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Development of carbohydrate-derived inhibitors of acid sphingomyelinase

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ABSTRACT

The acid sphingomyelinase is an emerging drug target, especially for inflammatory lung diseases. Presently, there are no directly-acting potent inhibitors available for cell-based studies. The potent inhibitor phosphatidylinositol-3,5-bisphosphate (PtdIns3,5P2) is not only unsuited for cell culture studies, but also does not provide hints for further structural improvements. In the SAR study described here, we replaced the inositolphosphate moiety by a carbohydrate derivative and the phosphatidic acid residue by an alkylsulfone ester. The resulting compound is more active than its parent compound and offers new means for further structural modification.

1. Introduction

The acid sphingomyelinase (aSMase) is an emerging drug target in a variety of diseases.[1–3] The enzyme is essential for infection of non-phagocytotic cells with *Neisseria gonorrhoeae*[4] and inhibition of aSMase prevents bacterial infections in a rat model of cystic fibrosis.[5] Formation of acute lung injury (ALI) elicited by endotoxin, acid instillation or platelet-activating factor (PAF)[6] and the formation of pulmonary emphysema,[7] is regulated by aSMase. Pharmacological or genetic inhibition of aSMase prevents apoptosis and degeneration of liver cells in a mouse model for Wilson's disease.[8] In addition, there are several reports that aSMase significantly contributes to the formation of atherosclerotic plugs.[9] The rapid advancement in dissecting the biological and patho-physiological roles of aSMase however, stands in sharp contrast to the lack of potent and selective inhibitors of this enzyme. For animal and cell culture studies, the tricyclic antidepressants desipramine and imipramine are commonly used, despite of their known indirect way of action[10] and despite their known lack of specificity towards other lysosomal sphingolipid hydrolases like acid ceramidase.[11]

Some time ago, we were able to identify phosphatidylinositol-3,5-bisphosphate (PtdIns3,5P_2) as the most potent inhibitor of aSMase (K_M = 0.53 μM).[12] Yet, this naturally occurring phospholipid has unfavourable biophysical characteristics like its fivefold negative charge and its two long fatty acid chains that would firmly fix it within cellular membranes. Moreover, the compound is a substrate for phospholipases A_1, A_2, C and D and phosphoinositide phosphatases, making it ill-suited for cell culture studies. In first structure–activity-relationship (SAR) studies, we[12,13] and Dawson and co-workers[14] found out that the inositol ring (number of phosphate groups, stereochemistry, and the existence of hydroxyl groups) is more or less invariant. In contrast, the phosphatidic acid residue could be replaced by a simple alkylsulfonyl residue, leading to inhibitors of the same potency, when compared to PtdIns3,5P_2. These inhibitors have improved biophysical properties and a marked cellular activity.[13] However, since PtdIns3,5P_2 is a naturally occurring lipid, involved in signal transduction, we envision the development of aSMase inhibitors that are less likely to interfere with phosphoinositide signalling and that may provide further possibilities for introducing structural modifications leading to more potent aSMase inhibitors.

2. Results and discussion

Preceding the identification of PtdIns3,5P_2, Sandhoff and colleagues found out that various nucleoside-3,5-bisphosphates inhibit the aSMase with IC_{50} values of ~5 μM, respectively.[15] In the light of our newer observations, clearly showing that the inhibitory potency of phosphoinositide analogs dramatically increases with lipophilicity, we were amazed by such a potent inhibition, despite of the relatively moderate lipophilicity of the nucleoside bisphosphates. Thus we wondered if carbohydrate analogues of PtdIns 3,5P_2 may be potent inhibitors of aSMase, too. While in the inositol moiety, the 1,3,5-trisphosphate motif is essentially in a *cis*, *cis* arrangement, the 3,5-bisphosphate motif at the ribose has a *trans* orientation. We believe that the *trans* orientation is possible only because of the flexibility at the primary 5′-carbon atom of the ribose scaffold. Because D-glucose is structurally very similar and is a very low-cost starting material and in addition the arsenal of established regio- and chemo-selective procedures at the glucose scaffold is nearly unlimited, we decided to synthesize PtdIns3,5P_2 analogs derived from glucose.

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In order to mimic the 1,3,5-trisphosphate and the 3,5-bisphosphate patterns of PtdIns3,5P_2 and of the nucleoside inhibitors diisopropylidene glucofuranose **1** was chosen as a starting material, allowing for modification at the 3-position. The starting material was benzylated in quantitative yield to give **2**, which then was converted into 3-*O*-benzyl-D-glucose **3**, upon treatment with acetic acid (Fig. 1).[16] Benzyl-protection at the anomeric position gave **4** in moderate yield. In the next step, alkyl sulfonyl groups were regioselectively attached to the 6-O-position to form **5a–d**. Each of the compounds then was treated with an excess of phosphoramidate and the P(III) intermediates were converted into the protected phosphates **6a–d** by oxidation, using hydroperoxide. The final deprotection by catalytic hydrogenation afforded the phosphoinositide-analogs **7a–d**.

When the analogs **7a–d** were tested as inhibitors of aSMase, we noticed a strong correlation between inhibition and increasing alkyl chain length. Fortunately, the carbohydrate analogue **7d** (IC_{50} = 0.44 μM) is approximately twice as potent when compared to PtdIns3,5P_2, itself (Table 1). In analogy to PtdIns3,5P_2, the IC_{50} values for neutral sphingomyelinase (nSMase) inhibition by compounds **7a-d** are all >100 μM, respectively (data not shown).

In order to learn more about the SAR of the carbohydrate derivatives, we furthermore envisioned the synthesis of the unlipidated scaffold D-glucose-2,4,6-trisphosphate **9a** and its 1,3-dideoxy-analog **9b** (see Figs. 2 and 3). Previous studies in our group had shown that *myo*-inositol 1,3,5-trisphosphate (IC_{50} = 27 μM), but not cyclohexane-*cis*, *cis*-1,3,5-trisphosphate (IC_{50} >100 μM), shows moderate inhibition of aSMase.[13] We wondered whether in contrast to the inositol ring, the hydroxyl groups of the glucose ring would not play a role for aSMase inhibition. The synthesis of **9a** started from the intermediate **4**, which was exhaustively phosphorylated under the conditions described above (Fig. 2). The yield for this one-pot reaction leading to **8** was only 32%. In the final step, the benzyl protecting groups were removed in a single quantitative step using hydrogen on palladium-charcoal catalyst, to afford **9a**. The synthesis of the dideoxy-analog **9b** started from D-glucose diacetonide **1**, which was de-hydroxylated using the Barton–McCombie procedure.[17] After acidic deprotection the resulting 3-deoxy-D-glucopyranose **12** was peracetylated and transformed into the glycosyl bromide (Fig. 3). The latter was de-functionalized to yield the tetrahydropyrane derivative **14**.[18] This compound was deprotected using cation exchange resin. The triol **15** then was phosphorylated according to the known procedure and was quantitatively deprotected to give the final product **9b**. To our surprise, **9a** as well as **9b** only marginally inhibited aSMase, with IC_{50} values ≫50 μM, respectively (data not shown).

Inhibitors of aSMase are potentially worthwhile as suppressors of apoptosis in a variety of inflammatory diseases. In order to test whether the inhibitor **7d** is active in cell culture, we stimulated apoptosis in cultured HEK-293 cells using 10^{-8} M dexamethasone. When 1 μM or 2 μM of the inhibitor together with the dexamethasone were added to the culture media, the apoptosis was gradually suppressed down to the level of untreated cells (Fig. 4).

Table 1
Inhibition of aSMase from rat brain

Compds	Inhibition IC_{50}[a] (μM)
7a	39.6 (±0.68)
7b	4.16 (±0.07)
7c	2.34 (±0.07)
7d	0.44 (±0.01)

[a] Values are means of three experiments, standard deviation is given in parentheses.

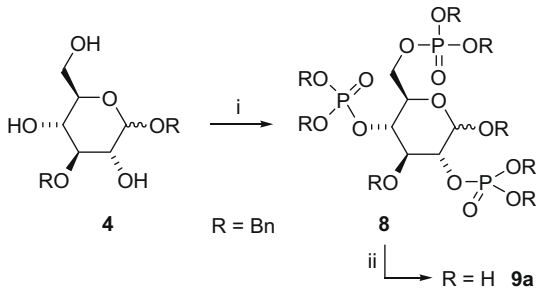

Figure 2. Reagents and conditions: (i) $(BnO)_2PN(iPr)_2$, tetrazole, CH_3CN, rt, 24 h, then *t*BuOOH, 0 °C to rt, 30 min; (ii) H_2, Pd/C, MeOH/H_2O (4:1), rt, 16 h.

3. Conclusions

Despite the fact that aSMase is emerging as an important drug target, there are no potent and selective inhibitors that can be used in cell culture. We have synthesized the first selective aSMase inhibitor based on a carbohydrate scaffold. The inhibitor significantly differs from the structure of PtdIns3,5P_2, is more active than its parent compound and provides a new starting point for SAR studies leading to more potent inhibitors that may replace the tricyclic antidepressants in cell culture and model animal studies. Indeed, we were able to show that the carbohydrate-based inhibitor is active in cell culture and inhibits dexamethasone-induced apoptosis. However, whether the inhibitor affects the lysosomal aSMase or the secreted aSMase only will be a matter of future investigations (Table 1).

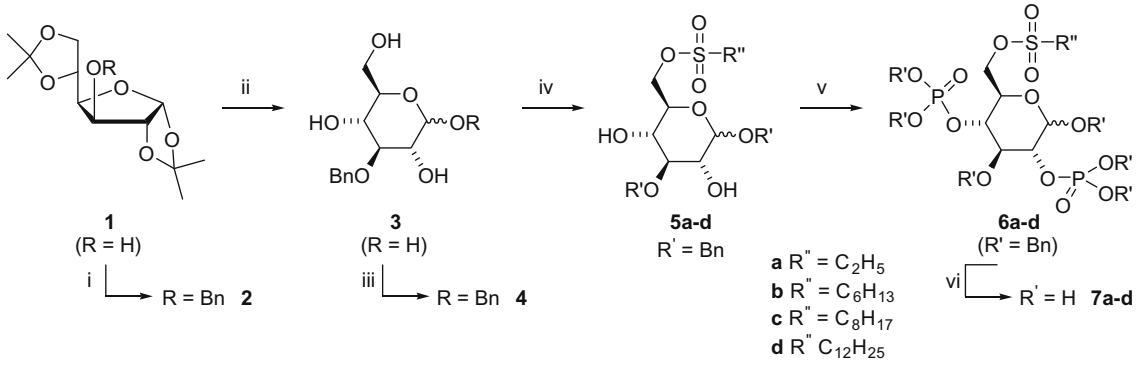

Figure 1. Reagents and conditions: (i) NaH, BnBr, DMF, 0 °C–rt, 3 h; (ii) AcOH (90%), 105 °C, 45 min; (iii) BnOH, AcCl, rt, 24 h; (iv) R'SO_2Cl, NEt_3, CH_2Cl_2, rt, 16 h; (v) $(BnO)_2PN(iPr)_2$, tetrazole, CH_3CN, rt, 24 h, then *t*BuOOH, 0 °C to rt, 30 min; (vi) H_2, Pd/C, MeOH/H_2O (4:1), rt, 16 h.

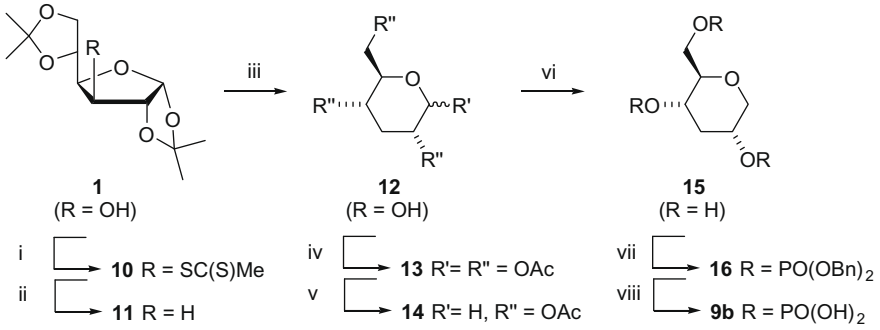

Figure 3. Reagents and conditions: (i) MeI, NaH, CS_2; (ii) Bu_3SnH; (iii) AcOH (90%), 105 °C, 45 min; (iv) Ac_2O, pyridine; (v) HBr, then Bu_3SnH; (vi) Na, MeOH; (vii) $(BnO)_2PN(iPr)_2$, tetrazole, CH_3CN, rt, 24 h, then *t*BuOOH, 0 °C to rt, 30 min; (viii) H_2, Pd/C, MeOH/H_2O (4:1), rt, 16 h.

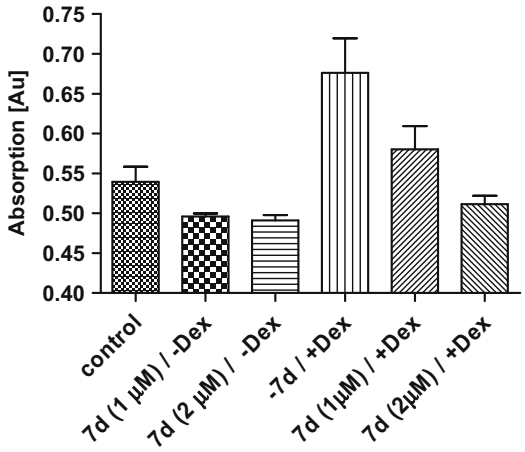

Figure 4. The aSMase inhibitor **7d** inhibits dexamethasone-induced apoptosis in HEK-293 cells. Absorption from a DNA fragmentation ELISA. Apoptosis was stimulated using 10^{-8} M dexamethasone for 12 h. Control: untreated cells.

4. Experimental

4.1. Enzyme preparation and activity assays

4.1.1. Partial purification of aSMase and nSMase

The isolation and partial purification of aSMase has been slightly modified from a method described previously.[19] Briefly, aSMase was obtained as follows: One rat brain (stripped rat brains, Pel-Freez Biologicals, Rogers, AR, USA) was homogenized in NaOAc buffer (10 mL, 100 mM, pH 5.0), containing Triton X-100 (0.1%, v/v) and protease inhibitor mix. The brain tissues were homogenized for three rounds of 10 passes each. Afterwards, the crude homogenate was centrifuged to remove debris (1000*g*, 15 min). The supernatant was centrifuged at 35,000*g* for 45 min and was filtered trough a sterile filter (∅ = 0.45 μm). The enzyme preparation was stored at −80 °C or loaded onto a strong anion exchange column (Mono Q 5/50 GL, GE Healthcare). For nSMase containing microsomes, one rat brain was homogenized with a Teflon pestle in Tris/HCl buffer (10 mL, 50 mM, pH7.4) containing EDTA (2 mM), EGTA (5 mM), DTT (5 mM), β-mercaptoethanol (5 mM) and a protease inhibitor mix (Roche) after washing with isotonic sodium chloride solution. The crude homogenate was centrifuged (1000*g*, 15 min) to remove debris. The supernatant was centrifuged at 100,000*g*, 90 min, and the pellet was solubilized in homogenisation buffer containing Triton X-100 (1.7%, v/v). After rocking at 4 °C for 2 h, the microsome preparation was stored at −80 °C or loaded onto a strong anion exchange column (Mono Q 5/50 GL, GE Healthcare). For both sphingomyelinases, the column was flushed with five column volumes (CV) of equilibration buffer (nSMase: 20 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, protease inhibitor mix, pH 7.4; aSMase: 50 mM NaOAc, 1 mM EDTA, 1 mM EGTA, protease inhibitor mix, pH 5.0). After sample loading, the column was washed with 10CV of equilibration buffer, followed by a linear gradient (3CV) of washing buffer (0–100%, equilibration buffer, containing 1 M sodium chloride) and maintained for 8CV at washing buffer (100%). The final elution was carried out with a 10CV gradient Triton X-100 (0–1.1%, v/v) in washing buffer.

4.1.2. Sphingomyelinase assays

The micellar nSMase assays using ^{14}C-labeled sphingomyelin as a substrate were performed as described before.[19] The fluorescent aSMase assay was performed in a 384-well-plate using the 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU-PC) substrate.[13,20] Reaction mixtures consisted of HMU-PC-substrate (13.3 μL), reaction-buffer (13.3 μL total, containing 100 mM NaOAc, pH 5.2, 0.2% (w/v) Na-TC, 0.02% (w/v), NaN_3 0.2% (v/v) Triton X-100) and enzyme preparation (13.3 μL). The inhibitors, which are well-soluble, were taken from 1 mM stock solutions (100 mM NaOAc, pH 4.5). Inhibitors were added in various concentrations and the reactions were incubated for 3 h at 37 °C in a plate reader (FLUOstar OPTIMA, BMG labtech, Offenburg, Germany). The fluorescence of 6-hexadecanoylamino-4-methylumbelliferone (HMU) was measured (λ_{ex} = 380 nm, λ_{em} = 460 nm) in real time. Michaelis–Menten analysis was done using radio-labelled sphingomyelin. The assays using either the HMU-PC or the ^{14}C-sphingomyelin as a substrate gave the same results.

4.2. Apoptosis assay

DNA fragmentation ELISA: First, the kinetics of DNA fragmentation after dexamethasone donation were measured in the lysate and in the supernatant, respectively. Between 6 h and 8 h, there was a steep increase in absorbance in the probes from the supernatant; this is typical for apoptosis (data not shown). The apoptosis assay was performed according to the manufacturer's protocol (Roche Cat. No. 11 585 045). Briefly, cells were harvested and suspended in culture medium (2×10^5 cells per mL) containing BrdU labelling solution (10 μM final concentration) and plated in a 96-well cell culture dish (~0.5×10^3 cells per well). After 16 h, cells were washed and new media was added. Then, cells were treated with dexamethasone (10^{-8} M) and **7d** (2 μM). After 7 h of incubation, the supernatant (100 μL) was collected and added to a 96-well plate containing immobilized anti-BrdU antibody. After incubation, removal of the supernatant and extensive washing, the secondary antibody and the TMB substrate were added and absorbance was measured at 370 nm (FLUOstar OPTIMA, BMG Labtech). The experiment was performed in quintuplicate.

4.3. Synthesis

The syntheses of **4**[16] and **15**[18] have been described previously. Synthetic protocols can be found in the Supplementary data.

General methods and material: All solvents employed were of reagent grade and were dried by refluxing over appropriate drying agents. All reactions were performed under argon unless stated otherwise. All reactions were monitored by thin-layer chromatography (TLC) by using Silica Gel 60 F_{254} and the compounds were detected with UV light (254 nm) or by spraying the plates with Seebach solution (25 g $MoO_3 \times H_3PO_4 \times H_2O$, 10 g $Ce(IV)SO_4 \times 4H_2O$, 60 ml sulfuric acid and 905 ml of water) or by sulfuric acid solution (5% sulfuric acid in ethanol) followed by heating at 250 °C. Flash chromatography was performed on silica gel MERCK 60 (0.040–0.063 mm). Organic phases were dried over $MgSO_4$ or Na_2SO_4 and solvents were evaporated under reduced pressure while maintaining the water bath temperature between 40 and 45 °C. NMR spectra (^1H, ^{13}C, APT, and ^{31}P) were recorded in $CDCl_3$, CD_3OD, CD_3CN or D_2O on Bruker DPX-300 NMR spectrometer at 300 MHz (^1H), 75 MHz (^{13}C, APT), and 121 MHz (^{31}P), temperature 25 °C. Chemical shifts are given in ppm with TMS as internal standard ($\delta = 0.00$); ^{31}P, 85% H_3PO_4 ($\delta = 0.00$) as external standard. Mass spectra were recorded with a Hewlett–Packard GCMS 5995-A spectrometer.

4.3.1. 1,5-Anhydro-3-deoxy-2,4,6-dibenzylphosphoryl-D-glucitol (16)

To a stirred solution of 1,5-anhydro-3-deoxy-D-glucitole **15** (0.17 g, 0.72 mmol) in acetonitrile and 1*H*-tetrazole (0.45 M, 8.00 ml, 3.61 mmol) was added *N*,*N*-Diisopropyl-*O*,*O*-dibenzyl phosphoramidite (1.25 g, 3.61 mmol) in an argon atmosphere. The reaction mixture was stirred at room temperature for 24 h. The solution was cooled to 0 °C and *t*BuOOH (670 µl) was added and stirred for 30 min at room temperature. It was diluted with chloroform (150 ml), washed with saturated sodium bicarbonate, water and brine, dried over anhydrous magnesium sulfate and concentrated in vacuum. The crude product was purified with a short column chromatography on SiO_2 gel (cyclohexane/ethyl acetate 1:1 (v/v)). Evaporation of the solvent gave the product in 10% (0.07 g) yield. ^1H NMR (300 MHz, CD_3OD): $\delta = 1.65$ (q, $J = 11.3$ Hz, 1H); 2.53–2.64 (m, 1H); 3.13 (t, $J = 10.5$ Hz, 1H); 3.84 (ddd, $J = 1.5$, 5.2, 10.7 Hz, 1H); 3.96–4.25 (m, 5H); 4.95–5.07 (m, 12H); 7.25–7.38 (m, 30H) ppm. ^{13}C NMR (75 MHz, CD_3OD): $\delta = 38.5$ (d, $J = 4.7$ Hz); 67.3 (d, $J = 5.6$ Hz); 70.2 (d, $J = 5.7$ Hz); 70.8; 70.9; 71.1; 71.2; 71.2; 71.3; 79.0 (dd, $J = 3.3$, 6.5 Hz); 129.1; 129.2; 129.4; 129.4; 129.7; 129.8; 129.9; 130.0; 136.9; 136.9; 137.0; 137.1; 137.1; 137.2 ppm. ^{31}P NMR (121 MHz, CD_3OD): $\delta = -1.19$ (1P); −0.91 (1P); −0.17 (1P) ppm. HRMS for: $C_{48}H_{52}O_{13}P_3$ $[M+H]^+$ calcd: 929.2615, found: 929.2622.

4.3.2. 1,5-Anhydro-3-deoxy-D-glucitole-2,4,6-triphosphate (9b)

A mixture of the 3-deoxy-2,4,6-dibenzylphosphoryl-D-glucopyranositole **16** and 10% Pd on carbon in 25 ml methanol and water ($MeOH/H_2O$ 4:1 (v/v)) was stirred under H2 (1 atm) overnight. The solution was filtered and concentrated in vacuum. The residue was diluted with water and lyophilized twice. ^1H NMR (300 MHz, D_2O): $\delta = 1.63$ (q, $J = 11.4$ Hz, 1H); 2.56–2.69 (m, 1H); 3.21 (t, $J = 10.6$ Hz, 1H); 3.39 (dd, $J = 3.4$, 9.5 Hz, 1H); 3.85–4.20 (m, 5H) ppm. ^{13}C NMR (75 MHz, D_2O): $\delta = 37.6$ (d, $J = 3.6$ Hz); 64.2 (d, $J = 5.1$ Hz); 68.0 (t, $J = 5.9$ Hz); 69.4 (d, $J = 5.5$ Hz); 78.3 (t, $J = 8.6$ Hz) ppm. ^{31}P NMR (121 MHz, D2O): $\delta = -0.51$ (1P); 0.03 (1P); 1.03 (1P) ppm. HRMS for: $C_6H_{16}O_{13}P_3$ $[M+H]^+$ calcd: 388.9798, found: 388.9801.

4.3.3. General procedure for preparation of 1,3-di-*O*-benzyl-6-*O*-alkylsulfonyl-D-glucose (5a–d)

To a stirred solution of 1,3-di-*O*-benzyl-D-glucose **4** (1.0 equiv) and triethylamine (3.0 equiv) in dichloromethane (5 ml) was added the corresponding sulfonyl chloride (1.1 equiv) at 0 °C. The reaction mixture was warmed up from 0 °C to room temperature overnight and diluted with diethyl ether. The mixture was washed successively with 100 ml each of water, 1 M HCl, water, saturated sodium bicarbonate solution and brine. The solution dried over anhydrous sodium sulfate. Filtration and concentration of the organic layer gave an oily residue, which was purified by silica gel chromatography (cyclohexane/ethyl acetate 4:1 (v/v)).

4.3.4. 1,3-Di-*O*-benzyl-6-*O*-ethylsulfonyl-α/β-D-glucose (5a)

The desired product was obtained as a colourless oil (92 mg, 73%). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.43$ (dt, $J = 2.2$, 7.4 Hz, 3H); 2.52 (s, 2H); 3.17 (q, $J = 7.4$ Hz, 2H); 3.50–3.74 (m, 3H); 4.30–4.47 (m, 2H); 4.76 (td, $J = 2.7$, 11.4 Hz, 2H); 4.89–5.05 (m, 2H); 7.30–7.40 (m. 10H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 8.1$; 44.9; 68.1; 68.2; 69.1; 69.7; 70.2; 71.2; 72.6; 73.5; 74.3; 74.8; 75.1; 82.5; 83.2; 98.0; 101.7; 127.9; 128.0; 128.1; 128.2; 128.2; 128.6; 136.7; 136.7; 138.3; 138.4 ppm. HRMS for: $C_{22}H_{28}O_8SNa$ $[M+Na]^+$ calcd: 475.1397, found: 475.1407.

4.3.5. 1,3-Di-*O*-benzyl-6-*O*-hexylsulfonyl-D-glucose (5b)

The desired product was obtained as a colourless oil (70 mg, 31%). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.89$ (t, $J = 6.8$ Hz, 3H); 1.27–1.34 (m, 4H); 1.41 (tt, $J = 4.9$, 9.6 Hz, 2H); 1.86 (ddd, $J = 5.0$, 7.64, 18.2 Hz, 2H); 2.14–2.55 (br s, 2H); 3.08–3.17 (m, 2H); 3.49–3.58 (m, 1H); 3.59–3.72 (m, 2H); 3.83 (ddd, $J = 1.8$, 4.5, 9.6 Hz, 1H); 4.31 (dd, $J = 1.9$, 11.3 Hz, 1H); 4.41 (dd, $J = 4.6$, 11.3 Hz, 1H); 4.58 (d, $J = 11.7$ Hz, 1H); 4.75 (dd, $J = 5.5$, 11.6 Hz, 2H); 4.99 (dd, $J = 5.5$, 7.4 Hz, 2H); 7.30–7.40 (m, 10H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 13.9$; 22.2; 23.3; 27.8; 31.1; 50.4; 67.9; 69.1; 69.7; 70.2; 72.6; 75.1; 82.5; 98.0; 127.9; 128.2; 128.3; 128.6; 136.6; 138.4 ppm. HRMS for: $C_{26}H_{35}O_8S$ $[M-H]^-$ calcd: 507.2058, found: 507.2064.

4.3.6. 1,3-Di-*O*-benzyl-6-*O*-octylsulfonyl-D-glucose (5c)

The desired product was obtained as a colourless oil (46 mg, 62%). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.88$ (t, $J = 6.8$ Hz, 3H); 1.24–1.33 (m, 8H); 1.37–1.45 (m, 2H); 1.79–1.93 (m, 2H); 2.44 (br s, 2H); 3.13 (ddd, $J = 3.3$, 6.3, 8.1 Hz, 2H); 3.49–3.63 (m, 2H); 3.64–3.72 (m, 1H); 3.83 (ddd, $J = 1.9$, 4.6, 9.7 Hz, 1H); 4.28–4.47 (m, 2H); 4.54 (td, $J = 2.0$, 11.3 Hz, 1H); 4.70–4.78 (m, 2H); 4.87–5.04 (m, 2H); 7.30–7.38 (m, 10H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.0$; 22.5; 23.4; 28.1; 28.9; 31.6; 50.4; 67.9; 69.1; 69.8; 70.2; 72.6; 75.1; 82.5; 98.0; 127.9; 128.0; 128.2; 128.2; 128.3; 128.6; 136.6; 138.4 ppm. HRMS for: $C_{28}H_{41}O_8S$ $[M+H]^+$ calcd: 537.2517, found: 537.2523.

4.3.7. 1,3-Di-*O*-benzyl-6-*O*-dodecylsulfonyl-D-glucose (5d)

The desired product was obtained as a colourless oil (107 mg, 40%). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.91$ (t, $J = 6.7$ Hz, 3H); 1.24–1.35 (m, 16H); 1.42 (dd, $J = 7.1$, 13.5 Hz, 2H); 1.81–1.94 (m, 2H); 2.15–2.78 (m, 2H); 3.07–3.24 (m, 2H); 3.49–3.67 (m, 2H); 3.67–3.75 (m, 1H); 3.85 (ddd, $J = 1.9$, 4.6, 9.7 Hz, 1H); 4.27–4.48 (m, 2H); 4.60 (dd, $J = 2.0$, 12.3 Hz, 1H); 4.72–4.80 (m, 2H); 4.91–5.11 (m, 2H); 7.35–7.42 (m, 10H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.1$; 22.6; 23.4; 28.1; 29.0; 29.2; 29.3; 29.4; 29.5; 31.8; 50.4; 67.9; 69.1; 69.8; 70.2; 72.6; 75.1; 82.5; 98.0; 127.9; 128.2; 128.3; 128. 6; 136.7; 138.4 ppm. HRMS for: $C_{32}H_{47}O_8S$ $[M-H]^-$ calcd: 591.2997, found: 591.3003.

4.3.8. General procedure for preparation 1,3-di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-alkylsulfonyl-D-glucose (6a–d)

To a stirred solution of the corresponding 1,3-di-*O*-benzyl-6-*O*-sulfonyl-D-glucose derivatives (**5a–d**) (1.0 equiv) in acetonitrile and 1*H*-tetrazole (0.45 M, 3.5 equiv) was added *N*,*N*-diisopropyl-*O*,*O*-dibenzylphosphoramidite (3.5 equiv) in an argon atmosphere. The reaction mixture was stirred at room temperature for 24 h. The solution was cooled to 0 °C and *t*BuOOH (3.5 equiv) was added and stirred for 30 min at room temperature. It was diluted with chloroform (50 ml), washed with saturated sodium bicarbonate, water and brine, dried over anhydrous magnesium sulfate and concentrated in vacuum. The crude product was purified with a short column chromatography on SiO_2 gel (cyclohexane/ethyl acetate 3:1 (v/v)).

4.3.9. 1,3-Di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-ethylsulfonyl-α/β-D-glucose (6a)

The desired product was obtained as a colourless oil (106 mg, 54%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38 (dt, *J* = 4.1, 7.4, 7.4 Hz, 3H); 3.11 (dq, *J* = 4.2, 7.4 Hz, 2H); 3.77 (dd, *J* = 8.5, 17.4 Hz, 1H); 3.99–4.12 (m, 1H); 4.28–4.56 (m, 3.5H); 4.59–4.72 (m, 2H); 4.73–5.02 (m, 11H); 5.27 (d, *J* = 3.6 Hz, 0.5H); 7.20–7.35 (m, 30H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 8.0; 44.9; 67.5; 67.8; 68.5 (d, *J* = 4.3 Hz); 69.2; 69.3; 69.3; 69.4; 69.5; 69.5; 69.7; 69.7–69.9 (m); 70.4; 70.9; 72.7 (d, *J* = 4.0 Hz); 74.0; 74.6 (d, *J* = 6.3 Hz); 74.9 (d, *J* = 6.3 Hz); 75.0; 76.3 (d, *J* = 6.0 Hz); 78.0 (d, *J* = 6.1 Hz); 81.0 (t, *J* = 3.8 Hz); 96.1; 99.4 (d, *J* = 3.5 Hz); 127.5; 127.7; 128.0; 128.1; 128.2; 128.3; 128.4; 128.4; 128.5; 135.2; 135.5; 135.5; 135.6; 135.7; 135.8; 136.2; 136.5; 137.5; 137.7 ppm. ^{31}P NMR (121 MHz, $CDCl_3$): δ = −1.35 (1P); −1.31 (1P); −1.28 (1P); −1.10 (1P) ppm. HRMS for: $C_{50}H_{55}O_{14}P_2S$ $[M+H]^+$ calcd: 973.2782, found: 973.2788.

4.3.10. 1,3-Di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-hexylsulfonyl-D-glucose (6b)

The desired product was obtained as a colourless oil (104 mg, 76%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.87 (t, *J* = 6.8 Hz, 3H); 1.23–1.30 (m, 4H); 1.33–1.41 (m, 2H); 1.81 (td, *J* = 7.6, 12.7 Hz, 2H); 2.98–3.11 (m, 2H); 4.01 (dd, *J* = 8.7, 17.5 Hz, 2H); 4.23–4.35 (m, 3H); 4.45–4.51 (m, 2H); 4.69 (d, *J* = 11.7 Hz, 1H); 4.76–4.83 (m, 3H); 4.85–4.89 (m, 2H); 4.89–4.95 (m, 4H); 4.98–5.11 (m, 1H); 5.23 (d, *J* = 3.6 Hz, 1H); 7.25–7.40 (m, 30H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.9; 22.3; 23.3; 27.8; 31.1; 50.4; 67.5; 68.5 (d, *J* = 4.2 Hz); 69.2 (d, *J* = 5.6 Hz); 69.4 (d, *J* = 5.5 Hz); 69.7 (dd, *J* = 5.4, 7.0 Hz); 70.4; 76.3 (d, *J* = 5.6 Hz), 78.1 (dd, *J* = 3.4, 8.1 Hz); 96.1; 127.4; 127.5; 127.7; 127.9; 127.9; 128.1; 128.1; 128.2; 128.4; 128.5; 135.4; 135.4; 135.4; 135.4; 135.5; 135.5; 136.5; 137.8 ppm. ^{31}P NMR (121 MHz, $CDCl_3$): δ = −1.78 (1P); −1.64 (1P) ppm. HRMS for: $C_{54}H_{63}O_{14}P_2S$ $[M+H]^+$ calcd: 1029.3408, found: 1029.3420.

4.3.11. 1,3-Di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-octylsulfonyl-D-glucose (6c)

The desired product was obtained as a colourless oil (110 mg, 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92 (t, *J* = 6.7 Hz, 3H); 1.24–1.36 (m, 8H); 1.38–1.48 (m, 2H); 1.79–1.94 (m, 2H); 3.03–3.17 (m, 2H); 4.01–4.13 (m, 2H); 4.27–4.43 (m, 3H); 4.53 (dd, *J* = 2.1, 11.7 Hz, 2H); 4.74 (d, *J* = 11.7 Hz, 1H); 4.79–4.86 (m, 3H); 4.89–5.00 (m, 6H); 5.09 (dd, *J* = 6.5, 9.6 Hz, 1H); 5.28 (d, *J* = 3.6 Hz, 1H); 7.25–7.40 (m, 30H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.0; 22.5; 23.3; 28.1; 28.9 (d, *J* = 1.8 Hz); 31.6; 50.4; 67.5; 68.5 (d, *J* = 4.3 Hz); 69.2 (d, *J* = 5.6 Hz); 69.5 (d, *J* = 5.7 Hz); 69.7 (dd, *J* = 4.6, 5.3 Hz); 70.4; 74.9 (d, *J* = 4.4 Hz); 75.0; 76.3 (d, *J* = 5.7 Hz); 78.0 (dd, *J* = 3.2, 8.0 Hz); 96.0; 127.4; 127.5; 127.7; 127.9; 127.9; 128.1; 128.1; 128.4; 128.6; 135.3; 135.3; 135.3; 135.4; 135.4; 135.4; 135.5; 136.5; 137.7 ppm. ^{31}P NMR (121 MHz, $CDCl_3$): δ = −1.23 (1P); −1.09 (1P) ppm. HRMS for: $C_{56}H_{67}O_{14}P_2S$ $[M+H]^+$ calcd: 1057.3721, found: 1057.3733.

4.3.12. 1,3-Di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-dodecylsulfonyl-D-glucose (6d)

The desired product was obtained as a colourless oil (86 mg, 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, *J* = 6.7 Hz, 3H); 1.25–1.35 (m, 16H); 1.41 (dd, *J* = 10.4, 11.5 Hz, 2H); 1.85 (td, *J* = 7.5, 10.4 Hz, 2H); 3.02–3.22 (m, 2H); 3.46–4.25 (m, 3H); 4.25–4.42 (m, 2H); 4.48–4.55 (m, 1H); 4.67–5.08 (m, 12H); 5.26 (d, *J* = 3.6 Hz, 1H), 7.25–7.42 (m, 30H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1; 22.6; 23.3; 28.1; 29.0; 29.3; 29.5; 29.6; 31.8; 50.5; 67.5; 68.5 (d, *J* = 4.5 Hz); 69.2 (d, *J* = 5.6 Hz); 69.4 (d, *J* = 5.6 Hz); 69.7 (t, *J* = 5.0 Hz); 70.4; 74.9 (d, *J* = 4.4 Hz); 75.1; 76.3 (d, *J* = 5.9 Hz); 78.1 (dd, *J* = 3.4, 8.0 Hz); 96.0; 126.9; 127.6; 127.7; 127.9; 127.9; 128.1; 128.2; 128.4; 128.5; 135.3; 135.4; 135.4; 135.5; 135.5; 135.5; 136.5; 137.8 ppm. ^{31}P NMR (121 MHz, $CDCl_3$): δ = −1.16 (1P); −1.02 (1P) ppm. HRMS for: $C_{60}H_{74}O_{14}P_2SNa$ $[M+Na]^+$ calcd: 1135.4172, found: 1135.4167.

4.3.13. 1,3-Di-O-benzyl-2,4,6-dibenzylphosphoryl-D-glucose (8)

The desired product was obtained as a colourless oil (56 mg, 32%). ^{1}H NMR (300 MHz, CD_3OD): δ = 3.88–3.94 (m, 1H); 3.96–4.04 (m, 1H); 4.05–4.12 (m, 1H); 4.18–4.22 (m, 1H); 4.24–4.30 (m, 1H); 4.34–4.44 (m, 1H); 4.60–4.94 (m, 12H); 5.01 (s, 2H); 5.03 (s, 2H); 5.10 (d, *J* = 3.6 Hz, 1H); 7.08–7.36 (m, 40H) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ = 28.0; 71.2; 76.1; 76.2; 76.3; 78.0; 79.5; 97.3; 127.5; 127.6; 127.7; 127.8; 127.9; 128.0; 128.3; 128.4; 128.6; 135.3; 135.4; 135.5; 135.6; 135.7; 136.6; 137.6; 137.8 ppm. ^{31}P NMR (121 MHz, CD_3OD): δ = −1.30 (1P); −0.95 (1P); −0.18 (1P) ppm. HRMS for: $C_{62}H_{64}O_{15}P_3$ $[M+H]^+$ calcd: 1141.3453, found: 1141.3458.

4.3.14. General procedure for deprotection of benzylethers, synthesis of 1,3-di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-dodecylsulfonyl-D-glucose (7a–d)

A mixture of the 1,3-di-*O*-benzyl-2,4-dibenzylphosphoryl-6-*O*-alkylsulfonyl-D-glucose derivatives (**6a–d**) and 10% Pd on carbon in 50 ml methanol and water ($MeOH/H_2O$ 4:1 (v/v)) was stirred under H_2 (1 atm) overnight. The solution was filtered and concentrated in vacuum. The residue was diluted with water and lyophilized twice.

4.3.15. 6-*O*-Ethylsulfonyl-D-glucose-2,4-bisphosphate (7a)

The desired product was obtained as a colourless solid (42 mg, 89%). ^{1}H NMR (300 MHz, D_2O): δ = 1.26 (t, *J* = 7.4 Hz, 3H); 3.28 (dq, *J* = 2.4, 7.4 Hz, 2H); 3.72–4.02 (m, 3H); 4.10 (ddd, *J* = 1.8, 4.5, 9.4 Hz, 1H); 4.34 (ddt, *J* = 2.8, 7.1, 8.4 Hz, 1H); 4.45–4.56 (m, 1H); 5.30 (d, *J* = 3.3 Hz, 1H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 7.2; 44.6; 67.8 (d, *J* = 6.4 Hz); 68.8 (d, *J* = 5.0 Hz); 70.6 (d, *J* = 6.1 Hz); 72.2 (d, *J* = 6.1 Hz); 73.5 (dd, *J* = 5.9, 11.8 Hz); 74.0; 74.9 (d, *J* = 5.6 Hz); 78.4 (d, *J* = 6.3 Hz); 90.5; 94.8 (d, *J* = 4.4 Hz) ppm. ^{31}P NMR (121 MHz, D_2O): δ = −0.09 (1P); −0.04 (1P); 0.18 (1P); 0.23 (1P) ppm. HRMS for: $C_8H_{17}O_{14}P_2S$ $[M-H]^-$ calcd: 430.9820, found: 430.9818.

4.3.16. 6-*O*-Hexylsulfonyl-D-glucose-2,4-bisphosphate (7b)

The desired product was obtained as a colourless solid (45 mg, 85%). ^{1}H NMR (300 MHz, D_2O): δ = 0.74 (t, *J* = 7.1 Hz, 3H); 1.12–1.24 (m, 4H); 1.27–1.39 (m, 2H); 1.62–1.79 (m, 2H); 3.24–3.34 (m, 2H); 3.70–3.81 (m, 1H); 3.92 (td, *J* = 8.5, 12.7 Hz, 2H); 4.09 (ddd, *J* = 1.9, 4.7, 9.6 Hz, 1H); 4.32 (ddd, *J* = 5.3, 11.5, 14.6 Hz, 1H); 4.44–4.56 (m, 1H); 5.30 (d, *J* = 3.3 Hz, 1H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 13.2; 21.6; 22.6; 26.8; 30.2; 49.6; 67.8 (d, *J* = 6.3 Hz); 68.8; 70.7 (d, *J* = 5.5 Hz); 72.3 (d, *J* = 6.3 Hz)M; 73.5 (dd, *J* = 6.0, 11.1 Hz); 74.0; 74.9 (d, *J* = 5.5 Hz); 78.4 (d, *J* = 6.2 Hz);

90.5; 94.9 (d, J = 4.5 Hz) ppm. ^{31}P NMR (121 MHz, D_2O): δ = −0.06 (1P); 0.25 (1P) ppm. HRMS for: $C_{12}H_{25}O_{14}P_2S$ $[M-H]^-$ calcd: 487.0446, found: 487.0446.

4.3.17. 6-*O*-Octylsulfonyl-D-glucose-2,4-bisphosphate (7c)

The desired product was obtained as a colourless solid (38 mg, 67%). ^{1}H NMR (300 MHz, D_2O): δ = 0.74 (t, J = 6.6 Hz, 3H); 1.12–1.26 (m, 8H); 1.31 (dd, J = 6.7, 12.0 Hz, 2H); 1.64–1.78 (m, 2H); 3.12–3.32 (m, 2H), 3.69–3.82 (m, 1H); 3.92 (dd, J = 6.5, 13.6 Hz, 2H); 4.05–4.16 (m, 1H); 4.34 (dd, J = 4.5, 11.4 Hz, 1H), 4.42–4.54 (m, 1H), 5.31 (s, 1H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 13.5; 22.1; 22.7; 27.3; 28.2 (d, J = 4.1 Hz); 31.1; 49.7 (d, J = 3.7 Hz); 67.8 (d, J = 6.4 Hz); 68.8; 70.7; 72.3 (d, J = 5.7 Hz); 73.7 (dd, J = 5.4, 9.4 Hz); 75.0 (d, J = 4.9 Hz); 90.5; 94.9 (d, J = 2.1 Hz) ppm. ^{31}P NMR (121 MHz, D_2O): δ = −0.04 (1P); 0.23 (1P) ppm. HRMS for: $C_{14}H_{29}O_{14}P_2S$ $[M-H]^-$ calcd: 515.0759, found: 515.0758.

4.3.18. 6-*O*-Dodecylsulfonyl-D-glucose-2,4-bisphosphate (7d)

The desired product was obtained as a colourless solid (31 mg, 70%). ^{1}H NMR (300 MHz, D_2O): δ = 0.77 (t, J = 5.6 Hz, 3H); 1.13–1.25 (m, 16H); 1.29–1.40 (m, 2H); 1.71 (s, 2H); 3.19 (s, 2H); 3.63–3.83 (m, 1H); 3.87- 4.06 (m, 2H); 4.08–4.21 (m, 1H); 4.27–4.40 (m, 1H); 4.41–4.54 (m, 1H); 5.35 (m, 1H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 13.8; 22.6; 23.1; 28.0; 29.3; 29.5; 29.9; 32.0; 49.9 (d, J = 4.6 Hz); 67.9 (d, J = 6.1 Hz); 69.0 (d, J = 4.0 Hz); 70.7 (d, J = 7.3 Hz); 74.0; 75.1; 90.5 ppm. ^{31}P NMR (121 MHz, D_2O): δ = 0.77 (1P); 2.26 (1P) ppm. HRMS for: $C_{18}H_{37}O_{14}P_2S$ $[M-H]^-$ calcd: 571.1385, found: 571.1384.

4.3.19. D-Glucose-2,4,6-trisphosphate (9)

The desired product was obtained as a colourless oil (31 mg, 70%). ^{1}H NMR (300 MHz, D_2O): δ = 4.00 (dd, J = 4.8, 11.2 Hz, 2H); 4.05–4.16 (m, 4H); 5.39 (d, J = 2.5 Hz, 1H) ppm. ^{13}C NMR (75 MHz, D_2O): δ = 63.9 (d, J = 4.3 Hz); 68.7–69.1 (m); 73.5 (d, J = 6.3 Hz); 74.8 (d, J = 5.2 Hz); 90.6 ppm. ^{31}P NMR (121 MHz, D_2O): δ = −0.21 (1P); 0.81 (1P); 1.39 (1P) ppm. HRMS for: $C_6H_{14}O_5P_3$ $[M-H]^-$ calcd: 418.9551, found: 418.9545.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.030.

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