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Novel lipid A mimetics derived from pentaerythritol: synthesis and their potent agonistic activity

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Abstract—A pentaerythritol unit is substituted for glucosamine as its functional mimic at the reducing end of lipid A disaccharide. Two such pentaerythritol derived lipid A mimetics **3** and **4**, carrying three identical fatty acyl moieties, have been designed and synthesized. An efficient strategy, which involves simultaneous introduction of lipids onto two amino groups, is described for the synthesis of compounds **3** and **4**. The biological activity profile of these two novel lipid A mimetics **3** and **4** is surprisingly similar to the natural lipid A product isolated from *Salmonella minnesota* R595. An in vitro cell activation assay, using human adherent antigen presenting cells, has demonstrated that both **3** and **4** induce the secretion of high levels of cytokines such as tumour necrosis factor-α (TNF-α), IL-6 and IL-8. Also, in a totally synthetic liposomal vaccine system, **3** and **4** exhibit strong immunostimulatory adjuvant property in enhancing antigen specific T-cell activation.

1. Introduction

Lipid A is the lipid anchor of lipopolysaccharide (LPS), the outer cell membrane component of Gram-negative bacteria, and widely considered responsible for strongly signalling and activating the innate immunity in the host, following bacterial infection. Structural investigations have shown that lipid A is responsible for the biological activities of LPS such as the typical endotoxic reactions, fever, lethal shock as well as for its beneficial effects such as the adjuvant characteristics and anti-tumour properties.[1] However, because of their toxicity, neither LPS nor lipid A has so far been used for clinical applications. There have been continuing efforts, through design, synthesis and testing of structural analogues, to reduce the toxicity and enhance the beneficial effects of lipid A.

Structure–activity relationship of lipid A has been a subject of wide research interest over the last two decades.[1,2] It appears that an optimal number of lipid chains and chain length are required on the disaccharide backbone in order to exert strong endotoxic and related biological activities of lipid A. Recently, Seydel et al. observed that the agonistic and antagonistic activity of lipid A were governed by the intrinsic conformation of lipid A, which in turn was defined mainly by the number of charges and acyl chains of the molecule.[3–5] Furthermore, the recent discovery of mammalian Toll-like receptors (TLRs)[6] and their essential role[7] in innate and adaptive immune responses has led to a better understanding of LPS–lipid A functions: it has been suggested that lipid A is a ligand for Toll-like receptor 4 (TLR4), a pattern-recognition receptor involved in the mediation of immune responses to LPS/lipid A.[8,9]

Numerous analogues related to lipid A partial structures have been synthesized with the aim of separating unwanted endotoxic properties from potentially beneficial immunostimulatory effects.[10] Several reports[11] have described the design and synthesis of acyclic lipid A analogues to mimic the functions of lipid A: in these molecules one monosaccharide unit or the whole disaccharide backbone of lipid A has been replaced by an open chain spacer. Of particular interest is the recently reported lipid A agonist ER-112022,[11e] a symmetric phospholipid dimer connected by a non-carbohydrate backbone. The authors suggested that ER-112022 interacted directly with the LPS-signalling receptor and mediated the stimulatory effects through the TLR4 and MD-2 pathway in a CD14-independent manner.

The endotoxic effect of the lipid A from *Salmonella minnesota* R595 (**2**, Fig. 1) could be ameliorated by selective hydrolysis of the 1-phosphate group,[12] and the lipid A modified in this way, called monophosphoryl lipid A (**1**),[13] is an effective adjuvant in both prophylactic and therapeutic human vaccines.[13b] Lipid A preparations purified from bacterial cultures suffer from lack of consistency both in composition and performance. Its heterogeneity is a major cause of large batch-to-batch variations both in composition and activity. As a result, its use in vaccine formulations adds to the compliance requirements. We chose to pursue synthetic analogues[14] of

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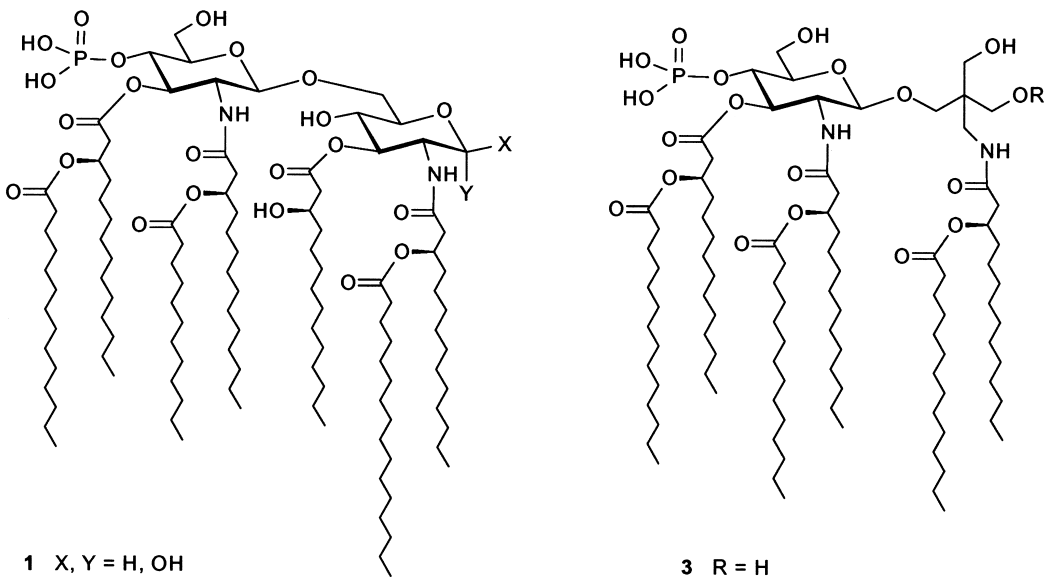


Figure 1. The main component (**2**) of the natural lipid A product isolated from *S. minnesota* R595 and its chemically modified monophosphoryl lipid A (**1**), and two lipid A mimetics **3** and **4**.

similar activity, which have the advantage of reproducibility and the consistency of pure single molecule. Such single molecule adjuvants fulfil the needs of regulatory compliance, which plays an important role in the approvability of a vaccine. An ideal synthetic adjuvant must have optimum activity balanced between benefits and toxicity, trigger a specific cytokine profile, which in turn directs the immune system to deliver an appropriate immune response to an accompanying antigen, and its synthesis should be adaptable for large-scale production. With this rationale, we have designed two lipid A mimetics (**3** and **4**, Fig. 1) with simplified structures as potential vaccine adjuvants. In this paper, we report the design, synthesis, potent agonistic activity, and adjuvant property of this two lipid A mimetics.

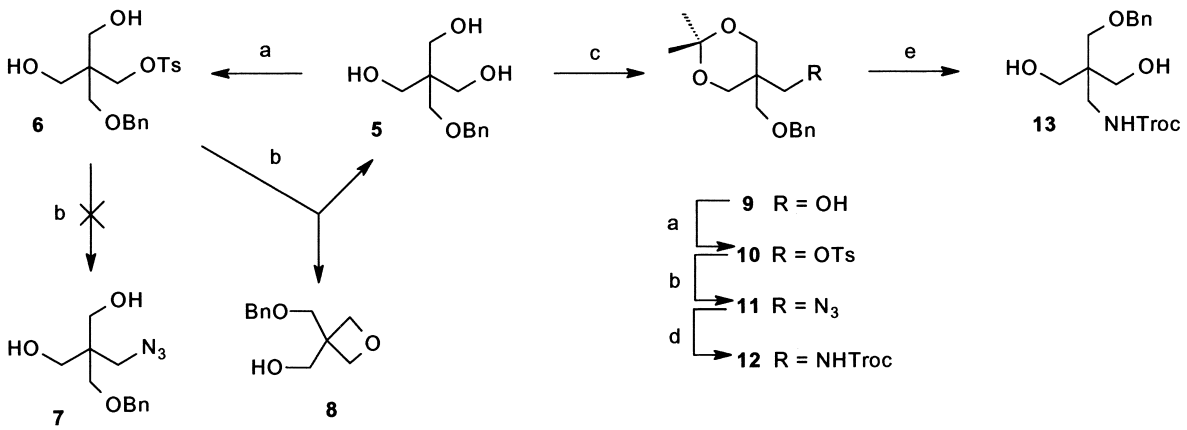
2. Results and discussion

In both structure **3** and **4**, the reducing end glucosamine of lipid A disaccharide has been replaced by an amine-substituted pentaerythritol unit. As a four directional core, pentaerythritol has been successfully employed for the synthesis of multi-functional dendrimers,[15] and as a molecular scaffold for combinatorial chemistry.[16] We perceive the amine-substituted pentaerythritol as a possible structural mimic for glucosamine since the four functional groups of the pentaerythritol core appear, from observing the molecular models, to represent the 2-amine as well as 1,4 and 6-hydroxy functional groups of glucosamine. Pentaerythritol not only simplifies the synthesis, but also adds stability to the entire molecule, as it holds the anomeric phosphate group well compared to that in glucosamine. Thus, structure **4** retains two phosphoryl groups, mimicking the natural diphosphoryl lipid A (**2**); and structure **3**, carrying only one phosphoryl group, is the structural mimetic of the chemically modified monophosphoryl lipid A (**1**). Both **3** and **4** carry three identical fatty acyl groups, which is an important feature required for large-scale synthesis of such molecules.

2.1. Synthesis

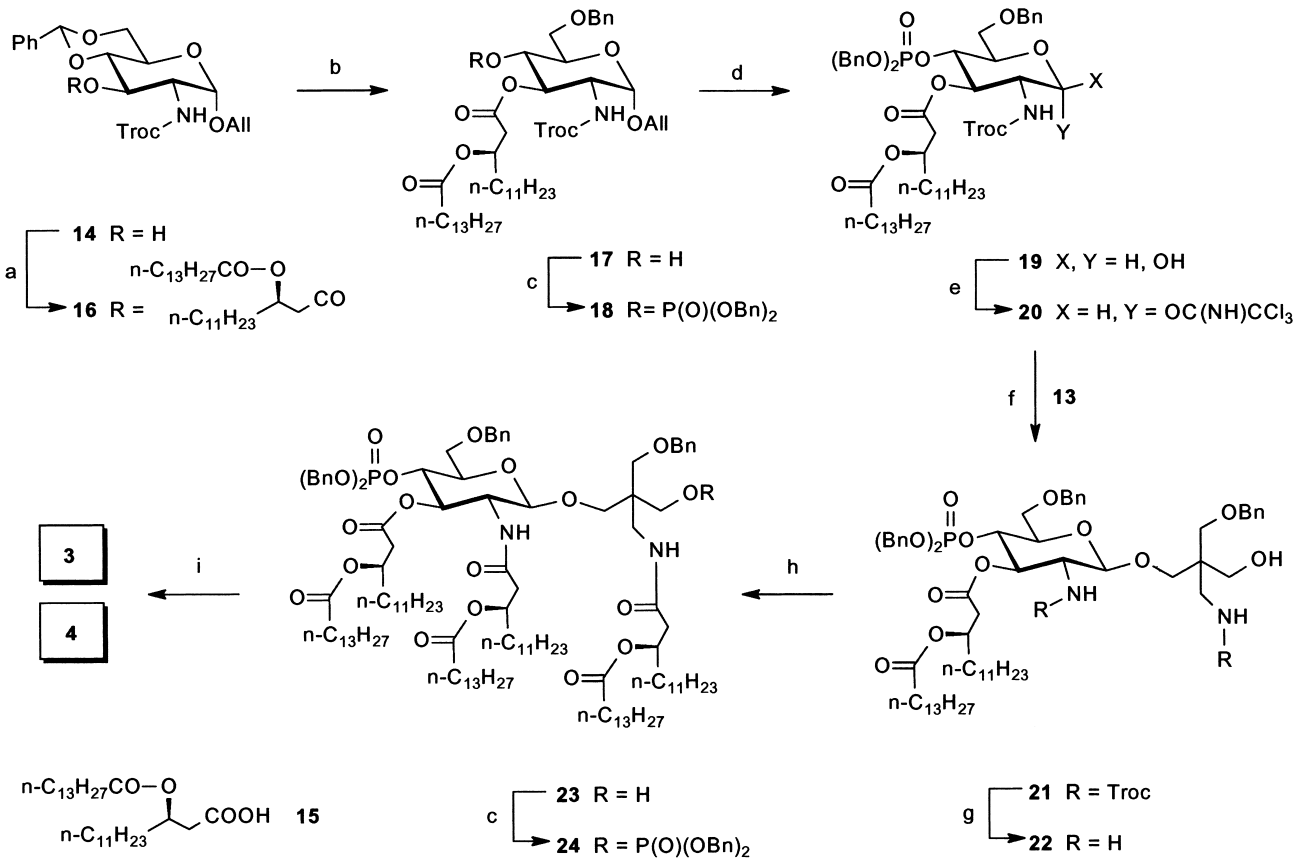
We have adapted a simplified strategy that requires the introduction of both *N*-acyl groups simultaneously and benzyl as a global protecting group. The preparation of the aglycone **13** is described in Scheme 1. The readily available mono-benzylated pentaerythritol **5**[17] is first converted to mono-tosylate **6** in 46% yield. Attempts to displace the tosyl group in **6** with sodium azide in two-phase medium in the presence of phase transfer catalyst have failed. Instead of producing the expected product **7**, the reaction results in the formation of **8** through an intra-molecular substitution,[18] together with some hydrolysed material **5**. To overcome this, compound **5** is first converted into its isopropylidene-protected derivative **9**, which is subsequently tosylated to give **10**. Under the same reaction conditions as above, azide ion displaces the tosyl group affording **11** in 70%, together with the recovery of 18% of the starting material. Treatment of **11** with Zn–HOAc, followed by reacting with trichloroethoxy chloroformate (Troc-Cl), provides **12** in 47% yield. Inspite of the moderate yield of the azide reduction, alternate conditions have not been tried since enough material of **12** has been obtained to carry out further synthesis. The removal of the isopropylidene group in **12** gives the desired glycosylation acceptor **13** in 90% yield.

O-Glycosyl trichloroacetimidate method[19] has been widely used for complex oligosaccharide synthesis, and trichloroethoxycarbonyl (Troc) group has been successfully employed as the amine protecting group for glucosamine to provide exclusively β-glycosyl linkages.[20,21] We have prepared trichloroacetimidate **20** as the glycosyl donor for the synthesis of lipid A mimetic **3** and **4** (Scheme 2). The coupling of the readily available glucosamine derivative **14**[22] and fatty acid **15**[23] is carried out in the presence of



Scheme 1. (a) Ts–Cl, Pyr., 0°C→rt, 46% for **6** and 98% for **10**; (b) NaN_3, aliquat, $NaHCO_3$, toluene–H_2O, reflux, 50% for **8** and 70% for **11**; (c) (i) $Me_2C(OMe)_2$, TsOH, CH_3CN, rt, (ii) TsOH·Et_3N, MeOH, reflux, 82% (for two steps); (d) (i) Zn, HOAc, rt, (ii) Troc-Cl, $NaHCO_3$, dioxane–H_2O, rt, 47% (two steps); (e) HOAc–H_2O (4:1), 60°C, 90%.

DCC and DMAP, which yields **16** in nearly quantitative yield. Selective reductive ring opening of 4,6-di-*O*-benzylidene in **16** by treatment with $NaBH_3CN$ and HCl(g)-saturated diethyl ether solution[24] results in the formation of **17** which is converted into 4-*O*-phosphorylated compound **18** through a two step procedure[25] (in 86% yield). Phosphite group is introduced by treating **17** with phosphoramidite and tetrazole, followed by the oxidation of phosphite to phosphate with *m*-chloroperbenzoic acid (*m*-CPBA). Removal of the allyl group at the anomeric position follows a two step procedure, first the isomerization of allyl double bond using iridium complex[26] followed by the hydrolysis of the isomerized aglycone to provide **19** in 76% yield. The conversion of **19** to the donor trichloroacetimidate **20** is effected by treatment with trichloroacetonitrile and cesium carbonate. The α-isomer (^{1}H NMR δ 6.47 ppm, d, J=3.5 Hz, 1H, H-1) is isolated in 76% yield. TLC showed a lower spot in the reaction mixture in about 5–10% yield, which is likely to be the β-isomer but has not been characterized. When this reaction is carried out using DBU



Scheme 2. (a) **15**, DCC, DMAP, CH_2Cl_2, rt, 98%; (b) $NaBH_3CN$, HCl(g)–Et_2O, THF, 0°C, 85%; (c) (i) $(BnO)_2PN(iPr)_2$, tetrazole, CH_2Cl_2, rt, (ii) *m*-CPBA, CH_2Cl_2, 0°C, 86% for **19** and 80% for **24** (two steps); (d) (i) [bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate, THF, rt, (ii) NBS, THF–H_2O, rt, 76% (two steps); (e) $CNCCl_3$, Cs_2CO_3, CH_2Cl_2, rt, 76%; (f) TMSOTf, CH_2Cl_2, rt, 81%; (g) Zn, HOAc, rt; (h) **15**, DCC, CH_2Cl_2, rt, 64% (two steps); (i) Pd–C, H_2, THF–HOAc, rt, 100% for **3** and 96% for **4**.

as the base, TLC indicates a more complex reaction profile while the yield of the α-isomer is lowered to about 50–60%.

The glycosylation reaction between donor **20** and acceptor **13** (at fourfold excess) in the presence of TMSOTf as catalyst has provided the expected glycoside **21** in 81% yield. No di-glycosylation product has been detected, probably due to an enormous steric crowding that would have ensued. Reductive cleavage of Troc-groups with Zn–HOAc (→**22**) and *N*-acylation with fatty acid **15** in the presence of DCC furnishes the crucial precursor **23** in 64% yield. For the synthesis of lipid A analogues, fatty acyl groups at both *N*-positions of lipid A disaccharide are usually introduced separately; in one similar instance, Johnson and coworkers[13a] tried the bis-*N*-acylation with the same fatty acid (**15**) in a single step, but obtained the desired product in very low yield (25–30%). The structure of **23** (and the initial glycosylation product **21**) has been confirmed by ^{1}H NMR spectral data: two sets of doublets at δ 4.35 and 4.65 (both J=8.0 Hz, each 0.5H, H-1) indicate the existence of two diastereoisomers in about 1:1 ratio, both as β-glycoside. Due to the loss of its symmetry in the pentaerythritol unit, compounds **21**–**24** and the final product **4** exist as a mixture of two diastereoisomers in about 1:1 ratio. Using the same reaction conditions for phosphate group introduction, compound **23** was converted to di-phosphate **24**. The global de-benzylation of **23** and **24** by hydrogenolysis in the presence of palladium on charcoal as catalyst provides, respectively, the designed lipid A mimetics **3** and **4**, and their structures have been confirmed by ^{1}H NMR and MS spectroscopic data.

2.2. Biological evaluation

LPS and its smaller structural component lipid A are well-known activators of innate immunity and thus display strong adjuvant activity and often acute toxicity to the host. As a strong immune stimulant, LPS or lipid A induces an acute inflammatory response in humans resulting in the release of cytokines and cellular mediators, including tumour necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8.[2] Endogenous production of TNF-α seems to be crucial for induction of normal immune responses against invading pathogens, although it also, when released in excess, plays a central role in LPS and lipid A associated endotoxicity. IL-6 and IL-8 play important roles in activating and attracting various types of cells at the site of invading organism. LPS and lipid A have also been shown to induce the production of IL-12 by dendritic cells and macrophages[27] and trigger the maturation of dendritic cells,[28] which as antigen presenting cells (APCs), are important in the development of adaptive immune responses. The synthetic lipid A mimetics **3** and **4** have been investigated for their potency to induce TNF-α, IL-6, and IL-8 in human GM-CSF and IL-4 activated APCs (Table 1). Even at concentration of 1 μg/mL, both **3** and **4** induce high level of cytokines TNF-α, IL-6 and IL-8, at magnitudes similar to that of detoxified lipid A product (R595 lipid A) isolated from *S. minnesota* R595.

The immune stimulatory (adjuvant) properties of **3** and **4** have been evaluated for their ability to induce antigen specific T-cell proliferation responses in comparison with the natural lipid A product, R595 lipid A (Fig. 2). A liposomal vaccine formulation[29] containing MUC1 derived 25 amino acid long lipopeptide BLP25,[30] and a lipid A analogue, either **3** or **4**, or the reference natural lipid A product, has been used to immunize mice. Preliminary results show that both **3** and **4** have demonstrated comparable adjuvant activity in promoting antigen specific T-cell responses. Significantly the monophosphorylated **3** exhibits higher potency in the activation of human APCs than the corresponding diphosphorylated analogue **4** (Table 1), while in the T-cell (C57B1/6) proliferation assay compound **4** shows slightly higher activity. Such change of potency level demonstrated by **3** and **4** might suggest subtle differences between the murine and human immune systems in response to lipid A molecules.

Table 1. Induction of cytokine TNF-α, IL-6 and IL-8 in matured human adherent APCs by synthetic lipid A mimetic **3**, **4**, and reference R595 lipid A (the natural detoxified lipid A product isolated from *S. minnesota* R595) at the concentration of 1.0 μg/mL

	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)
3	3713	5812	32,197
4	1513	1167	12,296
R595 lipid A	2742	5049	34,288
Medium	1	0	359

At the outset we were guided by the necessity to develop well-defined synthetic structures as adjuvants with consistent performance, rather than to achieve quantitative improvement to adjuvant activity. The function of an immunostimulatory adjuvant is to merely trigger innate immunity and to alert the immune system to a perception of 'pathogen invasion', a path that is becoming widely accepted as required for adaptive response to an accompanying antigen.[31] Adjuvant activity must be balanced and appropriate and not always measured in terms of 'more or less'. The present indications are that synthetic structure **3** and **4** exhibit adjuvant activity comparable to a natural product under clinical evaluation. A cytokine profile that is required to stimulate an immune system has been achieved by these less complex single synthetic structures. With these encouraging preliminary results, both compounds are currently under more detailed evaluation as potential

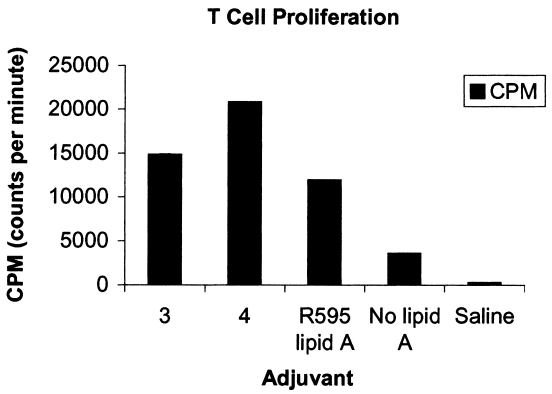


Figure 2. In vitro antigen specific proliferation of T-cells from C57Bl/6 mice immunized with a single dose of BLP25 liposomal vaccine formulation. The vaccine dose contains 0.2 μg of MUC1 derived 25-mer lipopeptide as an antigen and 0.1 μg of synthetic lipid A mimetic **3**, **4**, or reference R595 lipid A (the natural detoxified lipid A product isolated from *S. minnesota* R595) as an adjuvant.

adjuvants for vaccine formulations. Lien et al.[11e] have recently reported that an acyclic lipid A-like agonist ER-112022 activates the TLR4 signalling pathway. We believe that lipid A mimetic **3** and **4** also function by activating TLR4 in exhibiting their agonistic activities. Further studies are necessary in order to provide evidence of the underlying mechanism of their action.

3. Conclusions

In summary, we have demonstrated that, (a) a pentaerythritol unit can be a functional substitute for glucosamine in lipid A disaccharide, (b) the synthetic lipid A mimetics **3** and **4** exhibit biological activities similar to those of more complex mixtures of natural lipid A from *S. minnesota* R595, and, (c) both **3** and **4** can be easily synthesized in pure form.

There is a tremendous potential for molecular adjuvants since their activities can be reproduced impeccably. Unlike those derived from bacterial cultures, which are mixtures of several undefined structures, the synthetic structures described in this report are simpler to produce and constitute an excellent counterpart to molecular vaccines. Though a formal toxicity analysis of **3** and **4** has not yet been carried out, based on the tolerance by mice, they are expected to be at least comparable to the detoxified natural lipid A preparation from *S. minnesota* R595.

4. Experimental

4.1. General

All air and moisture sensitive reactions have been performed under nitrogen atmosphere. Anhydrous THF, DMF, acetonitrile, and dichloromethane are purchased from Aldrich and other dry solvents are prepared in accordance with standard procedures. ACS grade solvents are purchased from Fisher and used for chromatography without distillation. TLC plates (silica gel 60 F_{254}, thickness 0.25 mm, Merck) and flash silica gel 60 (35–75 μm) for column chromatography are purchased from Rose Scientific, Canada. ^{1}H NMR spectra are recorded on Brucker AM 300 MHz, Varian Unity 500 MHz, or Brucker DRX 600 MHz spectrometer with tetramethylsilane as internal standard. Chemical shifts are reported in ppm (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Optical rotations are measured on a Perkin–Elmer 241 Polarimeter at room temperature (20–22°C). Elemental analysis data are obtained from the Micro-analytical laboratory in the University of Alberta, Canada. Electron-spray mass spectrometric analyses (ESIMS) are performed, in positive mode, either on MS50B or MSD1 SPC mass spectrometer, and the data are reported in *m/z*.

4.2. Synthetic procedures

4.2.1. 2-(Benzyloxymethyl)-2-(toluene-4-sulfonyloxymethyl)-1,3-propanediol (6). Compound **5**[17] (500 mg, 3.21 mmol) is dissolved in dry pyridine (5 mL) and cooled to 0°C. *p*-Toluenesulfonyl chloride (0.51 g, 2.65 mmol) is added and the mixture stirred for 3 h while the temperature is allowed to warm up to room temperature. Pyridine is removed by co-distillation with toluene. The resultant crude solid is dissolved in dichloromethane (100 mL) and the solution is washed with saturated sodium chloride solution (30 mL). The organic layer is dried with sodium sulfate and concentrated. The residue, upon purification by flash chromatography (hexane–ethyl acetate, 1:1 and then 2:3) yields **6** (401 mg, 46%) as white powder. R_f 0.55 (hexane–ethyl acetate, 1:4). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.45 (s, 5H, CH_3, 2OH), 3.50 (s, 2H, CH_2), 3.62 (d, J=11.0 Hz, 2H), 3.67 (d, J=11.0 Hz, 2H), 4.15 (s, 2H, CH_2), 4.40 (s, 2H, CH_2Ph), 7.30 (m, 7H, Ar-H), 7.80 (m, 2H, Ar-H).

4.2.2. 2-(Benzyloxymethyl)-1,3-epoxy-2-(hydroxymethyl)-propane (8). Compound **6** (200 mg, 0.526 mmol) is dissolved in toluene (5 mL) and saturated $NaHCO_3$ solution (5 mL), sodium azide (262 mg, 4.04 mmol) and an aliquat of phase transfer catalyst (0.1 g, 0.11 mL, 0.247 mmol) are added. The mixture is maintained at reflux temperature for 16 h and let cool to room temperature. The organic layer is separated and the aqueous layer is extracted with ethyl acetate (10 mL×3). The combined organic phase is washed with cold water (10 mL), dried with sodium sulfate and the solvent removed under vacuum. The residue, upon purification by flash chromatography (hexane–ethyl acetate–methanol, 1:1:1% and then 1:1:5%), gives **8** (55 mg, 50%) and **5** (48 mg, 40%) as colorless syrup. R_f 0.50 (hexane–ethyl acetate–methanol, 1:1:2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (br s, 1H, OH), 3.80 (s, 2H, CH_2), 3.95 (s, 2H, CH_2), 4.43 (d, J=6.0 Hz, 2H), 4.49 (d, J=6.0 Hz, 2H), 4.57 (s, 2H), CH_2Ph). ESIMS calcd for $C_{12}H_{16}O_3$: 208.1; found: 231.1 (M+Na).

4.2.3. 5-(Benzyloxymethyl)-5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxane (9). Compound **5** (672 mg, 2.97 mmol) is dissolved in dry acetonitrile (10 mL) and 2,2-dimethoxypropane (560 mg, 0.66 mL, 5.35 mmol) and *p*-toluenesulfonic acid (56 mg, 0.279 mmol) are added. The mixture is stirred at room temperature for 1 h and then triethylamine (0.5 mL) is added to quench the reaction. The mixture is evaporated under vacuum and the residue purified by flash chromatography (hexane–ethyl acetate, 2:1) to obtain **9** (614 mg, 82%). R_f0.67 (hexane–ethyl acetate, 1:2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 2.40 (br s, 1H, OH), 3.59 (s, 2H, CH_2), 3.69 (s, 2H, CH_2), 3.74 (s, 4H, 2CH_2), 4.55 (s, 2H, CH_2Ph), 7.30 (m, 5H, Ar-H).

4.2.4. 5-(Benzyloxymethyl)-2,2-dimethyl-5-(toluene-4-sulfonyloxymethyl)-1,3-dioxane (10). To a cold (0°C) solution of **9** (572 mg, 2.26 mmol) in dry pyridine (3 mL), *p*-toluenesulfonyl chloride (517 mg, 2.71 mmol) is added and the mixture is stirred for 3 h. More *p*-toluenefulfonyl chloride (430 mg, 2.26 mmol) is added and the stirring is continued at room temperature overnight. Methanol (1 mL) is added to quench the reaction and the solvent is removed by co-distillation with toluene under vacuum. The residue is dissolved in dichloromethane (100 mL) and washed with saturated $NaHCO_3$ (30 mL). The organic layer is separated and the aqueous layer is extracted with dichloromethane (30 mL) and the combined organic layer is dried with

sodium sulfate and concentrated. The residue, upon purification by flash chromatography (hexane–ethyl acetate, 5:1), gives **10** (930 mg, 98%). R_f 0.65 (hexane–ethyl acetate, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 3.35 (s, 2H, CH_2), 3.63 (d, J=12.0 Hz, 2H), 3.72 (d, J=12.0 Hz, 2H), 4.20 (s, 4H, $2CH_2$), 4.50 (s, 2H, CH_2Ph), 7.30 (m, 7H, Ar-H), 7.78 (m, 2H, Ar-H). ESIMS calcd for $C_{22}H_{28}O_6S$: 420.2; found: 443.2 (M+Na).

4.2.5. 5-(Azidomethyl)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxane (11). Compound **10** (907 mg, 2.16 mmol) is dissolved in toluene (30 mL) and saturated $NaHCO_3$ (30 mL), sodium azide (561 mg, 8.63 mmol) and the phase transfer catalyst aliquat (433 mg, 0.49 mL, 1.08 mmol) are added. The mixture is refluxed for 16 h and more sodium azide (1.40 g, 21.60 mmol) is added. The reflux is continued for 24 h and let cool to room temperature. The organic layer is separated and the aqueous layer is extracted with ethyl acetate (30 mL×3). The combined organic layer is washed with water (30 mL), dried with sodium sulfate and the solvent is removed under vacuum. The residue, upon purification by flash chromatography (hexane–ethyl acetate, 8:1), gives **11** (440 mg, 70%) as colorless syrup and the starting material **10** (163 mg, 18%). R_f 0.34 (hexane–ethyl acetate, 6:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.42 (s, 6H, $2CH_3$), 3.40 (s, 2H, CH_2), 3.52 (s, 2H, CH_2), 3.64 (d, J=12.0 Hz, 2H), 3.73 (d, J=12.0 Hz, 2H), 4.50 (s, 2H, CH_2Ph), 7.30 (m, 5H, Ar-H). ESIMS calcd for $C_{15}H_{21}N_3O_3$: 291.2; found: 314.1 (M+Na).

4.2.6. 5-(Benzyloxymethyl)-2,2-dimethyl-5-(trichloroethoxycarbonylaminomethyl)-1,3-dioxane (12). Compound **11** (40 mg, 0.137 mmol) and zinc powder (1.0 g) in acetic acid (10 mL) is stirred at room temperature for 1 h. The solid is filtered and washed with acetic acid (10 mL), and the filtrate is concentrated under vacuum. The residue is dissolved in dioxane–sat. $NaHCO_3$ mixture (2:1, 6 mL, pH 8–9) and the mixture is stirred at room temperature for 6 h in the presence of 2,2,2-trichloroethoxylchloroformate (123 mg, 0.08 mL, 0.568 mmol). Dioxane is removed under vacuum followed by the addition of water (10 mL). The mixture is extracted with ethyl acetate (10 mL×3) and the organic layer is dried with sodium sulfate and concentrated under vacuum. The residue is purified by flash chromatography (hexane–ethyl acetate, 4:1) to obtain **12** (28 mg, 47%) as colorless syrup. R_f 0.17 (hexane–ethyl acetate, 6:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 3.32 (d, J=6.0 Hz, 2H, CH_2), 3.54 (s, 2H, CH_2), 3.67 (d, J=12.0 Hz, 2H), 3.75 (d, J=12.0 Hz, 2H), 4.57 (s, 2H), 4.72 (s, 2H), 5.50 (t, J=6.0 Hz, 1H, NH), 7.35 (m, 5H, Ar-H). ESIMS calcd for $C_{18}H_{24}Cl_3NO_5$: 439.1; found: 462.1 (M+Na), 464.1 (M+Na, ^{37}Cl).

4.2.7. 2-(Benzyloxymethyl)-2-(trichloroethoxycarbonylaminomethyl)-1,3-propanediol (13). Compound **12** (18.3 mg, 0.0417 mmol), in acetic acid and water (4:1, 10 mL), is stirred at 60°C for 45 min. The solvent is removed under vacuum and the residue is purified by flash chromatography (hexane–ethyl acetate, 1:1) to obtain **13** (15 mg, 90%) as white powder. R_f 0.27 (hexane–ethyl acetate, 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.03 (t, J=6.5 Hz, 2H, 2OH), 3.43 (s, 2H, CH_2), 3.44 (d, J=6.5 Hz, 2H, CH_2N), 3.51 (d, J=6.5 Hz, 4H, 2CH_2OH), 4.55 (s, 2H), 4.73 (s, 2H), 5.35 (t, J=6.5 Hz, 1H, NH), 7.35 (m, 5H, Ar-H). ESIMS calcd for $C_{15}H_{20}Cl_3NO_5$: 399.0; found: 422.0 (M+Na), 424.0 (M+Na, ^{37}Cl).

4.2.8. Allyl (*R*)-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (16). To a mixture of **14**[22] (312 mg, 0.65 mmol) and **15**[23] (200 mg, 0.44 mmol) in dry dichloromethane (5 mL), DCC (136 mg, 0.66 mmol) and 4-*N*,*N*-dimethylaminopyridine (DMAP, 27 mg, 0.22 mmol) are added. The reaction is stirred at room temperature for 4 h. The solid is filtered off and washed with ethyl acetate (5 mL). The filtrate is concentrated and the residue, upon purification by flash chromatography (hexane–ethyl acetate, 8:1), gives **16** (398 mg, 98%) as a colorless syrup. R_f 0.69 (hexane–ethyl acetate, 3:1). $[\alpha]_D^{22}$=+32.0 (*c* 0.5, chloroform). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, J=6.5 Hz, 6H, $2CH_3$), 1.25 (m, 38H, $19CH_2$), 1.52 (m, 4H, $2CH_2$), 2.16 (t, J=7.5 Hz, 2H, CH_2), 2.50 (dd, J=16.0, 6.0 Hz, 1H, C*H*H), 2.63 (dd, J=16.0, 6.0 Hz, 1H, C*H*H), 3.71 (dd, J=9.5, 9.5 Hz, 1H, H-4), 3.78 (dd, J=10.0, 10.0 Hz, 1H, H-6a), 3.94 (m, 1H, H-5), 3.98–4.08 (m, 2H, H-2, C*H*HCH=CH_2), 4.21 (m, 1H, CH*H*CH=CH_2), 4.29 (dd, J=10.0, 5.0 Hz, 1H, H-6b), 4.69, 4.76 (2d, J=12.0 Hz, each 1H, Troc-CH_2), 4.94 (d, J=3.6 Hz, 1H, H-1), 5.16 (m, 1H, lipid-3-H), 5.30 (m, 2H, CH=CH_2), 5.39 (dd, J=9.5, 9.5 Hz, 1H, H-3), 5.42 (d, J=10.0 Hz, 1H, NH), 5.53 (s, 1H, C*H*Ph), 5.90 (m, 1H, C*H*=CH_2), 7.30–7.35 (m, 15H, Ar-H). Anal. calcd for $C_{47}H_{74}Cl_3NO_{10}$ (919.46): C, 61.40; H, 8.11; N, 1.52; found: C, 61.40; H, 8.19; N, 1.58.

4.2.9. Allyl 6-*O*-benzyl-2-deoxy-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (17). A solution of **16** (1.45 g, 1.60 mmol) in dry THF (20 mL) and molecular sieves (4 Å, 3.0 g) is stirred at room temperature under nitrogen for 20 min. Sodium cyanoborohydride (1.0 g, 15.96 mmol) is added and the mixture is cooled to 0°C followed by drop wise addition of dry ethereal-HCl(g) till no gas is evolved. The mixture is poured into saturated sodium bicarbonate solution (50 mL) and extracted with dichloromethane (100 mL×3). The combined organic phase is washed with saturated sodium chloride solution (20 mL), dried with sodium sulphate and concentrated. The residue is purified by flash silica gel chromatography (hexane–ethyl acetate, 5:1 and 4:1) to obtain **17** (1.23 g, 85%) as colorless syrup. R_f 0.20 (hexane–ethyl acetate, 4:1). $[\alpha]_D^{20}$=+47.5 (*c* 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J=6.5 Hz, 6H, $2CH_3$), 1.25 (br s, 38H, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.28 (t, J=7.5 Hz, 2H, CH_2), 2.48 (dd, J=14.0, 4.0 Hz, 1H), 2.58 (dd, J=14.0, 7.5 Hz, 1H), 3.27 (d, J=3.5 Hz, 1H, OH), 3.70–3.86 (m, 4H), 3.92–4.03 (m, 2H), 4.58 (d, J=12.0 Hz, 1H), 4.64 (d, J=12.0 Hz, 1H), 4.66 (d, J=12.0 Hz, 1H), 4.76 (d, J=12.0 Hz, 1H), 4.92 (d, J=3.5 Hz, 1H, H-1), 5.13 (m, 2H), 5.19–5.31 (m, 2H, CH_2=CH), 5.40 (d, J=9.5 Hz, 1H, NH), 5.88 (m, 1H, CH_2=C*H*), 7.30 (m, 5H, Ar-H). ESIMS calcd for $C_{47}H_{76}Cl_3NO_{10}$: 919.5; found: 920.8 (M+H).

4.2.10. Allyl 6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (18). To compound **17** (1.20 g, 1.30 mmol) in dry dichloromethane (20 mL), 1*H*-tetrazole (273 mg, 3.90 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (900 mg, 0.875 mL, 2.61 mmol) are added and stirred at room temperature for 30 min. *m*-Chloroperbenzoic acid (*m*-CPBA, 1.63 g, 55%, 5.22 mmol) is added at 0°C and the mixture is stirred for 30 min. The mixture is poured into a solution of 10% sodium hydrogen sulfite (40 mL) and extracted with dichloromethane (40 mL×3). The organic layer is washed with saturated sodium bicarbonate solution (20 mL), dried with sodium sulfate and concentrated. The residue is purified by repeated flash chromatography (hexane–ethyl acetate, 4:1 and then 3:1) to obtain **18** (1.33 g, 86%) as colorless syrup. R_f 0.31 (hexane–ethyl acetate, 3:1). $[\alpha]_D^{20}=+35.0$ (*c* 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J*=6.5 Hz, 6H, $2CH_3$), 1.24 (br s, 38H, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.17 (t, *J*=7.0 Hz, 2H, CH_2), 2.41 (dd, *J*=16.5, 5.5 Hz, 1H), 2.51 (dd, *J*=16.5, 7.5 Hz, 1H), 3.66 (dd, *J*=11.0, 4.5 Hz, 1H), 3.74 (dd, *J*=11.0, 2.0 Hz, 1H), 3.91 (m, 1H), 4.00 (m, 2H), 4.20 (m, 1H), 4.44 (d, *J*=12.0 Hz, 1H), 4.53 (m, 1H, H-4), 4.54 (d, *J*=12.0 Hz, 1H), 4.63 (d, *J*=12.0 Hz, 1H), 4.88–4.95 (m, 5H), 5.11 (m, 1H), 5.20–5.32 (m, 2H, CH_2=CH), 5.35 (dd, *J*=10.5, 9.0 Hz, 1H, H-3), 5.41 (d, *J*=9.5 Hz, 1H, NH), 5.88 (m, 1H, CH_2=C*H*), 7.30 (m, 15H, Ar-H). ESIMS calcd for $C_{61}H_{89}Cl_3NO_{13}P$: 1179.6; found: 1181.0 (M+H).

4.2.11. 6-*O*-Benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α/β-D-glucopyranose (19). Into the suspension of [bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate (14 mg, 0.0165 mmol) in dry THF (5 mL) hydrogen gas is bubbled until a yellowish solution is formed (about 5 min), which is added to a solution of **18** (1.30 g, 1.10 mmol) in dry THF (10 mL). The mixture is stirred at room temperature for 2 h. Water (0.5 mL) and *N*-bromosuccinimide (NBS, 294 mg, 1.62 mmol) are added and the reaction is stirred further for 1 h. The residue obtained after solvent removal is dissolved in ethyl acetate (200 mL) and washed with saturated sodium bicarbonate solution (20 mL×2). The combined organic phase is dried with sodium sulfate and concentrated. The residue, upon purification by flash chromatography (hexane–ethyl acetate, 2:1), yields **19** (950 mg, 76%) as colorless syrup. R_f 0.31 (ethyl acetate–hexane, 1:2). $[\alpha]_D^{20}=+17.5$ (*c* 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J*=6.5 Hz, 6H, $2CH_3$), 1.24 (br s, 38H, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.18 (t, *J*=7.0 Hz, 2H, CH_2), 2.39 (m, 2H, CH_2), 3.59 (dd, *J*=11.0, 6.0 Hz, 1H), 3.71 (dd, *J*=11.0, 1.5 Hz, 1H), 3.94 (m, 1H), 4.16 (m, 1H), 4.40 (m, 3H), 4.49 (d, *J*=12.0 Hz, 1H), 4.65 (d, *J*=12.0 Hz, 1H), 4.72 (d, *J*=12.0 Hz, 1H), 4.90 (m, 4H), 5.09 (m, 1H), 5.39 (t, *J*=3.5 Hz, 1H, H-1), 5.37 (dd, *J*=10.0, 9.5 Hz, 1H, H-3), 5.70 (d, *J*=9.5 Hz, 1H, NH), 7.30 (m, 15H, Ar-H). ESIMS calcd for $C_{58}H_{85}Cl_3NO_{13}P$: 1139.5; found: 1141.0 (M+H).

4.2.12. 6-*O*-Benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranosyl trichloroacetimidate (20). To a solution of **19** (800 mg, 0.70 mmol) in dry dichloromethane (8 mL), trichloroacetonitrile (504 mg, 0.35 mL, 3.51 mmol) and cesium carbonate (343 mg, 1.05 mmol) are added. The mixture is stirred at room temperature for 1 h and the solid is filtered off through celite and washed well with dichloromethane. The filtrate is concentrated in vacuum and the residue, upon purification by flash chromatography (hexane–ethyl acetate, 4:1, with 0.5% of triethyl amine), gives **20** (685 mg, 76%) as colorless syrup. R_f 0.36 (hexane–ethyl acetate, 3:1). $[\alpha]_D^{20}=+12.5$ (*c* 0.4, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J*=6.5 Hz, 6H, $2CH_3$), 1.24 (br s, 38H, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.19 (t, *J*=7.0 Hz, 2H, CH_2), 2.46 (m, 2H, CH_2), 3.71 (m, 2H), 4.04 (m, 1H). 4.15 (ddd, *J*=1.0, 8.5, 3.5 Hz, 1H, H-2), 4.43 (d, *J*=12.0 Hz, 1H), 4.52 (d, *J*=12.0 Hz, 1H), 4.61 (d, *J*=12.0 Hz, 1H), 4.71 (ddd, *J*=9.5, 9.5, 9.5 Hz, 1H, H-4), 4.77 (d, *J*=12.0 Hz, 1H). 4.94 (m, 4H), 5.12 (m, 1H), 4.39 (dd, *J*=10.0, 9.5 Hz, 1H, H-3), 5.65 (d, *J*=8.5 Hz, 1H, NH), 6.47 (d, *J*=3.5 Hz, 1H, H-1), 7.32 (m, 15H, Ar-H), 8.72 (s, 1H, NH). ESIMS calcd for $C_{60}H_{85}Cl_6N_2O_{13}P$: 1282.4; found: 1284.0 (M+H).

4.2.13. 2-(Benzyloxymethyl)-3-hydroxy-2-(trichloroethoxycarbonylaminomethyl)-propan-1-yl 6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside (21). A solution of **20** (620 mg, 0.484 mmol) and **13** (750 mg, 1.936 mmol) in dry dichloromethane (15 mL) and molecular sieves (4 Å, 2.0 g) is stirred under nitrogen for 10 min at room temperature. A solution of TMSOTf (0.01 M in dichloromethane, 3.0 mL) is added drop wise in about 5 min. The mixture is stirred at room temperature for 1 h and saturated sodium bicarbonate solution (10 mL) is added to quench the reaction. Usual aqueous work-up and flash chromatography (hexane–acetone, 2.8:1 and 2:1) yields **21** (590 mg, 81%), colorless syrup of diastereomeric mixture in the ratio of about 1:1. R_f 0.27 (hexane–acetone, 2.5:1). $[\alpha]_D^{20}=-7.6$ (*c* 0.8, chloroform). ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J*=6.5 Hz, 6H, $2CH_3$), 1.25 (br s, 36H, $18CH_2$), 1.45 (m, 2H, CH_2), 1.58 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 2.23 (t, *J*=7.5 Hz, 2H, CH_2), 2.41 (m, 2H, CH_2), 3.10 (m, 0.5H), 3.30–3.62 (m, 7.5H), 3.68–3.83 (m, 2H), 4.40–4.56 (m, 7H), 4.65–4.80 (m, 5H), 4.90 (m, 5H), 5.19 (m, 2H), 5.53 (d, *J*=9.0 Hz, 0.5H, NH), 5.72 (m, 1.5H, NH), 7.30 (m, 20H, Ar-H). ESIMS calcd for $C_{73}H_{103}Cl_6N_2O_{17}P$: 1520.5; found: 1543.5 (M+Na, 42), 1544.4 (M+Na, ^{13}C-isotope, 34), 1545.5 (M+Na, ^{37}Cl-isotope, 100).

4.2.14. 2-(Benzyloxymethyl)-3-hydroxy-2-(aminomethyl)-propan-1-yl 6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-amino-β-D-glucopyranoside (22). To a solution of compound **21** (450 mg, 0.30 mmol) in acetic acid (50 mL) zinc powder (4.0 mg) is added. The mixture is stirred at room temperature for 1 h and filtered. The solid is washed with acetic acid (50 mL) and the filtrate was concentrated in vacuum. The residue is dissolved in dichloromethane (150 mL) and the solution is washed with saturated aqueous sodium bicarbonate solution (20 mL). After separating the organic layer, the aqueous phase is extracted with dichloromethane (20 mL×2). The combined organic phase

is dried with sodium sulphate and concentrated under vacuum to obtain the di-amine **22** (346 mg) which is used directly in the next step.

4.2.15. 2-(Benzyloxymethyl)-3-hydroxy-2-[(*R*)-3-tetradecanoyloxytetradecanamidomethyl]-propan-1-yl 6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamido]-β-D-glucopyranoside (23). A mixture of the di-amine **22** (346 mg), lipid acid **15**[23] (545 mg, 1.20 mmol) and DCC (371 mg, 1.80 mmol) in dry dichloromethane (10 mL) is stirred at room temperature for 20 h. Water (0.05 mL) is added and the reaction mixture is stirred for 10 min. The solid is filtered through a sintered glass funnel with a bed of sodium sulphate. The filtrate is concentrated and the residue purified by flash chromatography (hexane–acetone, 5:1 and 4.5:1) to obtain **23** (390 mg, 64% over two steps) as a colorless syrup. R_f 0.20 (hexane–acetone, 4:1). $[\alpha]_D^{20}=-9.4$ (*c* 0.5, chloroform). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, *J*=7.0 Hz, 18H, $6CH_3$), 1.25–1.50 (m, 112H, $56CH_2$), 1.58 (m, 11H), 1.71–1.81 (m, 3H), 1.97 (m, 1H, OH), 2.21 (t, *J*=7.5 Hz, 2H, CH_2), 2.24–2.63 (m, 10H, $5CH_2$), 3.06 (dd, *J*=14.0, 5.0 Hz, 0.5H), 3.17 (dd, *J*=14.0, 6.0 Hz, 0.5H), 3.30–3.40 (m, 3H), 3.43–3.53 (m, 2H), 3.57–3.63 (m, 2.5H), 3.67–3.80 (m, 2H), 3.87 (m, 1H), 3.97 (m, 0.5H), 4.35 (d, *J*=8.0 Hz, 0.5H, H-1), 4.38–4.53 (m, 5H), 4.65 (d, *J*=8.0 Hz, 0.5H, H-1), 4.90 (m, 4H), 5.07–5.24 (m, 4H), 6.04 (d, *J*=8.5 Hz, 0.5H, NH), 6.38 (d, *J*=7.5 Hz, 0.5H, NH), 6.65 (dd, *J*=6.5, 6.5 Hz, 0.5H, NH), 6.79 (dd, *J*=7.0, 6.0 Hz, 0.5H, NH), 7.30 (m, 20H, Ar-H). ESIMS calcd for $C_{123}H_{205}N_2O_{19}P$: 2045.5; found: 2068.5 (M+Na, 63), 2069.5 (M+Na, ^{13}C-isotope, 100).

4.2.16. 2-(Benzyloxymethyl)-3-*O*-(di-*O*-benzyl-phosphono)-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamidomethyl]-propan-3-ol-1-yl 6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzyl-phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (24). To a solution of compound **23** (220 mg, 0.108 mmol) in dry dichloromethane (5 mL), dibenzyl *N*,*N*-diisopropyl phosphoramidite (74.3 mg, 74.3 μL, 0.215 mmol) and 1*H*-tetrazole (22.7 mg, 0.324 mmol) are added and the mixture is stirred at room temperature for 30 min and then cooled to 0°C. *m*-Chloroperbenzoic acid (*m*-CPBA, 55%, 118 mg, 0.379 mmol) is added with stirring at 0°C for 30 min. The mixture is diluted with dichloromethane (100 mL) and washed with aqueous sodium bisulphite solution (10%, 20 mL). Following the separation of two layers, the aqueous layer is further extracted with dichloromethane (20 mL). The combined organic layer is washed with saturated sodium bicarbonate solution (20 mL) and again the aqueous layer is back extracted with dichloromethane (20 mL). The combined organic layer is dried with sodium sulphate and is concentrated under vacuum. The residue is purified by repeated flash chromatography (hexane–acetone, 5:1 followed by 4.5:1; dichloromethane–methanol, 100:1 followed by 100:1.5; hexane–ethyl acetate, 2:1 followed by 1.5:1) to obtain **24** (200 mg, 80%), colorless syrup, as a diastereomeric mixture in a ratio of about 1:1. R_f (upper spot) 0.29 and R_f (lower spot) 0.25 (hexane–acetone, 3:1). $[\alpha]_D^{20}$(~1:1 mixture)=−7.6 (*c* 0.5, chloroform). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (t, *J*=6.5 Hz, 18H, $6CH_3$), 1.30 (m, 108H, $54CH_2$), 1.48–1.70 (m, 18H), 2.10–2.53 (m, 11H), 2.90–3.35 (m, 5H), 3.55 (m, 2H), 3.75–3.90 (m, 4H), 4.00 (m, 1H), 4.36–4.52 (m, 6H), 4.85–5.01 (m, 8H), 5.08–5.22 (m, 4H), 6.30 (m, 1H, NH), 6.88 (d, *J*=8.5 Hz, 0.5H, NH), 7.00 (d, *J*=8.0 Hz, 0.5H, NH), 7.30 (m, 30H, Ar-H). ESIMS calcd for $C_{137}H_{218}N_2O_{22}P$: 2305.5; found: 2328.5 (M+Na, 78), 2329.5 (M+Na, ^{13}C-isotope, 100).

4.2.17. 2-(Hydroxymethyl)-3-hydroxy-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamidomethyl]-propan-1-yl 2-deoxy-4-*O*-(phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)-tetradecanoyl]-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamido]-β-D-glucopyranoside (3). A solution of compound **23** (96 mg, 0.047 mmol) in THF–HOAc (10:1, 77 mL) and palladium on charcoal (100 mg) is stirred under hydrogen atmosphere for 24 h. The catalyst is filtered and washed with chloroform–methanol (1:1, 30 mL). The filtrate is concentrated under vacuum and the residue is purified by flash chromatography (chloroform–methanol–water, 9:1:0 and then 4:1:0.1) to obtain **3** which is freeze-dried from *tert*-butanol to afford the product as white powder (80 mg, 100%). R_f 0.16 (chloroform–methanol–water–acetic acid, 6:1:0.1:0.1). $[\alpha]_D^{20}=-6.5$ (*c* 0.2, chloroform). ^{1}H NMR (600 MHz, $CDCl_3$–CD_3OD, 1:1): δ 0.89 (t, *J*=6.5 Hz, 18H, $6CH_3$), 1.26 (m, 114H, 57H), 1.60 (m, 12H, $6CH_2$), 2.30 (m, 6H, $3CH_2$), 2.37 (dd, *J*=15.0, 6.0 Hz, 1H), 2.45 (dd, *J*=15.0, 7.0 Hz, 1H), 2.50 (dd, *J*=15.0, 5.0 Hz, 1H), 2.54 (dd, *J*=15.0, 8.0 Hz, 1H), 2.57 (dd, *J*=15.0, 5.0 Hz, 1H), 2.67 (dd, *J*=15.0, 7.0 Hz, 1H), 3.04 (dd, *J*=14.0, 6.0 Hz, 1H), 3.18 (dd, *J*=14.0, 6.0 Hz, 1H), 3.25 (d, *J*=10.0 Hz, 1H), 3.29 (d, *J*=10.0 Hz, 1H), 3.36 (m, 3H), 3.37 (d, *J*=10.0 Hz, 1H), 3.64 (d, *J*=10.0 Hz, 1H), 3.77 (br d, *J*=12.0 Hz, 1H), 3.89 (dd, *J*=10.0, 9.0 Hz, 1H), 3.96 (br d, *J*=12.0 Hz, 1H), 4.25 (m, 1H, H-4), 4.43 (d, *J*=8.5 Hz, 1H, H-1), 5.07 (dd, *J*=10.0, 10.0 Hz, 1H, H-3), 5.17 (m, 2H), 5.23 (m, 1H). ESIMS calcd for $C_{95}H_{181}N_2O_{19}P$: 1685.3; found: 1686.3 (M+H), 1708.3 (M+Na), 1730.3 (M+2Na–H).

4.2.18. 2-(Hydroxymethyl)-3-phosphoryl-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamidomethyl]-propan-1-yl 2-deoxy-4-*O*-(phosphono)-3-*O*-[(*R*)-3-(tetradecanoyloxy)-tetradecanoyl]-2-[(*R*)-3-(tetradecanoyloxy)tetradecanamido]-β-D-glucopyranoside (4). Adapting the procedure described for **3**, compound **24** (104 mg, 0.045 mmol) is, following flash chromatographic purification (chloroform–methanol–water, 9:1:0 and then 6:4:0.5), converted to **4** (77 mg, 96%). R_f 0.50 (chloroform–methanol–water, 6:4:0.5). $[\alpha]_D^{20}=-3.0$ (*c* 0.2, chloroform). ^{1}H NMR (600 MHz, $CDCl_3$–CD_3OD, 1:1): δ 0.89 (t, *J*=6.5 Hz, 18H, $6CH_3$), 1.25 (m, 114H, $57CH_2$), 1.60 (m, 12H, $6CH_2$), 2.30 (m, 6H, $3CH_2$), 2.37–2.70 (m, 6H, $3CH_2$), 3.03 (d, *J*=14.0 Hz, 0.5H), 3.13 (d, *J*=14.0 Hz, 0.5H), 3.24 (d, *J*=14.0 Hz, 0.5H), 3.27 (d, *J*=14.0 Hz, 0.5H), 3.29–3.36 (m, 3H), 3.45 (br s, 1H), 3.55–3.95 (m, 6H), 4.06–4.32 (m, 2H), 5.14–5.27 (m, 4H). ESIMS calcd for $C_{95}H_{181}N_2O_{19}P$: 1685.3; found: 1686.3 (M+H), 1708.3 (M+Na), 1730.3 (M+2Na–H).

4.3. Bioassay

4.3.1. Measurement of cytokines secreted by human adherent antigen presenting cells. APCs isolated from

human peripheral blood by adherence to plastic are incubated for four days in complete RPMI-1640 medium with 5% human AB serum in the presence of GM-CSF (50 ng/mL) and IL-4 (10 ng/mL). The lipid A analogues are added at 1 μg/mL to the matured cells containing 10^6 cells. After 24 h incubation, the supernatants are harvested and the presence of the cytokines is determined using ELISA kits (BD Pharmingen). TNF-α, IL-6 and IL-8 levels are measured and the data are listed in Table 1.

4.3.2. Mice immunized with liposomal vaccines. Groups of C57Bl/6 mice are immunized intradermally with BLP25[30] liposomal vaccine containing 0.2 μg of MUC1-based 25-mer lipopeptide as an antigen and 0.1 μg of compound **3**, **4**, or the reference R595 lipid A as an adjuvant. Nine days after vaccine injection, mice are sacrificed and lymphocytes are taken from the draining lymph nodes (local response) to determine the immune response by measurement of antigen specific T-cell proliferation in vitro.

4.3.3. Measurement of T-cell proliferation. T-cell proliferation is evaluated using a standard ^{3}H thymidine incorporation assay. Briefly, nylon wool passed inguinal lymph node lymphocytes, at 0.25×10^6/well, pooled from each mouse group, are added to a culture containing naive mitomycin C-treated syngeneic splenocytes at 0.25×10^6/well, which serve as APCs. To each well 20 μg of MUC1-based 25-mer peptide[30] is added as boosting antigen. The culture is incubated for 72 h in a total volume of 300 μL/well, followed by the addition of 1 μCi of ^{3}H-thymidine in a volume of 50 μL. The plates are incubated for an additional 18–20 h. Cells are harvested and [^{3}H]dTh incorporation is measured by liquid scintillation counter. T-cell proliferation results corresponding to various liposomal vaccines adjuvanted with lipid A mimic **3**, **4**, or reference natural R595 lipid A are shown in Fig. 2.

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