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Syntheses of phosphatidyl-β-D-glucoside analogues to probe antigen selectivity of monoclonal antibody 'DIM21'

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ABSTRACT

Herein, we report the chemical syntheses of a series of phosphatidyl-β-D-glucoside (PtdGlc) analogues, including 6-*O*-Ac, *sn*-2-*O*-Me, phosphorothioate as well as phosphatidylgalactoside and -mannoside derivatives. In the key step, β-glycosyl H-phosphonate was condensed with enantiomerically pure diacylglycerol. Comparison of spectroscopic data with mono-acetylated PtdGlc from natural source confirmed the presence of an acetyl moiety at position 6. Furthermore, the reactivity of PtdGlc and its analogues toward monoclonal antibody 'DIM21' (MAb DIM21) was evaluated, revealing the crucial structural antigen features for successful MAb DIM21 binding.

1. Introduction

In 1970, Short and White[1] identified phosphatidylglucoside (**1**, PtdGlc) in the lipid fraction of *Staphylococcus aureus*. About 30 years later, the first isolation[2] from mammalian sources was reported by Nagatsuka et al.,[3] suggesting PtdGlc involvement in mammalian cell differentiation. More recently, Yamazaki et al.,[4] successfully produced a monoclonal antibody (MAb) against PtdGlc, called 'DIM21'. With the help of MAb DIM21, PtdGlc was visualized and isolated from rat embryonic brain tissue.[5] PtdGlc contained exclusively 18:0/20:0 fatty acid chains, rarely occurring in known mammalian lipids. The presence of both stereoisomers, PtdGlc (**1**) and its *sn*-2-epimer **3**, in the natural sample was recently confirmed.[6] Interestingly, besides PtdGlc also a 6-O-acetylated PtdGlc derivative (**2**) was isolated from rat embryonic brain tissue.[5] It was speculated that PtdGlc's unusual fatty acid chain pattern might be responsible for its presence within distinct membrane microdomains on astroglial cells. Moreover, it was suggested that PtdGlc plays an important role in glial cell development and differentiation.[5] However, the biological roles of PtdGlc and its derivatives in glial cells remain largely unknown.

Herein, we report the first syntheses of 6-O-acetylated PtdGlc (**2**) confirming the proposed structure of the natural compound. In addition, we synthesized a series of PtdGlc derivatives, including the phosphorothioate derivative (**4**), which was expected to exhibit an enhanced stability under physiological conditions. Furthermore, the *sn*-2-*O*-methyl PtdGlc (**5**), the phosphatidylgalactoside (**6**) and the phosphatidylmannoside (**7**) were also prepared (Scheme 1). We compared the reactivity of these compounds toward MAb DIM21 to clarify the key structural requirements for antigen recognition.

2. Results and discussion

2.1. Syntheses

Our synthetic plan toward PtdGlc (**1**) and its analogues **2**–**7**, required the separate preparation of stereochemically well-defined key intermediates. Subsequent condensation of these key intermediates, namely β-glycopyranosyl H-phosphonate and enantiomerically pure glycerol units, followed by selective removal of the protecting groups yielded the desired products. PtdGlc (**1**) and its stereoisomer **3** were prepared according to literature procedures[6] (see Schemes 2 and 3) by condensation of intermediate **21** with **10** and **15,** respectively.

O-Methylated glycerol unit **12** was prepared from the enantiomerically pure PMB protected glycerol **8**.[7] Silylation of the primary hydroxyl function was followed by methylation and desilylation, yielding intermediate **11**. DCC-mediated introduction of the stearic acid residue and carefully controlled removal of the PMB protection gave the desired glycerol unit **12**.

6-O-Acetylated glucose (**17**) and mannose (**19**) units were prepared via their α-trichloroacetimidates **16**[8] and **18,**[9] respectively.

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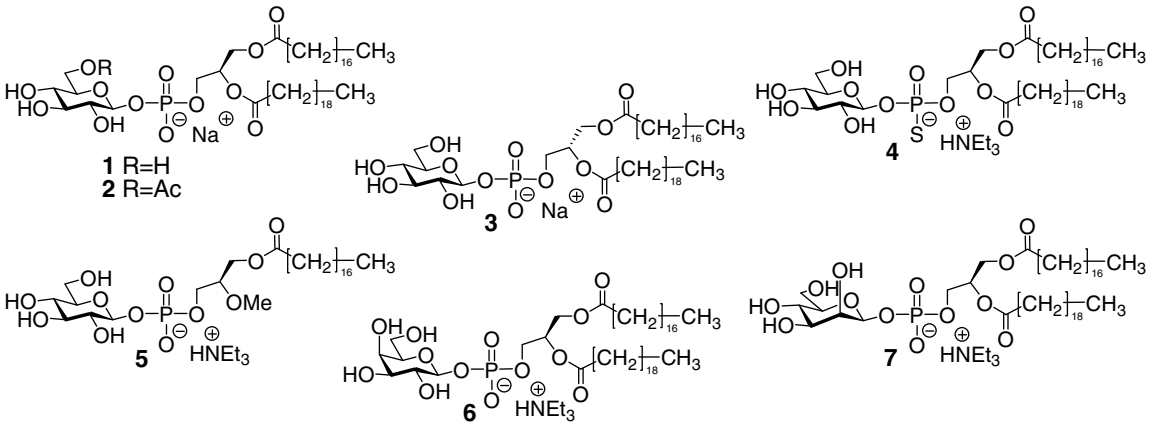


Scheme 1. Overview of synthesized phosphatidyl-β-D-glucoside (**1**) and its analogues (**2**–**7**).

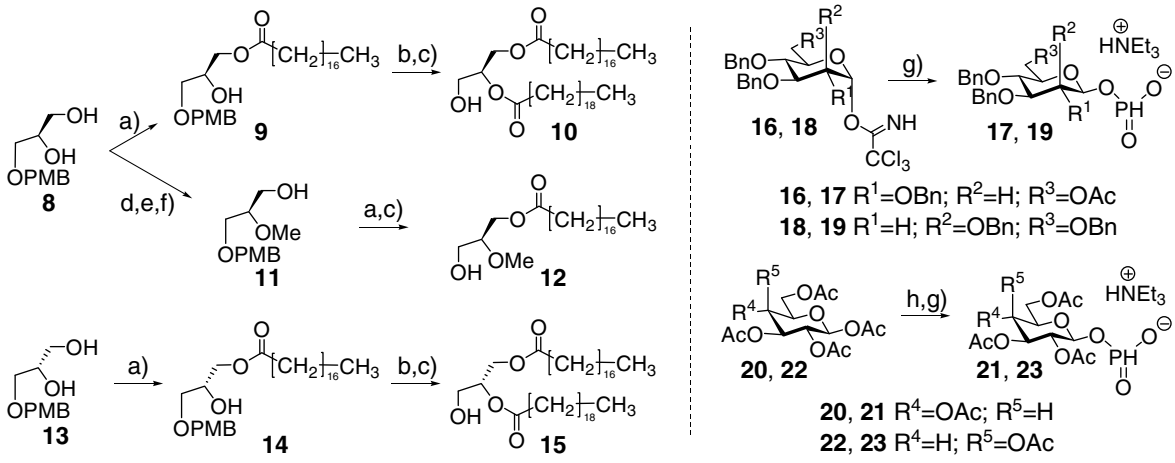
Treatment with phosphonic acid triggered trichloroacetimidate activation and β-phosphonate formation, similarly to previously reported phosphoric acid diester treatment.[10] However, H-phosphonates seemed to be more resistant to anomerization compared to their corresponding phosphates. The galactose H-phosphonate unit **23** was prepared via a two-step sequence from commercially available peracetate **22**. Firstly, **22** was converted to the corresponding *tert*-butyl orthoester under Lewis acid-mediated conditions.[11] Subsequently, autocatalytic reaction with phosphonic acid[12] gave desired β-phosphonate **23**. The glucose H-phosphonate unit **21** was prepared in an analogous manner.

Linkage of the carbohydrate and glycerol units was furnished in a unified manner with a 'one-pot' procedure through pivalyl chloride mediated H-phosphonate activation and subsequent iodine-mediated oxidation.[13] As a precursor of the phosphorothioate derivative **4**, compound **30** was synthesized from the coupling product of **21** and **10** by treatment with Beaucage reagent[14] as a sulfurizing agent. As expected, NMR revealed the presence of both diastereomers at the newly formed chiral phosphorothioate diester in a ratio close to 1:1.

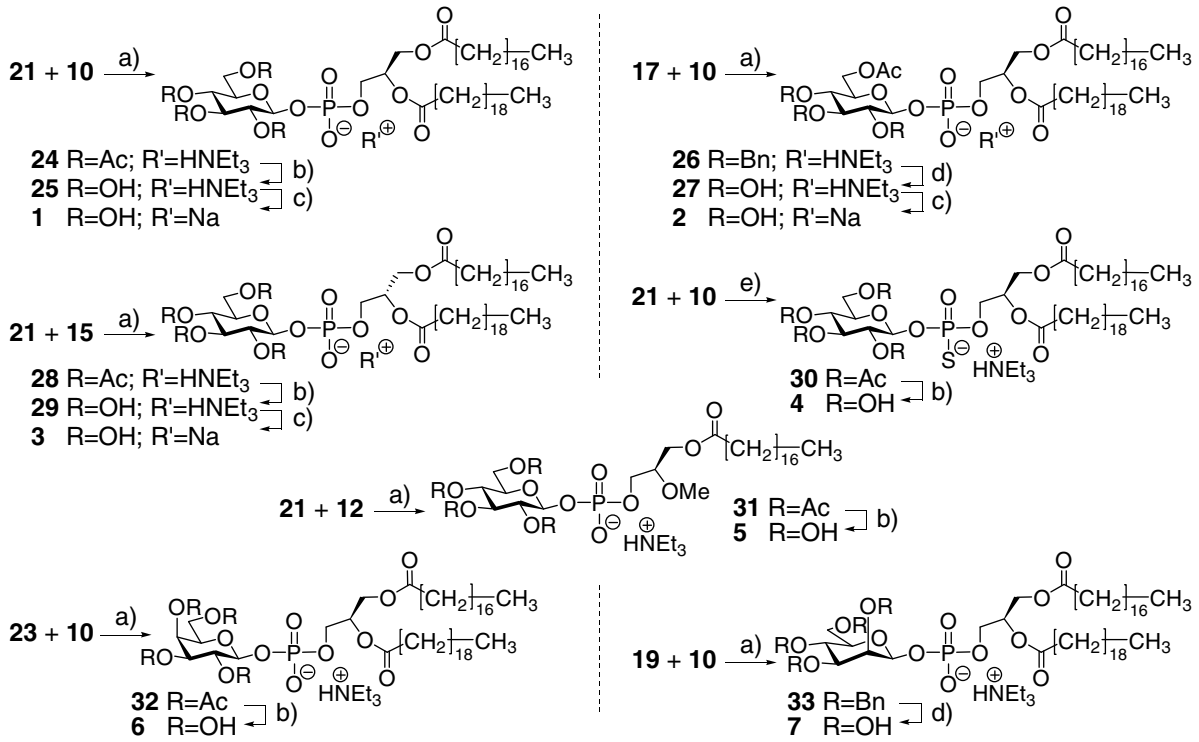
Final deprotection to yield compounds **4**–**6** required selective removal of the acetyl groups under conservation of the fatty acid esters. This apparently challenging strategy was chosen in order to develop a suitable approach to an initially proposed structure of PtdGlc, decorated with an arachidonic acid residue.[2] In addition, β-phosphate diesters are poorly tolerant to acidic conditions. Initial attempts to employ a catalytic amount of sodium methoxide were futile, showing poor selectivity, while guanidine/guanidinium nitrate[15] exhibited somewhat better selectivity. Most satisfactory was a modified hydrazine-mediated deacetylation protocol[16] yielding the desired products within 5 min in 43–78% yield. Perbenzylated intermediates **26** and **33** were cleanly deprotected with H_2 and Pd/C,[17] in contrast to the report of Ramirez et al.,[18] to furnish compounds **27** and **7**. In all cases, compounds **25**, **27**, **29**, and **4**–**7** were isolated as triethylammonium salts after chromatographic purification with $CHCl_3$–MeOH–Et_3N. Since natural PtdGlc and its derivatives were characterized as sodium salt, synthetic compounds **25**, **27**, and **29** were converted to the corresponding sodium salts **1**, **2** and **3** to allow structure verification. In general, compounds were poorly soluble in any organic solvents, for example, chloroform, methanol, DMSO or ether, as well as in water. NMR measurements were conducted with dilute solutions predominantly in DMSO-d_6.

2.2. Structure verification and characterization

In addition to PtdGlc (**1**), isolation of a slightly less polar novel compound from fetal rodent brain was reported.[5] MS spectroscopic data indicated the presence of an additional acetyl group. NMR data suggested the presence of an acetyl group at position 6 of the glucose moiety, due to a considerable low-field shift of protons at position 6 as well as 5 of the glucose moiety in contrast to PtdGlc



Scheme 2. Syntheses of glycerol units **10**, **12**, and **15** and carbohydrate units **17**, **19**, **21**, and **23**. Reagents and conditions: (a) DCC, DMAP, stearic acid; (b) DCC, DMAP, arachidic acid; (c) DDQ; (d) TBDPSCl, imidazole; (e) MeI, NaH; (f) TBAF, AcOH; (g) (i)—H_3PO_3; (ii)—NEt_3, 4 °C; (h) *t*-BuOH, DMAP, $AlCl_3$.



Scheme 3. Linkage and selective deprotection to yield PtdGlc (**1**) and its derivatives (**2**–**7**). Reagents and conditions: (a) (i)—PivCl, pyridine, 0 °C → rt; (ii)—I_2, pyridine/H_2O; (b) N_2H_4/HOAc = 4:1; (c) DOWEX 50WX8; (d) H_2, Pd/C; (e) (i)—PivCl, pyridine, 0 °C → rt; (ii)—Beaucage reagent.

(**1**). Comparison of the reported spectral data from natural source with synthetic 6-*O*-acetyl PtdGlc derivative (**2**) revealed good agreement of NMR- and MS-spectra, confirming the proposed structure of the natural sample.

Proton NMR spectrum of **25** (see Fig. 1B) acquired in DMSO-d_6 provided an interesting insight into the orientation of the hydroxyl groups of the glucose moiety. Namely, OH-2 exhibited, in contrast to OH-3, OH-4, and OH-6, an unusual low-field shift to 6.38 ppm. Saturation of the solvent signal via a selective DANTE pulse sequence affected OH-3, OH-4, and OH-6 signals strongly, consistent with a fast exchange process. On the contrary, the OH-2 signal showed little influence, indicating a slow exchange process. A possible explanation for these results would be the presence of an intramolecular hydrogen bond. This hypothesis is supported by computer simulations[19] of PtdGlc (**1**), proposing a distance of only 1.7 Å between the proton of OH-2 and one of the non-bridging oxygen of the phosphate moiety (see Fig. 1A). This structural feature resembles a six-membered ring in slightly distorted chair conformation, likely leading to a considerable reduction of the flexibility at the phosphate-sugar linkage. Nevertheless, to what extent this intramolecular hydrogen bond is present in water or in biological systems is unknown.

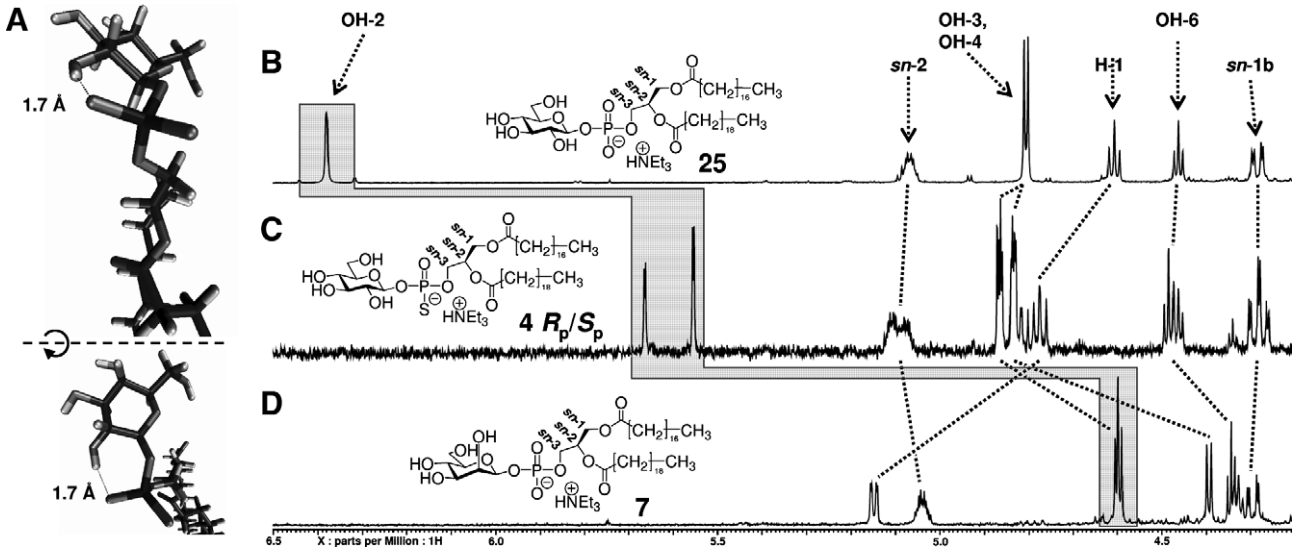


Figure 1. (A) Graphical representation of proposed intramolecular H-bond (dotted line) in PtdGlc (**1**); (B–D) 1H NMR in DMSO-d_6 (B) of PtdGlc (**25**); (C) of phosphorothioate derivative **4**; D) of manno derivative **7** (location of OH-2 indicated by gray area).

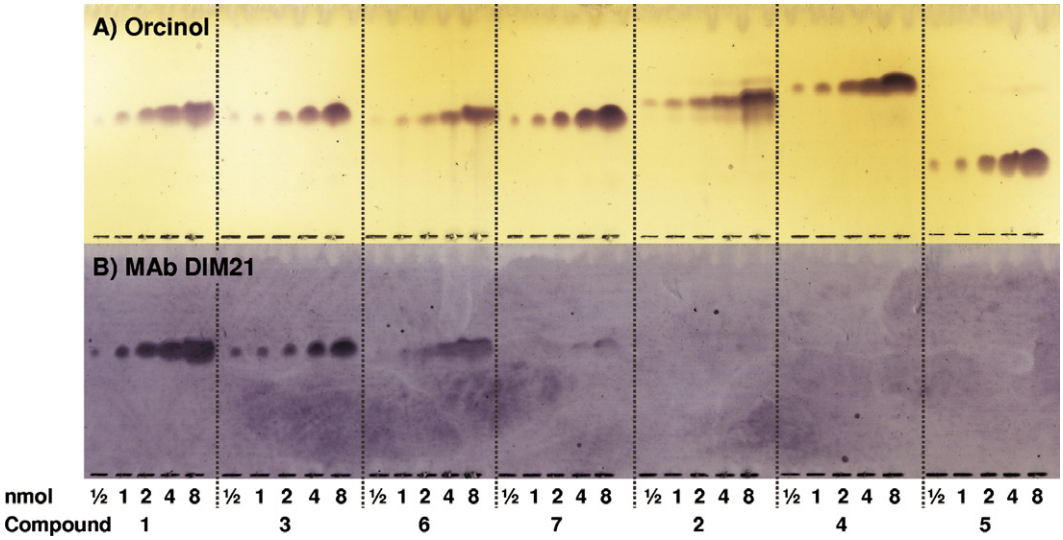


Figure 2. Determination of MAb DIM21 antigen specificity. TLC developed (A) with Orcinol for unspecific visualization; (B) by immunostaining assay to visualize MAb DIM21 immunoreactive spots.

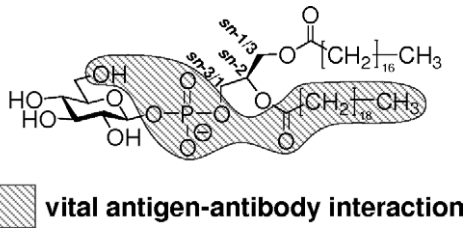


Figure 3. Visualization of proposed structure–immunoreactivity relationship map for MAb DIM21. Gray background pattern indicates essential area for MAb DIM21 binding.

This effect was observed with all synthetic PtdGlc derivatives bearing a glucose or galactose residue, except the phosphorothioate derivative (**4** R_p/S_p, see Fig. 1C), which exhibited a shift value of only 5.66 ppm for the minor and 5.56 ppm for the major diastereomer. This observation indicates the attenuated capacity of phosphorothioate to serve as a hydrogen bond acceptor, presumably due to the less electronegative nature of sulfur.[20]

Not surprisingly, the proton signal of OH-2 from phosphatidylmannoside (**7**, see Fig. 1D) resides at 4.60 ppm, being well within the range of hydroxyl group protons not involved in intramolecular hydrogen bonding. Hence, epimerization at C-2 seems to disfavor the formation of an intramolecular hydrogen bond, leading to full flexibility of the phosphate-sugar linkage.

2.3. Structure–activity realtionship (SAR) study

As previously reported,[4] MAb DIM21 did not show any cross reactivity with neutral phospholipids, like phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin, or glycosylceramide. MAb DIM21 reacted strongly with natural PtdGlc, a mixture of diastereomers, and exhibited some cross reactivity with phosphatidylinositol (PI) and phosphatidylglycerol (PGly). On the contrary, recent data[5] do not exhibit any cross reactivity of MAb DIM21 with PI or PGly at low nanomolar concentrations. Probably, the initially reported cross reactivity might have been caused by the presence of some immunoreactivities against these lipids in peroxidase-conjugated goat IgG fraction to mouse immunoglobulins (IgG, IgA, IgM). In order to gain a better understanding of MAb DIM21 antigen selectivity, we determined its immunoreactivity with our synthetic PtdGlc derivatives (see Fig. 2) at concentrations of 1/2–8 nmol by immunostaining assay.[4] Immunoreactivity of *sn*-1,2-di-*O*-acyl PtdGlc (**1**) was the strongest, tightly followed by its diastereomer **3** and phosphatidylgalactoside (**6**). In case of phosphatidylmannoside (**7**), immunoreactivity was considerably reduced, ensued by 6-*O*-acetyl PtdGlc (**2**) and phosphorothioatidylglucoside (**4**), exhibiting a further decrease in reactivity. Interestingly, *sn*-2-*O*-methyl PtdGlc (**5**) exhibited no detectable reactivity with MAb DIM21.

An overview of the immunological assay results was mapped as shown in Figure 3. The good recognition of phosphatidylgalactoside (**6**) and *sn*-2,3-di-*O*-acyl PtdGlc (**3**) by MAb DIM21 denotes that its interaction with the antigens is not sensitive to the stereochemistry at position 4 as well as *sn*-2. As for position 6, modification, for example, **2**, resulted in a largely abolished recognition by MAb DIM21. While steric reasons cannot be excluded, partial interruption of direct antigen-antibody interaction seems more likely to be responsible. This might also explain the absence of cross reactivity with PI and PGly at low concentrations. Although both posses very similar features, PGly totally lacks the OH-6 region and PI might not be able to present a suitable hydroxyl function in the required area, highlighting the importance of this position for antibody recognition.

Drastically reduced reactivity of phosphatidylmannoside (**7**) may derive from the altered orientation of the sugar residue due to the absence of the intramolecular hydrogen bond. The phosphate moiety itself seems to be essential for antigen recognition, as indicated by the strong reduction of antibody binding for both diastereomers of phosphorothioatidylglucoside (**4**). Predominantly, one of the diastereomers should be affected, in case primarily steric effects cause decreased recognition. Hence, only a reduction of about 50% should be observed, due to the presence of a close to 1:1 mixture of diastereomers. This is not in good agreement with experimental results, showing a much stronger reduction of antigen recognition. It seems more likely that recognition of both diastereomers is strongly affected, for example, due to a higher charge localization on the sulfur. While the remaining non-bridging oxygen of the phosphorothioate moiety is involved in the weakened intramolecular hydrogen bond, the negatively charged sulfur might not be able to form the necessary electrostatic or hydrogen bond with MAb DIM21 for binding. Consequently, antigen recognition of both diastereomers would be

largely abolished, as observed, indicating the great importance of the phosphate moiety in antigen recognition.

Intriguingly, conversion of ester at position *sn*-2 to ether, as in *sn*-2-*O*-methyl PtdGlc (**5**), widely eliminated antibody binding. It can only be speculated whether this is caused by the absence of vital hydrophobic interactions between the hydrocarbon tail of the arachidic acid residue and the antibody or due to the absence of an important antigen-antibody interaction at the *sn*-2 carbonyl group. Presumably, it might be a combination of both effects. Nevertheless, this strongly indicates the importance of the arachidic acid residue at *sn*-2 position of PtdGlc for antigen recognition.

3. Conclusion

MS and NMR spectroscopic data of natural mono-acetylated PtdGlc derivative[5] and synthetic 6-*O*-acetyl PtdGlc (**2**) were identical, thus confirming the proposed presence of the acetyl group at position 6 of the glucose moiety.

Furthermore, proton NMR data strongly suggested the presence of an intramolecular hydrogen bond at position OH-2. Computer simulations proposed one of the non-binding oxygen as a suitable partner. This prominent feature is conserved in most cases of glucose and galactose derivatives of PtdGlc, leading to a reduced flexibility at the phosphate moiety, likely contributing to its unique biological role. Interestingly, the presence of a phosphorothioate moiety (**4**) significantly weakened the intramolecular hydrogen bond, while it was totally abolished in case of the mannose derivate (**7**).

Concerning MAb DIM21, the phosphate moiety and the arachidic acid ester at position *sn*-2 of PtdGlc and its derivatives serve as the primary recognition site, additionally supported by the hydroxyl function at position 6. Disturbance at one of these three recognition sites will lead to considerable reduction or total abolishment of antigen recognition. Experimental data indicate that hydroxyl functions at position 4 as well as ester function at position *sn*-1/3 are less important for MAb DIM21 binding.

4. Experimental

Unless stated otherwise, all chemicals were purchased as reagent grade from commercial suppliers and used without further purification. Dry solvents were purchased from Kanto Chemical Co., Inc. and used as supplied. Analytical thin layer chromatography (TLC) was performed on Merck Silica gel 60 F_{256} plates, flash column chromatography on Kanto Chemical Co., Ltd silica gel 60N (40–100 mesh) using indicated solvent systems. Low resolution mass spectra (LRMS) were recorded on an AXIMA-LNR MALDI from Shimadzu/KRATOS (MALDI-TOF) or on a Jeol AccuTOF JMS-T700LCK (ESI-TOF). High resolution mass spectra (HRMS) were recorded on the ESI-TOF utilizing TFA as an internal standard. NMR spectra were obtained either with a JEOL EX-400, ECX-400 or JNM-ECA-600 spectrometer (^{1}H at 400/600, ^{13}C at 100/150 MHz and ^{31}P at 160/240 MHz) in the indicated solvents, with chemical shift referenced to residual non-deuterated solvent. Optical rotations were measured using a JASCO DIP-370 polarimeter.

4.1. General procedure: phosphonylation

A 5% solution of trichloroacetimidate (1.0 equiv) in dry THF was treated with phosphorous acid solution (7.1 equiv, 0.5 M in dry THF) and stirred for 5 min at rt. Subsequently, the reaction mixture was cooled to 0 °C, treated with triethylamine (21 equiv) and kept without stirring at 0 °C for 1 h. The precipitate was filtered off, and the filtrate was concentrated in vacuo. The residue was purified by preparative TLC (toluene/MeOH/NEt_3 = 3:1:2.5%).

4.1.1. 6-*O*-Acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl hydrogenphosphonate triethylammonium salt (17)

Prepared by phosphonylation of compound **16** (85 mg, 0.13 mmol) to give compound **17** as a colorless honey (30 mg, 0.046 mmol, 35% yield). $[\alpha]_D^{20}$ = 27 (*c* 3.2 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 7.38–7.26 (n.r., 15H; Bn), 7.07 (d, ^1J (H,P) = 641.3 Hz, 1H; HP), 5.17 (dd, *J* = 7.9 Hz, ^3J (H,P) = 9.2 Hz, 1H; H-1), 5.04 (d, *J* = 11.2 Hz, 1H; Bn), 4.92 (d, *J* = 10.7 Hz, 1H; Bn), 4.86 (d, *J* = 10.7 Hz, 1H; Bn), 4.79 (d, *J* = 10.5 Hz, 1H; Bn), 4.76 (d, *J* = 11.0 Hz, 1H; Bn), 4.56 (d, *J* = 11.0 Hz, 1H; Bn), 4.34 (d, *J* = 11.7 Hz, 1H; H-6b), 4.20 (d, *J* = 12.0 Hz, 1H; H-6a), 3.70 (dd, *J* = 8.5 Hz, *J* = 8.8 Hz, 1H; H-3), 3.59–3.58 (n.r., 2H; H-4, H-5), 3.51 (dd, *J* = 8.3 Hz, *J* = 8.5 Hz, 1H; H-2), 2.97 (q, *J* = 7.3 Hz, 6H; NEt_3), 2.01 (s, 3H; Ac), 1.25 (t, *J* = 7.3 Hz, 9H; NEt_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 170.69 (1C; Ac), 138.59, 138.41, 137.79 (3C; Bn-*ipso*), 128.51, 128.43, 128.27, 128.15, 127.95, 127.85, 127.70, 127.47 (15C; Bn), 97.44 (d, ^2J (C,P) = 3.3 Hz, 1C; C-1), 84.72 (1C; C-3), 82.78 (d, ^3J (C,P) = 5.0 Hz, 1C; C-2), 77.17 (1C; C-5), 75.86, 75.15, 74.80 (3C; Bn), 73.17 (1C; C-4), 63.10 (1C; C-6), 45.63 (3C; NEt_3), 21.22 (1C; Ac), 8.98 (3C; NEt_3); LRMS (MALDI-TOF, neg) calcd for $C_{35}H_{48}NO_9P$ $[M-NEt_3]^-$: 556.2, found: 556.3

4.1.2. 2,3,4,6-Tetra-*O*-benzyl-β-D-mannopyranosyl hydrogenphosphonate triethylammonium salt (19)

Prepared by phosphonylation of compound **18** (25 mg, 0.036 mmol) to give compound **19** as a colorless honey (13 mg, 0.019 mmol, 52% yield). $[\alpha]_D^{20}$ = 16 (*c* 1.6 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 7.40–7.18 (n.r., 20H; Bn), 6.92 (d, ^1J (H,P) = 628.8 Hz, 1H; HP), 5.73 (dd, *J* = 1.4 Hz, ^3J (H,P) = 8.7 Hz, 1H; H-1), 4.91 (d, *J* = 11.0 Hz, 1H; Bn), 4.78 (d, *J* = 12.5 Hz, 1H; Bn), 4.74 (d, *J* = 12.7 Hz, 1H; Bn), 4.63 (d, *J* = 12.0 Hz, 1H; Bn), 4.58 (s, 2H; Bn), 4.54 (d, *J* = 11.0 Hz, 1H; Bn), 4.49 (d, *J* = 12.0 Hz, 1H; Bn), 4.08–3.99 (n.r., 3H; H-3, H-4, H-5), 3.90 (dd, *J* = 1.5 Hz, *J* = 2.7 Hz, 1H; H-2), 3.77 (dd, *J* = 4.4 Hz, *J* = 10.7 Hz, 1H; H-6b), 3.71 (dd, *J* = 1.1 Hz, *J* = 10.9 Hz, 1H; H-6a), 2.93 (q, *J* = 7.3 Hz, 6H; NEt_3), 1.23 (t, *J* = 7.3 Hz, 9H; NEt_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 138.78, 138.65, 138.48 (4C; Bn-*ipso*), 128.29, 127.96, 127.90, 127.79, 127.56, 127.48 (20C; Bn), 93.35 (d, ^2J (C,P) = 2.5 Hz, 1C; C-1), 79.82 (1C; C-3), 75.49 (d, ^3J (C,P) = 5.8 Hz, 1C; C-2), 75.08, 73.54, 72.60, 72.14 (4C; Bn), 75.01 (1C; C-4), 73.10 (1C; C-5), 69.76 (1C; C-6), 45.65 (3C; NEt_3), 9.05 (3C; NEt_3); LRMS (MALDI-TOF, neg) calcd for $C_{40}H_{52}NO_8P$ $[M-NEt_3]^-$: 604.2, found: 604.3.

4.2. General procedure: condensation

A 4% solution of H-phosphonate (1.0–1.25 equiv) in dry THF was treated with pyridine (50 equiv) and cooled to 0 °C. Quick addition of a 2% solution of glycerol (1.0–1.25 equiv) in dry THF was followed by immediate activation of the condensation with pivalyl chloride (2.5 equiv) and removal of the ice-bath. The reaction mixture was stirred for 10 min at rt. Subsequently, oxidation was initiated by addition of iodine solution (2.0 equiv, 0.197 M in pyridine/water = 95:5) and the reaction mixture was stirred for 30 min at rt. The reaction mixture was quenched by addition of aq $Na_2S_2O_3$ solution (1 M). The water layer was washed twice with $CHCl_3$, and the combined organic layer was washed with NEt_3/H_2CO_3-buffer (1 M, pH 8.4), dried over Na_2SO_4, and concentrated in vacuo. The residue was subjected to flash chromatography on silica gel ($CHCl_3$/MeOH/NEt_3 = 7:1:1).

4.2.1. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) (6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)phosphate triethylammonium salt (26)

Prepared by condensation of compound **17** (15 mg, 23 μmol) with compound **10** (19 mg, 29 μmol) to give compound **26** as a

slightly yellow honey (16 mg, 12 μmol, 53% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 7.37–7.20 (n.r., 15H; Bn), 5.23–5.15 (m, 1H; *sn*-2), 5.11 (dd, J = 7.8 Hz, ^2J (H,P) = 7.8 Hz, 1H; H-1), 5.05 (d, J = 11.2 Hz, 1H; Bn), 4.90 (d, J = 11.0 Hz, 1H; Bn), 4.83 (d, J = 10.7 Hz, 1H; Bn), 4.75 (d, J = 10.3 Hz, 1H; Bn), 4.73 (d, J = 10.7 Hz, 1H; Bn), 4.54 (d, J = 11.0 Hz, 1H; Bn), 4.37 (dd, J = 3.4 Hz, J=11.7 Hz, 1H; H-6b), 4.31 (dd, J = 3.1 Hz, J = 12.1 Hz, 1H; *sn*-1b), 4.15 (dd, J = 4.0 Hz, J = 11.8 Hz, 1H; H-6a), 4.07 (dd, J = 7.0 Hz, J = 12.1 Hz, 1H; *sn*-1a), 3.99–3.93 (m, 2H; *sn*-3), 3.67 (dd, J = 8.8 Hz, J = 8.8 Hz, 1H; H-3), 3.59–3.56 (m, 1H; H-5), 3.52 (dd, J = 8.3 Hz, J = 10.0 Hz, 1H; H-4), 3.47 (dd, J = 8.3 Hz, J = 8.8 Hz, 1H; H-2), 2.98 (q, J = 7.3 Hz, 6H; NEt_3), 2.22 (t, J = 7.7 Hz, 4H; Stea-2, Ara-2), 1.99 (s, 3H; Ac), 1.56–1.54 (n.r., 4H; Stea-3, Ara-3), 1.26–1.17 (n.r., 69H; Stea-4-17, Ara-4-19, NEt_3), 0.85 (t, J = 6.8 Hz, 6H; Stea-18, Ara-20); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 173.35 (1C; Stea-1), 172.93 (1C; Ara-1), 170.67 (1C; Ac), 138.78, 138.47, 137.86 (3C; Bn-*ipso*), 128.52, 128.43, 128.24, 128.17, 127.98, 127.86, 127.69, 127.41 (15C; Bn), 98.43 (d, ^2J (C,P) = 4.2 Hz, 1C; C-1), 84.73 (1C; C-3), 82.70 (d, ^3J (C,P) = 7.5 Hz, 1C; C-2), 77.44 (1C; C-5), 75.89, 75.14, 74.62 (3C; Bn), 73.25 (1C; C-4), 70.70 (d, ^3J (C,P) = 10.0 Hz, 1C; *sn*-2), 63.95 (d, ^2J (C,P) = 5.0 Hz, 1C; *sn*-3), 63.12 (1C; *sn*-1), 62.89 (1C; C-6), 45.70 (3C; NEt_3), 34.61 (1C; Stea-2), 34.42 (1C; Ara-2), 32.26, 30.06, 29.88, 29.70, 29.51, 27.65, 23.05 (30C; Stea-4-17, Ara-4-19), 25.24 (2C; Stea-3, Ara-3), 21.22 (1C; Ac), 14.51 (2C; Stea-18, Ara-20), 8.95 (3C; NEt_3); LRMS (ESI-TOF, neg) calcd for $C_{76}H_{126}NO_{14}P$ [M−$HNEt_3$]$^-$: 1205.8, found: 1205.8.

4.2.2. (2-*O*-Methyl-1-*O*-stearyl-*sn*-glycer-3-yl) (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)phosphate triethylammonium salt (31)

Prepared by condensation of compound **21** (49 mg, 96 μmol) with compound **12** (29 mg, 77 μmol) to give compound **31** as a slightly yellow amorphous solid (46 mg, 52 μmol, 68% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 5.26 (dd, J = 8.1 Hz, ^3J (H,P) = 8.1 Hz, 1H; H-1), 5.19 (dd, J = 9.5 Hz, J = 9.5 Hz, 1H; H-4), 5.07 (dd, J = 9.8 Hz, J = 9.8 Hz, 1H; H-3), 5.00 (dd, J = 8.1 Hz, J = 9.5 Hz, 1H; H-2), 4.27 (dd, J = 3.3 Hz, J = 11.8 Hz, 1H; *sn*-1b), 4.21 (dd, J = 4.2 Hz, J = 12.5 Hz, 1H; H-6b), 4.14 (dd, J = 2.2 Hz, J = 12.5 Hz, 1H; H-6a), 4.05 (dd, J = 6.3 Hz, J = 11.7 Hz, 1H; *sn*-1a), 3.95 (ddd, J = 4.9 Hz, ^3J (H,P) = 6.3 Hz, J = 11.0 Hz, 1H; *sn*-3b), 3.86 (ddd, J = 4.5 Hz, ^3J (H,P) = 6.3 Hz, J = 12.2 Hz, 1H; *sn*-3a), 3.80 (ddd, J = 2.3 Hz, J = 4.0 Hz, J = 9.9 Hz, 1H; H-5), 3.60–3.57 (m, 1H; *sn*-2), 3.40 (s, 3H; OMe), 2.98 (q, J = 7.2 Hz, 6H; NEt_3), 2.29 (t, J = 7.6 Hz, 2H; Stea-2), 2.04, 2.03, 2.00, 1.97 (s, 12H; Ac), 1.60–1.54 (m, 2H; Stea-3), 1.27 (t, J = 7.2 Hz, 9H; NEt_3), 1.23 (n.r., 28H; Stea-4-17), 0.85 (t, J = 6.7 Hz, 3H; Stea-18); LRMS (ESI-TOF, neg) calcd for $C_{42}H_{78}NO_{16}P$ [M−$HNEt_3$]$^-$: 781.4, found: 781.3.

4.2.3. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) phosphate triethylammonium salt (32)

Prepared by condensation of compound **23** (71 mg, 14 μmol) with compound **10** (60 mg, 92 μmol) to give compound **32** as a slightly yellow amorphous solid (75 mg, 64 μmol, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 5.37 (dd, J = 2.0 Hz, 1H; H-4), 5.25 (d, J = 7.8 Hz, ^3J (H,P) = 7.8 Hz, 1H; H-1), 5.20 (n.r., 1H; *sn*-2), 5.19 (dd, J = 7.9 Hz, J = 10.1 Hz, 1H; H-2), 5.01 (dd, J = 3.5 Hz, J = 10.1 Hz, 1H; H-3), 4.35 (dd, J = 3.2 Hz, J = 12.0 Hz, 1H; *sn*-1b), 4.14 (dd, J = 7.1 Hz, J = 11.0 Hz, 1H; H-6b), 4.11 (dd, J = 7.1 Hz, J = 12.0 Hz, 1H; *sn*-1a), 4.06 (dd, J = 6.1 Hz, J = 11.0 Hz, 1H; H-6a), 4.01–3.93 (m, 3H; H-5, *sn*-3), 2.97 (q, J = 7.2 Hz, 6H; NEt_3), 2.26 (t, J = 7.4 Hz, 2H; Stea-2), 2.25 (t, J = 7.6 Hz, 2H; Ara-2), 2.11, 2.03, 2.00, 1.94 (s, 12H; Ac), 1.56–1.55 (n.r., 4H; Stea-3, Ara-3), 1.26–1.16 (n.r., 69H; Stea-4-17, Ara-4-19, NEt_3), 0.85 (t, J = 6.8 Hz, 6H; Stea-18, Ara-20); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 173.39 (1C; Stea-1), 172.95 (1C; Ara-1), 170.30, 170.20, 169.95, 169.76 (4C; Ac), 96.41 (d, ^2J (C,P) = 4.2 Hz, 1C; C-1), 71.22 (1C; C-3), 71.13 (1C; C-5), 70.57 (d, ^3J (C,P) = 7.5 Hz, 1C; *sn*-2), 69.74 (d, ^3J (C,P) = 7.5 Hz, 1C; C-2), 67.31 (1C; C-4), 63.98 (d, ^2J (C,P) = 4.2 Hz, 1C; *sn*-3), 63.05 (1C; *sn*-1), 61.35 (1C; C-6), 45.63 (3C; NEt_3), 34.61 (1C; Stea-2), 34.42 (1C; Ara-2), 32.25, 30.04, 29.86, 29.69, 29.50, 27.83, 23.04 (30C; Stea-4-17, Ara-4-19), 25.26 (1C; Stea-3), 25.23 (1C; Ara-3), 21.29, 21.03, 20.95 (4C; Ac), 14.49 (2C; Stea-18, Ara-20), 9.08 (3C; NEt_3); LRMS (ESI-TOF, neg) calcd for $C_{61}H_{114}NO_{17}P$ [M−$HNEt_3$]$^-$: 1061.7, found: 1061.6.

4.2.4. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) (2,3,4,6-tetra-*O*-benzyl-β-D-mannopyranosyl)phosphate triethylammonium salt (33)

Prepared by condensation of compound **19** (13 mg, 19 μmol) with compound **10** (15 mg, 23 μmol) to give compound **33** as a slightly yellow honey (11 mg, 8.1 μmol, 43% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 7.38–7.16 (n.r., 20H; Bn), 5.69 (d, ^3J (H,P) = 7.6 Hz, 1H; H-1), 5.20–5.18 (n.r., 1H; *sn*-2), 4.89 (d, J = 11.0 Hz, 1H; Bn), 4.74, 4.58 (s, 4H; Bn), 4.61 (d, J = 12.0 Hz, 1H; Bn), 4.52 (d, J = 11.5 Hz, 1H; Bn), 4.47 (d, J = 12.0 Hz, 1H; Bn), 4.34 (dd, J = 2.8 Hz, J = 12.1 Hz, 1H; *sn*-1b), 4.10 (dd, J = 7.0 Hz, J = 12.1 Hz, 1H; *sn*-1a), 4.04–3.92 (n.r., 6H; H-2, H-3, H-4, H-5, *sn*-3), 3.72–3.67 (m, 2H; H-6), 2.89 (q, J = 7.2 Hz, 6H; NEt_3), 2.24 (t, J = 7.3 Hz, 1H; Stea-2), 2.23 (t, J = 7.6 Hz, 1H; Ara-2), 1.54 (n.r., 2H; Stea-3, Ara-3), 1.24–1.17 (n.r., 73H; Stea-4-17, Ara-4-19, NEt_3), 0.87 (t, J = 6.7 Hz, 6H; Stea-18, Ara-20); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 173.34 (1C; Stea-1), 172.94 (1C; Ara-1), 138.75, 138.66, 138.53, 138.32 (4C; Bn-*ipso*), 128.34, 128.28, 128.03, 127.86, 127.82, 127.61, 127.47 (20C; Bn), 94.31 (d, ^2J (C,P) = 3.3 Hz, 1C; C-1), 79.69 (1C; C-3), 75.46 (d, ^3J (C,P) = 7.5 Hz, 1C; C-2), 75.10, 73.55, 72.62, 72.10 (4C; Bn), 74.94 (1C; C-4), 72.96 (1C; C-5), 70.63 (d, ^3J (C,P) = 7.5 Hz, 1C; *sn*-2), 69.83 (1C; C-6), 63.96 (d, ^2J (C,P) = 3.3 Hz, 1C; *sn*-3), 63.03 (1C; *sn*-1), 45.52 (3C; NEt_3), 34.62 (1C; Stea-2), 34.43 (1C; Ara-2), 32.26, 30.06, 29.88, 29.71, 29.50, 28.13, 23.06 (30C; Stea-4-17, Ara-4-19), 25.23 (2C; Stea-3, Ara-3), 14.51 (2C; Stea-18, Ara-20), 9.53 (3C; NEt_3); LRMS (ESI-TOF, neg) calcd for $C_{81}H_{130}NO_{13}P$ [M−$HNEt_3$]$^-$: 1253.8, found: 1253.8.

4.2.5. (*R*/*S*)-(2-*O*-Arachidyl-1-*O*-stearyl-sn-glycer-3-yl) (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)phosphorothioate triethylammonium salt (30 R_P/S_P)

A solution of compound **21** (49 mg, 96 μmol) in dry THF (2 mL) was treated with pyridine (310 μL, 3.9 mmol) and cooled to 0 °C. Quick addition of a solution of compound **10** (50 mg, 77 μmol) in dry THF (3 mL) was followed by immediate activation of the linkage with pivalyl chloride (24 μL, 0.19 mmol) and removal of the ice-bath. The reaction mixture was stirred for 10 min at rt. Subsequently, phosphorothioate formation was initiated by addition of a Beaucage reagent[14] solution (10 mL, 0.76 mmol, 76 mM in dry THF), and the reaction mixture was stirred for 7.5 min at rt. The reaction mixture was quenched by addition of NEt_3/H_2CO_3-buffer (1 M, pH 8.4), and extracted twice with $CHCl_3$. The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was subjected to flash chromatography on silica gel ($CHCl_3$/MeOH/NEt_3 = 25:1:0.5%) to give an inseparable mixture of compound **30** R_P and compound **30** S_P as a slightly yellow amorphous solid (72 mg, 61 μmol, 80% yield). $[\alpha]_D^{20}$ = 9.5 (*c* 8.3 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 5.42 (dd, J = 8.1 Hz, ^3J (H,P) = 11.0 Hz, 1H; H-1 (*R*/*S*)), 5.36 (dd, J = 8.2 Hz, ^3J (H,P) = 11.4 Hz, 1H; H-1 (*R*/*S*)), 5.24–5.19 (m, 4H; H-4 (*R*/*S*), *sn*-2 (*R*/*S*)), 5.08 (dd, J = 9.5 Hz, J = 9.8 Hz, 2H; H-3 (*R*/*S*)), 5.03–4.99 (m, 2H; H-2 (*R*/*S*)), 4.33 (dd, J = 3.2 Hz, J = 12.0 Hz, 1H; *sn*-1b (*R*/*S*)), 4.32 (dd, J = 2.9 Hz, J = 12.2 Hz, 1H; *sn*-1b (*R*/*S*)), 4.22 (dd, J = 4.0 Hz, J = 12.6 Hz, 2H; H-6b (*R*/*S*)), 4.17–4.07 (m, 8H; H-6a (*R*/*S*), *sn*-1a (*R*/*S*), *sn*-3 (*R*/*S*)), 3.78 (m, 2H; H-5 (*R*/*S*)), 2.90 (q, 12H; NEt_3), 2.27–2.22 (m, 8H; Stea-2 (*R*/

S), Ara-2 (*R*/*S*)), 2.05, 2.03, 2.02, 1.99, 1.99, 1.96, 1.96 (s, 24H; Ac (*R*/*S*)), 1.56 (n.r., 8H; Stea-3 (*R*/*S*), Ara-3 (*R*/*S*)), 1.24–1.20 (n.r., 138H; Stea-4-17 (*R*/*S*), Ara-4-19 (*R*/*S*), NEt_3), 0.85 (t, *J* = 6.8 Hz, 12H; Stea-18 (*R*/*S*), Ara-20 (*R*/*S*)); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 173.37, 173.35 (2C; Stea-1 (*R*/*S*)), 172.95, 172.91 (2C; Ara-1 (*R*/*S*)), 170.53, 170.03, 169.96, 169.71, 169.56, 169.50, 169.44 (8C; Ac (*R*/*S*)), 96.47 (d, ^2J (C,P) = 3.3 Hz, 2C; C-1 (*R*/*S*)), 73.21 (2C; C-4 (*R*/*S*)), 72.32, 72.19 (4C; C-5 (*R*/*S*)), 71.97 (d, ^3J (C,P) = 8.3 Hz, 2C; C-2 (*R*/*S*)), 71.75 (d, ^3J (C,P) = 9.2 Hz, 2C; C-2 (*R*/*S*)), 70.46 (d, ^3J (C,P) = 8.3 Hz, 1C; *sn*-2 (*R*/*S*)), 70.30 (d, ^3J (C,P) = 10.0 Hz, 1C; *sn*-2 (*R*/*S*)), 68.64, 68.55 (2C; C-3 (*R*/*S*)), 64.57 (d, ^2J (C,P) = 5.7 Hz, 1C; *sn*-3 (*R*/*S*)), 64.52 (d, ^2J (C,P) = 5.0 Hz, 1C; *sn*-3 (*R*/*S*)), 63.08, 62.89 (2C; *sn*-1 (*R*/*S*)), 62.18, 61.97 (2C; H-6 (*R*/*S*)), 46.09 (6C; NEt_3), 34.62, 34.60 (2C; Stea-2 (*R*/*S*)), 34.43, 34.39 (2C; Ara-2 (*R*/*S*)), 32.24, 30.03, 29.86, 29.68, 29.49, 23.03 (56C; Stea-4-17, Ara-4-19 (*R*/*S*)), 25.24 (2C; Stea-2 (*R*/*S*)), 25.22 (2C; Ara-2 (*R*/*S*)), 21.31, 21.11, 21.08, 20.96 (8C; Ac (*R*/*S*)), 14.48 (4C; Stea-18, Ara-20 (*R*/*S*)), 9.79 (6C; NEt_3); LRMS (ESI-TOF, neg) calcd for $C_{61}H_{114}NO_{16}PS$ $[M-HNEt_3]^-$: 1077.6, found: 1077.6.

4.3. General procedure: deacetylation

A 2% solution of acetylated phosphate diester (1.0 equiv) in a mixture of $CHCl_3$/MeOH = 1:2.5 was treated with fresh N_2H_4/HOAc solution (equal volume as $CHCl_3$; prepared by slow (Caution!—exothermic reaction) treatment of hydrazine monohydrate (2.04 g, 40.0 mmol) with glacial acetic acid (0.627 g, 10.4 mmol)) and stirred for 5 min at rt. Subsequently, the reaction mixture was quenched with NEt_3/H_2CO_3-buffer (1 M, pH 8.4). The resulting mixture was washed twice with $CHCl_3$/MeOH = 2:1, the combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by preparative TLC ($CHCl_3$/MeOH/NEt_3 = 5:1:1%) and subsequent gel filtration on LH-20 ($CHCl_3$/MeOH/NEt_3 = 1:1:0.5%).

4.3.1. (*R*/*S*)-(2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) β-D-glucopyranosyl phosphorothioate triethylammonium salt (4 R_P/S_P)

Prepared by deacetylation of compound **30** R_P/S_P (55 mg, 47 μmol) to give an inseparable mixture of **4** R_P and compound **4** S_P as a white amorphous solid (27 mg, 27 μmol, 57% yield). ^1H NMR (600 MHz, DMSO-d_6, 25 °C): δ = 5.66 (d, *J* = 2.2 Hz, 1H; OH-2 (*R*/*S*)), 5.56 (d, *J* = 2.2 Hz, 1H; OH-2 (*R*/*S*)), 5.11–5.10 (m, 1H; *sn*-2 (*R*/*S*)), 5.08–5.07 (m, 1H; *sn*-2 (*R*/*S*)), 4.87 (d, *J* = 4.9 Hz, 1H; OH-3 (*R*/*S*)), 4.86 (d, *J* = 4.9 Hz, 1H; OH-3 (*R*/*S*)), 4.84 (d, *J* = 4.4 Hz, 1H; OH-4 (*R*/*S*)), 4.83 (d, *J* = 4.9 Hz, 1H; OH-4 (*R*/*S*)), 4.82 (dd, *J* = 7.1 Hz, ^3J (H,P) = 8.8 Hz, 1H; H-1 (*R*/*S*)), 4.77 (dd, *J* = 7.7 Hz, ^3J (H,P) = 8.8 Hz, 1H; H-1 (*R*/*S*)), 4.48 (t, *J* = 6.0 Hz, 1H; OH-6 (*R*/*S*)), 4.46 (t, *J* = 6.3 Hz, 1H; OH-6 (*R*/*S*)), 4.29 (dd, *J* = 2.7 Hz, *J* = 12.1 Hz, 1H; *sn*-1b (*R*/*S*)), 4.27 (dd, *J* = 3.0 Hz, *J* = 11.8 Hz, 1H; *sn*-1b (*R*/*S*)), 4.09 (dd, *J* = 7.4 Hz, *J* = 11.8 Hz, 1H; *sn*-1a (*R*/*S*)), 4.07 (dd, *J* = 7.7 Hz, *J* = 12.1 Hz, 1H; *sn*-1a (*R*/*S*)), 3.93–3.91 (m, 2 H; *sn*-3b (*R*/*S*)), 3.87–3.86 (m, 1H; *sn*-3a (*R*/*S*)), 3.80–3.79 (m, 1H; *sn*-3a (*R*/*S*)), 3.66–3.59 (m, 2H; H-6b (*R*/*S*)), 3.46–3.40 (m, 2H; H-6a (*R*/*S*)), 3.32 (n.r., 12H; NEt_3), 3.17–3.11 (n.r., 2H; H-3 (*R*/*S*)), 3.09–3.05 (n.r., 4H; H-5 (*R*/*S*), H-4 (*R*/*S*)), 3.03–2.99 (n.r., 2H; H-2 (*R*/*S*)), 2.28 (t, 4H; Stea-2 (*R*/*S*)), 2.25 (t, 4H; Ara-2 (*R*/*S*)), 1.50–1.49 (n.r., 8H; Stea-3 (*R*/*S*), Ara-3 (*R*/*S*)), 1.23 (n.r., 138H; Stea-4-17 (*R*/*S*), Ara-4-19 (*R*/*S*), NEt_3), 0.85 (t, *J* = 6.9 Hz, 12H; Stea-18 (*R*/*S*), Ara-20 (*R*/*S*)); ^{31}P NMR (240 MHz, DMSO-d_6, 25°C): δ = −5.51, −4.42 (2P; P (*R*/*S*)); HRMS (ESI-TOF, neg) calcd for $C_{53}H_{106}NO_{12}PS$ $[M-HNEt_3]^-$: 909.5891, found: 909.5886.

4.3.2. (2-*O*-Methyl-1-*O*-stearyl-*sn*-glycer-3-yl) β-D-glucopyranosyl-phosphate triethylammonium salt (5)

Prepared by deacetylation of compound **31** (11 mg, 11 μmol) to give compound **5** as a white amorphous solid (6.9 mg, 8.7 μmol, 78% yield). ^1H NMR (600 MHz, DMSO-d_6, 25 °C): δ = 4.62 (dd, ^3J (H,P) = 6.9 Hz, *J* = 7.6 Hz, 1H; H-1), 4.16 (dd, *J* = 2.7 Hz, *J* = 11.7 Hz, 1H; *sn*-1b), 3.96 (dd, *J* = 6.2 Hz, *J* = 11.7 Hz, 1H; *sn*-1a), 3.74 (ddd, *J* = 5.5 Hz, ^3J (H,P) = 5.5 Hz, *J* = 11.0 Hz, 1H; *sn*-3b), 3.69 (ddd, *J* = 5.2 Hz, ^3J (H,P) = 6.2 Hz, *J* = 11.2 Hz, 1H; *sn*-3a), 3.64 (d, *J* = 11.7 Hz, 1H; H-6b), 3.52–3.49 (m, 1H; *sn*-2), 3.42 (dd, *J* = 6.2 Hz, *J* = 11.7 Hz, 1H; H-6a), 3.30 (s, 3H; OMe), 3.12 (dd, *J* = 8.2 Hz, *J* = 8.9 Hz, 1H; H-3), 3.09 (ddd, *J* = 2.3 Hz, *J* = 5.5 Hz, *J* = 9.4 Hz, 1H; H-5), 3.04 (dd, *J* = 8.9 Hz, *J* = 9.6 Hz, 1H; H-4), 3.00 (dd, *J* = 7.6 Hz, *J* = 8.9 Hz, 1H; H-2), 2.46 (q, *J* = 7.1 Hz, 6H; NEt_3), 2.29 (t, *J* = 7.2 Hz, 2H; Stea-2), 1.53–1.49 (m, 2H; Stea-3), 1.23 (n.r., 28H; Stea-4-17), 0.94 (t, *J* = 7.2 Hz, 9H; NEt_3), 0.85 (t, *J* = 7.2 Hz, 3H; Stea-18); ^{31}P NMR (240 MHz, DMSO-d_6, 25 °C): δ = −1.28 (1P; P); HRMS (ESI-TOF, neg) calcd for $C_{348}H_{70}NO_{12}P$ $[M-HNEt_3]^-$: 613.3353, found: 613.3344.

4.3.3. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) β-D-galacto-pyranosyl phosphate triethylammonium salt (6)

Prepared by deacetylation of compound **32** (26 mg, 22 μmol) to give compound **6** as a white amorphous solid (10 mg, 10 μmol, 43% yield). ^1H NMR (600 MHz, DMSO-d_6, 25 °C): δ = 6.23 (d, *J* = 1.5 Hz, 1H; OH-2), 5.08–5.05 (m, 1H; *sn*-2), 4.61 (d, *J* = 5.5 Hz, 1H; OH-3), 4.55 (dd, *J* = 6.6 Hz, ^3J (H,P) = 7.6 Hz, 1H; H-1), 4.54 (dd, *J* = 5.5 Hz, *J* = 6.6 Hz, 1H; OH-6), 4.29 (dd, *J* = 3.0 Hz, *J* = 12.1 Hz, 1H; *sn*-1b), 4.24 (d, *J* = 4.0 Hz, 1H; OH-4), 4.07 (dd, *J* = 7.3 Hz, *J* = 11.8 Hz, 1H; *sn*-1a), 3.79 (ddd, *J* = 5.3 Hz, ^3J (H,P) = 6.0 Hz, *J* = 11.3 Hz, 1H; *sn*-3b), 3.73 (ddd, *J* = 5.3 Hz, ^3J (H,P) = 6.0 Hz, *J* = 11.3 Hz, 1H; *sn*-3a), 3.59 (dd, *J* = 3.5 Hz, *J* = 4.0 Hz, 1H; H-4), 3.51 (ddd, *J* = 6.0 Hz, *J* = 6.6 Hz, *J* = 10.9 Hz, 1H; H-6b), 3.44 (ddd, *J* = 5.3 Hz, *J* = 5.8 Hz, *J* = 10.9 Hz, 1H; H-6a), 3.33–3.27 (n.r., 8H; H-2, H-5, NEt_3), 3.24 (ddd, *J* = 3.5 Hz, *J* = 5.8 Hz, *J* = 9.3 Hz, 1H; H-3), 2.28 (t, *J* = 7.1 Hz, 2H; Stea-2), 2.25 (t, *J* = 6.8 Hz, 2H; Ara-2), 1.52–1.47 (n.r., 4 H; Stea-3, Ara-4), 1.23 (s, 69H; Stea-4-17, Ara-4-19, NEt_3), 0.85 (t, *J* = 7.1 Hz, 6H; Stea-18, Ara-19); ^{31}P NMR (240 MHz, DMSO-d_6, 25 °C): δ = −0.88 (1P; P); HRMS (ESI-TOF, neg) calcd for $C_{53}H_{106}NO_{13}P$ $[M-HNEt_3]^-$: 893.6119, found: 893.6107.

4.4. General procedure: hydrogenation

A 0.5% solution of benzylated phosphate diester (1.0 equiv) in ethanol was hydrogenated in the presence of palladium on activated charcoal (1.7 equiv; 10% as Pd) under an atmosphere of H_2 at ambient pressure. The reaction mixture was stirred for 6 h, and subsequently filtered over Celite. The filtrate was concentrated in vacuo and the residue was purified by gel filtration on LH-20 ($CHCl_3$/MeOH/NEt_3 = 1:1:0.1%).

4.4.1. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) (6-*O*-acetyl-β-D-glucopyranosyl) phosphate triethylammonium salt (27)

Prepared by hydrogenation of compound **26** (15 mg, 11 μmol) to give compound **27** as a white solid (11 mg, 11 μmol, quant. yield). ^1H NMR (400 MHz, DMSO-d_6, 25 °C): δ = 6.31 (d, *J* = 2.7 Hz, 1H; OH-2), 5.10 (d, *J* = 5.4 Hz, 1H; OH-4), 5.07 (n.r., 1H; *sn*-2), 4.94 (d, *J* = 4.5 Hz, 1H; OH-3), 4.62 (dd, *J* = 7.6 Hz, ^3J (H,P) = 7.6 Hz, 1H; H-1), 4.29 (dd, *J* = 1.8 Hz, *J* = 11.7 Hz, 1H; *sn*-1b), 4.25 (d, *J* = 12.6 Hz, 1H; H-6b), 4.06 (dd, *J* = 7.0 Hz, *J* = 11.2 Hz, 1H; *sn*-1a), 3.97 (dd, *J* = 6.7 Hz, *J* = 11.7 Hz, 1H; H-6a), 3.79 (ddd, *J* = 5.6 Hz, ^3J (H,P) = 5.8 Hz, *J* = 11.5 Hz, 1H; *sn*-3b), 3.71 (ddd, *J* = 5.8 Hz, ^3J (H,P) = 5.8 Hz, *J* = 11.5 Hz, 1H; *sn*-3a), 3.44 (ddd, *J* = 1.8 Hz, *J* = 6.7 Hz, *J* = 8.1 Hz, 1H; H-5), 3.33 (n.r., 6H; NEt_3), 3.14 (ddd, *J* = 5.1 Hz, *J* = 8.8 Hz, *J* = 10.3 Hz, 1H; H-3), 3.05 (ddd, *J* = 5.8 Hz, *J* = 8.8 Hz, *J* = 9.9 Hz, 1H; H-4), 3.01 (ddd, *J* = 1.6 Hz, *J* = 7.6 Hz, *J* = 8.9 Hz, 1H; H-2), 2.26 (t, *J* = 6.7 Hz, 2H; Stea-2), 2.25 (t, *J* = 7.2 Hz, 2H; Ara-2), 2.00 (s, 3H; Ac), 1.50–1.49 (n.r., 4H; Stea-3, Ara-3), 1.23 (n.r., 60H; Stea-4-17, Ara-4-19), 1.05 (t, *J* = 7.0 Hz, 9H; NEt_3), 0.85 (t, *J* = 6.5 Hz, 6H; Stea-18, Ara-20); ^{31}P NMR (160 MHz, DMSO-d_6, 25 °C): δ = −1.63 (1P; P); HRMS (ESI-TOF, neg) calcd for $C_{55}H_{108}NO_{14}P$ $[M-HNEt_3]^-$: 935.6229, found: 935.6239.

4.4.2. (2-*O*-Arachidyl-1-*O*-stearyl-*sn*-glycer-3-yl) β-D-mannopyranosyl phosphate triethylammonium salt (7)

Prepared by hydrogenation of compound **33** (11 mg, 8.1 μmol) to give compound **7** as a white amorphous solid (7.8 mg, 7.8 μmol, 97% yield). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ = 5.15 (dd, J = 1.8 Hz, ^3J (H,P) = 8.3 Hz, 1H; H-1), 5.06–5.02 (m, 1H; *sn*-2), 4.60 (d, J = 4.0 Hz, 1H; OH-2), 4.59 (d, J = 5.0 Hz, 1H; OH-4), 4.39 (d, J = 6.6 Hz, 1H; OH-3), 4.34 (dd, J = 5.0 Hz, J = 5.0 Hz, 1H; OH-6), 4.30 (dd, J = 2.5 Hz, J = 12.1 Hz, 1H; *sn*-1 b), 4.08 (dd, J = 5.5 Hz, J = 11.1 Hz, 1H; *sn*-1a), 3.74 (ddd, J = 5.0 Hz, ^3J (H,P) = 5.8 Hz, J = 11.1 Hz, 1H; *sn*-3b), 3.68 (ddd, J = 5.0 Hz, ^3J (H,P) = 6.0 Hz, J = 10.8 Hz, 1H; *sn*-3a), 3.59 (ddd, J = 2.1 Hz, J = 5.4 Hz, J = 11.5 Hz, 1 H; H-6b), 3.57–3.56 (m, 1H; H-2), 3.49–3.35 (n.r., 4H; H-3, H-4, H-5, H-6a), 3.33 (n.r., 6H; NEt_3), 2.27 (t, J = 7.3 Hz, 2H; Stea-2), 2.25 (t, J = 7.6 Hz, 2H; Ara-2), 1.52–1.46 (n.r., 4H; Stea-3, Ara-3), 1.23 (n.r., 69H; Stea-4-17, Ara-4-19, NEt_3), 0.85 (t, J = 7.1 Hz, 6H; Stea-18, Ara-20); ^{31}P NMR (240 MHz, DMSO-d_6, 25 °C): δ = −2.52 (1P; P); HRMS (ESI-TOF, neg) calcd for $C_{53}H_{106}NO_{13}P$ $[M-HNEt_3]^-$: 893.6119, found: 893.6118.

4.5. General procedure: anion exchange

A 0.2% solution of phosphore diester triethylammonium salt (1.0 equiv) in a mixture of $CHCl_3$/MeOH = 1:1 was passed over an ion exchange resin column (Dowex 50WX8 sodium form, 100–200 mesh). Subsequently, the column was extensively rinsed with $CHCl_3$/MeOH = 1:1 mixture, and the combined eluate was concentrated in vacuo.

4.5.1. (2-*O*-Arachidyl-3-*O*-stearyl-*sn*-glycer-1-yl) β-D-glucopyranosyl phosphate sodium salt (3)

Prepared by anion exchange of compound **29** (3.5 mg, 3.5 μmol) to give compound **3** as a white amorphous solid (3.0 mg, 3.3 μmol, 94% yield). ^{1}H NMR (600 MHz, MeOH-d_4, 35 °C): δ = 5.25–5.22 (m, 1H; *sn*-2), 4.84 (dd, ^3J (H,P) = 7.1 Hz, J = 7.6 Hz, 1H; H-1), 4.44 (dd, J = 3.0 Hz, J = 12.1 Hz, 1H; *sn*-3b), 4.20 (dd, J = 7.1 Hz, J = 12.1 Hz, 1H; *sn*-3a), 4.07 (ddd, J = 5.7 Hz, ^3J (H,P) = 5.7 Hz, J = 11.6 Hz, 1H; *sn*-1b), 4.04 (ddd, J = 5.7 Hz, ^3J (H,P) = 5.7 Hz, J = 11.3 Hz, 1H; *sn*-1a), 3.84 (dd, J = 2.0 Hz, J = 12.1 Hz, 1H; H-6b), 3.65 (dd, J = 5.5 Hz, J = 12.1 Hz, 1H; H-6a), 3.37 (dd, J = 9.1 Hz, J = 9.1 Hz, 1H; H-3), 3.34–3.34 (m, 1H; H-5), 3.25 (dd, J = 9.6 Hz, J = 9.8 Hz, 1H; H-4), 3.22 (dd, J = 8.1 Hz, J = 9.1 Hz, 1H; H-2), 2.32 (t, J = 7.1 Hz, J = 7.6 Hz, 2H; Stea-2), 2.30 (t, J = 7.1 Hz, 2H; Ara-2), 1.62–1.57 (n.r., 4H; Stea-3, Ara-3), 1.28 (s, 60H; Stea-4-17, Ara-4-19), 0.89 (t, J = 6.8 Hz, 6H; Stea-18, Ara-20); ^{31}P NMR (240 MHz, MeOH-d_4, 35 °C): δ = -0.70 (1P; P); HRMS (ESI-TOF, neg) calcd for $C_{47}H_{90}O_{13}NaP$ $[M-Na]^-$: 893.6119, found: 893.6117.

4.6. Immunostaining

Samples were spotted with indicated concentration on two independent TLC plates (Polygram Sil G, Macherey-Nagel, Germany) and developed simultaneously with $CHCl_3$/MeOH/HOAc/water = 65:35:4:4. Subsequently, one TLC plate was sprayed with Orcinol reagent for visualization of sugar residue. The second TLC plate was soaked in 1% BSA in PBS for 1 h to block non-specific antibody binding, then reacted with DIM21 antibody, prepared according to literature procedure,[5] (40 μg/mL, diluted with 'Can Get Signal' solution 1 (Toyobo)) overnight. After three repeated washings with PBS for 5 min, the TLC plate was incubated with HRP-conjugated anti-mouse Igs (antibody to IgA, IgG, and IgM) (Cappel, 1:500 with 'Can Get Signal' solution 2 (Toyobo)) for at least 3 h. The immunoreactive spots were visualized by 4-chloro-1-naphthol substrate.

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Supplementary data

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