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Improving the immunostimulatory potency of diethanolamine-containing lipid A mimics

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ABSTRACT

Lipid A is the active principal of gram negative bacterial lipopolysaccharide (LPS) in the activation of Toll-like receptor 4 (TLR4). Given the important role TLR4 plays in innate immunity and the development of adaptive immune responses, ligands that can modulate TLR4-mediated signaling have great therapeutic potential. Recently, we have reported a series of monophosphorylated lipid A mimics as potential ligands of TLR4, in which a diethanolamine moiety is employed to replace the reducing end (D-glucosamine). In this paper, we describe the synthesis of two further diethanolamine-containing lipid A mimics, **3** and **4**, in an effort to mimic more closely the di-phosphate nature of natural lipid A. Both mimic **3**, with an additional phosphate on the diethanolamine acyclic scaffold, and mimic **4**, with a terminal carboxylic acid moiety as a phosphate bioisostere, serve to increase the potency of the immunostimulatory response induced, as measured by the induction of the cytokines TNF-α, IL-6, and IL-1β in the human monocytic cell line THP-1. In addition, mechanistic studies involving the known TLR4 antagonist lipid IVa confirm TLR4 as the target of the diethanolamine-containing lipid A mimics.

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1. Introduction

Toll-like receptor (TLR) 4 plays an important role in the innate immunity and the development of adaptive immune responses. The activation of TLR4 by Gram-negative bacterial lipopolysaccharide (LPS) has been extensively studied, and molecular mechanisms post-activation delineated.[1] The ability to regulate the induction of an adaptive immune response has made TLR4 an attractive target in terms of developing vaccine adjuvants.[2,3] Significant effort has thereby been directed towards the development of ligands for TLR4 via synthetic analogs and mimics of lipid A (**1**, Fig. 1), which is the active principal of LPS in triggering TLR4-mediated signaling.[4,5] The end goal of these efforts has been the maximization of the beneficial immunostimulatory activity, while minimizing the inherent endotoxic properties of LPS/lipid A.

TLR4 is expressed as a complex with the obligate accessory protein MD-2.[1] The structural basis of the recognition of lipid A/LPS by TLR4/MD-2 is highly complex, and has garnered considerable attention. The structural features important for optimal immunologic activity include the degree, pattern, and chain length of the lipid acylation.[6,7] The two phosphate groups in lipid A also greatly affect the immunologic activity of lipid A/LPS. Deletion of either of the phosphates reduces endotoxic activity ~100-fold, while elimination of both phosphates abolishes all activity.[8] Phosphate groups are not a strict requirement, however, as other negatively charged and acidic groups have been employed as bioisosteres of the phosphates, with only minor effects on activity noted.[9,10] Of note are the monophosphoryl lipid A (MPLA) derivatives, which are devoid of the anomeric phosphate.[11] These derivatives are known to have reduced toxicity, yet retain potent immunostimulatory activity.[11–13] Interestingly, MPLA has been shown to selectively activate the TLR4-TRAM-TRIF signaling pathway, but not the TLR4-MAL-MyD88 pathway, thus potentially accounting for the reduced toxicity.[14] These results, as well as structure–activity relationship data of other lipid A analogs,[15–18] have clearly shown the significance of the anomeric phosphate group in modulating the potency and the activity/endotoxicity profile of lipid A molecules. Our understanding of the recognition of lipid A/LPS by TLR4/MD-2 has improved as of late, thanks largely to a protein crystal structure of the receptor complex bound to LPS.[19] The two phosphate groups of lipid A have been shown to play an important role in receptor–ligand binding, and the subsequent receptor dimerization and activation. They provide ionic interactions with positively charged residues on both TLR4 and MD-2, and the adjacent TLR4 within the dimer complex.

As part of our continuing work on the design and synthesis of TLR4 ligands as immunostimulatory agents,[20–23] we recently reported a new class of lipid A mimics, as exemplified by **2** (Fig. 1), in which the reducing-end glucosamine residue of the natural disaccharide scaffold has been replaced by an acylated diethanolamine moiety.[24] Mimic **2** was shown to possess potent

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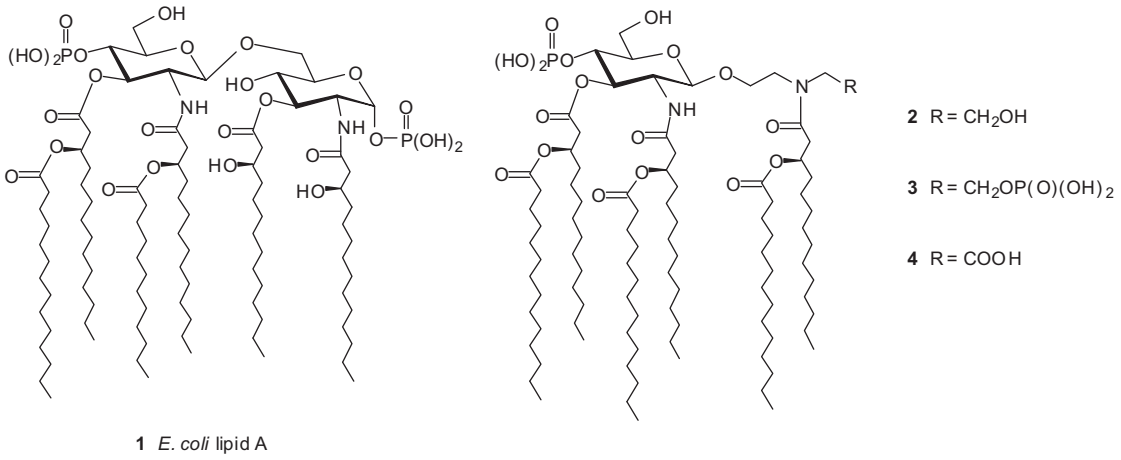


Figure 1. Structure of *E. coli* lipid A (**1**) and diethanolamine-containing lipid A mimics (**2**–**4**).

immunostimulatory activity in the human monocytic cell line THP-1, presumably stimulating TLR4. The therapeutic potential of compound **2** as an immunostimulant is currently under further investigation. Given the key role the two phosphate groups of lipid A play in the dimerization and activation of the TLR4/MD-2 receptor complex,[19] we have therefore designed and synthesized two more diethanolamine-containing lipid A mimics, **3** and **4** (Fig. 1) with the goal of further improving the potency of mimic **2**. Mimic **3** includes an additional phosphate on the diethanolamine scaffold while mimic **4** contains a terminal carboxylic acid moiety as a phosphate bioisostere. It is possible that mimics **3** and **4** may possess a higher binding affinity for the TLR4/MD-2 receptor complex, thus potentially increasing the potency of the induced immunostimulatory response. Herein, we report the synthesis and preliminary biological activity of lipid A mimics **3** and **4**.

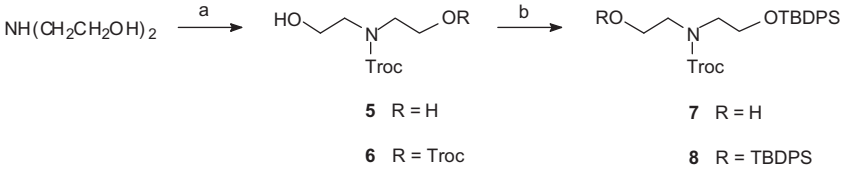
2. Results & discussion

2.1. Synthesis

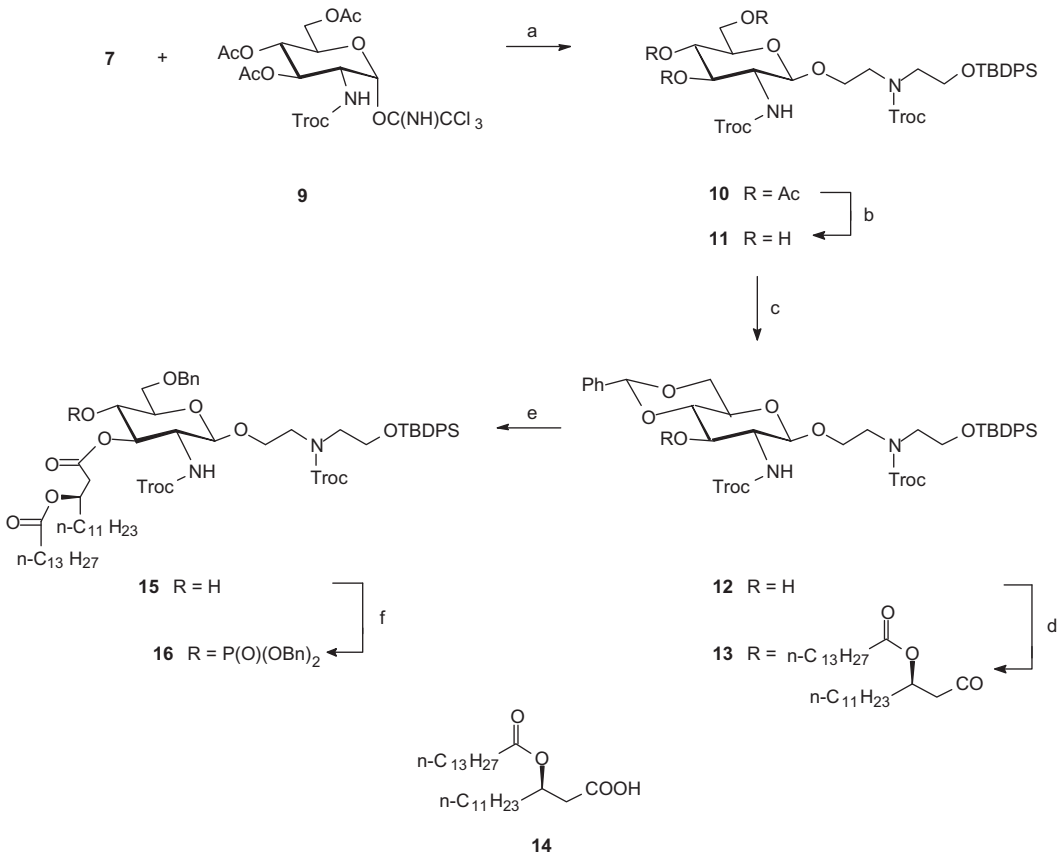
There is significant evidence indicating that the degree, pattern, and chain length of the lipid acylation in lipid A molecules are important factors contributing to their overall biological activity.[6,7] Our previous synthesis of lipid A mimic **2** installed a defined lipid onto the diethanolamine acyclic scaffold at the early stages, thus limiting the potential for incorporating variation in any of the above mentioned acyl characteristics.[17] We therefore chose to employ an alternate synthetic strategy for accessing lipid A mimics **3** and **4**, in an effort to ultimately allow for facile acyl chain variation in future structure–activity relationship investigations.

The synthesis began with the preparation of the diethanolamine-based glycosylation acceptor **7** (Scheme 1). In our previous synthesis of mimic **2** (Fig. 1), the terminal hydroxyl moiety of the diethanolamine acyclic scaffold was left free throughout the synthesis. This proved problematic at many stages of the synthesis, including a propensity to form undesired di-glycosylated products, issues with acylation selectivity, and interesting side reactions in which the solvent tetrahydrofuran molecule was ring-opened to form 1-hydroxybutyl adducts.[24] We therefore have chosen to keep the terminal hydroxyl moiety of the acyclic scaffold protected until the late stages of the synthesis. The amine moiety of diethanolamine was first protected as the 2,2,2-trichloroethoxy carbamate (Troc) to furnish **5** in 78% yield, along with a 7% yield of **6**, in which one of the β-hydroxy moieties has reacted to form the Troc carbonate. Attempts at the protection of one of the β-hydroxy moieties of **5** as the *tert*-butyldiphenyl silyl ether (TBDPS) yielded mono-silylated **7** and di-silylated **8** in 54% and 6%, respectively, with a 35% recovery of unreacted **5**.

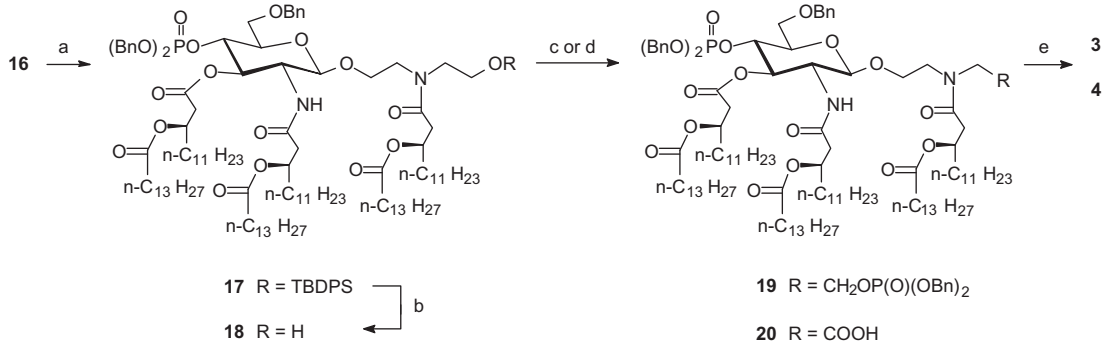
The trimethylsilyl trifluoromethanesulfonate catalyzed glycosylation of **7** with known imidate donor **9**[25] yielded glycoside **10** in 94% (Scheme 2). Similar to our previous work, compound **10** existed as a mixture of two rotational isomers in an approximate 1:1 ratio as a result of prohibited free rotation around the carbamate C–N bond. The desired β-glycosidic linkage in **10** was confirmed via NMR spectral data (^{1}H NMR: δ 4.53, d, J 8.5 Hz, H-1 from one isomer & δ 4.65, d, J 8.5 Hz, H-1 from the other isomer). Compound **10** was treated with guanidine nitrate–sodium methoxide at room temperature to give the O-deacetylated product in 91% yield, leaving the two N-Troc groups intact.[26] The acid catalyzed installation of the 4,6-di-*O*-benzylidene group furnished **12** in 87% yield. The *N*,*N*-dimethylaminopyridine (DMAP) and *N*,*N*′-diisopropylcarbodiimide (DIC) promoted acylation of the 3-OH moiety in **12** with dilipid acid **14**[27] yielded **13** in 93%. Regioselective benzylidene ring opening of **13** was brought about via sodium cyanoborohydride ($NaBH_3CN$) and an HCl(g)-infused diethyl ether solution at 0 °C to give **15**, with the free 4-OH, in 87%. Finally, reaction of **15** with 5-phenyl tetrazole (5-Ph-tetrazole) and dibenzyl *N*,*N*-diisopropylphosphoramidite [$(BnO)_2PN(iPr)_2$], followed by the *m*-chloroperbenzoic acid (*m*-CPBA) promoted oxidation at 0 °C furnished the desired protected phosphotriester functionality of advanced intermediate **16** in 85%. Following the removal of both N-Troc protection groups, the intermediate **16** would allow for



Scheme 1. Reagents and conditions: (a) Troc-Cl, $NaHCO_3$, H_2O, 78% for **5** and 7% for **6**; (b) TBDPS-Cl, Et_3N, CH_2Cl_2, 0 °C, 54% for **7** and 6% for **8** (35% recovery of **5**).



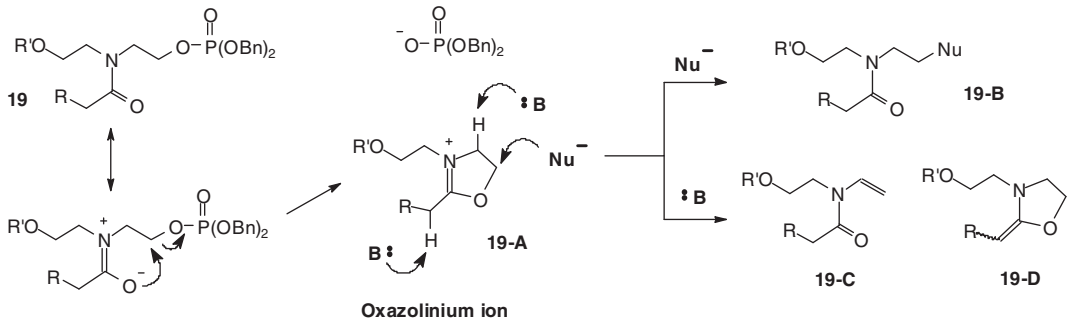
Scheme 2. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, 94%; (b) guanidinium nitrate, NaOMe/MeOH, CH_2Cl_2, 91%; (c) $PhCH(OMe)_2$, *p*-TsOH, CH_3CN, 87%; (d) **14**, DIC, DMAP, CH_2Cl_2, 93%; (e) $NaBH_3CN$, HCl(g)-Et_2O, THF, 0 °C; 87%; (f) (i) $(BnO)_2PN(iPr)_2$, 5-Ph-tetrazole, CH_2Cl_2, (ii) *m*-CPBA, 0 °C, 85%.



Scheme 3. Reagents and conditions: (a) (i) Zn dust, HOAc; (ii) **14**, HBTU, $(iPr)_2NEt$, DMF, 65%; (b) $(Bu)_4NF$, CH_2Cl_2, 90%; (c) (i) $(BnO)_2PN(iPr)_2$, 5-Ph-tetrazole, CH_2Cl_2, (ii) *m*-CPBA, 0 °C, to give **19**, 71%; (d) TEMPO, BAIB, CH_2Cl_2, H_2O, to give **20**, 83%; (e) H_2, Pd/C, THF, 70% for **3** and 77% for **4**.

chemoselective acylation of the two amine moieties, since the primary amine is anticipated to be more reactive toward an acylation reagent than the secondary amine due to the increased steric hindrance in the secondary amine.[31,32] Thus, **16** could serve as a common precursor to access molecules with different acylation patterns on the two amine groups.

Compound **16** was subjected to a zinc treatment in acetic acid to furnish the di-amine, which was immediately coupled with dilipid acid **14**[27] under the promotion of the peptide coupling reagent *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) to form **17** in 65% overall yield (Scheme 3). Deprotection of the acyclic scaffold hydroxyl via a tetrabutylammonium fluoride (TBAF) treatment furnished our previously reported intermediate **18**[17] in 90% yield. Compound **18** was subjected to a similar phosphoramidite treatment as **15** (Scheme 2) to install the desired phosphotriester functionality in **19** in a 71% yield. Compound **19** was found to be quite labile during chromatography purification or under storage in the freezer. We speculate that the decomposition of **19** may involve an intramolecular substitution forming the relatively stable oxazolinium ion **19-A**[24,28] (Scheme 4). Oxazolinium ions have been reported and structurally confirmed by NMR as intermediates for β-hydroxy alkyl amides, which react readily with a carboxylic acid to form esters under neutral condition.[28] The oxazolinium ion **19-A** may undergo nucleophilic attack or an elimination reaction leading to further decomposed products such as **19-B** to **19-D**. At this stage of the investigation, the structures of these decomposed products have not been determined. Practically, due to its relative instability, compound **19** had to be purified



Scheme 4. Proposed mechanism accounting for the ready decomposition of **19**.

quickly and immediately subjected to global deprotection via catalytic hydrogenation under atmospheric pressure in the presence of palladium on charcoal to yield **3**. The (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and bis(acetoxy)iodobenzene (BAIB) promoted oxidation of the acyclic scaffold free hydroxyl group in **18** furnished acid **20**, with which no stability issues were noted. A similar catalytic hydrogenation to remove benzyl protecting groups in acid **20**, afforded **4**. The structures of **3** and **4** were confirmed by ^{1}H NMR and MALDI-MS data.

2.2. Biological evaluations

The binding of agonistic ligands to the TLR4–MD-2 receptor complex leads to the release of various pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), as well as other immunomodulating mediators including interferon-β (IFN-β) and nitric oxide.[1] To evaluate the relative immunostimulatory activity of lipid A mimics **2**–**4**, we therefore measured the direct effects of the mimics on TNF-α, IL-6, and IL-1β production in phorbol 12-myristate 13-acetate (PMA) differentiated human monocytic cell line THP-1.

Lipid A mimics (**2**–**4**) were tested over a wide range of concentrations (10^{-4} μM–10 μM), and the cytokine responses follow a clear dose response relationship (Fig. 2). A maximum response level is achieved for all three cytokines at a stimulus concentration of between 0.1 μM and 1.0 μM, after which a further increase to a 10 μM stimulus concentration results in decreased responses. Significant levels of TNF-α and IL-1β are induced by all three mimics at concentrations in the 10^{-3} μM range. Measurable IL-1β is also observed for mimic **4** at a concentration as low as 10^{-4} μM. In general, both mimics **3** and **4** show increased potency over mimic **2**, with mimic **4** showing the greatest potency for stimulating production of all three cytokines. This trend is most evident in the IL-6 response data, in which mimics **3** and **4**, in comparison to **2**, exhibit an approximate 10-fold and 100-fold increase in potency, respectively. The results indicate that the phosphate/phosphate bioisostere group on the diethanolamine moiety plays an important role in the immunostimulatory potency of these lipid A mimics.

The suspected mode of action for lipid A mimics **2**–**4** is stimulation of the TLR4–MD-2 receptor complex. In an effort to confirm TLR4 as the target of our diethanolamine-containing lipid A mimics, we performed a competitive inhibition study with lipid IVa. Lipid IVa is the tetra-acyl biosynthetic precursor of lipid A, which has been shown to bind to the human TLR4/MD-2 receptor complex, yet not induce any activation.[29] It follows that lipid IVa is a potent antagonist of LPS-induced TLR4 activation in the human monocytic cell line THP-1.[30] We therefore tested the potential of lipid IVa to inhibit the TNF-α and IL-1β cytokine response of THP-1 cells to lipid A mimic **4** (Fig. 3). Co-treatment with lipid IVa significantly

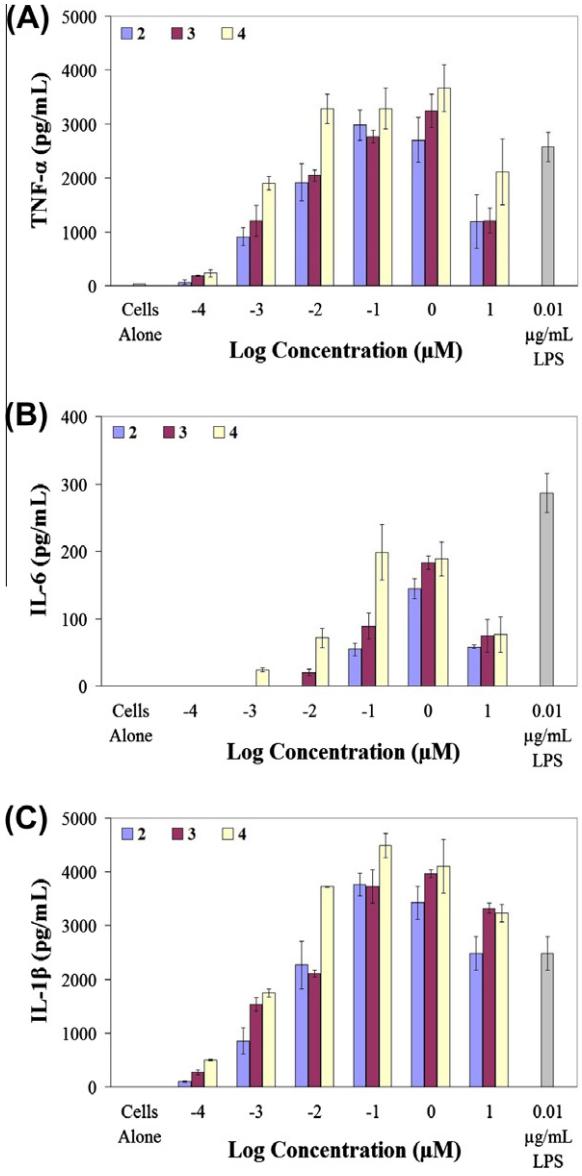


Figure 2. Cytokine production by THP-1 cells after stimulation with LPS and lipid A mimics (**2**–**4**). THP-1 cells were incubated for 24 h with increasing concentrations of **2**–**4**. TNF-α (A), IL-6 (B), and IL-1β (C) in cell supernatants were measured via ELISAs. The results are shown as the average of two separate experiments.

inhibits the production of both TNF-α and IL-1β induced by both LPS and mimic **4**. At a concentration of 0.1 μM of mimic **4**, at which point a maximum level of cytokine induction is expected, the pres-

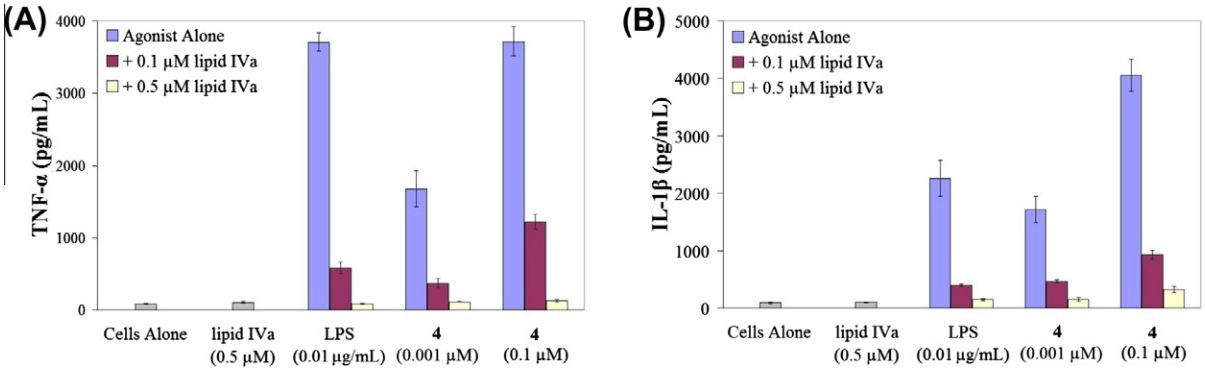


Figure 3. Inhibited cytokine production by THP-1 cells after stimulation with an agonist (LPS or lipid A mimic **4**) in the presence of lipid IVa. THP-1 cells were incubated for 24 h with either LPS or lipid A mimic **4** in the presence of increasing concentrations of lipid IVa. TNF-α (A) and IL-1β (B) in cell supernatants were measured via ELISAs. The results are shown as the average of two separate experiments.

ence of lipid IVa at 0.5 μM appears to have completely inhibited the production of TNF-α, while the production of IL-1β is still marginally measurable. In contrast, the presence of lipid IVa at 0.1 μM exhibits only partial antagonism against the production of both cytokines induced by **4** at a concentration of 0.1 μM. It is worth noting that complete inhibition of TNF-α and IL-1β production induced by 0.001 μM of **4** in the presence of 0.1 μM of lipid IVa (100-fold of **4**) is not observed, suggesting that mimic **4** may have a higher binding affinity with TLR4/MD-2 than lipid IVa. Based on these data, we conclude that compound **4** is an agonist of TLR4, and other diethanolamine-containing lipid A mimics are likely ligands of TLR4 as well.

In conclusion we have successfully synthesized two new members of diethanolamine-containing lipid A mimics, in which the previously free terminal hydroxyl moiety has now been replaced with either an additional phosphate, or a phosphate bioisostere carboxylic acid functionality. Structurally speaking, both the phosphate group in lipid A mimic **3**, and the acidic phosphate bioisostere in mimic **4** serve to increase the potency of the immunostimulatory response induced when compared to the terminal free hydroxyl in mimic **2**. Interestingly, the terminal acidic moiety of mimic **4** yields a higher potency than the phosphate moiety of mimic **3**. Competitive inhibition studies with lipid IVa, a known human TLR4 antagonist, confirm TLR4 as the target of diethanolamine-containing lipid A mimics. Further studies are underway to investigate the application of this novel class of lipid A mimics as immunostimulatory therapeutic agents.

3. Experimental

3.1. General methods

All air and moisture sensitive reactions were performed under nitrogen atmosphere. All commercial reagents were used as supplied. Anhydrous dichloromethane was distilled over calcium hydride, whereas anhydrous *N*,*N*-dimethylformamide (DMF) was purchased from Aldrich. ACS grade solvents were purchased from Fisher Scientific and used for chromatography without distillation. TLC plates (silica gel 60 F_{254}, thickness 0.25 mm) and silica gel 60 (40–63 μm) for flash column chromatography were purchased from SILICYCLE INC., Canada. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer. Tetramethylsilane (TMS, δ 0.00 ppm) or solvent peaks were used as internal standards for ^{1}H and ^{13}C NMR spectra. The chemical shifts were given in ppm and coupling constants in Hz indicated to a resolution of ±0.5 Hz. Multiplicity of proton signals is indicated as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet), br (broad). Structural assignments were made using standard gCOSY and gHSQC methodology. NMR peaks belonging to primary lipid chains are denoted with an L subscript, whereas those belonging to secondary lipid chains are denoted with an L′ subscript. MALDI mass spectra were measured on the Applied Biosystems Mariner 4700 System at the University of Western Ontario. Optical rotations were measured with Perkin Elmer 343 Polarimeter at 22 °C.

3.2. *N*,*N*-Bis(2-hydroxyethyl)-2,2,2-trichloroethoxymethanamide (5) & *N*-(2-|hydroxyethyl)-*N*-{2-(2,2,2-trichloroethoxycarbonyloxy)-ethyl}-2,2,2-trichloroethoxymethanamide (6)

To a solution of diethanolamine (1.10 g, 10.45 mmol) and sodium bicarbonate (3.07 g, 36.58 mmol) in water (70 mL), 2,2,2-trichloroethoxychloroformate (2.88 g, 13.59 mmol) was added drop wise in about 3 min. As the mixture was stirred at room temperature, a viscous insoluble oil formed and settled to the bottom of the reaction vessel. After stirring for 3 h, the mixture was extracted with EtOAc (3 × 135 mL), after which the combined organic layers were dried over Na_2SO_4 and concentrated. Purification via flash column chromatography (hexane/EtOAc/MeOH, 1:1:0.1) yielded **5** (2.29 g, 78%) and **6** (332 mg, 7%), both as colorless syrups.

For (**5**): R_f 0.34 (hexane/EtOAc/MeOH, 1:1:0.1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.52–3.63 (br m, 6H, $NCH_2 \times 2$, $OH \times 2$), 3.86–3.94 (m, 4H, $OCH_2 \times 2$), 4.78 (s, 2H, Troc-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 52.55 ($N\underline{C}H_2$), 53.26 ($N\underline{C}H_2$), 61.57 ($O\underline{C}H_2$), 62.04 ($O\underline{C}H_2$), 75.45 (Troc-$\underline{C}H_2$), 95.61 (Troc-$\underline{C}Cl_3$), 155.15 (C=O); ESI-MS (*m*/*z*) Calcd for $C_7H_{12}Cl_3NO_4$ $[M+Na]^+$: 301.9, found: 302.0.

For (**6**): R_f 0.65 (hexane/EtOAc/MeOH, 1:1:0.1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.82–1.95 (br s, 0.5H, OH from one isomer), 2.20–2.34 (br s, 0.5H, OH from one isomer), 3.58–3.61 (m, 2H, $HOCH_2\underline{C}H_2N$), 3.73–3.77 (m, 2H, $N\underline{C}H_2CH_2OTroc$), 3.82–3.88 (br m, 2H, $HO\underline{C}H_2$), 4.44–4.49 (m, 2H, $\underline{C}H_2OTroc$), 4.76–4.81 (m, 4H, Troc-$CH_2 \times 2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 47.45 ($HOCH_2\underline{C}H_2N$ from one isomer), 48.24 ($HOCH_2\underline{C}H_2N$ from one isomer), 51.10 ($N\underline{C}H_2CH_2OTroc$ from one isomer), 51.86 ($N\underline{C}H_2CH_2OTroc$ from one isomer), 61.22 ($HO\underline{C}H_2$ from one isomer), 61.27 ($HO\underline{C}H_2$ from one isomer), 66.66 ($\underline{C}H_2OTroc$ from one isomer), 66.91 ($\underline{C}H_2OTroc$ from one isomer), 75.20 (O-Troc-CH_2 from one isomer), 75.34 (O-Troc-$\underline{C}H_2$ from one isomer), 76.86 (N-Troc-$\underline{C}H_2$), 94.24 (O-Troc-$\underline{C}Cl_3$), 95.22 (N-Troc-$\underline{C}Cl_3$), 153.82 (O-Troc-C=O), 154.52 (N-Troc-C=O from one isomer), 155.05 (N-Troc-C=O from one isomer); ESI-MS (*m*/*z*) Calcd for $C_{10}H_{13}Cl_6NO_6$ $[M+Na]^+$: 475.9, found: 475.9.

3.3. *N*-(2-Hydroxyethyl)-*N*-{2-(*tert*-butyldiphenylsilyloxy)ethyl}-2,2,2-trichloroethoxymethanamide (7) & *N*,*N*-Bis{2-(*tert*-Butyldiphenylsilyloxy)ethyl}-2,2,2-trichloroethoxymethanamide (8)

To a cooled solution (ice water bath) of **5** (2.00 g, 7.13 mmol) and Et_3N (902 mg, 8.91 mmol) in CH_2Cl_2 (8 mL), *tert*-butyldiphenylsilyl chloride (2.45 g, 8.91 mmol) was added. After stirring at room temperature for 3 hours, the reaction was quenched with MeOH and concentrated. Flash column chromatography purification (hexane/acetone, 3.5: 1) yielded **7** (2.01 g, 54%) and **8** (321 mg, 6%), as well as recovered **5** (706 mg, 35%), all as colorless syrups.

For (**7**): R_f 0.38 (hexane/acetone, 3:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.09 (s, 9H, C(CH_3)$_3$), 2.69 (br s, 0.4H, OH from one isomer), 2.94 (br s, 0.6H, OH from one isomer), 3.56–3.64 (m, 4H, $NCH_2 \times 2$), 3.83–3.90 (m, 4H, $OCH_2 \times 2$), 4.68 (s, 1.2H, Troc-CH_2 from one isomer), 4.78 (s, 0.8H, Troc-CH_2 from one isomer), 7.40–7.48 (m, 6H, Ar-H), 7.67–7.70 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.10 (C(CH_3)$_3$ from one isomer), 19.13 (C(CH_3)$_3$ from one isomer), 26.82 (C(CH_3)$_3$), 50.77 (NCH_2CH_2OTBDPS from one isomer), 51.42 (NCH_2CH_2OTBDPS from one isomer), 51.46 ($HOCH_2CH_2N$ from one isomer), 52.44 ($HOCH_2CH_2N$ from one isomer), 61.54 (CH_2OTBDPS from one isomer), 61.72 (CH_2OTBDPS from one isomer), 62.18 ($HOCH_2$ from one isomer, 62.72 ($HOCH_2$ from one isomer), 75.15 (Troc-CH_2 from one isomer), 75.19 (Troc-CH_2 from one isomer), 95.30 (Troc-CCl_3 from one isomer), 95.57 (Troc-CCl_3 from one isomer), 127.84 (CH-Ar), 127.86 (CH-Ar), 129.93 (CH-Ar), 132.92 (C-Ar), 135.58 (CH-Ar), 154.40 (C=O from one isomer), 155.45 (C=O from one isomer); ESI-MS (*m*/*z*) Calcd for $C_{23}H_{30}Cl_3NO_4Si$ $[M-C_4H_9]^+$: 460.0, found: 460.4.

For (**8**): R_f 0.67 (hexane/acetone, 3:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.05 (s, 18H, C(CH_3)$_3 \times 2$), 3.61–3.69 (m, 4H, $NCH_2 \times 2$), 3.85–3.92 (m, 4H, $OCH_2 \times 2$), 4.75 (s, 2H, Troc-CH_2), 7.43–7.52 (m, 12H, Ar-H), 7.72–7.79 (m, 8H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.20 (C(CH_3)$_3$), 26.88 (C(CH_3)$_3$), 50.86 (NCH_2), 51.32 (NCH_2), 62.24 (OCH_2), 62.65 (OCH_2), 75.04 (Troc-CH_2), 95.62 (Troc-CCl_3), 127.78 (CH-Ar), 127.82 (CH-Ar), 127.85 (CH-Ar), 129.70 (CH-Ar), 129.82 (CH-Ar) 129.84 (CH-Ar), 133.35 (C-Ar), 133.44 (C-Ar), 133.59 (C-Ar), 135.59 (CH-Ar), 135.62 (CH-Ar), 154.27 (C=O); ESI-MS (*m*/*z*) Calcd for $C_{39}H_{48}Cl_3NO_4Si_2$ $[M-C_4H_9]^+$: 698.2, found: 698.5.

3.4. *N*-{2-(*tert*-Butyldiphenylsilyloxy)ethyl}-*N*-{2-[3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (10)

A solution of **7** (997 mg, 1.92 mmol) and imidate **9** (1.26 g, 2.02 mmol) in dry CH_2Cl_2 (8 mL) in the presence of molecular sieves (4Å, 4.0 g) was stirred under nitrogen at room temperature for 30 min. A solution of TMSOTf (0.05 M in dry CH_2Cl_2, 0.8 mL) was added drop wise in about 3 min. The mixture was stirred at room temperature for 1 h before a saturated sodium bicarbonate solution (15 mL) was added to quench the reaction. Solids were filtered out, and the filtrate was extracted with CH_2Cl_2 (3×30 mL). The combined organic phase was dried over Na_2SO_4, concentrated, and purified via flash column chromatography (hexane/acetone, 2.5: 1) to yield **10** (1.77 g, 94%) as a fluffy white solid. R_f 0.42 (hexane/acetone, 5:2); $[\alpha]_D^{22}$+ 0.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.04 (s, 9H, C(CH_3)$_3$), 2.01–2.09 (m, 9H, Ac $\times$ 3), 3.38–3.47 (m, 2H, NCH_2), 3.51–3.72 (m, 4H, H-2, H-5, NCH_2), 3.75–3.86 (m, 3H, ROCH*H*, CH_2OTBDPS), 3.94–4.04 (m, 1H, ROC*H*H), 4.08–4.13 (m, 1H, H-6b), 4.26 (dd, 0.5H, *J* 12.0, 12.0 Hz, H-6a from one isomer), 4.28 (dd, 0.5H, *J* 12.0, 12.0 Hz, H-6a from one isomer), 4.53 (d, 0.5H, *J* 8.5 Hz, H-1 from one isomer), 4.55 (d, 0.5H, *J* 12.5 Hz, Troc), 4.60 (d, 0.5H, *J* 12.0 Hz, Troc), 4.65 (d, 0.5H, *J* 8.5 Hz, H-1 from one isomer), 4.68 (d, 0.5H, *J* 12.5 Hz, Troc), 4.72 (d, 0.5H, *J* 12.5 Hz, Troc), 4.76 (d, 1.0H, *J* 12.0 Hz, Troc), 4.89 (d, 1.0H, *J* 12.0 Hz, Troc), 4.94 (d, 0.5H, *J* 8.0 Hz, NH from one isomer), 5.05 (dd, 0.5H, *J* 9.5, 9.5 Hz, H-4 from one isomer), 5.08 (dd, 0.5H, *J* 9.5, 9.5 Hz, H-4 from one isomer), 5.22 (dd, 0.5H, *J* 9.5, 9.5 Hz, H-3 from one isomer), 5.24 (dd, 0.5H, *J* 9.5, 9.5 Hz, H-3 from one isomer), 5.31 (d, 0.5H, *J* 8.0 Hz, NH from one isomer), 7.38–7.46 (m, 6H, Ar-H), 7.62–7.66 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.11 (C(CH_3)$_3$ from one isomer), 19.14 (C(CH_3)$_3$ from one isomer), 20.69 (C(CH_3)$_3$ from one isomer), 20.80 (C(CH_3)$_3$ from one isomer), 26.85 (COOCH_3), 48.26 (NCH_2), 48.33 (NCH_2), 50.22 (NCH_2), 51.30 (NCH_2), 61.90 (OCH_2), 61.94 (OCH_2), 61.98 (C-6), 62.48 (OCH_2), 66.89 (OCH_2), 68.50 (C-4 from one isomer), 68.58 (C-4 from one isomer), 71.79 (C-5 from one isomer), 71.84 (C-5 from one isomer), 71.89 (C-3 from one isomer), 72.39 (C-3 from one isomer), 74.32 (Troc-CH_2), 74.35 (Troc-CH_2), 75.01 (Troc-CH_2), 75.10 (Troc-CH_2), 95.38 (Troc-CCl_3), 95.45 (Troc-CCl_3), 95.51 (Troc-CCl_3), 95.68 (Troc-CCl_3), 99.61 (C-1 from one isomer), 100.99 (C-1 from one isomer), 127.80 (CH-Ar), 127.83 (CH-Ar), 127.84 (CH-Ar), 129.83 (CH-Ar), 129.88 (CH-Ar), 133.11 (C-Ar), 133.16 (C-Ar), 133.29 (C-Ar), 133.34 (C-Ar), 135.56 (CH-Ar), 135.57 (CH-Ar), 153.97 (C=O Troc), 153.99 (C=O Troc), 154.20 (C=O Troc), 154.67 (C=O Troc), 169.49 (C=O), 170.57 (C=O). 170.64 (C=O), 170.71 (C=O), 170.73 (C=O); MALDI-MS (*m*/*z*) Calcd for $C_{38}H_{48}Cl_6N_2O_{13}Si$ $[M+Na]^+$: 1001.09, found: 1001.08.

3.5. *N*-{2-(tert-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (11)

Guanidinium nitrate (1.87 g, 15.32 mmol) was dissolved in MeOH:CH_2Cl_2 (9:1, 150 mL) and sodium methoxide in methanol solution (0.5 M, 6 mL) was added. **10** (1.86 g, 1.89 mmol) was dissolved in the above solution and stirred at room temperature for 3 h. The mixture was then neutralized by adding weak acidic ion-exchange resin (Amberlite IRC-64). The resin was filtered and the filtrate concentrated. The residue was purified via flash column chromatography (CH_2Cl_2/MeOH, 13: 1) to afford **11** (1.48 g, 91%) as a white fluffy solid. R_f 0.29 (hexane/acetone, 3:2); $[\alpha]_D^{22}$ −12.5 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.04 (s, 9H, C(CH_3)$_3$), 3.25–3.67 (m, 8H, H-2, H-4, H-6b, H-6a, $NCH_2 \times 2$), 3.69–3.85 (m, 5H, H-3, H-5, ROCH*H*, CH_2OTBDPS), 3.87–3.99 (m, 1H, ROC*H*H), 4.43 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.54 (d, 0.6H, *J* 8.0 Hz, H-1 from one isomer), 4.61 (d, 0.6H, *J* 12.0 Hz, Troc), 4.63 (d, 0.6H, *J* 12.0 Hz, Troc), 4.69 (d, 1H, J 12.0 Hz, Troc), 4.71 (d, 0.4H, *J* 12.0 Hz, Troc), 4.74 (d, 0.4H, *J* 12.0 Hz, Troc), 4.78 (d, 1H, *J* 12.0 Hz, Troc), 4.99 (br s, 3H, OH $\times$ 3), 6.17 (d, 0.4H, *J* 8.0 Hz, NH from one isomer), 6.31 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.36–7.43 (m, 6H, Ar-H), 7.61–7.64 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.14 (C(CH_3)$_3$ from one isomer), 19.17 (C(CH_3)$_3$ from one isomer), 26.90 (C(CH_3)$_3$), 48.22 (NCH_2), 48.42 (NCH_2), 49.93 (NCH_2), 51.43 (NCH_2), 57.63 (C-2 from one isomer), 57.78 (C-2 from one isomer), 61.35 (OCH_2), 61.92 (OCH_2), 62.40 (C-6), 66.23 (OCH_2), 68.32 (OCH_2), 70.07 (C-4 from one isomer), 70.20 (C-4 from one isomer), 73.95 (C-5 from one isomer), 74.61 (Troc-CH_2), 74.72 (C-5 from one isomer), 75.09 (Troc-CH_2), 75.17 (Troc-CH_2), 75.50 (C-3 from one isomer), 75.63 (Troc-CH_2), 95.31 (Troc-CCl_3), 95.61 (Troc-CCl_3), 95.64 (Troc-CCl_3), 100.03 (C-1 from one isomer), 101.50 (C-1 from one isomer), 127.81 (CH-Ar), 127.87 (CH-Ar), 129.82 (CH-Ar), 129.90 (CH-Ar), 133.09 (C-Ar), 133.27 (C-Ar), 135.57 (CH-Ar), 154.46 (C=O Troc), 154.88 (C=O Troc), 155.17 (C=O Troc), 155.57 (C=O Troc); MALDI-MS (*m*/*z*) Calcd for $C_{32}H_{42}Cl_6N_2O_{10}Si$ $[M+Na]^+$: 875.06, found: 875.05.

3.6. *N*-{2-(tert-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[4,6-*O*-benzylidene-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (12)

To a solution of **11** (1.35 g, 1.58 mmol) in CH_3CN (8 mL), benzaldehyde dimethyl acetal (308 mg, 2.02 mmol) and *p*-toluene sulfonic acid (15 mg, 0.08 mmol) were added successively. The mixture was stirred at room temperature for 2 h before being quenched with Et_3N (0.5 mL), and concentrated. The residue was purified via flash column chromatography (hexane/acetone, 3:1) to yield **12** (1.29 g, 87%) as a white fluffy solid. R_f 0.46 (hexane/acetone, 5:2); $[\alpha]_D^{22}$ −17.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.05 (s, 9H, C(C$\underline{H}_3$)$_3$), 3.33–3.66 (m, 8H, H-2, H-4, H-5, OH, NC$\underline{H}_2$ × 2), 3.68–3.99 (m, 6H, H-3, H-6b, OC$\underline{H}_2$ × 2), 4.27 (dd, 0.4H, J 10.0, 10.0 Hz, H-6a from one isomer), 4.29 (dd, 0.6H, J 10.0, 10.0 Hz, H-6a from one isomer), 4.45 (d, 0.4H, J 8.0 Hz, H-1 from one isomer), 4.53 (d, 0.6H, J 8.0 Hz, H-1 from one isomer), 4.61 (d, 1.4H, J 12.0 Hz, Troc), 4.69 (0.6H, *J* 12.0 Hz, Troc), 4.72 (d, 0.6H, J 12.0 Hz, Troc), 4.74 (d, 1H, J 12.0 Hz, Troc), 4.81 (d, 0.4H, *J* 12.0 Hz, Troc), 5.23 (d, 0.4H, J 8.0 Hz, NH from one isomer), 5.50 (s, 0.4H, Ph-C$\underline{H}$ from one isomer), 5.52 (s, 0.6H, Ph-C$\underline{H}$ from one isomer), 5.74 (d, 0.6H, J 8.0 Hz, NH from one isomer), 7.33–7.44 (m, 9H, Ar-H), 7.47–7.51 (m, 2H, Ar-H), 7.61–7.65 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.15 (C$\underline{(CH_3)_3}$ from one isomer), 19.18 (C$\underline{(CH_3)_3}$ from one isomer), 26.89 (C(C$\underline{H}_3$)$_3$), 48.38 (NC$\underline{H}_2$), 48.45 (NC$\underline{H}_2$), 50.19 (NC$\underline{H}_2$), 51.35 (NC$\underline{H}_2$), 58.45 (C-2 from one isomer), 58.62 (C-2 from one isomer), 61.97 (OC$\underline{H}_2$), 62.51 (OC$\underline{H}_2$), 66.03 (C-5 from one isomer), 66.17 (C-5 from one isomer), 66.83 (OC$\underline{H}_2$), 68.55 (C-6), 70.74 (C-3 from one isomer), 71.81 (C-3 from one isomer), 74.66 (Troc-C$\underline{H}_2$), 75.06 (Troc-C$\underline{H}_2$), 75.15 (Troc-C$\underline{H}_2$), 81.40 (C-4), 95.38 (Troc-C$\underline{C}$l$_3$), 95.48 (Troc-C$\underline{C}$l$_3$), 95.70 (Troc-C$\underline{C}$l$_3$), 99.86 (C-1 from one isomer), 101.35 (C-1 from one isomer), 101.86 (Ph-C$\underline{H}$ from one isomer), 101.89 (Ph-C$\underline{H}$ from one isomer), 126.43 (C$\underline{H}$-Ar), 126.45 (C$\underline{H}$-Ar), 127.83 (C$\underline{H}$-Ar), 127.87 (C$\underline{H}$-Ar), 128.45 (C$\underline{H}$-Ar), 128.49 (C$\underline{H}$-Ar), 129.42 (C$\underline{H}$-Ar), 129.46 (C$\underline{H}$-Ar), 129.85 (C$\underline{H}$-Ar), 129.91 (C$\underline{H}$-Ar), 133.12 (C$\underline{}$-Ar), 133.14 (C$\underline{}$-Ar), 133.31 (C$\underline{}$-Ar), 133.33 (C$\underline{}$-Ar), 135.58 (C$\underline{H}$-Ar), 137.03 (C$\underline{}$-Ar), 154.05 (C=O Troc), 154.54 (C=O Troc), 154.85 (C=O Troc), 155.37 (C=O Troc); MALDI-MS (*m*/*z*) Calcd for $C_{39}H_{46}Cl_6N_2O_{10}Si$ $[M+Na-C_4H_9]^+$: 906.06, found: 906.12.

3.7. *N*-{2-(tert-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[4,6-*O*-benzylidene-2-deoxy-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (13)

A mixture of **12** (1.16 g, 1.23 mmol), dilipid acid **14** (590 mg, 1.30 mmol), *N*,*N*-dimethylaminopyridine (15 mg, 0.12 mmol) and *N*,*N*′-diisopropylcarbodiimide (235 mg, 1.85 mmol) in CH_2Cl_2 (7 mL) was stirred at room temperature for 4 h. Water (0.5 mL) was added and the mixture stirred for a further 1 h. The solids were then filtered through a scintered glass funnel with a bed of Na_2SO_4. The filtrate was concentrated and the residue purified by flash column chromatography (hexane/acetone, 4.5:1) to afford **13** (1.58 g, 93%) as a colorless syrup. R_f 0.38 (hexane/acetone, 4:1); $[\alpha]_D^{22}$ −14.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.91 (t, 6H, J 6.5 Hz, CH_3 × 2), 1.08 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.14–1.36 (br m, 38H, CH_2 × 19), 1.53–162 (br m, 4H, H-4$_L$, H-3$_{L'}$), 2.16–2.23 (m, 2H, H-2$_{L'}$), 2.53–2.66 (m, 2H, H-2$_L$), 3.46–3.68 (m, 7H, H-2, H-4, H-5, NC$\underline{H}_2$ × 2), 3.71–3.87 (m, 4H, H-6b, C$\underline{H}_2$OTBDPS, ROCH*H*), 3.94–4.04 (m, 1H, ROC*H*H), 4.33 (dd, 0.4H, J 10.0, 10.0 Hz, H-6a from one isomer), 4.34 (dd, 0.6H, *J* 10.0, 10.0 Hz, H-6a from one isomer), 4.55 (d, 0.4H, *J* 12.0 Hz, Troc), 4.59 (d, 0.6H, *J* 12.0 Hz, Troc), 4.61 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.63 (d, 0.6H, *J* 12.0 Hz, Troc), 4.69 (d, 0.6H, *J* 8.0 Hz, H-1 from one isomer), 4.72 (d, 0.4H, *J* 12.0 Hz, Troc), 4.74 (d, 0.6H, *J* 12.0 Hz, Troc), 4.78 (d, 0.6H, *J* 12.0 Hz, Troc), 4.80 (d, 0.4H, *J* 12.0 Hz, Troc), 4.87 (d, 0.4H, *J* 12.0 Hz, Troc), 5.18–5.26 (m, 1H, H-3$_L$), 5.32–5.38 (m, 1.4H, H-3, NH from one isomer), 5.50–5.54 (m, 1.6H, Ph-C$\underline{H}$, NH from one isomer), 7.34–7.49 (m, 11H, Ar-H), 7.58–7.69 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.18 (CH_3), 19.13 (C$\underline{(CH_3)_3}$ from one isomer), 19.17 (C$\underline{(CH_3)_3}$ from one isomer), 22.73 (CH_2), 25.01 (CH_2), 25.09 (CH_2), 26.86 (C(C$\underline{H}_3$)$_3$ from one isomer), 26.88 (C(C$\underline{H}_3$)$_3$ from one isomer), 29.16 (CH_2), 29.33 (CH_2), 29.35 (CH_2), 29.39 (CH_2), 29.400 (CH_2), 29.56 (CH_2), 29.58 (CH_2), 29.68 (CH_2), 29.70 (CH_2), 29.71 (CH_2), 29.73 (CH_2), 33.87 (CH_2), 33.92 (CH_2), 34.37 (CH_2), 39.22 (C-2$_L$ from one isomer), 39.32 (C-2$_L$ from one isomer), 48.35 (NC$\underline{H}_2$), 48.69 (NC$\underline{H}_2$), 50.42 (NC$\underline{H}_2$), 51.28 (NC$\underline{H}_2$), 56.85 (C-2), 61.96 (OC$\underline{H}_2$), 62.51 (OC$\underline{H}_2$), 66.30 (C-5 from one isomer), 66.35 (C-5 from one isomer), 67.57 (OC$\underline{H}_2$), 68.55 (C-6), 68.65 (OC$\underline{H}_2$), 69.96 (C-3$_L$ from one isomer), 69.99 (C-3$_L$ from one isomer), 70.99 (C-3 from one isomer), 71.27 (C-3 from one isomer), 74.41 (Troc-CH_2), 74.44 (Troc-CH_2), 75.04 (Troc-CH_2), 75.09 (Troc-CH_2), 78.74 (C-4 from one isomer), 78.80 (C-4 from one isomer), 95.42 (Troc-CCl_3), 95.51 (Troc-CCl_3), 95.69 (Troc-CCl_3), 100.95 (C-1 from one isomer), 101.47 (Ph-C$\underline{H}$), 101.84 (C-1 from one isomer), 126.16 (C$\underline{H}$-Ar), 127.80 (C$\underline{H}$-Ar), 127.84 (C$\underline{H}$-Ar), 128.27 (C$\underline{H}$-Ar), 129.18 (C$\underline{H}$-Ar), 129.82 (C$\underline{H}$-Ar), 129.86 (C$\underline{H}$-Ar), 133.17 (C$\underline{}$-Ar), 133.20 (C$\underline{}$-Ar), 133.32 (C$\underline{}$-Ar), 133.36 (C$\underline{}$-Ar), 135.58 (C$\underline{H}$-Ar), 136.82 (C$\underline{}$-Ar), 154.01 (C=O Troc), 154.19 (C=O Troc), 154.36 (C=O Troc), 154.50 (C=O Troc), 170.01 (C=O), 170.06 (C=O), 173.41 (C=O), 173.50 H (C=O); MALDI-MS (*m*/*z*) Calcd for $C_{67}H_{98}Cl_6N_2O_{13}Si$ $[M+Na]^+$: 1399.49, found: 1399.50.

3.8. *N*-{2-(*tert*-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[6-*O*-benzyl-2-deoxy-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (15)

A solution of **13** (1.51 g, 1.09 mmol) in dry THF (10 mL) and molecular sieves (4 Å, 4.0 g) was stirred at room temperature under nitrogen for 30 min. Sodium cyanoborohydride (550 mg, 8.75 mmol) was added and the mixture cooled to 0 °C, followed by the drop wise addition of a dry, saturated ethereal-HCl(g) solution until no further gas was evolved. The mixture was poured into a saturated sodium bicarbonate solution (100 mL) and solids were filtered out before removal of the THF in vacuo. The resulting solution was extracted with EtOAc (3 × 100 mL), with the combined organic phase dried over Na_2SO_4 and concentrated. Flash column chromatography of the residue (hexane/acetone, 4:1) afforded **15** (1.31 g, 87%) as a colorless syrup. R_f 0.31 (hexane/acetone, 4:1); $[\alpha]_D^{22}$ −6.6 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.91 (t, 6H, *J* 6.5 Hz, CH_3 × 2), 1.07 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.24–1.38 (br m, 38H, CH_2 × 19), 1.54–1.68 (br m, 4H, H-4$_L$, H-3$_{L'}$), 2.31 (t, 2H, *J* 7.5 Hz, H-2$_{L'}$), 2.48–2.61 (m, 2H, H-2$_L$), 3.46–3.72 (m, 8H, H-2, H-4, H-5, OH, NC$\underline{H}_2$ × 2), 3.75–3.86 (m, 5H, H-6b, H-6a, C$\underline{H}_2$OTBDPS, ROCH*H*), 3.94–4.05 (m, 1H, ROC*H*H), 4.42 (d, 0.4H, *J* 8.5 Hz, H-1 from one isomer), 4.48 (d, 0.6H, *J* 12.0 Hz, Troc), 4.52–4.65 (m, 2.6H, H-1 from one isomer, Ph-C$\underline{H}_2$), 4.68 (d, 1H, *J* 12.0 Hz, Troc), 4.72 (d, 0.6H, *J* 12.0 Hz, Troc), 4.76 (d, 0.4H, *J* 12.0 Hz, Troc), 4.80 (d, 0.4H, *J* 12.0 Hz, Troc), 4.89 (d, 1H, *J* 12.0 Hz, Troc), 5.00 (dd, 0.4H, *J* 10.0, 10.0 Hz, H-3 from one isomer). 5.02 (dd, 0.6H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 5.13–5.20 (m, 1H, H-3$_L$), 5.26 (d, 0.4H, *J* 8.0 Hz, NH from one isomer), 5.49 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.30–7.47 (m, 11H, Ar-H), 7.63–7.68 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.14 (CH_3), 19.11 (C$\underline{(CH_3)_3}$ from one isomer), 19.15 (C$\underline{(CH_3)_3}$ from one isomer), 22.70 (CH_2), 24.98 (CH_2), 25.14 (CH_2), 26.86 (C(C$\underline{H}_3$)$_3$), 29.15 (CH_2), 29.30 (CH_2), 29.36 (CH_2), 29.38 (CH_2), 29.51 (CH_2), 29.53 (CH_2), 29.55 (CH_2), 29.65 (CH_2), 29.66 (CH_2), 29.68 (CH_2), 29.72 (CH_2), 31.93

(CH_2), 31.94 (CH_2), 34.51 (CH_2), 34.64 (CH_2), 40.09 (C-2_L), 48.29 (N$\underline{C}H_2$), 48.36 (N$\underline{C}H_2$), 50.22 (N$\underline{C}H_2$), 51.20 (N$\underline{C}H_2$), 55.70 (C-2), 61.89 (O$\underline{C}H_2$), 62.48 (O$\underline{C}H_2$), 66.86 (O$\underline{C}H_2$), 68.29 (O$\underline{C}H_2$), 66.71 (C-6 from one isomer), 66.73 (C-6 from one isomer), 70.10 (C-4), 71.00 (C-3_L from one isomer), 71.03 (C-3_L from one isomer), 73.69 (Ph-$\underline{C}H_2$), 74.36 (Troc-CH_2), 74.38 (Troc-CH_2), 74.51 (C-5 from one isomer), 74.63 (C-5 from one isomer), 75.03 (Troc-CH_2), 75.11 (Troc-CH_2), 75.65 (C-3 from one isomer), 76.12 (C-3 from one isomer), 95.43 (Troc-CCl_3), 95.58 (Troc-CCl_3), 95.61 (Troc-CCl_3), 95.71 (Troc-CCl_3), 100.17 (C-1 from one isomer), 101.44 (C-1 from one isomer), 127.67 (C$\underline{H}$-Ar), 127.69 (C$\underline{H}$-Ar), 127.77 (C$\underline{H}$-Ar), 127.81 (C$\underline{H}$-Ar), 128.46 (C$\underline{H}$-Ar), 129.78 (C$\underline{H}$-Ar), 129.82 (C$\underline{H}$-Ar), 133.20 (C-Ar), 133.36 (C-Ar), 135.57 (C$\underline{H}$-Ar), 137.80 (C-Ar), 137.85 (C-Ar), 154.05 (C=O Troc), 154.20 (C=O Troc), 154.38 (C=O Troc), 154.58 (C=O Troc), 171.48 (C=O), 174.37 (C=O); MALDI-MS (m/z) Calcd for $C_{67}H_{100}Cl_6N_2O_{13}Si$ $[M+Na]^+$: 1401.50, found: 1401.45.

3.9. *N*-{2-(*tert*-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzylphosphono)-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyloxy]-ethyl}-2,2,2-trichloroethoxymethanamide (16)

To a solution of **15** (1.25 g, 0.90 mmol) in dry CH_2Cl_2 (8 mL), 5-phenyltetrazole (265 mg, 1.80 mmol) and *N*,*N*-diisopropylphosphoramidite (0.62 mL, 1.80 mmol) were added. The mixture was stirred at room temperature for 1 h and then cooled to 0 °C before the addition of *m*-chloroperbenzoic acid (505 mg, 77% purity, 2.25 mmol). The mixture was stirred at 0 °C for 1 h and then poured into a saturated sodium bicarbonate solution (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was dried over Na_2SO_4, concentrated, and purified by flash column chromatography (hexane/acetone, 4:1) to give **16** (1.26 g, 85%) as a colorless syrup. R_f 0.40 (hexane/EtOAc, 5:2); $[\alpha]_D^{22}$ −2.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, 6H, *J* 6.5 Hz, $CH_3 \times 2$), 1.04 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.17–1.32 (br m, 38H, $CH_2 \times 19$), 1.43–1.56 (br m, 4H, H-4_L, H-$3_{L'}$), 2.18–2.25 (t, 2H, J 7.5 Hz, H-$2_{L'}$), 2.38–2.47 (m, 2H, H-2_L), 3.39–3.63 (m, 7H, H-2, H-5, H-6b, $NCH_2 \times 2$), 3.68–3.82 (m, 4H, H-6a, C$\underline{H}_2$OTBDPS, ROC$\underline{H}$H), 3.94–4.02 (m, 1H, ROC$\underline{H}$H), 4.40–4.54 (m, 4H, H-4, Ph-C$\underline{H}_2$, Troc), 4.57 (d, 0.4H, *J* 12.0 Hz, Troc), 4.59 (d, 0.6H, *J* 12.0 Hz, Troc), 4.64 (d, 0.6H, *J* 12.0 Hz, Troc), 4.68 (d, 0.4H, *J* 12.0 Hz, Troc), 4.71 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.73 (d, 0.4H, *J* 12.0 Hz, Troc) 4.75 (d, 0.6H, *J* 8.0 Hz, H-1 from one isomer), 4.80 (d, 0.6H, *J* 12.0 Hz, Troc), 4.86–4.93 (m, 4H, (PhC$\underline{H}_2$O)$_2$P), 5.14–5.23 (m, 1H, H-3_L), 5.34–5.40 (m, 1H, H-3), 5.43 (d, 0.4H, *J* 8.0 Hz, NH from one isomer), 5.55 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.22–7.32 (m, 13H, Ar-H), 7.35–7.44 (m, 8H, Ar-H), 7.60–7.66 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.20 (CH_3), 19.13 (C$\underline{(CH_3)_3}$ from one isomer), 19.16 (C$\underline{(CH_3)_3}$ from one isomer), 22.75 (CH_2), 25.05 (CH_2), 25.16 (CH_2), 26.88 (C(C$\underline{H}_3$)$_3$ from one isomer), 26.90 (C(C$\underline{H}_3$)$_3$ from one isomer), 29.21 (CH_2), 29.37 (CH_2), 29.41 (CH_2), 29.58 (CH_2), 29.59 (CH_2), 29.62 (CH_2), 29.68 (CH_2), 29.70 (CH_2), 29.72 (CH_2), 29.74 (CH_2), 31.97 (CH_2), 34.34 (CH_2), 34.49 (CH_2), 39.37 (C-2_L from one isomer), 39.61 (C-2_L from one isomer), 48.27 (N$\underline{C}H_2$), 48.62 (N$\underline{C}H_2$), 50.30 (N$\underline{C}H_2$), 51.17 (N$\underline{C}H_2$), 56.51 (C-2 from one isomer), 56.54 (C-2 from one isomer), 61.92 (O$\underline{C}H_2$ROCH$_2$CH$_2$NCH$_2$-$\underline{C}H_2$OTBDPS from one isomer), 62.49 (O$\underline{C}H_2$), 67.44 (O$\underline{C}H_2$), 68.46 (C-6), 68.58 (O$\underline{C}H_2$), 69.61–69.73 (m, (PhC$\underline{H}_2$O)$_2$P), 69.99 (C-3_L from one isomer), 70.12 (C-3_L from one isomer), 72.27 (C-3 from one isomer), 72.62 (C-3 from one isomer), 73.46 (Ph-$\underline{C}H_2$), 74.00 (d, *J* 5.5 Hz, C-4 from one isomer), 74.05 (d, *J* 5.5 Hz, C-4 from one isomer), 74.14 (C-5 from one isomer), 74.19 (C-5 from one isomer), 74.44 (Troc-CH_2), 75.01 (Troc-CH_2), 75.06 (Troc-CH_2), 95.46 (Troc-CCl_3), 95.48 (Troc-CCl_3), 95.50 (Troc-CCl_3), 95.69 (Troc-CCl_3), 99.77 (C-1 from one isomer), 100.58 (C-1 from one isomer), 127.65 (C$\underline{H}$-Ar), 127.80 (C$\underline{H}$-Ar), 127.84 (C$\underline{H}$-Ar), 128.04 (C$\underline{H}$-Ar), 128.11 (C$\underline{H}$-Ar), 128.14 (C$\underline{H}$-Ar), 128.38 (C$\underline{H}$-Ar), 128.63 (C$\underline{H}$-Ar), 128.67 (C$\underline{H}$-Ar), 129.81 (C$\underline{H}$-Ar), 129.84 (C$\underline{H}$-Ar), 133.19 (C-Ar), 133.21 (C-Ar), 133.34 (C-Ar), 133.37 (C-Ar), 135.59 (C$\underline{H}$-Ar), 138.00 (C-Ar), 138.02 (C-Ar), 154.02 (C=O Troc), 154.22 (C=O Troc), 154.49 (C=O Troc), 170.28 (C=O), 170.32 (C=O), 173.61 (C=O), 173.75 (C=O); MALDI-MS (m/z) Calcd for $C_{81}H_{113}Cl_6N_2O_{16}PSi$ $[M+Na]^+$: 1661.56, found: 1661.48.

3.10. *N*-{2-(*tert*-Butyldiphenylsilyloxy)-ethyl}-*N*-{2-[6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzylphosphono)-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (17)

To a solution of **16** (440 mg, 0.27 mmol) in glacial acetic acid (5 mL), zinc powder (analytical grade, <10 μ, 750 mg) was added and the mixture was stirred at room temperature for 45 min. The mixture was then filtered, and the solids were washed with acetic acid (10 mL). The filtrate was slowly poured into a saturated sodium bicarbonate solution (300 mL) and then extracted with CH_2Cl_2 (3 × 150 mL). The combined organic phase was washed with further saturated sodium bicarbonate solution (100 mL), dried over Na_2SO_4, and concentrated to give the crude di-amine (338 mg, 98%) as a colorless syrup.

A solution of the crude di-amine (337 mg) in DMF (2 mL) was added to a mixture of dilipid acid **14** (283 mg, 0.62 mmol), HBTU (352 mg, 0.94 mmol) and DIPEA (0.16 mL, 0.94 mmol) in DMF (5 mL). The resulting mixture was stirred at room temperature for 18 h before being poured into a separatory funnel with water (50 mL), and then extracted with Et_2O (3 × 75 mL). The combined organic phase was washed with a cold saturated sodium chloride solution (3 × 8 mL), dried over Na_2SO_4, and concentrated. Flash column chromatography of the residue (hexane/EtOAc, 2.5:1) afforded **17** (381 mg, 67%) as a colorless syrup. R_f 0.35 (hexane/acetone, 4:1); $[\alpha]_D^{22}$ −1.5 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, 18H, *J* 6.5 Hz, $CH_3 \times 6$), 1.03 (s, 9H, C(C$\underline{H}_3$)$_3$), 1.16–1.38 (br m, 114H, $CH_2 \times 57$), 1.52–1.68 (br m, 12H, H-$4_L \times 6$, H-$3_{L'} \times 6$), 2.17–2.51 (m, 11H, H-$2_L \times 5$, H-$2_{L'} \times 6$), 2.75 (dd, 0.4H, *J* 15.5, 6.5 Hz, H-2_{LB} from one isomer), 2.87 (dd, 0.6H, *J* 15.5, 5.0 Hz, H-2_{LA} from one isomer), 3.07–3.18 (m, 0.4H, H-2 from one isomer), 3.41–3.79 (m, 10.6H, H-2 from one isomer, H-5, H-6b, H-6a, $NCH_2 \times 2$, C$\underline{H}_2$OTBDPS, ROC$\underline{H}$H), 3.82–3.91 (m, 1H, ROC$\underline{H}$H), 4.36–4.51 (m, 3H, H-4, Ph-C$\underline{H}_2$), 4.66 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.84–4.92 (m, 4H, (PhC$\underline{H}_2$O)$_2$P), 5.04–5.22 (m, 3.6H, H-1 from one isomer, H-$3_L \times 3$), 5.29 (dd, 0.6H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 5.73 (dd, 0.4H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 6.15 (d, 0.4H, *J* 8.0 Hz, NH from one isomer), 6.73 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.22–7.32 (m, 13H, Ar-H), 7.34–7.44 (m, 8H, Ar-H), 7.58–7.64 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.15 (CH_3), 19.08 (C$\underline{(CH_3)_3}$ from one isomer), 19.15 (C$\underline{(CH_3)_3}$ from one isomer), 22.73 (CH_2), 25.06 (CH_2), 25.09 (CH_2), 25.15 (CH_2), 25.18 (CH_2), 25.25 (CH_2), 25.33 (CH_2), 25.42 (CH_2), 25.83 (CH_2), 26.85 (C(C$\underline{H}_3$)$_3$ from one isomer), 26.90 (C(C$\underline{H}_3$)$_3$ from one isomer), 29.17 (CH_2), 29.23 (CH_2), 29.27 (CH_2), 29.31 (CH_2), 29.35 (CH_2), 29.41 (CH_2), 29.43 (CH_2), 29.49 (CH_2), 29.54 (CH_2), 29.56 (CH_2), 29.61 (CH_2), 29.64 (CH_2), 29.68 (CH_2), 29.73 (CH_2), 29.76 (CH_2), 31.97 (CH_2), 34.04 (CH_2), 34.12 (CH_2), 34.33 (CH_2), 34.43 (CH_2), 34.49 (CH_2), 34.60 (CH_2), 34.700(CH_2), 38.04 (C-2_L), 38.13 (C-2_L), 39.11 (C-2_L), 39.26 (C-2_L), 40.74 (C-2_L), 41.25 (C-2_L), 45.65 (N$\underline{C}H_2$), 47.09 (N$\underline{C}H_2$), 48.29 (N$\underline{C}H_2$), 50.45 (N$\underline{C}H_2$), 54.92 (C-2 from one isomer), 56.65 (C-2 from one isomer), 61.61 (O$\underline{C}H_2$), 62.64 (O$\underline{C}H_2$), 63.42 (C-6 from one isomer), 67.22 (C-6 from one isomer), 68.21 (O$\underline{C}H_2$), 68.43 (O$\underline{C}H_2$), 69.46–69.67 (m, (PhC$\underline{H}_2$O)$_2$P), 69.99 (C-3_L), 70.07 (C-3_L), 70.15 (C-3_L), 70.68 (C-3_L),

71.22 (C-3_L), 71.70 (C-3 from one isomer), 72.35 (C-3_L), 72.87 (C-3 from one isomer), 73.30 (Ph-$\underline{C}H_2$ from one isomer), 73.32 (Ph-$\underline{C}H_2$ from one isomer), 73.73 (C-5 from one isomer), 73.98 (C-5 from one isomer), 74.09 (d, *J* 5.5 Hz, C-4 from one isomer), 74.49 (d, *J* 5.5 Hz, C-4 from one isomer), 99.43 (C-1 from one isomer), 100.77 (C-1 from one isomer), 127.56 (CH-Ar), 127.60 (CH-Ar), 127.74 (CH-Ar), 127.77 (CH-Ar), 127.87 (CH-Ar), 127.90 (CH-Ar), 127.99 (CH-Ar), 128.10 (CH-Ar), 128.12 (CH-Ar), 128.31 (CH-Ar), 128.53 (CH-Ar), 128.56 (CH-Ar), 129.74 (CH-Ar), 129.77 (CH-Ar), 129.88 (CH-Ar), 129.90 (CH-Ar), 132.94 (C-Ar), 133.36 (C-Ar), 133.45 (C-Ar), 135.51 (CH-Ar), 135.54 (CH-Ar), 138.08 (C-Ar), 169.70 (C=O), 170.03 (C=O), 170.24 (C=O), 170.41 (C=O), 170.50 (C=O), 170.74 (C=O), 173.14 (C=O), 173.29 (C=O), 173.40 (C=O), 173.45 (C=O), 173.52 (C=O), 174.18 (C=O); MALDI-MS (*m*/*z*) Calcd for $C_{131}H_{215}N_2O_{18}PSi$ $[M+Na]^+$: 2186.54, found: 2186.48.

3.11. *N*-(2-Hydroxyethyl)-*N*-{2-[6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzylphosphono)-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (18)

To a solution of **17** (890 mg, 0.41 mmol) in CH_2Cl_2 (3 mL) and glacial acetic acid (0.1 mL), a tetrabutylammonium fluoride solution (1 M in THF, 0.62 mL) was added, and the mixture was stirred at room temperature for 18 h. The mixture was the poured into a saturated sodium bicarbonate solution (30 mL), extracted with CH_2Cl_2 (3 × 50 mL), dried over Na_2SO_4, and concentrated. Flash column chromatography of the residue (hexane/EtOAc/MeOH, 3:1:0.1) yielded **18** (715 mg, 90%) as a colorless syrup. Spectroscopic data are identical with those reported previously.[17]

3.12. *N*-{2-(di-*O*-Benzylphosphono)-ethyl}-*N*-{2-[6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzylphosphono)-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (19)

To a solution of **18** (85 mg, 0.044 mmol) in dry CH_2Cl_2 (1.5 mL), 5-phenyltetrazole (13 mg, 0.088 mmol) and *N*,*N*-diisopropylphosphoramidite (30 μL, 0.088 mmol) were added. The mixture was stirred at room temperature for 30 min and then cooled to 0 °C before the addition of *m*-chloroperbenzoic acid (22 mg, 77% purity, 0.099 mmol). The mixture was stirred at 0 °C for 30 min before being poured into a 10% sodium thiosulphate solution (10 mL) and then extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phase was washed with a saturated sodium bicarbonate solution (15 mL), dried over Na_2SO_4, concentrated, and purified by repeated flash chromatography (hexane/EtOAc, 3:2 → 1:1) to give **19** (69 mg, 71%) as a colorless syrup. R_f 0.28 (hexane/EtOAc, 3:1; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.86 (t, 18H, *J* 6.5 Hz, CH_3 × 6), 1.14–1.39 (br m, 114H, CH_2 × 57), 1.50–1.69 (br m, 12H, H-4_L × 6, H-$3_{L'}$ × 6), 2.18–2.57 (m, 11H, H-2_L × 5, H-$2_{L'}$ × × 6), 2.68 (dd, 0.4H, *J* 15.5, 6.5 Hz, H-2_{LB} from one isomer), 2.78 (dd, 0.6H, *J* 15.5, 5.0 Hz, H-2_{LA} from one isomer), 3.20–3.37 (m, 2.6H, H-2 from one isomer, NCH_2), 3.43–3.48 (m, 1H, H-6b), 3.51–3.70 (m, 4.4H, H-2 from one isomer, H-5, NCH_2, ROCH*H*), 3.75–3.87 (m, 2H, H-6a, ROC*H*H), 4.06–4.16 (m, 2H, $C\underline{H}_2$O-P(O)(OBn)$_2$), 4.40–4.52 (m, 3H, H-4, Ph-$C\underline{H}_2$), 4.72 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.89–4.94 (m, 4H, (Ph$C\underline{H}_2$O)$_2$P), 4.97–5.04 (m, 4H, CH_2OP(O)(O$C\underline{H}_2$Ph)$_2$), 5.07 (d, 0.6H, *J* 8.0 Hz, H-1 from one isomer), 5.09–5.23 (m, 3H, H-3_L × 3), 5.39 (dd, 0.4H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 5.67 (dd, 0.6H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 6.59 (d, 0.4H. J 8.0 Hz, NH from one isomer), 6.88 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.18–7.42 (m, 25H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.15 (CH_3), 22.71 (CH_2), 25.05 (CH_2), 25.08 (CH_2), 25.15 (CH_2), 25.17 (CH_2), 25.24 (CH_2), 25.35 (CH_2), 25.43 (CH_2), 25.81 (CH_2), 24.84 (CH_2), 25.88 (CH_2), 29.15 (CH_2), 29.21 (CH_2), 29.25 (CH_2), 29.301 (CH_2), 29.33 (CH_2), 29.40 (CH_2), 29.43 (CH_2), 29.47 (CH_2), 29.52 (CH_2), 29.56 (CH_2), 29.62 (CH_2), 29.64 (CH_2), 29.68 (CH_2), 29.73 (CH_2), 29.77 (CH_2), 31.96 (CH_2), 34.02 (CH_2), 34.12 (CH_2), 34.35 (CH_2), 34.43 (CH_2), 34.49 (CH_2), 34.61 (CH_2), 34.70 (CH_2), 37.89 (C-2_L), 38.15 (C-2_L), 39.04 (C-2_L), 39.16 (C-2_L), 40.80 (C-2_L), 41.20 (C-2_L), 46.15 (NCH_2), 46.22 (NCH_2) 46.52 (NCH_2), 48.63 (NCH_2), 55.12 (C-2 from one isomer), 56.16 (C-2 from one isomer), 64.59 (d, *J* 5.5 Hz, CH_2OP(O)(OBn)$_2$ from one isomer), 65.87 (d, *J* 5.5 Hz, CH_2OP(O)(OBn)$_2$ from one isomer), 67.34 (C-6), 68.48 (ROCH_2), 68.52 (ROCH_2), 69.41–69.66 (m, PhCH_2O)$_2$P, CH_2OP(O)(OCH_2Ph)$_2$), 69.96 (C-3_L), 70.04 (C-3_L), 70.16 (C-3_L), 70.68 (C-3_L), 71.26 (C-3_L), 71.83 (C-3_L), 71.93 (C-3 from one isomer), 72.71 (C-3 from one isomer), 73.32 (Ph-$\underline{C}H_2$), 73.81 (C-5 from one isomer), 73.83 (C-5 from one isomer), 74.19 (d, *J* 5.5 Hz, C-4 from one isomer), 74.37 (d, *J* 5.5 Hz, C-4 from one isomer), 99.58 (C-1 from one isomer), 100.07 (C-1 from one isomer), 127.55 (CH-Ar), 127.58 (CH-Ar), 127.72 (CH-Ar), 127.75 (CH-Ar), 127.85 (CH-Ar), 127.90 (CH-Ar), 127.97 (CH-Ar), 128.11 (CH-Ar), 128.12 (CH-Ar), 128.31 (CH-Ar), 128.52 (CH-Ar), 128.54 (CH-Ar), 129.75 (CH-Ar), 129.78 (CH-Ar), 129.87 (CH-Ar), 129.90 (CH-Ar), 132.94 (C-Ar), 133.36 (C-Ar), 133.47 (C-Ar), 135.52 (CH-Ar), 135.54 (CH-Ar), 138.08 (C-Ar), 169.72 (C=O), 170.05 (C=O), 170.27 (C=O), 170.44 (C=O), 170.51 (C=O), 170.79 (C=O), 173.18 (C=O), 173.35 (C=O), 173.45 (C=O), 173.48 (C=O), 173.59 (C=O), 174.18 (C=O). Decomposition issues prevented the acquiring of optical rotation and mass spectral data.

3.13. *N*-{Carboxymethyl}-*N*-{2-[6-*O*-benzyl-2-deoxy-4-*O*-(di-*O*-benzylphosphono)-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (20)

To a solution of **18** (80 mg, 0.042 mmol) in CH_2Cl_2 (3 mL) and water (0.5 mL), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 3 mg, 0.017 mmol) and bis(acetoxy)iodobenzene (54 mg, 0.168 mmol) were added. The mixture was stirred at room temperature for 2 h before being poured into a 10% sodium thiosulphate solution (10 mL), and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were dried over Na_2SO_4, concentrated, and purified by repeated flash chromatography (hexane/EtOAc/MeOH, 3:1:0.2) to afford **20** (67 mg, 83%) as a colorless syrup. R_f 0.35 (hexane/EtOAc, 3:1); $[\alpha]_D^{22}$ −0.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, 18H, *J* 6.5 Hz, CH_3 × 6), 1.16–1.39 (br m, 114H, CH_2 × 57), 1.48–1.64 (br m, 12H, H-4_L × 6, H-$3_{L'}$ × 6), 2.19–2.52 (m, 11H, H-2_L × 5, H-$2_{L'}$ × 6), 2.63 (dd, 0.4H, *J* 15.5, 6.5 Hz, H-2_{LB} from one isomer), 2.86 (dd, 0.6H, *J* 15.5, 5.0 Hz, H-2_{LA} from one isomer), 3.27–3.34 (m, 0.6H, H-2 from one isomer), 3.44–3.68 (m, 5H, H-5, H-6b, ROCH$_2$C$\underline{H}_2$N, ROCH*H*), 3.70–3.78 (m, 1.4H, H-2 from one isomer, H-6a), 3.87–3.98 (m, 1H, ROC*H*H), 4.00–4.21 (m, 2H, NC$\underline{H}_2$COOH), 4.40–4.53 (m, 3H, H-4, Ph-$C\underline{H}_2$), 4.59 (d, 0.4H, *J* 8.0 Hz, H-1 from one isomer), 4.83–4.92 (m, 4H, (PhC$\underline{H}_2$O)$_2$P), 4.98 (d, 0.6H, *J* 8.0 Hz, H-1 from one isomer), 5.09–5.24 (m, 3H, H-3_L × 3), 5.28 (dd, 0.4H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 5.61 (dd, 0.6H, *J* 10.0, 10.0 Hz, H-3 from one isomer), 6.32 (d, 0.4H, *J* 8.0 Hz, NH from one isomer), 6.67 (d, 0.6H, *J* 8.0 Hz, NH from one isomer), 7.21–7.34 (m, 15H, Ar-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.12 (CH_3), 22.71 (CH_2), 25.05 (CH_2), 25.14 (CH_2), 25.22 (CH_2), 25.32 (CH_2), 25.38 (CH_2), 25.58 (CH_2), 29.21 (CH_2), 29.24 (CH_2), 29.28 (CH_2), 29.32 (CH_2), 29.39 (CH_2), 29.40 (CH_2), 29.42 (CH_2), 29.45 (CH_2), 29.47 (CH_2), 29.49 (CH_2), 29.52 (CH_2), 29.57 (CH_2), 29.60 (CH_2), 29.62 (CH_2), 29.66 (CH_2), 29.68 (CH_2), 29.71

(CH_2), 29.73 (CH_2), 29.75 (CH_2), 31.95 (CH_2), 31.97 (CH_2), 34.27 (CH_2), 34.31 (CH_2), 34.44 (CH_2), 34.50 (CH_2), 34.54 (CH_2), 34.62 (CH_2), 37.86 (C-2_L), 37.94 (C-2_L), 39.19 (C-2_L), 39.40 (C-2_L), 40.87 (C-2_L), 41.45 (C-2_L), 47.59 (N$\underline{C}H_2$), 48.94 (N$\underline{C}H_2$), 49.20 (N$\underline{C}H_2$), 51.54 (N$\underline{C}H_2$), 55.05 (C-2 from one isomer), 55.97 (C-2 from one isomer), 67.63 (O$\underline{C}H_2$), 68.40 (C-6), 69.25 (O$\underline{C}H_2$), 69.52–69.63 (m, Ph$\underline{C}H_2O)_2$P), 70.15 (C-3_L), 70.18 (C-3_L), 70.44 (C-3_L), 70.87 (C-3_L), 71.16 (C-3_L), 71.80 (C-3_L), 72.09 (C-3 from one isomer), 72.62 (C-3_L), 73.35 (Ph-$\underline{C}H_2$ from one isomer), 73.44 (Ph-$\underline{C}H_2$ from one isomer), 73.70 (C-5 from one isomer), 73.74 (C-5 from one isomer), 74.22 (d, *J* 5.5 Hz, C-4 from one isomer), 74.30 (d, *J* 5.5 Hz, C-4 from one isomer), 99.76 (C-1 from one isomer), 100.50 (C-1 from one isomer), 127.59 (C$\underline{H}$-Ar), 127.64 (C$\underline{H}$-Ar), 127.72 (C$\underline{H}$-Ar), 127.80 (C$\underline{H}$-Ar), 127.99 (C$\underline{H}$-Ar), 128.04 (C$\underline{H}$-Ar), 128.10 (C$\underline{H}$-Ar), 128.13 (C$\underline{H}$-Ar), 128.33 (C$\underline{H}$-Ar), 128.38 (C$\underline{H}$-Ar), 128.54 (C$\underline{H}$-Ar), 128.56 (C$\underline{H}$-Ar), 128.59 (C$\underline{H}$-Ar), 128.60 (C$\underline{H}$-Ar), 128.64 (C$\underline{H}$-Ar), 128.66 (C$\underline{H}$-Ar), 135.55 (C-Ar), 135.61 (C-Ar), 137.67 (C-Ar), 137.98 (C-Ar), 169.90 (C=O), 170.35 (C=O), 170.79 (C=O), 170.93 (C=O), 171.00 (C=O), 171.25 (C=O), 173.23 (C=O), 173.39 (C=O), 173.75 (C=O), 173.78 (C=O), 173.91 (C=O); MALDI-MS (*m*/*z*) Calcd for $C_{115}H_{195}N_2O_{19}P$ $[M+Na]^+$: 1962.39, found: 1962.30.

3.14. *N*-{2-Phosphonoethyl}-*N*-{2-[2-deoxy-4-*O*-phosphono-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (3)

To a solution of **19** (53 mg, 0.027 mmol) in freshly distilled THF (45 mL), palladium on charcoal (5%, 26 mg) was added and the mixture was stirred at room temperature under a hydrogen atmosphere (balloon) for 24 h. The mixture was filtered, and the filtrate concentrated. The residue was purified by flash column chromatography ($CHCl_3$/MeOH, 9:1 and then $CHCl_3$/MeOH/H_2O, 3:1:0.2) to afford **3** (28 mg, 70%) as white fluffy solid after being freeze dried from a dioxane–$CHCl_3$ mixture (95:5). R_f 0.37 ($CHCl_3$/MeOH/H_2O/NH_4OH, 3:2:0.2:0.2); $[\alpha]_D^{22}$ −0.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.89 (t, 18H, *J* 6.5 Hz, $CH_3 \times 6$), 1.22–1.43 (br m, 114H, $CH_2 \times 57$), 1.54–1.68 (br m, 12H, H-$4_L \times 6$, H-$3_{L'} \times 6$), 2.20–2.82 (br m, 12H, H-$2_L \times 6$, H-$2_{L'} \times 6$), 3.68–4.16 (br m, 12H, H-2, H-5, H-6b, H-6a, $NCH_2 \times 2$, $OCH_2 \times \times 2$), 4.48–4.70 (br m, 2H, H-1, H-4), 5.06–5.38 (br m, 4H, H-3, H-$3_L \times 3$); MALDI-MS (*m*/*z*) Calcd for $C_{94}H_{180}N_2O_{21}P_2$ $[M+Na]^+$: 1758.24, found: 1758.20.

3.15. *N*-{Carboxymethyl}-*N*-{2-[2-deoxy-4-*O*-phosphono-3-*O*-((*R*)-3-tetradecanoyloxytetradecanoyl)-2-((*R*)-3-tetradecanoyloxytetradecanamido)-β-D-glucopyranosyloxy]-ethyl}-(*R*)-3-tetradecanoyloxytetradecanamide (4)

In a similar manner as described for preparation of **3**, a solution of **20** (35 mg, 0.018 mmol) and palladium on charcoal (5%, 20 mg) in freshly distilled THF (40 mL) was stirred under a hydrogen atmosphere (balloon) at room temperature for 24 h. The mixture was filtered, the filtrate concentrated, and the resulting residue was purified by flash column chromatography ($CHCl_3$/MeOH, 9:1 and then $CHCl_3$/MeOH/H_2O, 3:1:0.1) to yield **4** (23 mg, 77%) as a white fluffy solid after being freeze-dried from a dioxane–$CHCl_3$ mixture (95:5). R_f 0.41 ($CHCl_3$/MeOH/H_2O, 3:1:0.1); $[\alpha]_D^{22}$ −0.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.80 (t, 18H, *J* 6.5 Hz, $CH_3 \times 6$), 1.12–1.33 (br m, 114H, $CH_2 \times 57$), 1.46–1.62 (br m, 12H, H-$4_L \times 6$, H-$3_{L'} \times 6$), 2.16–2.31 (m, 9H, H-$2_L \times 3$, H-$2_{L'} \times 6$), 2.33–2.65 (m, 3H, H-$2_L \times 3$), 3.45–3.72 (br m, 7H, H-2, H-5, H-6b, H-6a, ROCH$_2$C$\underline{H}_2$N, ROC$\underline{H}$H), 3.75–3.98 (br m, 3H, NC$\underline{H}_2$COOH, ROC$\underline{H}$H), 4.41–4.55 (br m, 2H, H-1, H-4), 5.04–5.20 (br m, 4H, H-3, H-$3_L \times 3$); MALDI-MS (*m*/*z*) Calcd for $C_{94}H_{177}N_2O_{19}P$ $[M+Na]^+$: 1692.25, found: 1692.17.

3.16. Reagents for biological experiments

Escherichia coli LPS 011:B4 was obtained from Sigma. Each of synthetic lipid A mimics **2**–**4** were reconstituted in 20% DMSO in phosphate buffered saline (PBS) with brief sonication, aliquoted and stored at −80 °C. A fresh aliquot was used for each individual experiment. Solution concentrations were set such that the total addition of DMSO never exceeded 0.5% to avoid toxic effects. THP-1 cells were obtained from American Type Culture Collection (ATCC). RPMI-1640 media, fetal bovine serum, and antibiotic-antimycotic 100× were obtained from Gibco BRL. Phorbol 12-myristate 13 acetate (PMA) was purchased from Sigma, dissolved in DMSO, aliquoted and stored at −80 °C. Lipid IVa was purchased from Peptide Institute, Inc. (Osaka, Japan), and was dissolved in DMSO.

3.17. Cell maintenance

THP-1 cells were maintained at 37 °C and 5% CO_2 atmosphere in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic 100×. Cell counting was performed using a Beckman Coulter ViCell X-R instrument, with viability being determined through the trypan blue cellular exclusion method.

3.18. Cytokine induction and measurement

THP-1 pre-monocytic cells were plated at 0.5×10^6 cells well^{-1} in 6-well tissue culture plates containing the RPMI media further supplemented with 25 ng mL^{-1} of PMA. After 48 h, the media was removed and the now adhered monocytic THP-1 cells were washed with PBS. The wells were then refilled with serum-free RPMI media, incubated for 3 h, and then exposed to stimuli. After 24 h stimulation, culture supernatants were collected and stored frozen (−80 °C) until assayed for cytokine production.

All cytokine ELISAs were performed in 96-well MaxiSorp plates. Ready-Set-Go! ELISA kits (eBioscience) were used for cytokine quantification of human TNF-α, IL-6, and IL-1β according to the manufacturer's instructions. The absorbance was measured at 450 nm with wavelength correction set to 540 nm using a microplate reader (BMG Labtech). All cytokine values were measured in duplicate, and are presented as the mean ± SD of two separate experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.024.

References and notes

1. Akira, S.; Yamamoto, M. *Adv. Exp. Med. Biol.* **2010**, *667*, 59.
2. Jiang, Z.-H.; Koganty, R. R. *Curr. Med. Chem.* **2003**, *10*, 1423.
3. Ulevitch, R. J. *Nat. Rev. Immunol.* **2004**, *4*, 512.
4. Westphal, O.; Lüdertitz, O. *Z. Naturforsch.* **1952**, *7*, 148.
5. Miyake, K. *J. Endotoxin Res.* **2006**, *12*, 195.
6. Hajjar, A. M.; Ernst, R. K.; Tsai, J. H.; Wilson, C. B.; Miller, S. I. *Nat. Immunol.* **2002**, *3*, 354.
7. Kusumoto, S.; Fukase, K.; Fukase, Y.; Kataoka, M.; Yoshizaki, H.; Sato, K.; Oikawa, M.; Suda, Y. *J. Endotoxin Res.* **2003**, *9*, 361.
8. Rietschel, E. T.; Kirikae, T.; Schade, F. U.; Mamat, U.; Schmidt, G.; Loppnow, H.; Ulmer, A. J.; Zahringer, U.; Seydel, U.; Di Padova, F.; Schreier, M.; Brade, H. *FASEB J.* **1994**, *8*, 217.
9. Ulmer, A. J.; Heine, H.; Feist, W.; Kusumoto, S.; Kusama, T.; Brade, H.; Schade, U.; Rietschel, E. T.; Flad, H. D. *Infect. Immun.* **1992**, *60*, 3309.
10. Kataoka, M.; Hashimoto, M.; Suda, Y.; Kusumoto, S.; Fukase, S. *Heterocycles* **2006**, *69*, 395.
11. Johnson, D. A.; Keegan, D. S.; Sowell, C. G.; Livesay, M. T.; Johnson, C. L.; Taubner, L. M.; Harris, A.; Myers, K. R.; Thompson, J. D.; Gustafson, G. L.;

Rhodes, M. J.; Ulrich, J. T.; Ward, J. R.; Yorgensen, Y. M.; Cantrell, J. L.; Brookshire, V. G. *J. Med. Chem.* **1999**, *42*, 4640.
12. Myers, K. R.; Truchot, A. T.; Ward, J.; Hudson, Y.; Ulrich, J. T. In *Cellular and Molecular Aspects of Endotoxin Reactions*; Nowotny, A., Spitzer, J. J., Ziegler, E. J., Eds.; Endotoxin Research Series; Elsevier: Amsterdam, 1990; Vol. 1, pp 145–156.
13. Ulrich, J. T., Myers, K. R., Eds.Vaccine Design: The Subunit and Adjuvant Approach; Powell, M. F., Newman, M. J., Eds.; Plenum: New York, 1995; pp 495–524.
14. Mata-Haro, V.; Cekic, C.; Martin, M.; Chitton, D. M.; Casella, C. R.; Mitchell, T. C. *Science* **2007**, *316*, 1628.
15. Gaekwad, J.; Zhang, Y.; Zhang, W.; Reeves, J.; Wolfert, M. A.; Boons, G. J. *J. Biol. Chem.* **2010**, *285*, 29375.
16. Zhang, Y.; Gaekwad, J.; Wolfer, M. A.; Boons, G. J. *J. Am. Chem. Soc.* **2007**, *129*, 5200.
17. Kusumoto, S.; Fukase, K. *Chem. Rec.* **2006**, *6*, 333.
18. Fujimoto, Y.; Adachi, Y.; Akamatsu, M.; Fukase, Y.; Kataoka, M.; Suda, Y.; Fukase, K.; Kusumoto, S. *J. Endotoxin. Res.* **2005**, *11*, 341.
19. Park, B. S.; Song, D. H.; Kim, H. M.; Choi, B. S.; Lee, J. O. *Nature* **2009**, *458*, 1191.
20. Jiang, Z.-H.; Budzynski, W. A.; Skeels, L. N.; Krantz, M. J.; Koganty, R. R. *Tetrahedron* **2002**, *58*, 8833.
21. Jiang, Z.-H.; Bach, M. V.; Budzynski, W. A.; Krantz, M. J.; Koganty, R. R.; Longenecker, B. M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2193.
22. Jiang, Z.-H.; Budzynski, W. A.; Qiu, D.; Yalamati, D.; Koganty, R. R. *Carbohydr. Res.* **2007**, *342*, 784.
23. Lewicky, J. D.; Ulanova, M.; Jiang, Z.-H. *Carbohydr. Res.* **2011**, *346*, 1705.
24. Lewicky, J. D.; Ulanova, M.; Jiang, Z.-H. *RSC Adv.* **2012**, *2*, 1917.
25. Dullenkopf, W.; Castro-Palomino, J. C.; Manzoni, L.; Schmidt, R. R. *Cabohydr. Res.* **1996**, *296*, 135.
26. Ellervik, U.; Magnusson, G. *Tetrahedron Lett.* **1997**, *38*, 1627.
27. Kiso, M.; Tanaka, S.; Fujita, M.; Fujishima, Y.; Ogawa, Y.; Hasagawa, A. *Carbohydr. Res.* **1987**, *162*, 247.
28. Stanssens, D.; Hermanns, R.; Wories, H. *Prog. Org. Coat.* **1993**, *22*, 379.
29. Saitoh, S.; Akashi, S.; Yamada, T.; Tanimura, N.; Kobayashi, M.; Konno, K.; Fukase, K.; Kusumoto, Y.; Kosagi, A.; Miyake, K. *Int. Immunol.* **2004**, *16*, 961.
30. Golenbock, D. T.; Hampton, R. Y.; Qureshi, N.; Takayama, K.; Raetz, C. R. H. *J. Biol. Chem.* **1991**, *266*, 19490.
31. O'Sullivan, M. C.; Dalrymple, D. M. *Tetrahedron Lett.* **1995**, *36*, 3451.
32. Kim, T. H.; Yang, G.-Y. *Tetrahedron Lett.* **2002**, *43*, 9553.