyme system and in an ovarian carcinoma cell line.

Results and discussion

Chemistry

10 Synthesis of monoesters **1a,b**

Compounds **1a,b** were efficiently prepared starting from the known 2-*O*-(2',3',4',6'-tetra-*O*-acetyl- β -D-glucopyranosyl)-*sn*-glycerol^{23a,b} (Scheme 1). Its *Pseudomonas cepacia* lipase (LPS) mediated transesterification in organic solvent, by means of a procedure already used for similar substrates employing 2,2,2-trifluoroethyl (TFE) esters as acyl carriers,²⁴ allowed the selective introduction of the desired acyl chain (octadecanoyl or decanoyl) at C-1 of the *sn*-glycerol moiety in good yields. The configurations of the obtained **4a,b** were confirmed by chemical correlation with known compounds²⁴ (*vide infra*). After conventional tritylation of the remaining primary free hydroxyl, the obtained fully protected glucosylglycerols **5a,b** were converted with reasonable yields into the fully deacetylated compound **6a,b** by treatment with hydrazine hydrate in aqueous ethanol for the selective removal of the sugar acetlys.²⁵ Selective TEMPO oxidation of the glucose primary hydroxyl group yielded **7a,b** which were finally transformed into the desired compounds **1a,b** by treatment with an acidic resin in dichloromethane.



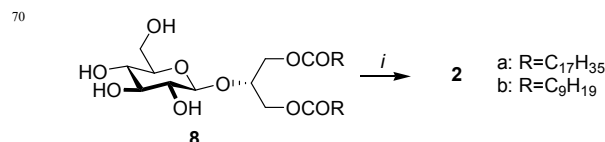
Scheme 1 *i.* *Pseudomonas cepacia* lipase (Amano PS), Py, TFE-octadecanoate or -decanoate, 45°C, (74-82%); *ii.* Ph₃CCl, Py, 100°C, (70-97%); *iii.* Hydrazine hydrate, EtOH aq. (45-56%); *iv.* TEMPO, NaClO/NaClO₂, CH₃CN, 0.67M phosphate buffer (pH 6.7), RT, (97-98%); *v.* DOWEX H⁺, CH₂Cl₂ (76-81%).

50 Configuration assignment of compounds **4a,b**

Compound **4a** was transformed into the 3-*O*-acetyl derivative by acetic anhydride/pyridine treatment. The obtained compound was identical to the known²⁴ 1-*O*-octadecanoyl-3-*O*-acetyl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl- β -D-glucopyranosyl)-*sn*-glycerol, confirming the 2*S* configuration of compound **4a**. As the enzymatic transesterifications employed to obtain the monoesters **4a** and **4b** differed just in the acyl carrier used, only the configuration of compound **4a** was assigned, assuming the same 2*S* configuration also for compound **4b**.

Synthesis of diesters **2a,b**

The glucuronide diester **2a** was obtained^{14b} by regioselective TEMPO oxidation of the primary hydroxyl of the known^{14a} 1,3-di-*O*-octadecanoyl-2-*O*- β -D-glucopyranosyl-*sn*-glycerol **8a**. Similarly, the didecanoate **8b**, obtained from the known 2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl- β -D-glucopyranosyl)-*sn*-glycerol^{14c} (see experimental), was efficiently oxidized to **2b** (Scheme 2).



Scheme 2 *i.* TEMPO, NaClO/NaClO₂, CH₃CN, 0.67M phosphate buffer (pH 6.7), RT, (43-89%).

Synthesis of glucuronides **3a,b**

Known glucuronides **3a**²⁶ and **3b**²⁷ (see ESI) were obtained in good yields by regioselective TEMPO oxidation²⁶ of decyl β -D-glucopyranoside²⁸ or commercial octyl β -D-glucopyranoside, respectively.

85 Cell free evaluation of Akt inhibition

The prepared glucuronides, with the exception of compound **2a** that was not soluble in DMSO, were tested for *in vitro* inhibitory activity against Akt (Akt1), using an *in vitro* ELISA kinase assay.

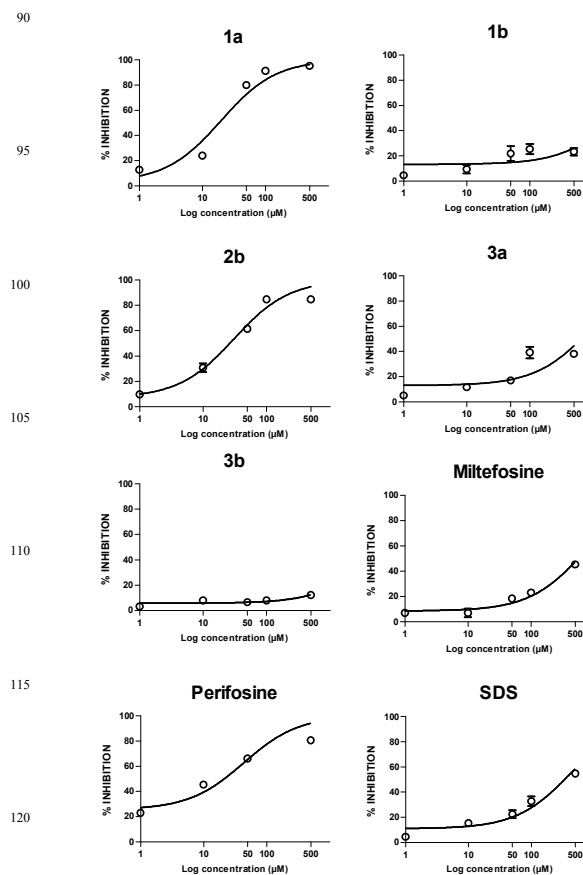


Fig. 2 *In vitro* PKB1 Kinase Assay (ELISA) of compounds **1a,b**, **2b**, **3a,b**, miltefosine, perifosine and SDS at the indicated concentrations. Concentration-response (%inhibition) analysis.

Fig. 2 shows the effect of compounds **1a-b**, **2b**, **3a-b**, tested at five different (1, 10, 50, 100 and 500 μM) concentrations compared to the alkylphospholipids (ALPs) miltefosine (general inhibitor of the PI3K/Akt pathway) and perifosine (Akt inhibitor targeting the PH domain)²⁹ and the surfactant sodium dodecyl sulfate (SDS).

The results show that the Akt1 activity is poorly influenced by compound **3b**, while the other compounds showed a concentration-dependent inhibitory effect, **1a** and **2b** being the most potent inhibitors of Akt1 activity (IC_{50} = 19.71 μM and 30.75 μM respectively, Table 1). Compounds **1b** and **3a** elicited a concentration-dependent inhibition of Akt1, however they displayed only slight efficacy, even at the maximum concentration: 24% and 39%, respectively. The IC_{50} values (Table 1) and the maximal efficacy indicated that the effect is higher for long than for short acyl chains (**1a** vs **1b** and **3a** vs **3b**) and for the presence of a second acyl chain (**1b** vs **2b**, Table 1). These results are in agreement with other data by our group.¹⁸

The data obtained (Table 1 and Figure 2) for Miltefosine as well as SDS didn't exhibit any significant inhibition of Akt in the ELISA test (only a very weak inhibition was observed at very high concentrations, i.e. 500 μM). On the contrary, Perifosine displayed a significant inhibition with an IC_{50} value of about 40 μM , thus suggesting a specificity for the effect induced by glucuronides **1a** and **2b** and a potency that is comparable with that of Perifosine.

Table 1 Compound concentration producing 50% inhibition of PKB1 activity in ELISA assay.

Compound	IC_{50} (μM)	95% Confidence Intervals
1a	19.71	13.85-28.04
1b	>100	
2b	30.75	22.91-41.26
3a	>100	
3b	>100	
Miltefosine	>100	
Perifosine	43.35	22.65-83.17
SDS	>100	

Cellular studies

The antiproliferative activity of the glucuronide compounds was examined in the IGROV-1 ovarian carcinoma cell line which is characterized by heterozygous mutation (Het c.955_958delACTT) of the dual-specificity phosphatase PTEN, a negative regulator of Akt. A 72 h exposure to the compounds resulted in a concentration-dependent inhibition of IGROV-1 cell growth for three of the novel glucuronides (Fig. 3). In particular, the inhibitory effect was evident for compound **1a** and **2b**. In fact the IC_{50} values ($\pm\text{SD}$) were 49.0 ± 7.6 and 156.7 ± 23.0 (SD) μM , respectively. The **3a** and **3b** compounds induced a slight inhibition of IGROV-1 cell growth after 72 h exposure only when a concentration of 300 μM was used. A modest inhibition of proliferation was also observed when cells were exposed to the **1b** compound, the IC_{50} value being around 300 μM . Since the **3a** and **3b** compounds do not contain glycerol in their structure, such data suggest that the presence of glycerol might facilitate growth inhibition (**1b** vs **3a**). Moreover, long chains seem to enhance the activity because the **1a** compound carries a longer chain than **1b**. Further effort is required to optimize the growth inhibition properties of this class of compounds. Indeed, an analysis of inhibition of cell growth in cells exposed for 24 h to the studied compounds in serum-free medium indicated an increased potency for the two active compounds. Under these conditions, the IC_{50} values ($\pm\text{SD}$) of compound **1a** and **2b** were 3.35 ± 0.35 and 9.40

± 5.0 μM , respectively. This evidence raises the possibility that binding of the compounds to serum components can reduce their cellular uptake or affect their stability.

To confirm the influence of serum presence in medium on cellular sensitivity to AKT inhibitors, we also tested the sensitivity of IGROV-1 cells to Perifosine in the presence or absence of serum in the medium (Table 2). Under our experimental conditions, we found that the growth inhibitory effect of the compound was favored in serum-free medium, thus implying that serum affects the stability of Perifosine. However, whereas for Perifosine a 2.8 fold increase in the anti-proliferative activity was observed upon incubation in serum-free medium, the fold-change for the novel compounds was higher, implying that they might be less stable or less prone to accumulate in the cells than perifosine in the presence of serum.

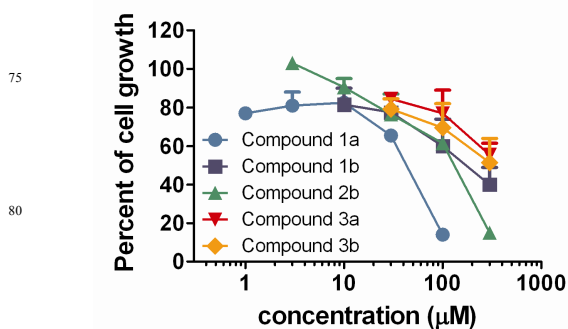


Fig. 3 Cell sensitivity of human ovarian carcinoma cells (IGROV-1) as evaluated by growth inhibition assays. Cells were seeded and 24 h later they were exposed to the novel compounds for 72 h. At the end of incubation with the compounds, cells were counted with a cell counter. Results from a representative experiment are shown.

Table 2. Sensitivity of IGROV-1 ovarian carcinoma cells to perifosine in the presence of serum or in serum-free medium.^a

FBS	IC_{50} (μM); $\pm\text{SD}$
+	2.21 ± 0.69
-	0.80 ± 0.29

^a Cell sensitivity was assessed by growth inhibition assays in which cells were exposed to perifosine for 72 h in serum (FBS)-containing medium or for 24 h in serum-free medium. Cells were counted 72 h after treatment start. IC_{50} represents the perifosine concentration producing 50% inhibition of cell growth. The reported values are the mean \pm standard deviation of 3 independent experiments.

Conclusions

New glucuronosyldiacylglycerols were prepared and their inhibitory activity against Akt, both in an isolated enzyme system and in an ovarian carcinoma cell line, was tested.

The two compounds exhibiting the best target inhibition activity in cell-free assay, i.e. compounds **1a** and **2b**, were found to be endowed with antiproliferative activity in ovarian carcinoma cells. Although inhibition of proliferation was observed, further efforts are needed to increase the potency of the compounds in *in vitro* cultured cells. Overall, our data by showing a clear modulation of Akt inhibition in relation to chain length and number, and also to the presence of glycerol in cell experiments, provide insights into the understanding of the structural features needed to achieve Akt inhibition.

Experimental

Chemical procedures

Materials and equipments

Pseudomonas cepacia lipase (LPS, lipase PS, specific activity 30.5 triacetin units/mg solid), from Amano Pharmaceutical Co. (Mitsubishi Italia), was supported on celite.²⁴ 2-*O*-(2',3',4',6'-Tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol,^{23a,b} 2-*O*-(2',3',4',6'-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol,^{14c} 1,3-di-*O*-octadecanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (8a),^{14a} decyl β-D-glucuronopyranoside (3a),²⁶ octyl β-D-glucuronopyranoside (3b)^{26,27} and the acyl carriers (trifluoroethyldecanoate and -octadecanoate)²⁴ were synthesized according to literature procedures. The acidic Dowex[®] Marathon[™] C sodium form resin was activated by washing it with 1M HCl and with distilled water prior to use. Optical rotations were determined on a Perkin–Elmer 241 polarimeter 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) or by a Biotage Isolera[™] Prime flash purification system (Biotage-Uppsala, Sweden). TLC analysis was carried out on silica gel plate (Merck 60F254) 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 ¹³C 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 ¹³C NMR spectra at 125.76 MHz were done for 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 CHCl₃, CH₃OD or pyridine fixed at 7.26, 3.30 ppm and 7.19 ppm (higher field signal), respectively, for ¹H NMR spectra and relative to CDCl₃ fixed at 77.0 ppm (central line), CD₃OD at 49.0 ppm (central line) or pyridine at 123.0 ppm (higher field signal, central line) for ¹³C 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 Finnigan 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. ¹H and ¹³C NMR and MS analysis confirmed purity and identity of all synthesized compounds.

Chemistry

Synthesis of monoesters 1a and 1b

1-*O*-Acyl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerols (4a,b)
2-*O*-(2',3',4',6'-Tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol^{23a,b} (0.50 g, 1.18 mmol) was dissolved in dry pyridine (5 mL) and trifluoroethyl octadecanoate (2.60 g, 7.10 mmol) and LPS (2.50 g) were added in the order. The suspension was stirred at 45 °C and monitored by TLC (CH₂Cl₂:CH₃OH 95:5 v/v). After

18 h the reaction was stopped and filtered to remove the enzyme which was washed with pyridine and methanol. The solvent was evaporated under vacuum and the crude purified by flash chromatography (hexane:EtOAc from 90:10 to 20:80, v/v) to yield 1-*O*-octadecanoyl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol (4a) as an amorphous solid (0.60 g, 0.87 mmol, 74% yield). Mp 95.1–95.4 °C; [α]_D²⁰ = -4.7 (CHCl₃, c 0.5); ¹H NMR (CDCl₃): δ = 0.87 (t, 3H, J = 7.0 Hz, CH₃), 1.19–1.34 (m, 28H, 14 CH₂), 1.61 (m, 2H, CH₂), 2.00 (s, 3H, COCH₃), 2.03 (s, 3H, COCH₃), 2.05 (s, 3H, COCH₃), 2.09 (s, 3H, COCH₃), 2.30 (t, 2H, J = 7.6 Hz, CH₂), 3.58–3.69 (m, 2H, H-3a and H-3b), 3.76 (ddd, 1H, J_{5',6'a} = 5.6 Hz, J_{5',6'b} = 2.6 Hz, J_{4',5'} = 9.7 Hz, H-5'), 3.88 (m, 1H, H-2), 4.05–4.11 (m, 2H, H-1a and H-1b), 4.16 (dd, 1H, J_{6'a,6'b} = 12.2 Hz, H-6'a), 4.21 (dd, 1H, H-6'b), 4.61 (d, 1H, J_{1',2'} = 8.0 Hz, H-1'), 5.01 (dd, 1H, J_{2',3'} = 9.7 Hz, H-2'), 5.05 (dd, 1H, J_{3',4'} = 9.7 Hz, H-4'), 5.22 (dd, 1H, H-3'); ¹³C-NMR (CDCl₃): δ = 14.1 (CH₃), 20.5–20.07 (4 COCH₃), 22.7 (CH₂), 24.9 (CH₂), 28.9–30.0 (12 CH₂), 31.9 (CH₂), 34.1 (CH₂), 61.9 (C6'), 62.8 (C3), 63.1 (C1), 68.4 (C4'), 71.2 (C2'), 72.0 (C5'), 72.5 (C3'), 81.5 (C2), 101.2 (C1'), 169.2, 169.4, 170.2, 170.6 and 173.4 (5 CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 711.3 [M+Na]⁺, calcd for C₃₅H₆₀O₁₃, m/z 688.40 [M]. The same enzymatic procedure on the same amount of substrate, using trifluoroethyldecanoate (1.80 g, 7.08 mmol) as acyl carrier afforded after flash chromatography (hexane: EtOAc from 70:30 to 30:70, v/v) 1-*O*-decanoyl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol (4b) as an oil: (0.56 g, 0.97 mmol, 82% yield). [α]_D²⁰ = -1.0 (CHCl₃, c 1.0); ¹H-NMR (CDCl₃): δ = 0.87 (t, 3H, J = 7.0 Hz, CH₃), 1.20–1.35 (m, 12H, 6 CH₂), 1.61 (m, 2H, CH₂), 2.00 (s, 3H, COCH₃), 2.03 (s, 3H, COCH₃), 2.04 (s, 3H, COCH₃), 2.09 (s, 3H, COCH₃), 2.30 (t, 2H, J = 7.6 Hz, CH₂), 3.57–3.69 (m, 2H, H-3a and H-3b), 3.76 (ddd, 1H, J_{5',6'a} = 5.6 Hz, J_{5',6'b} = 2.3 Hz, J_{4',5'} = 9.8 Hz, H-5'), 3.88 (m, 1H, H-2), 4.05–4.12 (m, 2H, H-1a and H-1b), 4.16 (dd, 1H, J_{6'a,6'b} = 12.3 Hz, H-6'a), 4.20 (dd, 1H, H-6'b), 4.61 (d, 1H, J_{1',2'} = 8.0 Hz, H-1'), 5.00 (dd, 1H, J_{2',3'} = 9.8 Hz, H-2'), 5.04 (dd, 1H, J_{3',4'} = 9.8 Hz, H-4'), 5.21 (dd, 1H, H-3'); ¹³C-NMR (CDCl₃): δ = 14.1 (CH₃), 20.5–20.7 (4 COCH₃), 22.7 (CH₂), 24.9 (CH₂), 29.0–29.5 (4 CH₂), 31.8 (CH₂), 34.1 (CH₂), 61.9 (C6'), 62.8 (C3), 63.1 (C1), 68.4 (C4'), 71.2 (C2'), 72.0 (C5'), 72.5 (C3'), 81.5 (C2), 101.2 (C1'), 169.2, 169.4, 170.2, 170.6 and 173.4 (5 CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 599.3 [M+Na]⁺, calcd for C₂₇H₄₄O₁₃, m/z 576.28 [M].

1-*O*-Acyl-3-*O*-trityl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerols (5a,b)

Compound 4a (0.47 g, 0.68 mmol) was dissolved in 5 mL of dry pyridine and trityl chloride (0.38 g, 1.36 mmol) was added. The reaction mixture was heated at 100 °C and stirred under argon for 3h (TLC, hexane: EtOAc 60:40 v/v). The solvent was evaporated under reduced pressure and the obtained crude compound was submitted to flash chromatography (hexane: EtOAc 75:25, v/v, 120 1% TEA) to yield 1-*O*-octadecanoyl-3-*O*-trityl-2-*O*-(2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol (5a) (0.61 g, 0.66 mmol, 97% yield) as an oil. [α]_D²⁰ = -3.3 (CHCl₃, c 1.0); ¹H-NMR (Pyd₃): δ = 0.85 (t, 3H, J = 6.5 Hz, CH₃), 1.16–1.33 (m, 28H, 14 CH₂), 1.64 (m, 2H, CH₂), 1.96 (s, 3H, COCH₃), 1.99 (s, 3H, COCH₃), 2.01 (s, 3H, COCH₃), 2.15 (s, 3H, COCH₃), 2.35 (t, 2H, J = 7.5 Hz, CH₂), 3.44 (dd, 1H, J_{3a,2} = 6.0 Hz, J_{3a,3b} = 9.4 Hz, H-3a), 3.57 (dd, 1H, J_{3b,2} = 5.1 Hz, H-3b), 4.13 (ddd, 1H, J_{5',6'a} = 2.0 Hz, J_{5',6'b} = 4.3 Hz, J_{4',5'} = 9.6 Hz, H-5'), 4.33 (dd, 1H, J_{6'a,6'b} = 12.1 Hz, H-6'a), 4.38 (m, 1H, H-2), 4.49 (dd, 1H, J_{1a,2} = 5.8 Hz, J_{1a,1b} = 11.6 Hz, H-1a), 4.56 (dd, 1H, H-6'a), 4.58 (dd, 1H, J_{1b,2} = 3.5 Hz, H-1b), 5.19 (d, 1H, J_{1',2'} = 8.0 Hz, H-1'), 5.48 (dd, 1H, J_{2',3'} = 9.6 Hz, H-2'), 5.50 (dd, 1H, J_{3',4'} = 9.6 Hz, H-4'), 5.77 (dd, 1H, H-3'), 7.26 (dd, 3H, J = 7.3 Hz, Ph), 7.35 (dd, 6H, J = 7.4 Hz, Ph), 7.65 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₃): δ = 13.8

(CH₃), 19.7-20.3 (4 COCH₃), 22.4 (CH₂), 24.7 (CH₂), 28.6-28.8 (12 CH₂), 31.6 (CH₂), 33.8 (CH₂), 62.1 (C6'), 63.4 (C3), 63.5 (C1), 68.7 (C4'), 71.6 (C2'), 71.7 (C5'), 72.9 (C3'), 77.0 (C2), 86.7 (OCPh₃), 100.6 (C1'), 127.0 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.0 (3 C, Ph), 169.1, 169.3, 169.8, 170.0 and 172.8 (5 CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 953.5 [M+Na]⁺, calcd for C₅₄H₇₄O₁₃, m/z 930.51 [M].

With the same procedure, starting from **4b** (0.51 g, 0.88 mmol), *l*-O-decanoyl-3-O-trityl-2-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-sn-glycerol (**5b**) (0.51 g, 0.62 mmol, 70% yield) was obtained as an oil. [α]_D²⁰ = -3.4 (CHCl₃, c 1.0); ¹H-NMR (Pyd₅): δ = 0.85 (t, 3H, J = 7.0 Hz, CH₃), 1.14-1.30 (m, 12H, 6 CH₂), 1.63 (m, 2H, CH₂), 1.96 (s, 3H, COCH₃), 2.00 (s, 3H, COCH₃), 2.01 (s, 3H, COCH₃), 2.14 (s, 3H, COCH₃), 2.34 (t, 2H, J = 7.5 Hz, CH₂), 3.43 (dd, 1H, J_{3a,2} = 6.0 Hz, J_{3a,3b} = 9.6 Hz, H-3a), 3.57 (dd, 1H, J_{3b,2} = 5.2 Hz, H-3b), 4.13 (ddd, 1H, J_{5',6'a} = 2.5 Hz, J_{5',6'b} = 4.4 Hz, J_{4',5'} = 9.5 Hz, H-5'), 4.33 (dd, 1H, J_{6'a,6'b} = 12.2 Hz, H-6'a), 4.38 (m, 1H, H-2), 4.49 (dd, 1H, J_{1a,2} = 6.0 Hz, J_{1a,1b} = 11.6 Hz, H-1a), 4.55 (dd, 1H, H-6'a), 4.58 (dd, 1H, J_{1b,2} = 3.6 Hz, H-1b), 5.19 (d, 1H, J_{1',2'} = 8.0 Hz, H-1'), 5.48 (dd, 1H, J_{2',3'} = 9.5 Hz, H-2'), 5.51 (dd, 1H, J_{3',4'} = 9.5 Hz, H-4'), 5.77 (dd, 1H, H-3'), 7.26 (dd, 3H, J = 7.3 Hz, Ph), 7.35 (dd, 6H, J = 7.4 Hz, Ph), 7.64 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 19.6-20.4 (4 COCH₃), 22.4 (CH₂), 24.7 (CH₂), 28.4-29.6 (4 CH₂), 31.6 (CH₂), 33.8 (CH₂), 62.0 (C6'), 63.4 (C3), 63.5 (C1), 68.7 (C4'), 71.6 (C2'), 71.7 (C5'), 72.9 (C3'), 77.0 (C2), 86.6 (OCPh₃), 100.6 (C1'), 127.0 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.0 (3 C, Ph), 169.1, 169.3, 169.8, 170.0 and 172.8 (5 CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 841.3 [M+Na]⁺, calcd for C₄₆H₅₈O₁₃, m/z 818.39 [M].

1-O-Acyl-3-O-trityl-2-O-β-D-glucopyranosyl-sn-glycerols (6a,b)

Compound **5a** (0.60 g, 0.64 mmol) was dissolved in 6.4 mL of aq. ethanol (85%). Hydrazine mono-hydrate (0.32 g, 6.4 mmol) was added and the reaction mixture was stirred at 45 °C overnight (TLC, CH₂Cl₂:CH₃OH 95:5 v/v). The solvent was evaporated under a stream of N₂ and the crude purified by flash chromatography (CH₂Cl₂:CH₃OH 95:5, v/v) to yield *l*-O-octadecanoyl-3-O-trityl-2-O-β-D-glucopyranosyl-sn-glycerol (**6a**) (0.22 g, 0.29 mmol, 45% yield) as an oil: [α]_D²⁰ = +6.3 (CHCl₃, c 1.0); ¹H-NMR (Pyd₅): δ = 0.85 (t, 3H, J = 7.0 Hz, CH₃), 1.15-1.31 (m, 28H, 14 CH₂), 1.61 (m, 2H, CH₂), 2.32 (m, 2H, CH₂), 3.54 (dd, 1H, J_{3a,2} = 6.6 Hz, J_{3a,3b} = 9.4 Hz, H-3a), 3.66 (dd, 1H, J_{3b,2} = 4.7 Hz, H-3b), 3.92 (m, 1H, H-5'), 3.99 (m, 1H, H-2'), 4.19-4.27 (m, 2H, H-3' and H-4'), 4.34 (m, 1H, H-6'a), 4.46 (m, 1H, H-6'b), 4.49 (m, 1H, H-2), 4.66-4.72 (m, 2H, H-1a and H-1b), 5.04 (d, 1H, J_{1',2'} = 7.7 Hz, H-1'), 7.23 (dd, 3H, J = 7.3 Hz, Ph), 7.32 (dd, 6H, J = 7.4 Hz, Ph), 7.64 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 22.4 (CH₂), 24.7 (CH₂), 28.8-29.7 (12 CH₂), 31.6 (CH₂), 33.9 (CH₂), 62.4 (C6'), 63.7 (C3), 63.8 (C1), 71.2 (C3' or C4'), 74.7 (C2'), 76.0 (C2), 77.9 (C3' or C4' and C5'), 86.6 (OCPh₃), 104.3 (C1'), 126.9 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.2 (3 C, Ph), 173.0 (CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 785.3 [M+Na]⁺, calcd for C₄₆H₆₆O₉, m/z 762.47 [M].

With the same procedure starting from **5b** (0.47 g, 0.58 mmol) *l*-O-decanoyl-3-O-trityl-2-O-β-D-glucopyranosyl-sn-glycerol (**6b**) (0.21 g, 0.32 mmol, 56% yield) was obtained as an oil: [α]_D²⁰ = +7.8 (CHCl₃, c 1.0); ¹H-NMR (Pyd₅): δ = 0.83 (t, 3H, J = 7.0 Hz, CH₃), 1.07-1.28 (m, 12H, 6 CH₂), 1.60 (m, 2H, CH₂), 2.31 (m, 2H, CH₂), 3.54 (dd, 1H, J_{3a,2} = 6.5 Hz, J_{3a,3b} = 9.4 Hz, H-3a), 3.66 (dd, 1H, J_{3b,2} = 4.7 Hz, H-3b), 3.92 (m, 1H, H-5'), 3.98 (m, 1H, H-2'), 4.18-4.26 (m, 2H, H-3' and H-4'), 4.33 (dd, 1H, J_{5',6'a} = 4.9 Hz, J_{5',6'b} = 11.5 Hz, H-6'a), 4.45 (dd, 1H, J_{5',6'a} = 2.2 Hz, H-6'b), 4.49 (m, 1H, H-2), 4.65-4.71 (m, 2H, H-1a and H-1b), 5.03 (d, 1H, J_{1',2'} = 7.7 Hz, H-1'), 7.23 (dd, 3H, J = 7.3 Hz, Ph), 7.32

(dd, 6H, J = 7.4 Hz, Ph), 7.63 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 22.4 (CH₂), 24.7 (CH₂), 28.7-29.3 (4 CH₂), 31.5 (CH₂), 33.8 (CH₂), 62.4 (C6'), 63.7 (C3), 63.8 (C1), 71.2 (C3' or C4'), 74.7 (C2'), 76.0 (C2), 77.9 (C3' or C4' and C5'), 86.6 (OCPh₃), 104.3 (C1'), 126.9 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.2 (3 C, Ph), 173.0 (CO); ESI-MS (CH₃OH, positive-ion mode): m/z = 673.2 [M+Na]⁺, calcd for C₃₈H₅₀O₉, m/z 650.35 [M].

1-O-Acyl-3-O-trityl-2-O-β-D-glucuronopyranosyl-sn-glycerols (7a,b)

To a solution of compounds **6a** (0.22 g, 0.29 mmol) in a 55:45 mixture of CH₃CN and 0.67 M phosphate buffer (3 mL, pH 6.7), TEMPO (0.01 g, 0.061 mmol), NaClO₂ (20% aqueous solution, 0.4 mL) and NaClO₂ (15% aqueous solution, 0.025 mL) were added in the order. After stirring for 3 hours (TLC, CH₂Cl₂:CH₃OH 90:10 v/v), acetonitrile was removed under reduced pressure and the aqueous phase was extracted with Et₂O. The organic layers were assembled, dried over anhydrous Na₂SO₄, filtered and evaporated to give the desired *l*-O-octadecanoyl-3-O-trityl-2-O-β-D-glucuronopyranosyl-sn-glycerol (**7a**) (0.22 g, 0.28 mmol, 97% yield) as an amorphous solid. Mp: 98 - 99 °C; [α]_D²⁰ = -11.1 (CHCl₃:CH₃OH 65:35, c 1.0); ¹H-NMR (Pyd₅): δ = 0.85 (t, 3H, J = 7.0 Hz, CH₃), 1.13-1.35 (m, 28H, 14 CH₂), 1.59 (m, 2H, CH₂), 2.28 (m, 2H, CH₂), 3.56 (dd, 1H, J_{3a,2} = 6.6 Hz, J_{3a,3b} = 9.2 Hz, H-3a), 3.68 (dd, 1H, J_{3b,2} = 4.4 Hz, H-3b), 4.03 (dd, J_{1',2'} = 7.6 Hz, J_{2',3'} = 8.0 Hz, 1H, H-2'), 4.25 (m, 1H, H-3'), 4.32-4.42 (m, 2H, H-4' and H-5'), 4.54 (m, 1H, H-2), 4.60-4.71 (m, 2H, H-1a and H-1b), 5.06 (d, 1H, H-1'), 7.19 (dd, 3H, J = 7.4 Hz, Ph), 7.31 (dd, 6H, J = 7.4 Hz, Ph), 7.63 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 22.4 (CH₂), 24.7 (CH₂), 28.8-29.7 (12 CH₂), 31.6 (CH₂), 33.8 (CH₂), 63.5 (C1 and C3), 73.1 (C4' or C5'), 74.4 (C2'), 75.5 (C2), 76.5 (C4' or C5'), 77.6 (C3'), 86.6 (OCPh₃), 103.7 (C1'), 126.8 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.1 (3 C, Ph), 173.0 (CO), 174.3 (CO); ESI-MS (CH₃OH, negative-ion mode): m/z = 775.5 [M-1], calcd for C₄₆H₆₄O₁₀, m/z 776.45 [M].

With the same procedure, starting from **6b** (0.21 g, 0.32 mmol), *l*-O-decanoyl-3-O-trityl-2-O-β-D-glucuronopyranosyl-sn-glycerol (**7b**) (0.21 g, 0.31 mmol, 98 % yield) was obtained as an amorphous solid. Mp: 154 - 155 °C; [α]_D²⁰ = -11.3 (CHCl₃:CH₃OH 65:35, c 1.0); ¹H-NMR (Pyd₅): δ = 0.84 (t, 3H, J = 7.0 Hz, CH₃), 1.07-1.28 (m, 12H, 6 CH₂), 1.57 (m, 2H, CH₂), 2.26 (m, 2H, CH₂), 3.56 (dd, 1H, J_{3a,2} = 6.6 Hz, J_{3a,3b} = 9.0 Hz, H-3a), 3.64 (dd, 1H, J_{3b,2} = 4.2 Hz, H-3b), 3.99 (dd, J_{1',2'} = 7.8 Hz, J_{2',3'} = 8.5 Hz, 1H, H-2'), 4.16-4.27 (m, 3H, H-3', H-4' and H-5'), 4.57 (m, 1H, H-2), 4.65 (dd, 2H, J_{1'a,2} = 4.8 Hz, J_{1'a,1'b} = 11.5 Hz, H-1a), 4.70 (dd, 2H, J_{1'b,2} = 4.0 Hz, H-1b), 5.00 (d, 1H, H-1'), 7.20 (dd, 3H, J = 7.4 Hz, Ph), 7.31 (dd, 6H, J = 7.4 Hz, Ph), 7.62 (d, 6H, J = 7.8 Hz, Ph); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 22.4 (CH₂), 24.6 (CH₂), 28.7-29.3 (4 CH₂), 31.5 (CH₂), 33.8 (CH₂), 63.2 (C1), 63.4 (C3), 73.2 (C4' or C5'), 74.3 (C2'), 74.9 (C2), 76.0 (C4' or C5'), 77.7 (C3'), 86.6 (OCPh₃), 103.1 (C1'), 126.8 (3 CH, Ph), 127.8 (6 CH, Ph), 128.7 (6 CH, Ph), 144.1 (3 C, Ph), 173.0 (CO), 175.6 (CO); ESI-MS (CH₃OH, negative-ion mode): m/z = 663.2 [M-1], calcd for C₃₈H₄₈O₁₀, m/z 664.32 [M].

1-O-acyl-2-O-β-D-glucuronopyranosyl-sn-glycerols (1a,b)

Compound **7a** (0.11 g, 0.14 mmol) was dissolved in CH₂Cl₂ (1.5 mL) and Dowex[®] Marathon[™] C, H⁺ form (0.15 g), was added (methanol washing of the resin was not done, see materials, to avoid methyl ester formation). The reaction was stirred overnight at room temperature (TLC, CH₂Cl₂:CH₃OH 90:10 v/v) obtaining a white suspension. The reaction mixture was filtered and the residue washed with CH₂Cl₂ which was eliminated. The remaining solid was then washed with AcOEt and the washings dried over Na₂SO₄ and evaporated under reduced pressure

yielding the desired pure *1-O-octadecanoyl-2-O-β-D-glucuronopyranosyl-sn-glycerol* (**1a**) (0.06 g, 0.11 mmol, 76% yield) as a white solid. Mp: 127 - 128 °C; $[\alpha]_D^{20} = -29.3$ (CHCl₃:CH₃OH 65:35, *c* 0.5); ¹H-NMR (Pyd₅): δ = 0.85 (t, 3H, J = 7.0 Hz, CH₃), 1.13-1.31 (m, 28H, 14 CH₂), 1.64 (m, 2H, CH₂), 2.37 (m, 2H, CH₂), 4.10 (dd, J_{1,2} = 7.8 Hz, J_{2,3} = 8.9 Hz, 1H, H-2'), 4.16 (dd, 1H, J_{3a,2} = 5.5 Hz, J_{3a,3b} = 11.5 Hz, H-3a), 4.23 (dd, 1H, J_{3b,2} = 4.9 Hz, H-3b), 4.33 (dd, 1H, J_{3',4'} = 8.9 Hz, H-3'), 4.52 (m, 1H, H-2), 4.60 (dd, 1H, J_{4',5'} = 8.9 Hz, H-4'), 4.66 (d, 1H, H-5'), 4.69-4.77 (m, 2H, H-1a and H-1b), 5.22 (d, 1H, H-1'); ¹³C-NMR (Pyd₅): δ = 13.8 (CH₃), 22.4 (CH₂), 24.7 (CH₂), 28.8-29.5 (12 CH₂), 31.6 (CH₂), 33.9 (CH₂), 62.2 (C3), 63.9 (C1), 72.9 (C4'), 74.3 (C2'), 77.3 (C3' and C5'), 78.7 (C2), 104.5 (C1'), 172.1 (CO), 173.1 (CO); ESI-MS (CH₃OH, negative-ion mode): *m/z* = 533.3 [M-1]⁻, calcd for C₂₇H₅₀O₁₀, *m/z* 534.34 [M].

With the same procedure, starting with **7b** (0.14 g, 0.21 mmol) and washing the residue with AcOEt:iPrOH 1:1, *1-O-decanoyl-2-O-β-D-glucuronopyranosyl-sn-glycerol* (**1b**) (0.07 g, 0.17 mmol, 81% yield) was obtained as a white sticky solid. $[\alpha]_D^{20} = -31.6$ (CHCl₃:CH₃OH 65:35, *c* 1.0); ¹H-NMR (Pyd₅): δ = 0.82 (t, 3H, J = 7.0 Hz, CH₃), 1.09-1.27 (m, 12H, 6 CH₂), 1.62 (m, 2H, CH₂), 2.36 (m, 2H, CH₂), 4.09 (dd, J_{1,2} = 7.8 Hz, J_{2,3} = 8.4 Hz, 1H, H-2'), 4.15 (dd, 1H, J_{3a,2} = 5.5 Hz, J_{3a,3b} = 11.4 Hz, H-3a), 4.21 (dd, 1H, J_{3b,2} = 4.8 Hz, H-3b), 4.32 (dd, 1H, J_{3',4'} = 8.9 Hz, H-3'), 4.51 (m, 1H, H-2), 4.58 (dd, 1H, J_{4',5'} = 8.9 Hz, H-4'), 4.64 (d, 1H, H-5'), 4.68-4.76 (m, 2H, H-1a and H-1b), 5.20 (d, 1H, H-1'); ¹³C-NMR (Pyd₅): δ = 13.7 (CH₃), 22.4 (CH₂), 24.7 (CH₂), 28.7-29.2 (4 CH₂), 31.5 (CH₂), 33.8 (CH₂), 62.2 (C3), 63.8 (C1), 72.8 (C4'), 74.3 (C2'), 77.2 (C5'), 77.3 (C3'), 78.7 (C2), 104.5 (C1'), 172.1 (CO), 173.1 (CO); ESI-MS (CH₃OH, negative-ion mode): *m/z* = 421.5 [M-1]⁻, Calcd for C₁₉H₃₄O₁₀, *m/z* 422.22 [M].

Configuration assignment of compound 4a

Compound **4a** (0.027 g, 0.04 mmol) was dissolved in dry pyridine (1 mL) and acetic anhydride (0.5 g, 4.9 mmol) was added. The reaction was stirred at room temperature and stopped after 3 hours (TLC, hexane: EtOAc 60:40 v/v). After usual work-up the crude was purified by flash chromatography (hexane:EtOAc 70:30 v/v) and the obtained pure compound (0.024 g, 0.033 mmol, 82% yield) resulted identical to the known *1-O-octadecanoyl-3-O-acetyl-2-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-sn-glycerol*.²⁴ Oil; $[\alpha]_D^{20} = -8.7$ (CHCl₃, *c* 1); ¹H NMR (CDCl₃): δ = 0.86 (t, 3H, J = 7.0 Hz, CH₃), 1.19-1.33 (m, 28H, 14 CH₂), 1.59 (m, 2H, CH₂), 1.98 (s, 3H, COCH₃), 2.00 (s, 3H, COCH₃), 2.01 (s, 3H, COCH₃), 2.04 (s, 3H, COCH₃), 2.06 (s, 3H, COCH₃), 2.29 (t, 2H, J = 7.6 Hz, CH₂), 3.67 (ddd, 1H, J_{5',6'a} = 2.4 Hz, J_{5',6'b} = 5.1 Hz, J_{4',5'} = 10.0 Hz, H-5'), 4.04 (m, 1H, H-2), 4.06-4.20 (m, 5H, H-1a, H-1b, H-3a, H-3b and H-6'a), 4.22 (dd, 1H, J_{6'a,6'b} = 12.3 Hz, H-6'b), 4.61 (d, 1H, J_{1,2} = 7.9 Hz, H-1'), 4.96 (dd, 1H, J_{2,3} = 9.6 Hz, H-2'), 5.04 (dd, 1H, J_{3',4'} = 9.6 Hz, H-4'), 5.17 (dd, 1H, H-3'); ¹³C-NMR (CDCl₃): δ = 14.1 (CH₃), 20.4-20.08 (5 COCH₃), 22.7 (CH₂), 24.8 (CH₂), 28.9-29.9 (12 CH₂), 31.9 (CH₂), 34.1 (CH₂), 62.0 (C6'), 63.1 (C1), 63.3 (C3), 68.4 (C4'), 71.3 (C2'), 71.9 (C5'), 72.7 (C3'), 75.6 (C2), 100.8 (C1'), 169.1, 169.3, 170.2, 170.5, 170.6 and 173.3 (6 CO); ESI-MS (CH₃OH, positive-ion mode): *m/z* = 753.5 [M+Na]⁺, calcd for C₃₇H₆₂O₁₄, *m/z* 730.41 [M].

Synthesis of diesters 2a and 2b

1,3-Di-*O*-octadecanoyl-2-*O*-β-D-glucuronopyranosyl-*sn*-glycerol (**2a**)

*1,3-Di-*O*-octadecanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol* **8a**^{14a} (0.055 g, 0.07 mmol) was suspended in 1 mL of a 55:45 mixture of CH₃CN and 0.67M phosphate buffer (pH 6.7) and TEMPO (0.008 g, 0.05 mmol), NaClO₂ (20% aqueous solution, 0.3 mL) and NaClO (15% aqueous solution, 0.015 mL) were added. After

stirring overnight at room temperature (TLC, CH₂Cl₂:CH₃OH 90:10 v/v), Na₂S₂O₃ 0.5 M was added and the aqueous phase was acidified with HCl and extracted with Et₂O. The organic layers were assembled, dried over anhydrous Na₂SO₄, filtered and evaporated under reduced pressure to give *1,3-di-*O*-octadecanoyl-2-*O*-β-D-glucuronopyranosyl-*sn*-glycerol* (**2a**) (0.024 g, 0.03 mmol, 43% yield)^{14b} as a white solid. Mp: 158 - 159 °C. ¹H-NMR (CDCl₃:CD₃OD:D₂O, 65:35:6, 315 K): δ = 0.82-0.87 (m, 6H, 2 CH₃), 1.18-1.31 (m, 56H, 28 CH₂), 1.53-1.61 (m, 4H, 2 CH₂), 2.27-2.32 (m, 4H, 2 CH₂), 3.24 (dd, 1H, J_{1,2} = 7.8 Hz, J_{2,3} = 9.1 Hz, H-2'), 3.42 (dd, 1H, J_{3',4'} = 9.0 Hz, H-3'), 3.45 (dd, 1H, J_{4',5'} = 9.5 Hz, H-4'), 3.56 (d, 1H, H-5'), 4.17-4.23 (m, 4H, H-2, H-1a, H-3a, and H-1b or H-3b), 4.30 (m, 1H, H-1b or H-3b), 4.41 (d, 1H, H-1'); ¹³C-NMR (CDCl₃:CD₃OD:D₂O, 65:35:6, 315 K): δ = 14.3 (2 CH₃), 23.1 (2 CH₂), 25.3 (2 CH₂), 29.5-30.2 (24 CH₂), 32.4 (2 CH₂), 34.6 (2 CH₂), 63.2 (C1 or C3), 64.0 (C1 or C3), 72.5 (C4'), 73.8 (C2'), 75.2 (C2), 75.6 (C5'), 76.6 (C3'), 103.2 (C1'), 174.8 (2 CO); ESI-MS (CH₃OH, negative-ion mode): *m/z* = 799.7 [M-1]⁻, Calcd for C₄₅H₈₄O₁₁, *m/z* 800.60 [M].

1,3-Di-*O*-decanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (**8b**)

*2-*O*-(2,3,4,6-Tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol*^{14c} (0.26 g, 0.47 mmol) was dissolved in dry CH₂Cl₂ (2.5 mL) and cooled at -10 °C. Decanoyl chloride (0.22 g, 1.18 mmol) as a 15% (v/v) CH₂Cl₂ solution and pyridine (0.23 mL, 2.8 mmol) as a 10% (v/v) CH₂Cl₂ 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 stopped after 50 min diluting with CH₂Cl₂ (15 mL). The solution was washed with 1M HCl (10 mL), water (10 mL), NaHCO₃ saturated solution (10 mL), and water (2 x 10 mL) in the order and the aqueous phases re-extracted with CH₂Cl₂ (2 x 15 mL). The collected organic layers were dried over Na₂SO₄, evaporated under reduced pressure and the crude residue submitted to flash chromatography (petroleum ether:EtOAc 80:20 v/v) affording *1,3-di-*O*-decanoyl-2-*O*-(2',3',4',6'-tetra-*O*-chloroacetyl-β-D-glucopyranosyl)-*sn*-glycerol* (0.24 g, 0.28 mmol, 60% yield), oil, $[\alpha]_D^{20} = -3.3$ (CHCl₃, *c* 1.0); ¹H-NMR (CDCl₃): δ = 0.85-0.90 (m, 6H, 2 CH₃), 1.20-1.34 (m, 24H, 12 CH₂), 1.56-1.64 (m, 4H, 2 CH₂), 2.27-2.33 (m, 4H, 2 CH₂), 3.83 (ddd, 1H, J_{5',6'a} = 2.5 Hz, J_{5',6'b} = 5.1 Hz, J_{4',5'} = 10.0 Hz, H-5'), 3.98 (s, 2H, ClCH₂), 4.01 (m, 2H, ClCH₂), 4.04 (m, 2H, ClCH₂), 4.05-4.27 (m, 5H, H-1a, H-1b, H-3a, H-3b and H-2), 4.14 (s, 2H, ClCH₂), 4.29 (dd, 1H, J_{6'a,5'} = 2.5 Hz, J_{6'a,6'b} = 12.3 Hz, H-6'a), 4.36 (dd, 1H, J_{6'b,5'} = 5.1 Hz, H-6'b), 4.72 (d, 1H, J_{1,2} = 7.9 Hz, H-1'), 5.05 (dd, 1H, J_{2,3} = 9.6 Hz, H-2'), 5.14 (dd, 1H, J_{3',4'} = 9.6 Hz, H-4'), 4.32 (dd, 1H, H-3'); ¹³C-NMR (CDCl₃): δ = 14.1 (2 CH₃), 22.6 (2 CH₂), 24.8 (2 CH₂), 29.1-29.4 (8 CH₂), 31.8 (2 CH₂), 34.0 (CH₂), 34.1 (CH₂), 40.1, 40.2, 40.3 and 40.5 (4 CH₂Cl), 62.7 (C1 and C3), 63.1 (C6'), 69.6 (C4'), 71.3 (C5'), 72.2 (C2'), 73.7 (C3'), 75.9 (C2), 100.2 (C1'), 165.9, 166.2, 166.9, 167.0 (4 CO), 173.4 (2 CO); ESI-MS (CH₃OH, negative-ion mode): *m/z* = 867.1 [M-1]⁻, Calcd for C₃₇H₅₈Cl₄O₁₄, *m/z* 868.25 [M].

The obtained didecanoate (0.23 g, 0.26 mmol) was dissolved in EtOAc:CH₃OH (7 mL, 1:1 v/v) and hydrazine acetate (0.366 g, 3.98 mmol) was added. The reaction was stirred under Ar atmosphere at room temperature overnight and monitored by TLC (CH₂Cl₂:CH₃OH, 95:5 v/v). The solvent was evaporated under reduced pressure and the crude residue subjected to repeated flash column chromatography (CH₂Cl₂:CH₃OH, 95:5 - 90:10 v/v) followed by recrystallization from ethanol to remove hydrazine impurities yielding pure *1,3-di-*O*-decanoyl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol* (**8b**) (0.063 g, 0.11 mmol, 43% yield) as a white solid. Mp: 85 °C (from ethanol); $[\alpha]_D^{20} = -8.2$ (CHCl₃, *c* 1.0); ¹H-NMR (CDCl₃): δ = 0.86-0.91 (m, 6H, 2 CH₃), 1.19-

1.35 (m, 24H, 12 CH₂), 1.56-1.65 (m, 4H, 2 CH₂), 2.28-2.37 (m, 4H, 2 CH₂), 3.32-3.44 (m, 2H, H-2' and H-5'), 3.51-3.58 (m, 2H, H-3' and H-4'), 3.78 (m, 1H, H-6'a), 3.89 (m, 1H, H-6'b), 4.04 (m, 1H, H-2), 4.14-4.22 (m, 2H, H-1a and H-3a), 4.27 (dd, 1H, ⁵J_{1b/3b,2} = 5.0 Hz, J_{1b/3b,1a/3a} = 11.5 Hz, H-1b or H-3b), 4.35 (dd, 1H, J_{1b/3b,2} = 3.8 Hz, J_{1b/3b,1a/3a} = 11.8 Hz, H-1b or H-3b), 4.41 (d, 1H, J_{1',2'} = 7.7 Hz, H-1'); ¹³C-NMR (CDCl₃): δ = 14.1 (2 CH₃), 22.7 (2 CH₂), 24.8 (2 CH₂), 29.1-29.4 (8 CH₂), 31.8 (2 CH₂), 34.1 (CH₂), 34.2 (CH₂), 62.3 (C6'), 63.1 (C1 and C3), 70.1 (C3' or C4'), 73.5 (C2'), 75.8 (C5'), 76.0 (C2), 76.2 (C3' or C4'), 103.3 (C1'), 173.7 (CO), 174.1 (CO). ESI-MS (CH₃OH, positive-ion mode): m/z = 585.4 [M+Na]⁺, calcd for C₂₉H₅₄O₁₀, m/z 562.37 [M].

1,3-Di-*O*-decanoyl-2-*O*-β-D-glucuronopyranosyl-*sn*-glycerol (2b)

Starting from **8b** (0.05 g, 0.09 mmol), with the same procedure above reported for **2a**, 1,3-di-*O*-decanoyl-2-*O*-β-D-glucuronopyranosyl-*sn*-glycerol (**2b**) (0.046 g, 0.08 mmol, 89% yield) was obtained as an oil. [α]_D²⁰ = -18.9 (CH₃OH, c 1.0); ¹H-NMR (CD₃OD): δ = 0.85-0.92 (m, 6H, 2 CH₃), 1.22-1.36 (m, 24H, 12 CH₂), 1.54-1.64 (m, 4H, 2 CH₂), 2.29-2.37 (m, 4H, 2 CH₂), 3.32 (dd, 1H, J_{1',2'} = 8.0 Hz, J_{2',3'} = 8.5 Hz, H-2'), 3.41 (dd, 1H, J_{3',4'} = 9.0 Hz, H-3'), 3.47 (dd, 1H, J_{4',5'} = 9.0 Hz, H-4'), 3.65 (br d, 1H, H-5'), 4.19-4.26 (m, 4H, H-2, H-1a, H-3a, and H-1b or H-3b), 4.31 (m, 1H, H-1b or H-3b), 4.46 (d, 1H, H-1'); ¹³C-NMR (CD₃OD): δ = 14.5 (2 CH₃), 23.7 (2 CH₂), 26.0 (2 CH₂), 30.2-30.6 (8 CH₂), 33.1 (2 CH₂), 34.9 (2 CH₂), 63.9 (C1 or C3), 64.7 (C1 or C3), 73.5 (C4'), 74.8 (C2'), 75.9 (C2), 76.4 (s, C5'), 77.6 (C3'), 104.3 (C1'), 175.1 (CO), 175.2 (CO), 176.9 (br s, CO); ESI-MS (CH₃OH, negative-ion mode): m/z = 575.3 [M-1]⁻, Calcd for C₂₉H₅₂O₁₁, m/z 576.35 [M].

Akt Inhibition Assays

Akt1 ELISA activity assay. The inhibitory activity of compounds **1a-b**, **2b**, **3a-b**, Miltefosine (Sigma), Perifosine (Sigma) and SDS (Sigma) was tested employing the CycLex AKT/PKB kinase Assay/Inhibitor Screening Kit (CycLex, Eppendorf, Milano, Italy). Plates were pre-coated with "AKTide-2T" which can be efficiently phosphorylated by Akt1. The detector antibody specifically detects the phosphorylated "AKTide-2T". Particularly, to perform the test, samples **1a-b**, **2b**, **3a-b** and SDS were dissolved in DMSO (note that **2a** was insoluble in this solvent), and miltefosine and perifosine in water. The prepared solutions were then diluted in Kinase Buffer to a final concentration of 500, 100, 50, 10 and 1 μM. Compounds were added together with constitutive active form of human Akt1 (25 m units/well), and allowed to phosphorylate the bound substrate following the addition of Mg²⁺ and ATP. The amount of phosphorylated substrate was measured by binding it with horseradish peroxidase conjugate of an anti-phospho-AKTide-2T monoclonal antibody, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine from the colourless reduced form to the yellow oxidized product, after the addition of the stopping reagent. The absorbance of the resulting solution is determined spectrophotometrically at λ = 450 nm, and it is related to Akt1 activity in the tested solution. Staurosporine (Sigma-Aldrich, Milano, Italy) at the final concentration of 1 μM was employed as "inhibitor control" as indicated in the assay protocol. Each experiment was performed in triplicate.

Cellular studies

Compounds preparation. Perifosine was dissolved in H₂O at 20 mM. All tested compounds were easily dissolved in 100% DMSO at 50 mM. **Cell culture and cell growth assay.** The human ovarian carcinoma IGROV-1 cell line³⁰ was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in

5% CO₂ atmosphere. For cell growth inhibition assays, cells were plated in 12 well-plates at 10000 cells/cm² in complete medium. The day after seeding, cells were exposed to solvent (DMSO) or to different concentration of the novel compounds for 72 h. For tests in serum-free medium, the day after seeding complete medium was substituted with serum-free medium and the cells were exposed to the compounds. Twenty four h later drug-containing medium was replaced with complete medium. Cells were harvested using trypsin and counted 96 h after seeding by a Coulter Counter (Z1, Beckman Coulter). Each experiment was performed three times. The percentages of inhibition in drug-treated versus solvent-treated samples are reported in dose-response curves. IC₅₀ represents the drug concentration inhibiting growth by 50%.

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Notes and references

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