Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

Cite this: *Org. Biomol. Chem.*, 2015, **13**, 1091

Anionic glycolipids related to glucuronosyldiacylglycerol inhibit protein kinase Akt†

Maria Vetro,[a] Barbara Costa,[b] Giulia Donvito,[b] Noemi Arrighetti,[c] Laura Cipolla,[b] Paola Perego,*[c] Federica Compostella,[a] Fiamma Ronchetti[a] and Diego Colombo*[a]

Received 28th July 2014,
Accepted 6th November 2014

DOI: 10.1039/c4ob01602e

www.rsc.org/obc

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. The Akt inhibitory effect of the 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, Fig. 1) are unusual acidic glycoglycerolipids found in bacteria,[1] fungi,[2] algae[3] 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 to be related to conditions of reduced phosphorous availability in which they replace phospholipids for their biological functions.[4,6] GlcADGs participate in this lipid remodelling as the better-known anionic sulfolipids, sulfoquinovosyldiacylglycerols (SQDGs, Fig. 1),[7,8] sharing also the same biosynthetic pathway which requires a common SQDG synthase.[4] In contrast to GlcADGs, in the last few years both natural and synthetic sulfoquinovosylacylglycerols have been tested for their antitumor, antiviral, anti-inflammatory, immunosuppressive and other bioactivities.[9–13] This prompted us to prepare some SQDG analogues (Fig. 1) based on 2-*O*-β-D-glucosylglycerol, which were tested as inhibitors of tumor-promoters in cancer prevention studies.[14a,15] These unnatural sulfolipids, similar to other glucose-based compounds,[16] display a structure which can be related to that of 3-phosphorylated phosphatidylinositol (PI3P, Fig. 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] Thus they are currently investigated as potential inhibitors of protein kinase B (PKB or Akt),[18] a kinase involved in sustaining multiple aggressive 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)P_3 and PI(3,4)P_2, are generated by PI3K and, at the plasma membrane, they can bind the pleckstrin homology (PH) domain promoting kinase activation.[17a,20]

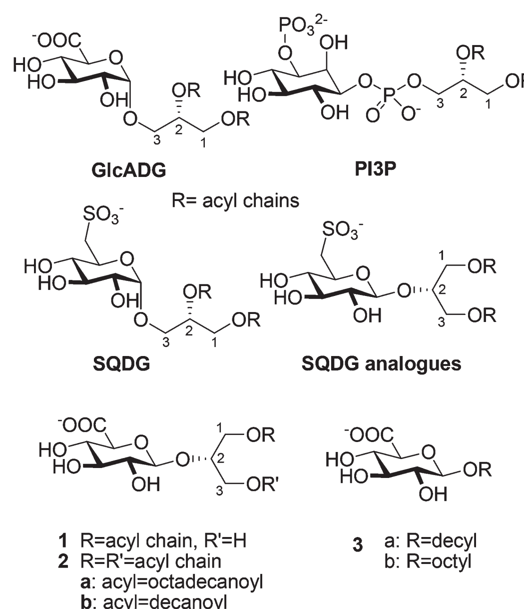


Fig. 1 Structures of PI3P, related natural anionic glycolipids and synthetic analogues.

[a]Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Via Saldini 50, 20133 Milano, Italy.
E-mail: diego.colombo@unimi.it; Fax: +39 02 50316036; Tel: +39 02 50316039
[b]Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, P.za Scienza 2, 20126 Milano, Italy
[c]Molecular Pharmacology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, via Amadeo 42/ via Venezian 1, 20133 Milan, Italy.
E-mail: paola.perego@istitutotumori.mi.it; Fax: +39 0223902692;
Tel: +39 0223902237

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra of the prepared compounds. See DOI: 10.1039/c4ob01602e

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

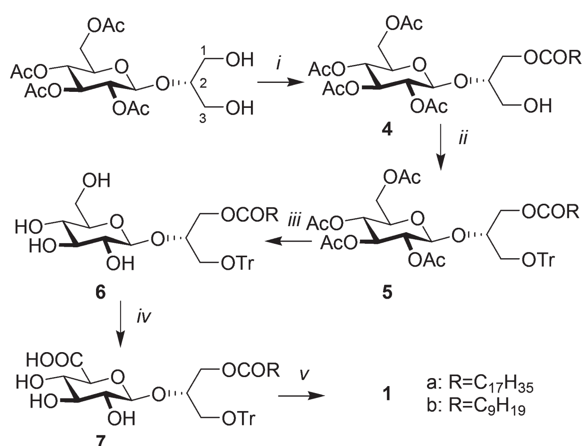
Many proteins with the PH domain are known, but only a few of them are regulated by the direct binding of 3-phosphoinositides.[17a] For this reason, even if different types of small molecules have been synthesized as Akt inhibitors (*e.g.* ATP mimetics and allosteric inhibitors), PH binders (such as perifosine, 3-DPI and 3-DPIEL) are thought as more selective potential Akt inhibitors.[19,21,22]

Here we describe the synthesis of the new glucuronosyldiacylglycerol (GlcADG) analogues **1a,b** and **2a,b**, carrying acyl chains which differ in length and number (Fig. 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.* SO_3^-) for interaction with the PI3P binding pocket of the 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

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 an organic solvent, by means of a procedure already used for similar substrates employing 2,2,2-trifluoroethyl (TFE) esters as acyl carriers,[24] allowing 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 compounds[24] (*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 compounds **6a,b** by treatment with hydrazine hydrate in aqueous ethanol for selective removal of the sugar acetyls.[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) Ph_3CCl, Py, 100 °C, (70–97%); (iii) hydrazine hydrate, EtOH aq. (45–56%); (iv) TEMPO, $NaClO/NaClO_2$, CH_3CN, 0.67 M phosphate buffer (pH 6.7), RT, (97–98%); (v) DOWEX H^+, CH_2Cl_2 (76–81%).

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 known 1-*O*-octadecanoyl-3-*O*-acetyl-2-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-*sn*-glycerol,[24] 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 for compound **4b** also.

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).

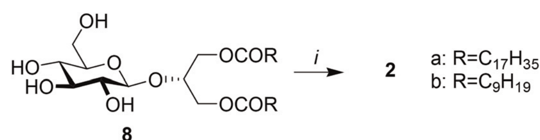
Synthesis of glucuronides 3a,b. Known glucuronides **3a**[26] and **3b**[27] (see ESI†) were obtained in good yields by regioselective TEMPO oxidation[26] of decyl β-D-glucopyranoside[28] and commercial octyl β-D-glucopyranoside, respectively.

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 shows the effects 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



Scheme 2 (i) TEMPO, $NaClO/NaClO_2$, CH_3CN, 0.67 M phosphate buffer (pH 6.7), RT, (43–89%).

This journal is © The Royal Society of Chemistry 2015

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

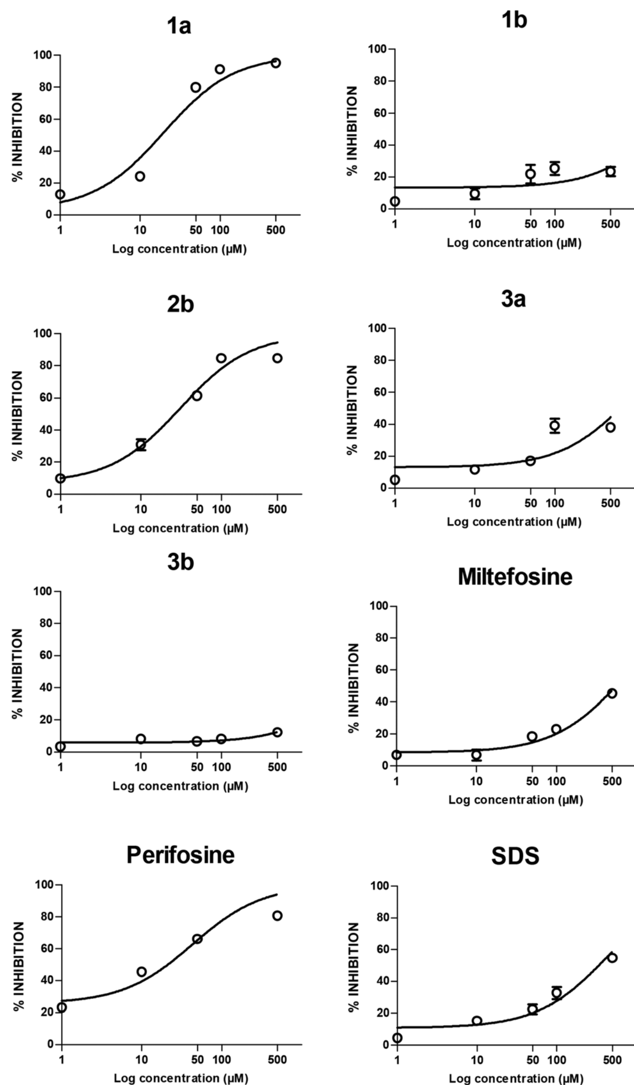


Fig. 2 PKB1 kinase assay (ELISA) of compounds **1a**,**b**, **2b**, **3a**,**b**, miltefosine, perifosine and SDS at the indicated concentrations. Concentration–response (%inhibition) analysis.

Table 1 Compound concentration producing 50% inhibition of PKB1 activity in the 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	

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 obtained by our group.[18]

The data obtained (Table 1 and Fig. 2) for miltefosine as well as SDS did not 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). In contrast, 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.

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 compounds **1a** and **2b**. In fact, the IC_{50} values (±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 a 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 a serum-free medium indicated an increased potency for the two active compounds. Under these conditions, the IC_{50} values (±SD) of compounds **1a** and **2b** were 3.35 ± 0.35 and 9.40 ± 5.0 μM, respectively. This evidence

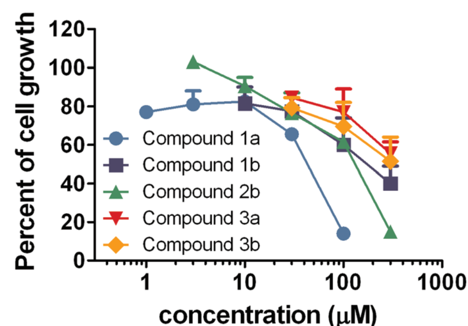


Fig. 3 Cell sensitivity of human ovarian carcinoma cells (IGROV-1) as evaluated by growth inhibition assays. Cells were seeded and 24 h later they were exposed to the novel compounds for 72 h. At the end of incubation with the compounds, cells were counted with a cell counter. Results from a representative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

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

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) ±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 started. IC_{50} represents the perifosine concentration producing 50% inhibition of cell growth. The reported values are the mean ± standard deviation of 3 independent experiments.

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 the serum presence in a medium on cellular sensitivity towards Akt inhibitors, we also tested the sensitivity of IGROV-1 cells towards perifosine in the presence and 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 a serum-free medium, thus implying that serum affects the stability of perifosine. However, for perifosine a 2.8 fold increase in the antiproliferative activity was observed upon incubation in a 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.

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 a 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 structural features needed to achieve Akt inhibition.

Experimental

Chemical procedures

Materials and equipment. *Pseudomonas cepacia* lipase (LPS, lipase PS, specific activity 30.5 triacetin units per 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 1 M 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 of 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 a silica gel plate (Merck 60F254) developed with 50% sulfuric acid or an 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 was 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 recorded for all the new compounds. The signals were unambiguously assigned by 2D COSY and HSQC experiments (standard Bruker pulse program). Chemical shifts were reported as δ (ppm) relative to residual $CHCl_3$, CH_3OD or pyridine fixed at 7.26, 3.30 ppm and 7.19 ppm (higher field signal), respectively, for ^1H NMR spectra and relative to $CDCl_3$ fixed at 77.0 ppm (central line), CD_3OD at 49.0 ppm (central line) or pyridine at 123.0 ppm (higher field signal, central line) for ^{13}C NMR spectra; scalar coupling constants were 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 an 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 a capillary temperature of 250 °C. Data were processed using the Finnigan Xcalibur software system. ^1H and ^{13}C NMR and MS analysis confirmed the 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 (CH_2Cl_2–CH_3OH 95 : 5 v/v). After 18 h the reaction was stopped and the

 This journal is © The Royal Society of Chemistry 2015

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

suspension was filtered to remove the enzyme which was washed with pyridine and methanol. The solvent was evaporated under vacuum and the crude compound was 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; $[\alpha]_D^{20} = -4.7$ ($CHCl_3$, *c* 0.5); ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, 3H, J = 7.0 Hz, CH_3), 1.19–1.34 (m, 28H, 14 CH_2), 1.61 (m, 2H, CH_2), 2.00 (s, 3H, $COCH_3$), 2.03 (s, 3H, $COCH_3$), 2.05 (s, 3H, $COCH_3$), 2.09 (s, 3H, $COCH_3$), 2.30 (t, 2H, J = 7.6 Hz, CH_2), 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′); ^{13}C-NMR ($CDCl_3$): δ = 14.1 (CH_3), 20.5–20.07 (4 COCH_3), 22.7 (CH_2), 24.9 (CH_2), 28.9–30.0 (12 CH_2), 31.9 (CH_2), 34.1 (CH_2), 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 (CH_3OH, positive-ion mode): m/z = 711.3 $[M + Na]^+$, calcd for $C_{35}H_{60}O_{13}$, m/z 688.40 [M]. The same enzymatic procedure on the same amount of substrate, using trifluoroethyldecanoate (1.80 g, 7.08 mmol) as the 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). $[\alpha]_D^{20} = -1.0$ ($CHCl_3$, *c* 1.0); ^{1}H-NMR ($CDCl_3$): δ = 0.87 (t, 3H, J = 7.0 Hz, CH_3), 1.20–1.35 (m, 12H, 6 CH_2), 1.61 (m, 2H, CH_2), 2.00 (s, 3H, $COCH_3$), 2.03 (s, 3H, $COCH_3$), 2.04 (s, 3H, $COCH_3$), 2.09 (s, 3H, $COCH_3$), 2.30 (t, 2H, J = 7.6 Hz, CH_2), 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′); ^{13}C-NMR ($CDCl_3$): δ = 14.1 (CH_3), 20.5–20.7 (4 COCH_3), 22.7 (CH_2), 24.9 (CH_2), 29.0–29.5 (4 CH_2), 31.8 (CH_2), 34.1 (CH_2), 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 (CH_3OH, positive-ion mode): m/z = 599.3 $[M + Na]^+$, calcd for $C_{27}H_{44}O_{13}$, 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 3 h (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, 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. $[\alpha]_D^{20} = -3.3$ ($CHCl_3$, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.85 (t, 3H, J = 6.5 Hz, CH_3), 1.16–1.33 (m, 28H, 14 CH_2), 1.64 (m, 2H, CH_2), 1.96 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$), 2.15 (s, 3H, $COCH_3$), 2.35 (t, 2H, J = 7.5 Hz, CH_2), 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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 19.7–20.3 (4 COCH_3), 22.4 (CH_2), 24.7 (CH_2), 28.6–28.8 (12 CH_2), 31.6 (CH_2), 33.8 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, positive-ion mode): m/z = 953.5 $[M + Na]^+$, calcd for $C_{54}H_{74}O_{13}$, m/z 930.51 [M].

With the same procedure, starting from **4b** (0.51 g, 0.88 mmol), 1-*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. $[\alpha]_D^{20} = -3.4$ ($CHCl_3$, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.85 (t, 3H, J = 7.0 Hz, CH_3), 1.14–1.30 (m, 12H, 6 CH_2), 1.63 (m, 2H, CH_2), 1.96 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$), 2.14 (s, 3H, $COCH_3$), 2.34 (t, 2H, J = 7.5 Hz, CH_2), 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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 19.6–20.4 (4 COCH_3), 22.4 (CH_2), 24.7 (CH_2), 28.4–29.6 (4 CH_2), 31.6 (CH_2), 33.8 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, positive-ion mode): m/z = 841.3 $[M + Na]^+$, calcd for $C_{46}H_{58}O_{13}$, 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, CH_2Cl_2–CH_3OH 95 : 5 v/v). The solvent was evaporated under a stream of N_2 and the crude compound was purified by flash chromatography (CH_2Cl_2–CH_3OH 95 : 5, v/v) to yield 1-*O*-octadecanoyl-3-*O*-trityl-2-*O*-β-D-glucopyranosyl-*sn*-glycerol (**6a**) (0.22 g, 0.29 mmol, 45% yield) as an oil: $[\alpha]_D^{20}$ = +6.3 ($CHCl_3$, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.85 (t, 3H, J = 7.0 Hz, CH_3), 1.15–1.31 (m, 28H, 14 CH_2), 1.61 (m, 2H, CH_2), 2.32 (m, 2H, CH_2), 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

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 22.4 (CH_2), 24.7 (CH_2), 28.8–29.7 (12 CH_2), 31.6 (CH_2), 33.9 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, positive-ion mode): *m*/*z* = 785.3 $[M + Na]^+$, calcd for $C_{46}H_{66}O_9$, *m*/*z* 762.47 [M].

With the same procedure staring from **5b** (0.47 g, 0.58 mmol) 1-*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: $[\alpha]^{20}_D$ = +7.8 ($CHCl_3$, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.83 (t, 3H, *J* = 7.0 Hz, CH_3), 1.07–1.28 (m, 12H, 6 CH_2), 1.60 (m, 2H, CH_2), 2.31 (m, 2H, CH_2), 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, $J_{6'a,6'b}$ = 11.5 Hz, H-6′a), 4.45 (dd, 1H, $J_{5',6'ab}$ = 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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 22.4 (CH_2), 24.7 (CH_2), 28.7–29.3 (4 CH_2), 31.5 (CH_2), 33.8 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, positive-ion mode): *m*/*z* = 673.2 $[M + Na]^+$, calcd for $C_{38}H_{50}O_9$, *m*/*z* 650.35 [M].

*1-O-Acyl-3-O-trityl-2-O-β-D-glucuronopyranosyl-sn-glycerols (**7a, b**).* To a solution of compound **6a** (0.22 g, 0.29 mmol) in a 55 : 45 mixture of CH_3CN and 0.67 M phosphate buffer (3 mL, pH 6.7), TEMPO (0.01 g, 0.061 mmol), $NaClO_2$ (20% aqueous solution, 0.4 mL) and $NaClO_2$ (15% aqueous solution, 0.025 mL) were added in the order. After stirring for 3 hours (TLC, CH_2Cl_2–CH_3OH 90 : 10 v/v), acetonitrile was removed under reduced pressure and the aqueous phase was extracted with Et_2O. The organic layers were assembled, dried over anhydrous Na_2SO_4, filtered and evaporated to give the desired 1-*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; $[\alpha]^{20}_D$ = −11.1 ($CHCl_3$–CH_3OH 65 : 35, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.85 (t, 3H, *J* = 7.0 Hz, CH_3), 1.13–1.35 (m, 28H, 14 CH_2), 1.59 (m, 2H, CH_2), 2.28 (m, 2H, CH_2), 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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 22.4 (CH_2), 24.7 (CH_2), 28.8–29.7 (12 CH_2), 31.6 (CH_2), 33.8 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, negative-ion mode): *m*/*z* = 775.5 $[M - 1]^-$, calcd for $C_{46}H_{64}O_{10}$, *m*/*z* 776.45 [M].

With the same procedure, starting from **6b** (0.21 g, 0.32 mmol), 1-*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; $[\alpha]^{20}_D$ = −11.3 ($CHCl_3$–CH_3OH 65 : 35, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.84 (t, 3H, *J* = 7.0 Hz, CH_3), 1.07–1.28 (m, 12H, 6 CH_2), 1.57 (m, 2H, CH_2), 2.26 (m, 2H, CH_2), 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); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 22.4 (CH_2), 24.6 (CH_2), 28.7–29.3 (4 CH_2), 31.5 (CH_2), 33.8 (CH_2), 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 (O*C*Ph_3), 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 (CH_3OH, negative-ion mode): *m*/*z* = 663.2 $[M - 1]^-$, calcd for $C_{38}H_{48}O_{10}$, *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 CH_2Cl_2 (1.5 mL) and Dowex® Marathon™ C, H^+ form (0.15 g), was added (methanol washing of the resin was not done, see Materials and equipment, to avoid methyl ester formation). The reaction was stirred overnight at room temperature (TLC, CH_2Cl_2–CH_3OH 90 : 10 v/v) obtaining a white suspension. The reaction mixture was filtered and the residue was washed with CH_2Cl_2 which was eliminated. The remaining solid was then washed with AcOEt and the washings were dried over Na_2SO_4 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; $[\alpha]^{20}_D$ = −29.3 ($CHCl_3$–CH_3OH 65 : 35, *c* 0.5); ^{1}H-NMR (Pyd_5): δ = 0.85 (t, 3H, *J* = 7.0 Hz, CH_3), 1.13–1.31 (m, 28H, 14 CH_2), 1.64 (m, 2H, CH_2), 2.37 (m, 2H, CH_2), 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′); ^{13}C-NMR (Pyd_5): δ = 13.8 (CH_3), 22.4 (CH_2), 24.7 (CH_2), 28.8–29.5 (12 CH_2), 31.6 (CH_2), 33.9 (CH_2), 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 (CH_3OH, negative-ion mode): *m*/*z* = 533.3 $[M - 1]^-$, calcd for $C_{27}H_{50}O_{10}$, *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. $[\alpha]^{20}_D$ = −31.6 ($CHCl_3$–CH_3OH 65 : 35, *c* 1.0); ^{1}H-NMR (Pyd_5): δ = 0.82 (t, 3H, *J* = 7.0 Hz, CH_3), 1.09–1.27 (m, 12H, 6 CH_2), 1.62 (m, 2H, CH_2), 2.36 (m, 2H, CH_2), 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),

 This journal is © The Royal Society of Chemistry 2015

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

5.20 (d, 1H, H-1′); ^{13}C-NMR (Pyd$_5$): δ = 13.7 (CH_3), 22.4 (CH_2), 24.7 (CH_2), 28.7–29.2 (4 CH_2), 31.5 (CH_2), 33.8 (CH_2), 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 (CH_3OH, negative-ion mode): m/z = 421.5 [M − 1]$^-$, Calcd for $C_{19}H_{34}O_{10}$, 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 the usual work-up the crude compound 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 to be 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 ($CHCl_3$, c 1); ^{1}H NMR ($CDCl_3$): δ = 0.86 (t, 3H, J = 7.0 Hz, CH_3), 1.19–1.33 (m, 28H, 14 CH_2), 1.59 (m, 2H, CH_2), 1.98 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$), 2.04 (s, 3H, $COCH_3$), 2.06 (s, 3H, $COCH_3$), 2.29 (t, 2H, J = 7.6 Hz, CH_2), 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′); ^{13}C-NMR ($CDCl_3$): δ = 14.1 (CH_3), 20.4–20.08 (5 COCH_3), 22.7 (CH_2), 24.8 (CH_2), 28.9–29.9 (12 CH_2), 31.9 (CH_2), 34.1 (CH_2), 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 (CH_3OH, positive-ion mode): m/z = 753.5 [M + Na]$^+$, calcd for $C_{37}H_{62}O_{14}$, 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 CH_3CN and 0.67 M phosphate buffer (pH 6.7) and TEMPO (0.008 g, 0.05 mmol), $NaClO_2$ (20% aqueous solution, 0.3 mL) and NaClO (15% aqueous solution, 0.015 mL) were added. After stirring overnight at room temperature (TLC, CH_2Cl_2–CH_3OH 90 : 10 v/v), 0.5 M $Na_2S_2O_3$ was added and the aqueous phase was acidified with HCl and extracted with Et_2O. The organic layers were assembled, dried over anhydrous Na_2SO_4, 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. ^{1}H-NMR ($CDCl_3$–CD_3OD–D_2O, 65 : 35 : 6, 315 K): δ = 0.82–0.87 (m, 6H, 2 CH_3), 1.18–1.31 (m, 56H, 28 CH_2), 1.53–1.61 (m, 4H, 2 CH_2), 2.27–2.32 (m, 4H, 2 CH_2), 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′); ^{13}C-NMR ($CDCl_3$–CD_3OD–D_2O, 65 : 35 : 6, 315 K): δ = 14.3 (2 CH_3), 23.1 (2 CH_2), 25.3 (2 CH_2), 29.5–30.2 (24 CH_2), 32.4 (2 CH_2), 34.6 (2 CH_2), 63.2 (C1 or C3), 64.0 (C1 or C3), 72.5 (C4′), 73.8 (C2′), 75.2 (C2), 75.6 (s, C5′), 76.6 (C3′), 103.2 (C1′), 174.8 (2 CO); ESI-MS (CH_3OH, negative-ion mode): m/z = 799.7 [M − 1]$^-$, Calcd for $C_{45}H_{84}O_{11}$, 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 CH_2Cl_2 (2.5 mL) and cooled at −10 °C. Decanoyl chloride (0.22 g, 1.18 mmol) as a 15% (v/v) CH_2Cl_2 solution and pyridine (0.23 mL, 2.8 mmol) as a 10% (v/v) CH_2Cl_2 solution were added in the order and the mixture was stirred at −10 °C under an Ar atmosphere. The reaction was monitored by TLC (petroleum ether–EtOAc, 70 : 30 v/v) and stopped after 50 min diluting with CH_2Cl_2 (15 mL). The solution was washed with 1 M HCl (10 mL), water (10 mL), $NaHCO_3$ saturated solution (10 mL), and water (2 × 10 mL) in the order and the aqueous phases were re-extracted with CH_2Cl_2 (2 × 15 mL). The collected organic layers were dried over Na_2SO_4, evaporated under reduced pressure and the crude residue was 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 ($CHCl_3$, c 1.0); ^{1}H-NMR ($CDCl_3$): δ = 0.85–0.90 (m, 6H, 2 CH_3), 1.20–1.34 (m, 24H, 12 CH_2), 1.56–1.64 (m, 4H, 2 CH_2), 2.27–2.33 (m, 4H, 2 CH_2), 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, $ClCH_2$), 4.01 (m, 2H, $ClCH_2$), 4.04 (m, 2H, $ClCH_2$), 4.05–4.27 (m, 5H, H-1a, H-1b, H-3a, H-3b and H-2), 4.14 (s, 2H, $ClCH_2$), 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′); ^{13}C-NMR ($CDCl_3$): δ = 14.1 (2 CH_3), 22.6 (2 CH_2), 24.8 (2 CH_2), 29.1–29.4 (8 CH_2), 31.8 (2 CH_2), 34.0 (CH_2), 34.1 (CH_2), 40.1, 40.2, 40.3 and 40.5 (4 CH_2Cl), 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 (CH_3OH, negative-ion mode): m/z = 867.1 [M − 1]$^-$, Calcd for $C_{37}H_{58}Cl_4O_{14}$, m/z 868.25 [M].

The obtained didecanoate (0.23 g, 0.26 mmol) was dissolved in EtOAc–CH_3OH (7 mL, 1 : 1 v/v) and hydrazine acetate (0.366 g, 3.98 mmol) was added. The reaction was stirred under an Ar atmosphere at room temperature overnight and monitored by TLC (CH_2Cl_2–CH_3OH, 95 : 5 v/v). The solvent was evaporated under reduced pressure and the crude residue was subjected to repeated flash column chromatography (CH_2Cl_2–CH_3OH, 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 ($CHCl_3$, c 1.0); ^{1}H-NMR ($CDCl_3$): δ = 0.86–0.91 (m, 6H, 2 CH_3), 1.19–1.35 (m, 24H, 12 CH_2), 1.56–1.65 (m, 4H, 2 CH_2), 2.28–2.37 (m, 4H, 2 CH_2), 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, $J_{1b/3b,2}$ = 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,

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

$J_{1',2'}$ = 7.7 Hz, H-1′); ^{13}C-NMR ($CDCl_3$): δ = 14.1 (2 CH_3), 22.7 (2 CH_2), 24.8 (2 CH_2), 29.1–29.4 (8 CH_2), 31.8 (2 CH_2), 34.1 (CH_2), 34.2 (CH_2), 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 (CH_3OH, positive-ion mode): *m*/*z* = 585.4 $[M + Na]^+$, calcd for $C_{29}H_{54}O_{10}$, *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 as reported above 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. $[\alpha]_D^{20}$ = −18.9 (CH_3OH, *c* 1.0); ^{1}H-NMR (CD_3OD): δ = 0.85–0.92 (m, 6H, 2 CH_3), 1.22–1.36 (m, 24H, 12 CH_2), 1.54–1.64 (m, 4H, 2 CH_2), 2.29–2.37 (m, 4H, 2 CH_2), 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′); ^{13}C-NMR (CD_3OD): δ = 14.5 (2 CH_3), 23.7 (2 CH_2), 26.0 (2 CH_2), 30.2–30.6 (8 CH_2), 33.1 (2 CH_2), 34.9 (2 CH_2), 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 (CH_3OH, negative-ion mode): *m*/*z* = 575.3 $[M - 1]^-$, Calcd for $C_{29}H_{52}O_{11}$, *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, respectively. Compounds were added together with the constitutive active form of human Akt1 (25 m units per well), and allowed to phosphorylate the bound substrate following the addiction of Mg^{2+} and ATP. The amount of phosphorylated substrate was measured by binding it with the horseradish peroxidase conjugate of an anti-phospho-AKTide-2T monoclonal antibody, which then catalyzes the conversion of the chromogenic substrate tetramethylbenzidine 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 the “inhibitor control” as indicated in the assay protocol. Each experiment was performed in triplicate.

Cellular studies

Compound preparation. Perifosine was dissolved in H_2O 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 line[30] was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 atmosphere. For cell growth inhibition assays, cells were plated in 12-well plates at 10 000 cells cm^{-2} in a complete medium. The day after seeding, cells were exposed to the solvent (DMSO) or to different concentrations of the novel compounds for 72 h. For tests in a serum-free medium, the day after seeding complete medium was substituted with a serum-free medium and the cells were exposed to the compounds. Twenty-four hours later the drug-containing medium was replaced with a complete medium. Cells were harvested using trypsin and counted 96 h after seeding using 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. IC_{50} represents the drug concentration inhibiting growth by 50%.

Acknowledgements

This work has been supported by Fondazione Cariplo, grant no. 2011-0490. The authors thank Mr Andrea Lorenzi for MS technical support.

Notes and references

1 G. Holzl and P. Dormann, *Prog. Lipid Res.*, 2007, **46**, 225.
2 T. Fontaine, C. Lamarre, C. Simenel, K. Lambou, B. Coddeville, M. Delepierre and J. P. Latgé, *Carbohydr. Res.*, 2009, **344**, 1960.
3 W. Eichenberger and C. Gribi, *Phytochemistry*, 1997, **45**, 1561.
4 Y. Okazaki, H. Otsuki, T. Narisawa, M. Kobayashi, S. Sawai, Y. Kamide, M. Kusano, T. Aoki, M. Y. Hirai and K. Saito, *Nat. Commun.*, 2013, **4**, 1510.
5 B. Cao, X. Q. Chen, Y. Yamaryo-Botte, M. B. Richardson, K. L. Martin, G. N. Khairallah, T. W. T. Rupasinghe, R. M. O'Flaherty, R. A. J. O'Hair, J. E. Ralton, P. K. Crellin, R. L. Coppel, M. J. McConville and S. J. Williams, *J. Org. Chem.*, 2013, **78**, 2175.
6 A. Semeniuk, C. Sohlenkamp, K. Duda and Georg Hölzl, *J. Biol. Chem.*, 2014, **289**, 10104.
7 Z. Zhang, H. Liao and W. J. Lucas, *J. Integr. Plant Biol.*, 2014, **56**, 192.
8 Y. Nakamura, *Prog. Lipid Res.*, 2013, **52**, 43.
9 N. Shaikh, D. Colombo, F. Ronchetti and M. Dangate, *C. R. Chim.*, 2013, **16**, 850.
10 K. Matsuki, A. Tanabe, A. Hongo, F. Sugawara, K. Sakaguchi, N. Takahashi, N. Sato and H. Sahara, *Cancer Sci.*, 2012, **103**, 1546.
11 E. Plouguerné, L. M. de Souza, G. L. Sassaki, J. F. Cavalcanti, M. T. Villela Romanos, B. A. da Gama,

 This journal is © The Royal Society of Chemistry 2015

Published on 06 November 2014. Downloaded by Universita Studi di Milano on 08/01/2015 10:32:04.

R. C. Pereira and E. Barreto-Bergter, *Mar. Drugs*, 2013, **11**, 4628.

12 A. Bruno, C. Rossi, G. Marcolongo, A. Di Lena, A. Venzo, C. P. Berrie and D. Corda, *Eur. J. Pharmacol.*, 2005, **524**, 159.

13 T. Tanaka, H. Kitamura, H. Sahara, A. Imai, Y. Itoh, I. Honma, E. Sato, K. Kobayashi, T. Maeda, M. Takenouchi, K. Ohta, F. Sugawara, K. Sakaguchi, A. Ando, H. Inoko, N. Sato and T. Tsukamoto, *Transplant. Immunol.*, 2007, **18**, 67.

14 (*a*) M. Dangate, L. Franchini, F. Ronchetti, T. Arai, A. Iida, H. Tokuda and D. Colombo, *Bioorg. Med. Chem.*, 2009, **17**, 5968; (*b*) The lower yield obtained for **2a** (see Experimental) was due to the low solubility of the starting material **8a** in the reaction conditions. Also **2a** was scarcely soluble and its was possible to record its NMR spectra only in a mixture of deuterated solvents (see Experimental) using a very diluted solution (about 0.5 mg mL^{-1}) and heating at 315 K. This hampered to detect in the ^{13}C spectrum of **2a** the carbonyl resonance of the carboxyl group that, for the corresponding didecanoate **2b**, appeared as a broadened singlet (see Experimental and ESI†). For the same reason the optical rotation of **2a** was not reported (*c*) D. Colombo, F. Compostella, F. Ronchetti, A. Scala, L. Toma, H. Tokuda and H. Nishino, *Bioorg. Med. Chem.*, 1999, **7**, 1867.

15 M. Dangate, L. Franchini, F. Ronchetti, T. Arai, A. Iida, H. Tokuda and D. Colombo, *Eur. J. Org. Chem.*, 2009, 6019.

16 L. Cipolla, C. Redaelli, F. Granucci, G. Zampella, A. Zaza, R. Chisci and F. Nicotra, *Carbohydr. Res.*, 2010, **345**, 1291.

17 (*a*) B. Vanhaesebroeck, L. Stephens and P. Hawkins, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 195; (*b*) D. J. Gillooly, A. Simonsen and H. Stenmark, *Biochem. J.*, 2001, **355**, 249; (*c*) D. Karathanassis, R. V. Stahelin, J. Bravo, O. Perisic, C. M. Pacold, W. W. Cho and R. L. Williams, *EMBO J.*, 2002, **21**, 5057; (*d*) S. Jean and A. A. Kiger, *J. Cell Sci.*, 2014, **127**, 923.

18 L. Gabrielli, I. Calloni, G. Donvito, B. Costa, N. Arrighetti, P. Perego, D. Colombo, F. Ronchetti, F. Nicotra and L. Cipolla, *Eur. J. Org. Chem.*, 2014, 5962.

19 G. Cassinelli, V. Zuco, L. Gatti, C. Lanzi, N. Zaffaroni, D. Colombo and P. Perego, *Curr. Med. Chem.*, 2013, **20**, 1923.

20 S-B. Rong, Y. Hu, I. Enyedy, G. Powis, E. J. Meuillet, X. Wu, R. Wang, S. Wang and A. P. Kozikowski, *J. Med. Chem.*, 2001, **44**, 898.

21 C. Redaelli, F. Granucci, F. De Gioia and L. Cipolla, *Mini-Rev. Med. Chem.*, 2006, **6**, 1127.

22 I. Hers, E. E. Vincent and J. M. Tavaré, *Cell. Signalling*, 2011, **23**, 1515.

23 (*a*) D. Colombo, F. Ronchetti, A. Scala, I. M. Taino, F. Marinone Albini and L. Toma, *Tetrahedron: Asymmetry*, 1994, **5**, 1377; (*b*) F. Marinone Albini, C. Murelli, G. Patritti and M. Rovati, *Synth. Commun.*, 1994, **24**, 1651.

24 D. Colombo, F. Ronchetti, A. Scala, I. M. Taino and L. Toma, *Tetrahedron: Asymmetry*, 1996, **7**, 771.

25 E. Manzo, M. L. Ciavatta, D. Pagano and A. Fontana, *Tetrahedron Lett.*, 2012, **53**, 879.

26 M. Barbier, T. Breton, K. Servat, E. Grand, B. Kokoh and J. Kovensky, *J. Carbohydr. Chem.*, 2006, **25**, 253.

27 N. Ferlin, D. Grassi, C. Ojeda, M. J. L. Castro, E. Grand, A. Fernández Cirelli and J. Kovenskya, *Carbohydr. Res.*, 2008, **343**, 839.

28 V. Adasch, B. Hoffmann, W. Milius, G. Platz and G. Voss, *Carbohydr. Res.*, 1998, **314**, 177.

29 (*a*) W. J. van Blitterswijk and M. Verheij, *Biochim. Biophys. Acta*, 2013, **1831**, 663; (*b*) J. J. Gills and P. A. Dennis, *Curr. Oncol. Rep.*, 2009, **11**, 102; (*c*) J. Fensterle, B. Aicher, I. Seipelt, M. Teifel and J. Engel, *Anti-Cancer Agents Med. Chem.*, 2014, **14**, 629.

30 P. Perego, S. Romanelli, N. Carenini, I. Magnani, R. Leone, A. Bonetti, A. Paolicchi and F. Zunino, *Ann. Oncol.*, 1998, **9**, 423.