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Monophosphoryl lipid A analogues with varying 3-O-substitution: synthesis and potent adjuvant activity

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Abstract—Structurally defined immunostimulatory adjuvants play important roles in the development of new generation vaccines. Here described are the syntheses of three monophosphoryl lipid A analogues (**1**–**3**) with different substitution at 3-O-position of the reducing sugar and their potent immunostimulatory adjuvant activity. The syntheses involve the preparation of glycosylation acceptors benzyl 3,4-di-*O*-benzyl-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (**16**) and benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (**17**). The glycosylation reactions between the donor 4,6-di-*O*-benzylidene-2-deoxy-2-(2′,2′,2′-trichloroethoxycarbonylamino)-α-D-glucopyranosyl trichloroacetimidate (**21**) and acceptors **16** and **17** provide the desired β-(1→6)-linked disaccharides **22** and **23**, respectively. Selective reductive ring opening of the 4,6-di-*O*-benzylidene group, installation of a phosphate group to the 4′-hydroxyl group, and the final global debenzylation produce the designed monophosphoryl lipid A analogues **1**–**3**. All three synthetic analogues induce antigen specific T-cell proliferation and interferon-gamma (IFN-γ) production in ex vivo experiments with a totally synthetic liposomal vaccine system. The immunostimulatory potency of compound **1**–**3** is in the same order of magnitude as that of the detoxified natural lipid A product isolated from *Salmonella minnesota* R595 (R595 lipid A). The substituent at the 3-O-position of the reducing sugar does not have much effect on the adjuvant activity of monophosphoryl lipid A analogues. The preliminary lethal toxicity study indicates that the 3-O-acylated hepta-acyl monophosphoryl lipid A may not be more toxic than its 3-O-deacylated hexa-acyl analogue.

Keywords: Vaccine adjuvant; Immunostimulant; Monophosphoryl lipid A; Glycolipid; Cytokine

1. Introduction

Successful vaccination against infectious or neoplastic diseases is to prime the host's immune system to generate an efficient defence and memory response. Generation of strong immune responses to poorly immunogenic antigens requires the help of an immunostimulatory adjuvant. Current understanding of the role of vaccine adjuvants is that they serve as danger signals, which are detected by a group of pattern recognition receptors such as Toll-like receptors (TLRs) expressed on macrophages and dendritic cells.[1] The activation of TLRs triggers the activation of antigen presentation cells (APCs) and the secretion of inflammatory cytokines and chemokines, leading to the subsequent development of a strong and specific acquired immunity. Interests in developing novel vaccine adjuvants continue to grow in recent years,[2–4] particularly in the context of developing new type of vaccines capable of eliciting T_H1 immune responses.[5,6]

Lipid A is the active principle of lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria. Lipid A has strong immunostimulatory activity, but its high toxicity prevents its use in clinical

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practice. However, the endotoxic effect of lipid A can be largely reduced by selective hydrolysis of the anomeric phosphate group while the immunostimulatory property of the molecule remains unaffected.[7] The promising immunostimulatory adjuvant MPL®, monophosphoryl lipid A, is the natural lipid A product isolated from *Salmonella minnesota* R595 and detoxified by selective hydrolysis of the anomeric phosphate group.[8] Currently, MPL® is under extensive clinical evaluation for both prophylactic and therapeutic human vaccine use.[9]

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. On the other hand, synthetic lipid A analogues are pure material of single molecule, which are advantageous in achieving reproducibility and consistency with respect to product manufacturing and performance. In order to develop synthetic lipid A adjuvants with reduced toxicity and enhanced beneficial immunostimulatory effect, various lipid A analogues have been designed and synthesized by us[10,11] and many others.[12–17] As part of our continuous cancer vaccine program,[4,18,19] we report here the syntheses and immunostimulatory property of three monophosphoryl lipid A analogues (**1**–**3**) (Chart 1) with different 3-O-substitute groups.

2. Results and discussion

2.1. Syntheses of monophosphoryl lipid A analogues 1–3

Synthetic strategies for lipid A molecules[15,20–23] incorporate different protecting groups and glycosylation methods in constructing the β-(1→6)-linked diglucosamine unit. Here we use the benzyl group as the global protecting group and the trichloroacetimidate as the glycosyl-

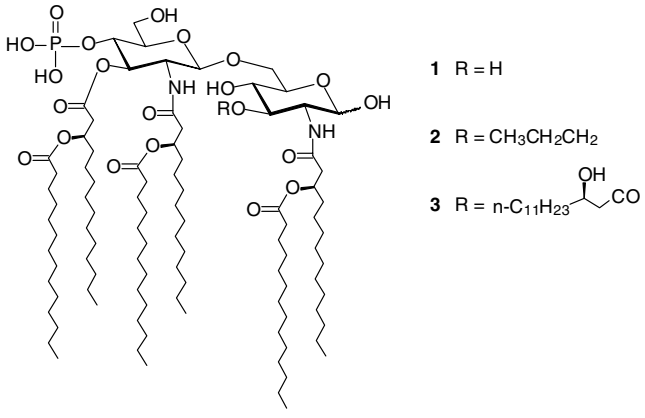
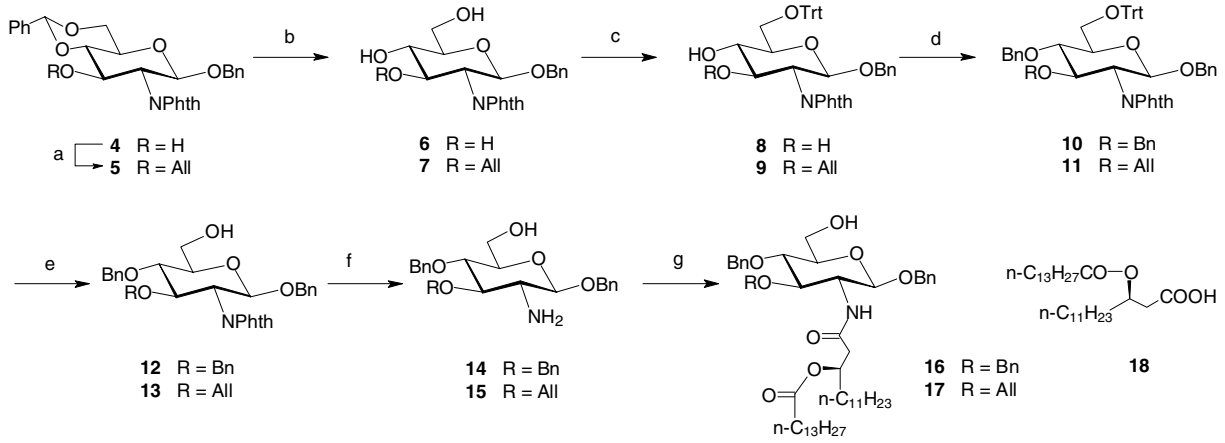


Chart 1. Monophosphoryl lipid A analogues (**1**–**3**) with different 3-O-substitution.

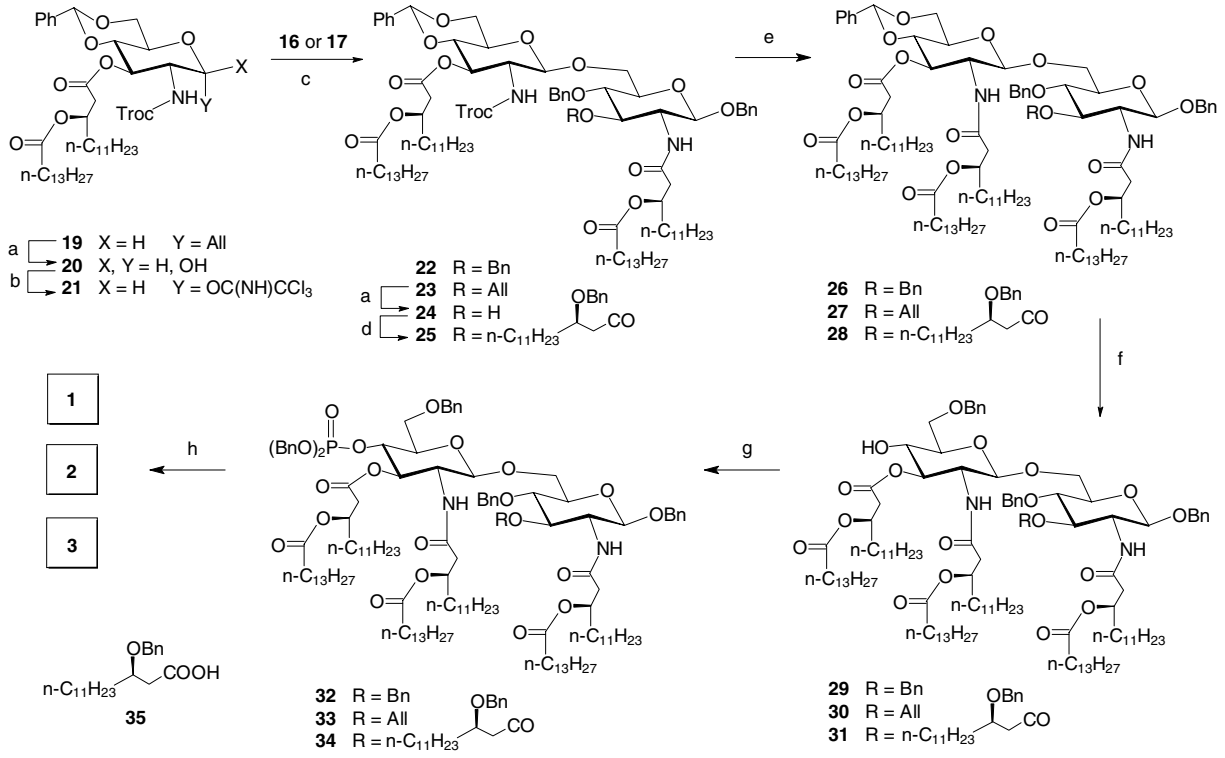
ation donor.[24] The preparation of glycosylation acceptors **16** and **17** is shown in Scheme 1. The readily available glucosamine derivative **6**[25] is selectively protected with the trityl group at the 6-O-position to give **8**, which is then treated with benzyl bromide and sodium hydride to provide **10** in high yield. The removal of the 6-*O*-trityl group (→**12**) and the phthalimide function affords the free amine **14**, which is then coupled with fatty acid **18**[26] in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) to provide the glycosylation acceptor **16** in 82% yield. In order to attach a lipid chain at the 3-O-position for the synthesis of compound **3**, glycosylation acceptor **17** with an allyl group at the 3-O-position has been prepared. This allyl group can be converted to a propyl group upon catalytic hydrogenation at the final debenzylation step, allowing for the straightforward preparation of compound **2**. Thus, glucosamine derivative **4**[25] is treated with allyl bromide and sodium hydride to give **5**, which upon treatment with aqueous acid at 65 °C provides the 3-*O*-allyl protected intermediate **7**. Following the same reaction sequence as described for the synthesis of **16**, compound **7** is converted to acceptor **17** in an overall good yield through the following intermediates: 6-*O*-trityl-protected **9**, 4-O-benzylated **11**, 6-O-detritylated **13** and the free amine **15**.

The 2,2,2-trichloroethoxycarbonyl (Troc) group is an efficient amine-protection group, which can be cleaved by reductive β-elimination.[27] The Troc group has been widely employed in the syntheses of β-glycosides of glucosamine derivatives because of its neighbouring group participating capacity.[11,28,29] Here we have prepared the glycosyl donor **21** with *N*-Troc protection for the stereoselective synthesis of β-(1→6)-linked diglucosamine unit (Scheme 2). The previously reported building block **19**[11,22] is converted to the reducing end derivative **20** in 82% yield following the two-step procedure: first the isomerization of the allyl double bond using the iridium complex,[26] [bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate, and then hydrolysis of the isomerized aglycone in the presence of *N*-bromosuccinimide (NBS).

The conversion of compound **20** to trichloroacetimidate **21**[21] is effected by treating with trichloroacetonitrile and diazabicyclo[5,4,0]undec-7-ene (DBU) in 81% as a single α-isomer (^{1}H NMR, δ 6.42, d, J 4.0 Hz, H-1). The glycosylation reaction of **21** with either **16** or **17** in the presence of $BF_3{\cdot}OEt_2$ as the catalyst gives the desired disaccharide **22** or **23** in good yield. The newly formed β-linkage is confirmed by ^{1}H NMR data in both **22** (δ 4.52, d, J 8.0 Hz, 1H, H-1′; δ 4.89, d, J 8.0 Hz, 1H, H-1) and **23** (δ 4.51, d, J 8.0 Hz, 1H, H-1′; δ 4.88, d, J 8.0 Hz, 1H, H-1). The allyl group at the 3-O-position in **23** is then removed to give **24**, which is subsequently coupled with (*R*)-3-benzyloxytetradecanoic acid **35**[30] in the presence of DCC and 4-*N*,*N*′-dimethylaminopyridine (DMAP) to afford **25** in 71%.



Scheme 1. Reagents and conditions: (a) AllBr, NaH, DMF, 82%; (b) HOAc–H_2O, 65 °C, 95% for **7**; (c) Trt–Cl, DMAP, pyridine, 40 °C, 90% for **8** and 79% for **9**; (d) BnBr, NaH, DMF, 90% for **10** and 57% for **11**; (e) HOAc–H_2O–AllOH, 110 °C, 70% for **12** and 87% for **13**; (f) $H_2NNH_2{\cdot}H_2O$, EtOH, reflux, 97% for **14** and 77% for **15**; (g) **18**, DCC, CH_2Cl_2, 82% for **16** and 80% for **17**.



Scheme 2. Reagents and conditions: (a) (i) [bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate, THF; (ii) NBS, THF–H_2O, 82% for **20** and 62% for **24**; (b) Cl_3CCN, DBU, CH_2Cl_2, 81%; (c) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 60% for **22** and 88% for **23**; (d) **35**, DCC, DMAP, CH_2Cl_2, 71%; (e) (i) Zn dust, HOAc; (ii) **18**, DCC, CH_2Cl_2, 60% for **26**, 56% for **27** and 72% for **28**; (f) $NaBH_3CN$, HCl(g)–Et_2O, THF, 0 °C, 83% for **29**, 67% for **30** and 71% for **31**; (g) (i) $(BnO)_2PN(^iPr)_2$, tetrazole, CH_2Cl_2; (ii) *m*-CPBA, CH_2Cl_2, 0 °C, 85% for **32**, 61% for **33**, and 59% for **34**; (h) H_2, Pd/C, THF–HOAc, 62% for **1**, 95% for **2**, and 75% for **3**.

Removal of the *N*-Troc protecting group in **22**, **23** and **25**, followed by coupling with fatty acid **18** (Scheme 1) in the presence of DCC, provides compounds **26**, **27** and **28**, respectively, in 56–72%. With all the acyl chains installed in proper positions of the disaccharide backbone, the next task is to introduce the phosphate group at 4′-O-position. Regioselective reductive ring opening of the 4,6-di-*O*-benzylidene group in **26**–**28** by treating with $NaBH_3CN$ and HCl(g)-saturated diethyl ether solution[31] at 0 °C results in the formation of 4′-free hydroxyl derivatives **29**–**31**. Treatment of compounds **29**–**31** with phosphoramidite and tetrazole, followed by oxidizing with *m*-chloroperbenzoic acid (*m*-CPBA), furnishes phosphates **32**–**34**. The two-step phosphorylation proce-

dure,[32] through phosphite to phosphate, is highly efficient for all three transformations, as indicated by TLC profile of these reactions. The typical isolated yield for this phosphorylation reaction is in the range of 59–85%. The reduced yield in some cases is the result of multiple chromatographic purification procedures, which are often required in order to obtain highly pure material. Finally, the removal of all benzyl groups in **32**–**34** and the reduction of the allyl double bond in **33** are facilitated by catalytic hydrogenation over Pd/C in THF–HOAc to give target compounds **1**–**3**, respectively, in 62–95% yield. The synthesis and immunostimulant activity of structure **1** as its triethylammonium salt was reported earlier by Johnson et al.[15]

2.2. Biological evaluation

The immuno-adjuvant activity of monophosphoryl lipid A analogues **1**–**3** has been evaluated in a totally synthetic BLP25 liposomal vaccine system in comparison with the natural lipid A product, R595 lipid A, which is purified from the bacteria *S. minnesota* R595. The BLP25 liposomal vaccine formulation contains a MUC1-derived 25 amino acid lipopeptide as the antigen,[18] with one of the synthetic compounds (**1**–**3**) as adjuvant. Mice are immunized with a single dose of this vaccine formulation containing 40 μg of the antigen and 20 μg of the adjuvant. Lymphocytes obtained from draining lymph nodes of the sacrificed mice are re-activated by the same antigen and immune responses are measured by T-cell proliferation (blastogenesis) and the secretion of the cytokine interferon-gamma (IFN-γ) (Fig. 1). All three synthetic analogues **1**–**3** demonstrate potent adjuvant activity in the same order of magnitude as R595 lipid A in promoting antigen specific T-cell response (CPM, counts per minute) and IFN-γ production (pg/mL). The same vaccine construct without a monophosphoryl lipid A analogue as an adjuvant fails to induce antigen specific T-cell proliferation and IFN-γ production. Compound **3** with an (*R*)-3-hydroxytetradecanoyl group at the 3-O-position appears to be the most active one in terms of inducing antigen specific T-cell response while compound **2** with a propyl group at the 3-O-position has lower activity. The differences in both CPM and IFN-γ levels induced by these compounds indicate that the substituent at the 3-O-position of monophosphoryl lipid A molecules affects the potency of their immunostimulating activity. However, the differences in both CPM and IFN-γ levels are relatively small; therefore, the effect exhibited by this 3-O-substituent on the adjuvant activity of these molecules is probably not significant.

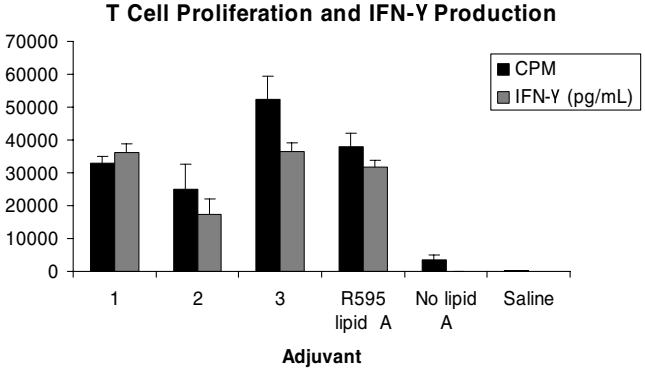


Figure 1. T-Cell proliferation and IFN-γ production.

The 3-O-acylated monophosphoryl lipid A analogue **3** shows very good adjuvant activity, and structurally it has seven fatty acyl chains of uniform 14 carbon length. Thus, compound **3** is of particular interest as a vaccine adjuvant for further evaluation. A preliminary lethal toxicity study was carried out for compound **3** in comparison with natural product R595 lipid A of which the main component is the 3-O-deacylated hexa-acyl monophosphoryl lipid A with its six fatty acyl chains each having 12–16 carbon atoms.[15] The actinomycin D-sensitized[33] C57 black mice are injected with different doses of monophosphoryl lipid A analogue **3** or R595 lipid A. The mice injected with 50 μg of compound **3** have all survived while the mice injected with the same dose of R595 lipid A have all died. Two thirds of the mice have also died when they were injected with a 10 μg dose of R595 lipid A. This finding is a bit surprising since the removal of the 3-*O*-acyl group of structurally diverse lipid A molecules is believed to reduce lipid A toxicity.[34,35] The manufacturing process of R595 lipid A includes a basic hydrolysis step, which is supposed to have selectively removed the (*R*)-3-hydroxytetradecanoyl group at the 3-O-position of the main component.[35] The reduced toxicity of R595 lipid A has been partially attributed to the removal of this 3-*O*-acyl group during the manufacturing process. Our data suggest that the 3-O-acylated hepta-acyl monophosphoryl lipid A may not be more toxic than the 3-O-deacylated hexa-acyl analogue. In order to unravel the effect of the 3-O-substituent on the toxicity profile of monophosphoryl lipid A molecules, further toxicology investigation is needed.

In summary, we have described a straightforward synthesis of three monophosphoryl lipid A analogues with different substitution groups at the 3-O-position of the reducing sugar. The strategy is geared towards the incorporation of different acyl groups at 2-N-, 3-O-, 2′-N- and 3′-O-positions of the lipid A disaccharide backbone. In a totally synthetic liposomal vaccine formulation, all three monophosphoryl lipid A analogues (**1**–**3**) show strong adjuvant activity in promoting T-cell proliferation and IFN-γ production. Their immunostimulatory potency is in the same level as that of the detoxified lipid A product purified from *S. minnesota* R595. The preliminary lethal toxicity study indicates that 3-O-acylated hepta-acyl monophosphoryl lipid A molecules may not

be more toxic than their 3-O-deacylated hexa-acyl analogues.

3. Experimental

3.1. Synthesis

3.1.1. General methods. All air and moisture sensitive reactions have been performed under nitrogen atmosphere. Anhydrous tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), acetonitrile and CH_2Cl_2 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, E. 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 parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br (broad). Protons of disaccharide backbone are indicated by regular number (1–6) for the reducing end sugar, while for the non-reducing end sugar they are indicated by prime (1′–6′). Optical rotations are measured on a Perkin–Elmer 241 Polarimeter at room temperature (20–22 °C). Elemental analysis data are obtained from the Microanalytical laboratory in the University of Alberta, Canada. Electron-spray ionization mass spectrometric analyses (ESIMS) are performed either on MS50B or MSD1 SPC mass spectrometer, and the data are reported in *m*/*z*.

3.1.2. Benzyl 2-deoxy-2-phthalimido-6-*O*-triphenylmethyl-β-D-glucopyranoside (8). To a soln of **6** (1.0 g, 3.50 mmol) in dry pyridine (10 mL), triphenylmethyl chloride (836 mg, 3.0 mmol) and DMAP (30.5 mg, 0.25 mmol) were added. The mixture was stirred at room temperature for 20 h. Additional trityl chloride (418 mg, 1.25 mmol) and DMAP (30.5 mg, 0.25 mmol) were added and the mixture was stirred at 40 °C for 4 h. The solvent was removed by co-distillation with toluene and the residue was purified by flash chromatography (hexane–EtOAc, 1:1) to give **8** (1.42 g, 88%). R_f 0.36 (hexane–EtOAc, 1:1); $[\alpha]_D^{22}$ −48.3 (*c* 0.6, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.70 (br s, 1H, OH), 3.00 (br s, 1H, OH), 3.44–3.53 (m, 2H, H-6a/b), 3.59 (m, 1H, H-5), 3.65 (dd, $J_{4,5}$ 9.5, $J_{4,3}$ 9.0 Hz, 1H, H-4), 4.21 (dd, $J_{2,3}$ 9.5, $J_{2,1}$ 8.0 Hz, 1H, H-2), 4.33 (dd, $J_{2,3}$ 9.5, $J_{4,3}$ 9.0 Hz, 1H, H-3), 4.55 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.95 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 5.23 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 7.10–7.80 (m, 24H, Ar–H). Anal. Calcd for $C_{40}H_{35}NO_7 \cdot 1.3H_2O$ (641.72): C, 72.23; H, 5.70; N, 2.10. Found: C, 72.24; H, 5.92; N, 1.83.

3.1.3. Benzyl 3-*O*-allyl-2-deoxy-6-*O*-triphenylmethyl-2-phthalimido-β-D-glucopyranoside (9). The soln of **4** (2.02 g, 4.14 mmol) in dry DMF (15 mL) was added dropwise within 10 min to a mixture of sodium hydride (230 mg, 9.58 mmol), allyl bromide (0.75 g, 0.50 mL, 6.21 mmol) and dry DMF (20 mL). The reaction mixture was stirred at room temperature for 3 h and then MeOH (1.0 mL) was added and stirred for 15 min. DMF was removed under high vacuo, followed by aqueous work-up. The residue was purified by flash chromatography (hexane–EtOAc, 5:1) to give **5** as syrup (1.79 g, 82%). Compound **5** (5.79 g, 11.0 mmol) was treated with acetic acid–water (4:1, 130 mL) at 65 °C for 6 h. The solvent was removed and the residue was purified by flash chromatography (hexane–EtOAc, 1:2) to give **7** as syrup (4.91 g, 95%). In a similar way as described for the preparation of **8**, compound **7** (4.79 g, 10.91 mmol) was converted to **9** (5.87 g, 79%). R_f 0.66 (hexane–EtOAc, 1:2); $[\alpha]_D^{22}$ −37.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.71 (d, *J* 2.8 Hz, 1H, OH), 3.46 (m, 2H, H-6a/b), 3.59 (m, 1H, H-5), 3.80 (m, 1H, H-4), 3.95 (m, 1H, CH*H*CH=CH_2), 4.15 (dd, $J_{3,2}$ 10.0, $J_{3,4}$ 8.5 Hz, 1H, H-3), 4.16 (m, 1H, CH*H*CH=CH_2), 4.25 (dd, $J_{3,2}$ 10.0, $J_{2,1}$ 8.0 Hz, 1H, H-2), 4.55 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.84 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.85 (m, 1H, CH*H*=CH), 5.02 (m, 1H, CH*H*=CH), 5.19 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 5.59 (m, 1H, CH_2=C*H*), 7.09–7.90 (m, 24H, Ar–H). Anal. Calcd for $C_{43}H_{39}NO_7$ (681.78): C, 75.75; H, 5.76; N, 2.04. Found: C, 75.37; H, 5.67; N, 2.04.

3.1.4. Benzyl 2-deoxy-3,4-di-*O*-benzyl-2-phthalimido-6-*O*-triphenylmethyl-β-D-glucopyranoside (10). The soln of **8** (1.34 g, 2.09 mmol) in dry DMF (8 mL) was added dropwise to the mixture of sodium hydride (120 mg, 5.02 mmol) and benzyl bromide (0.86 g, 0.60 mL, 5.02 mmol) in dry DMF (10 mL). The reaction mixture was stirred at room temperature for 1 h and treated further with an additional amount of benzyl bromide (0.43 g, 0.30 mL, 2.51 mmol) and sodium hydride (60 mg, 2.51 mmol). The reaction mixture was allowed to stir for another 2 h. Methanol (2 mL) was then added and the mixture was stirred for 10 more minutes. The reaction was then poured into ice water (100 mL) and extracted with diethyl ether (60 mL × 3). The combined ether layer was washed with ice water (15 mL × 3), dried with sodium sulfate and concentrated. The residue was purified by flash chromatography (hexane–EtOAc, 5:1) to give **10** (1.55 g, 90%). R_f 0.60 (hexane–EtOAc, 3:1); $[\alpha]_D^{22}$ +5.5 (*c* 0.8, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.32 (dd, *J* 10.0, $J_{6a,5}$ 4.5 Hz, 1H, H-6a), 3.60 (m, 1H, H-5), 3.68 (dd, *J* 10.0, $J_{6b,5}$ 1.8 Hz, 1H, H-6b), 4.03 (dd, $J_{2,3}$ 9.5, $J_{2,1}$ 8.2 Hz, 1H, H-2), 4.33 (m, 2H, H-3, H-4), 4.43 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.47 (d, *J* 10.0 Hz,

1H, CH*H*Ph), 4.61 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.72 (d, *J* 10.0 Hz, 1H, CH*H*Ph), 4.80 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.94 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 5.20 (d, $J_{1,2}$ Hz, 1H, H-1), 6.84–7.80 (m, 34H, Ar–H). Anal. Calcd for $C_{54}H_{47}NO_7 \cdot 1.3H_2O$ (821.97): C, 76.72; H, 5.91; N, 1.66. Found: C, 76.56; H, 6.13; N, 1.52.

3.1.5. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-6-*O*-triphenylmethyl-2-phthalimido-β-D-glucopyranoside (11). In a similar way as described for the preparation of **10**, compound **9** (3.80 g, 5.57 mmol) was converted to **11** (2.45 g, 57%). R_f 0.67 (hexane–EtOAc, 2:1); $[\alpha]_D^{22}$ −37.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.32 (dd, *J* 10.0, $J_{6a,5}$ 3.5 Hz, 1H, H-6a), 3.62 (m, 1H, H-5), 3.69 (dd, *J*10.0, $J_{6b,5}$ 1.0 Hz, 1H, H-6b), 3.93 (m, 1H, CH*H*CH=CH_2), 3.96 (m, 1H, H-4), 4.25 (m, 1H, CH*H*CH=CH_2), 4.27 (dd, $J_{2,3}$ 10.5, $J_{2,1}$ 8.5 Hz, 1H, H-2), 4.42 (dd, $J_{2,3}$ 10.5, $J_{3,4}$ 8.5 Hz, 1H, H-3), 4.44 (d, *J* 10.0 Hz, 1H, CH*H*Ph), 4.66 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.70 (d, *J* 10.0 Hz, 1H, CH*H*Ph), 4.83 (m, 1H, CH*H*=CH), 4.99 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 5.02 (m, 1H, CH*H*=CH), 5.27 (d, $J_{1,2}$ 8.5 Hz, 1H, H-1), 5.59 (m, 1H, CH_2=C*H*), 6.92–7.90 (m, 29H, Ar–H). Anal. Calcd for $C_{50}H_{45}NO_7 \cdot 0.5H_2O$ (771.91): C, 76.90; H, 5.94; N, 1.79. Found: C, 76.72; H, 6.11; N, 1.78.

3.1.6. Benzyl 2-deoxy-3,4-di-*O*-benzyl-2-phthalimido-β-D-glucopyranoside (12). The soln of **10** (1.42 g, 1.73 mmol) in acetic acid–water (4:1, 60 mL) was stirred at 110 °C for 1 h. The solvent was removed by co-distillation with toluene and the residue was purified by flash chromatography (hexane–EtOAc, 2:1) to give **12** (700 mg, 70%). R_f 0.31 (hexane–EtOAc, 2:1); $[\alpha]_D^{22}$ +16.0 (*c* 0.25, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.90 (dd, *J* 6.5, *J* 6.5 Hz, 1H, OH), 3.54 (m, 1H, H-5), 3.73 (dd, $J_{4,5}$ 9.5, $J_{4,3}$ 9.0 Hz, 1H, H-4), 3.78 (m, 1H, H-6a), 3.93 (m, 1H, H-6b), 4.19 (dd, $J_{2,3}$ 10.0, $J_{2,1}$ 8.5 Hz, 1H, H-2), 4.36 (dd, $J_{3,2}$ 10.0, $J_{3,4}$ 9.0 Hz, 1H, H-3), 4.43 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.50 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.73 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.76 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.79 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.90 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 5.20 (d, $J_{1,2}$ 8.5 Hz, 1H, H-1), 6.80–7.80 (m, 19H, Ar–H). Anal. Calcd for $C_{35}H_{33}NO_7 \cdot 0.8H_2O$ (579.65): C, 70.76; H, 5.87; N, 2.35. Found: C, 70.74; H, 6.14; N, 2.20.

3.1.7. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (13). In a similar way as described for the preparation of **12**, compound **11** (1.50 g, 1.94 mmol) was converted to **13** (0.90 g, 87%). R_f 0.33 (hexane–EtOAc, 2:1); $[\alpha]_D^{22}$ −16.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.90 (dd, *J* 6.0, *J* 6.0 Hz, 1H, OH), 3.52 (m, 1H, H-6a), 3.65 (dd, *J* 9.5, *J* 8.5 Hz, 1H, H-4), 3.75 (m, 1H, H-5), 3.90 (m, 2H, H-6b, CH*H*CH=CH_2), 4.20 (m, 2H, H-3, CH*H*CH=CH_2), 4.28 (dd, $J_{2,3}$ 10.0, $J_{2,1}$ 8.0 Hz, 1H, H-2), 4.52 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.68 (d, *J* 10.5 Hz, 1H, CH*H*Ph), 4.79 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.80 (m, 1H, CH*H*=CH), 4.84 (d, *J* 10.5 Hz, 1H, CH*H*Ph), 5.00 (m, 1H, CH*H*=CH), 5.23 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 5.55 (m, 1H, CH_2=C*H*), 7.10–7.85 (m, 14H, Ar–H). Anal. Calcd for $C_{31}H_{31}NO_7 \cdot 0.7H_2O$ (529.59): C, 68.67; H, 6.02; N, 2.58. Found: C, 68.46; H, 5.93; N, 2.53.

3.1.8. Benzyl 2-amino-2-deoxyl-3,4-di-*O*-benzyl-β-D-glucopyranoside (14). To the soln of **12** (0.60 g, 1.04 mmol) in 95% ethanol (40 mL) was added hydrazine monohydrate (2.06 g, 2.0 mL, 41.2 mmol). The mixture was refluxed for 2 h and then the solvent was removed under diminished pressure. The residue was purified by flash chromatography (1–2% MeOH in CH_2Cl_2) to give **14** (450 mg, 97%). R_f 0.20 (2% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$ −9.4 (*c* 0.35, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.75 (br s, 3H, OH, NH_2), 2.92 (dd, $J_{2,3}$ 9.0, $J_{2,1}$ 8.0 Hz, 1H, H-2), 3.43 (m, 1H, H-5), 3.49 (dd, $J_{4,3} = J_{4,5}$ 9.5 Hz 1H, H-4), 3.66 (dd, $J_{3,4}$ 9.5, $J_{3,2}$ 9.0 Hz, 1H, H-3), 3.76 (dd, *J* 12.0, $J_{6a,5}$ 5.0 Hz, 1H, H-6a), 3.91 (dd, *J* 12.0, $J_{6b,5}$ 2.5 Hz, 1H, H-6b), 4.39 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 4.63 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.70 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.74 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.86 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.88 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.99 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 7.35 (m, 15H, Ar–H). Anal. Calcd for $C_{27}H_{31}NO_5$ (449.55): C, 72.14; H, 6.95; N, 3.15. Found: C, 72.34; H, 7.15; N, 3.12.

3.1.9. Benzyl 3-*O*-allyl-2-amino-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (15). In a similar way as described for the preparation of **14**, compound **13** (0.90 g, 1.70 mmol) was converted to **15** (525 mg, 77%). R_f 0.28 (3% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$ −17.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.75 (s, 3H, NH_2, OH), 2.87 (dd, *J* 9.5, 8.0 Hz, 1H, H-2), 3.35 (dd, *J* 9.5, 9.5 Hz, 1H, H-4), 3.36 (m, 1H, H-5), 3.57 (dd, *J* 9.5, 9.5 Hz, 1H, H-3), 3.72 (dd, *J* 12.0, 4.0 Hz, 1H, H-6a), 3.88 (dd, *J* 12.0, 2.5 Hz, 1H, H-6b), 4.24 (m, 1H, CH*H*CH=CH_2), 4.36 (d, *J* 8.0 Hz, 1H, H-1), 4.42 (m, 1H, CH*H*CH=CH_2), 4.62 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.64 (d, *J* 1.0 Hz, 1H, CH*H*Ph), 4.82 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.88 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 5.18–5.33 (m, 2H, CH_2=CH), 5.97 (m, 1H, CH_2=C*H*), 7.30 (m, 10H, Ar–H).

3.1.10. Benzyl 2-deoxy-3,4-di-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (16). To the soln of compound **14** (410 mg, 0.913 mmol) in dry CH_2Cl_2 (30 mL), compound **18** (623 mg, 1.37 mmol) and DCC (564 mg, 2.74 mmol) were added. The mixture was stirred at room temperature for 24 h. The solid was filtered off and washed with CH_2Cl_2 (4 mL). The filtrate was concentrated and the residue purified by flash chromatography (0.5–1% MeOH in CH_2Cl_2) to give **16** (664 mg, 82%). R_f 0.33 (2% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$

−3.2 (*c* 0.6, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, *J* 7.0 Hz, 6H, $2CH_3$), 1.25 (m, 38H, $19CH_2$), 1.55 (m, 4H, $2CH_2$), 1.89 (dd, *J* 7.0, 6.0 Hz, 1H, OH), 2.15 (m, 2H, CH_2), 2.27 (dd, *J* 15.0, 5.5 Hz, 1H, CH*H*), 2.36 (dd, *J* 15.0, 6.0 Hz, 1H, CH*H*), 3.46 (m, 1H, H-5), 3.52 (m, 1H, H-4), 3.59 (dd, *J* 10.0, 9.0 Hz, 1H, H-3), 3.70 (m, 1H, H-6a), 3.86 (m, 1H, H-6b), 4.10 (dd, *J* 10.0, 8.0 Hz, 1H, H-2), 4.60 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.64 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.65 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.81 (d, *J* 11.5 Hz, 2H, 2CH*H*Ph), 4.83 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.95 (d, *J* 8.0 Hz, 1H, H-1), 5.04 (m, 1H, lipid-3-H), 5.92 (d, *J* 8.0 Hz, 1H, NH), 7.30 (m, 15H, Ar–H). Anal. Calcd for $C_{55}H_{83}NO_8$ (886.26): C, 74.47; H, 9.44; N, 1.58. Found: C, 74.25; H, 9.44; N, 1.64.

3.1.11. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (17). In a similar way as described for the preparation of **16**, compound **15** (510 mg, 1.28 mmol) was coupled with **18** (870 mg, 1.92 mmol) in the presence of DCC (659 mg, 3.20 mmol) to give **17** (853 mg, 80%) after flash chromatographic purification (2–5% acetone in $CHCl_3$). R_f 0.38 (2% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$ −6.0 (*c* 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J* 6.5 Hz, 6H, $2CH_3$), 1.25 (br s, 38H, $19CH_2$), 1.59 (m, 4H, $2CH_2$), 1.86 (t, *J* 7.0 Hz, 1H, OH), 2.23 (t, *J* 7.5 Hz, 2H, CH_2), 2.37 (dd, *J* 15.0, 5.5 Hz, 1H, CH*H*), 2.48 (dd, *J* 15.0, 5.5 Hz, 1H, CH*H*), 3.40 (m, 2H, H-2, H-5), 3.52 (dd, *J* 9.5, 8.5 Hz, 1H, H-4), 3.70 (m, 1H, H-6a), 3.85 (m, 1H, H-6b), 4.00 (dd, *J* 10.0, 8.5 Hz, 1H, H-3), 4.14 (m, 1H, CH*H*CH=CH_2), 4.26 (m, 1H, CH*H*CH=CH_2), 4.59 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.63 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.82 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.83 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.96 (d, *J* = 8.0 Hz, 1H, H-1), 5.08 (m, 1H, lipid-3-H), 5.13 (m, 1H, CH*H*=CH), 5.23 (m, 1H, CH*H*=CH), 5.88 (m, 1H, C*H*=CH_2), 6.00 (d, *J* 8.0 Hz, 1H, NH), 7.35 (m, 10H, Ar–H). Anal. Calcd for $C_{51}H_{81}NO_8 \cdot 0.7H_2O$ (836.20): C, 72.17; H, 9.78; N, 1.65. Found: C, 72.07; H, 9.81; N, 1.72.

3.1.12. 2-Deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α/β-D-glucopyranose (20). [Bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate (37 mg, 0.044 mmol) was suspended in dry THF (5 mL) and hydrogen gas was bubbled in for 5 min to give a yellowish soln, which was added to the soln of **19** (400 mg, 0.44 mmol) in dry THF (5 mL). The mixture was stirred at room temperature for 2 h. Water (0.5 mL) and *N*-bromosuccinimide (NBS, 117 mg, 0.66 mmol) were then added and the reaction was stirred for 1 hour longer. The remainder obtained from solvent removal was dissolved in EtOAc (200 mL) and washed with saturated sodium bicarbonate soln (20 mL × 2). Combined organic layers were dried with sodium sulfate and concentrated. The residue was purified by flash chromatography (hexane–EtOAc, 4:1 and 3:1) to give **20** (314 mg, 82%) as an anomeric mixture (α/β, 4:1). R_f 0.36 (hexane–EtOAc, 3:1); $[\alpha]_D^{22}$ −9.6 (*c* 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) for the α-isomer: δ 0.88 (t, *J* 6.5 Hz, 6H, $2CH_3$), 1.24 (m, 38H, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.16 (t, *J* 7.5 Hz, 2H, CH_2), 2.49 (dd, *J* 15.0, 5.0 Hz, 1H, CH*H*), 2.60 (dd, *J* 15.0, 7.0 Hz, 1H, CH*H*), 3.65 (d, *J* 4.0 Hz, 1H, OH), 3.70 (dd, *J* 9.5, 9.5 Hz, 1H, H-4), 3.77 (dd, *J* 10.0, 10.0 Hz, 1H, H-6a), 4.03 (m, 1H, H-2), 4.17 (m, 1H, H-5), 4.28 (dd, *J* = 10.0, 4.5 Hz, 1H, H-6b), 4.67, 4.75 (2d, *J* = 12.0 Hz, each 1H, Troc–CH_2), 5.15 (m, 1H, lipid-3-H), 5.35 (dd, *J* 4.0, 4.0 Hz, 1H, H-1), 5.43 (dd, *J* 9.5, 9.5 Hz, 1H, H-3), 5.51 (s, 1H, C*H*Ph), 5.81 (d, *J* 10.0 Hz, 1H, NH), 7.32–7.47 (m, 5H, Ar–H). Anal. Calcd for $C_{44}H_{70}Cl_3NO_{10}$ (879.39): C, 60.10; H, 8.02; N, 1.59. Found: C, 60.11; H, 8.09; N, 1.61.

3.1.13. 2-Deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranosyl trichloroacetimidate (21). To the soln of **20** (2.50 g, 2.88 mmol) in dry CH_2Cl_2 (30 mL), trichloroacetonitrile (8.64 g, 6.0 mL, 60.0 mmol) and DBU (10 drops) were added. The mixture was stirred at room temperature for 2 h and concentrated under diminished pressure (not to dryness). The residue was purified by flash chromatography (hexane–EtOAc–Et_3N, 6:1:1% and 5:1:1%) to give **21** (2.40 g, 81%). R_f 0.25 (hexane–EtOAc, 8:1); $[\alpha]_D^{22}$ +35.0 (*c* 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, *J* 7.0 Hz, 6H, $2CH_3$), 1.25 (m, 38 Hz, $19CH_2$), 1.50 (m, 4H, $2CH_2$), 2.20 (t, *J* 7.5 Hz, 2H, CH_2), 2.56 (dd, *J* 15.5, 5.5 Hz, 1H, CH*H*), 2.65 (dd, *J* 15.5, 7.0 Hz, 1H, CH*H*), 3.81 (dd, *J* 10.0, 10.0 Hz, 1H, H-4), 3.83 (dd, *J* 10.0, 10.0 Hz, 1H, H-6a), 4.06 (m, 1H, H-5), 4.25 (ddd, *J* 10.0, 9.0, 4.0 Hz, 1H, H-2), 4.36 (dd, *J* 10.0, 5.0 Hz, 1H, H-6b), 4.63, 4.78 (2d, *J* 12.0 Hz, each 1H, Troc–CH_2), 5.18 (m, 1H, lipid-3-H), 5.45 (dd, *J* 10.0, 10.0 Hz, 1H, H-3), 5.56 (d, *J* 9.0 Hz, 1H, NH), 5.58 (s, 1H, C*H*Ph), 6.42 (d, *J* 4.0 Hz, 1H, H-1), 7.30–7.45 (m, 5H, Ar–H), 8.73 (s, H, NH). Anal. Calcd for $C_{46}H_{70}Cl_6N_2O_{10}$ (1023.78): C, 53.97; H, 6.89; N, 2.74. Found: C, 53. 80; H, 6.77; N, 2.80.

3.1.14. Benzyl 2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl}-3,4-di-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (22). To the soln of **16** (290 mg, 0.328 mmol) and **21** (503 mg, 0.492 mmol) in dry CH_2Cl_2 (6 mL) were added molecular sieves (4 Å, 0.5 g). The mixture was stirred under nitrogen at room temperature for 20 min. Trifluoroboron etherate soln (0.1 M in CH_2Cl_2, 1.3 mL) was added dropwise within 20 min. The mixture was stirred for 1 h and then poured

into saturated sodium bicarbonate soln (10 mL) and extracted with CH_2Cl_2 (20 mL × 3). Combined organic layers were dried with sodium sulfate and concentrated. The residue was purified by silica gel chromatography (0.5–1% MeOH in CH_2Cl_2) to give **22** (457 mg, 80%). R_f 0.21 (3% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −17.8 (*c* 0.6, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, *J* 7.0 Hz, 12H, $4CH_3$), 1.25 (m, 76H, $38CH_2$), 1.52 (m, 8H, $4CH_2$), 2.15 (m, 4H, $2CH_2$), 2.26, 2.35 (2dd, *J* 14.0, 6.0 Hz, each 1H, CH_2), 2.48 (dd, *J* 15.0, 5.5 Hz, 1H, CH*H*), 2.58 (dd, *J* 15.0, 7.0 Hz, 1H, CH*H*), 3.34–3.78 (m, 8H, H-2, H-3, H-4, H-5, H-6a, H-2′, H-4′, H-6′a), 4.02–4.13 (m, 2H, H-6b, H-5′), 4.30 (dd, *J* 10.5, 5.0 Hz, 1H, H-6′b), 4.52 (d, *J* 8.0 Hz, 1H, H-1′), 4.57–4.90 (m, 8H, $3CH_2$Ph, Troc–CH_2), 4.89 (d, *J* 8.0 Hz, 1H, H-1) 5.02 (m, 1H, lipid-3-H), 5.15 (m, 3H, NH, H-3′, lipid-3-H), 5.55 (s, 1H, C*H*Ph), 6.00 (d, *J* 8.0 Hz, 1H, NH), 7.25–7.45 (m, 20H, Ar–H). Anal. Calcd for $C_{99}H_{151}Cl_3N_2O_{17}$ (1747.64): C, 68.04; H, 8.71; N, 1.60. Found: C, 67.92, H, 8.85, N, 1.64.

3.1.15. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (23). In a similar method as described for the preparation of **22**, compound **23** was prepared by reacting imidate **21** (1.15 g, 1.12 mmol) and the glycosylation acceptor **17** (652 mg, 0.75 mmol) in the presence of catalyst $BF_3{\cdot}OEt_2$ (0.15 M in CH_2Cl_2, 3.5 mL). Purification by flash chromatography (1–2% acetone in $CHCl_3$) yielded **23** (1.30 g, 83%). R_f 0.36 (6% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −18.6 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (t, *J* 6.5 Hz, 12H, $4CH_3$), 1.22 (br s, 76H, $38CH_2$), 1.53 (m, 8H, $4CH_2$), 2.15 (t, *J* 7.5 Hz, 2H, CH_2), 2.20 (t, *J* 7.5H, 2H, CH_2), 2.32 (dd, *J* 14.0, 5.5 Hz, 1H, CH*H*), 2.42 (dd, *J* 14.0, 6.0 Hz, 1H, CH*H*), 2.47 (dd, *J* 15.0, 5.0 Hz, 1H, CH*H*), 2.57 (dd, *J* 15.0, 7.0 Hz, 1H, CH*H*), 3.34–4.21 (m, 12H, H-2, H-3, H-4, H-5, 2H-6, H-2′, H-4′, H-5′, H-6′a, $CH_2CH{=}CH_2$), 4.30 (dd, *J* 10.0, 5.0 Hz, 1H, H-6′b), 4.51 (d, *J* 8.5 Hz, 1H, H-1′), 4.57 (m, 4H, 2CH*H*Ph, Cl_3CCH_2O), 4.78 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.85 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.88 (d, *J* 8.0 Hz, 1H, H-1), 5.00–5.25 (m, 5H, H-3′, 2 lipid-3-H, $CH_2{=}$CH), 5.45 (s, 1H, C*H*Ph), 5.85 (m, 1H, C*H*=CH_2), 6.00 (d, *J* 8.0 Hz, 1H, NH), 7.30 (m, 15H, Ar–H). Anal. Calcd for $C_{95}H_{149}Cl_3N_2O_{17}$ (1697.58): C, 67.21; H, 8.85; N, 1.65. Found: C, 66.99; H, 8.96; N, 1.65.

3.1.16. Benzyl 4-*O*-benzyl-2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (24). In a similar way as described for the preparation of **20**, compound **23** (350 mg, 0.195 mmol) was converted to **24** (200 mg, 62%). R_f 0.30 (5% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −25.7 (*c* 0.83, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, *J* 6.5 Hz, 12H, $4CH_3$), 1.25 (br s, 76H, $38CH_2$), 1.55 (m, 8H, $4CH_2$), 1.70 (s, 1H, OH), 2.17 (t, *J* 7.0 Hz, 2H, CH_2), 2.26 (t, *J* 7.0 Hz, 2H, CH_2), 2.43 (m, 2H, CH_2), 2.50 (dd, *J* 14.0, 5.5 Hz, 1H, CH*H*), 2.60 (dd, *J* 15.0, 7.5 Hz, 1H, CH*H*), 3.40–3.90 (m, 9H, H-2, H-3, H-4, H-5, H-6a, H-2′, H-4′, H-5′, H-6′a), 4.13 (d, *J* 10.0 Hz, 1H, H-6b), 4.34 (dd, *J* 10.0, 5.0 Hz, 1H, H-6′b), 4.51, 4.52 (2d, *J* 8.5 Hz, each 1H, H-1, H-1′), 4.60 (d, *J* 12.5 Hz, 1H, CH*H*Ph), 4.66 (m, 3H, CH*H*Ph, Cl_3CCH_2O), 4.90 (d, *J* 12.5 Hz, 1H, CH*H*Ph), 4.98 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 5.04–5.25 (m, 3H, H-3′, 2 lipid-3-H), 5.49 (s, 1H, C*H*Ph), 6.02 (d, *J* 5.0 Hz, 1H, NH), 7.40 (m, 15H, Ar–H). Anal. Calcd for $C_{92}H_{145}Cl_3N_2O_{17}{\cdot}0.8H_2O$ (1657.52): C, 66.09; H, 8.84; N, 1.67. Found: C, 66.06; H, 8.84; N, 1.64.

3.1.17. Benzyl 4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (25). To the mixture of compound **24** (670 mg, 0.405 mmol), **35** (270 mg, 0.81 mmol), DCC (208 mg, 1.01 mmol) and DMAP (25 mg, 0.20 mmol) was added dry CH_2Cl_2 (10 mL). The reaction mixture was stirred at room temperature for 72 h. The solid was filtered and washed with CH_2Cl_2. The filtrate was concentrated under diminished pressure and the residue was purified by flash chromatography (CH_2Cl_2–hexane–acetone, 2:1:3%; and 1% MeOH in CH_2Cl_2) to give **25** (570 mg, 71%). R_f 0.60 (CH_2Cl_2–hexane–acetone, 10:5:1); $[\alpha]_D^{22}$ −20.0 (*c* 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, *J* 7.0 Hz, 15H, $5CH_3$), 1.25 (m, 74H, $37CH_2$), 1.53 (m, 10H, $5CH_2$), 2.17 (t, *J* 7.5 Hz, 2H, CH_2), 2.22 (dd, *J* 15.0, 6.0 Hz, 1H, CH*H*), 2.24 (t, *J* 7.5 Hz, 2H, CH_2), 2.34 (dd, *J* 15.0, 6.5 Hz, 1H, CH*H*), 2.45 (dd, *J* 16.0, 5.0 Hz, 1H, CH*H*), 2.50 (dd, *J* 15.5, 5.5 Hz, 1H, CH*H*), 2.55 (dd, *J* 16.0, 7.5 Hz, 1H, CH*H*), 2.59 (dd, *J* 15.5, 7.5 Hz, 1H, CH*H*), 3.38 (ddd, *J* 10.0, 10.0, 5.0 Hz, 1H, H-5′), 3.56 (m, 2H, H-4, H-5), 3.62 (m, 1H, H-2), 3.64 (dd, *J* 10.0, 10.0 Hz, 1H, H-4′), 3.69 (dd, *J* 11.0, 5.0 Hz, 1H, H-6a), 3.76 (dd, *J* 10.0, 10,0 Hz, 1H, H-6′a), 3.83 (m, 1H, lipid-3-H), 3.95 (m, 1H, H-2′), 4.05 (br d, *J* 11.0 Hz, 1H, H-6b), 4.32 (dd, *J* 10.0, 5.0 Hz, 1H, H-6′b), 4.45 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.48 (d, *J* 11.0 Hz, 2H, 2CH*H*Ph), 4.51 (d, *J* 8.0 Hz, 1H, H-1), 4.59 (d, *J* 8.0 Hz, 1H, H-1′), 4.60–4.67 (m, 4H, 2CH*H*Ph, Cl_3CCH_2O), 4.85 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 5.01 (m, 1H, lipid-3-H), 5.12 (d, *J* 9.0 Hz, 1H, NH), 5.19 (m, 4H, H-3, H-3′, 2 lipid-3-H), 5.48 (s, 1H, C*H*Ph), 5.71 (d, *J* 8.0 Hz, 1H, NH), 7.20–7.45 (m, 20H, Ar–H). Anal. Calcd for $C_{113}H_{177}Cl_3N_2O_{19}$ (1974.00): C, 68.76; H, 9.04; N, 1.42. Found: C, 68.68; H, 9.10; N, 1.39.

3.1.18. Benzyl 2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-3,4-di-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (26). Compound **22** (224 mg, 0.126 mmol) was treated with zinc dust (5.0 g) in acetic acid–EtOAc (4:1, 300 mL) at room temperature for 24 h. The solid was filtered and washed with CH_2Cl_2, and the filtrate concentrated under diminished pressure. The residue was re-dissolved in CH_2Cl_2 (200 mL) and the soln washed with saturated aq bicarbonate soln (20 mL). The organic layer was dried with sodium sulfate and concentrated to give the free amine (192 mg, 95%).

The free amine (175 mg, 0.11 mmol), **18** (101 mg, 0.22 mmol) and DCC (68 mg, 0.33 mmol) were dissolved in dry CH_2Cl_2 (5 mL) and the mixture was stirred at room temperature for 48 h. The solid was filtered and washed with CH_2Cl_2. The filtrate was concentrated under diminished pressure and the residue purified by repeated flash chromatography (CH_2Cl_2–hexane–acetone, 2:1:3%, and CH_2Cl_2–MeOH, 100:1) to give **26** (150 mg, 67%). R_f 0.27 (2% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$ −14.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, *J* 7.0 Hz, 18H, $6CH_3$), 1.25 (m, 114H, $57CH_2$), 1.53 (m, 12H, $6CH_2$), 2.15 (t, *J* 7.0 Hz, 4H, $2CH_2$), 2.23–2.39 (m, 6H, $3CH_2$), 2.56 (dd, *J* 15.5, 5.5 Hz, 1H, CH*H*), 2.60 (dd, *J* 15.5, 7.0 Hz, 1H, CH*H*), 3.37–4.00 (m, 9H, H-2, H-3, H-4, H-5, H-6a, H-2′, H-4′, H-5′, H-6′a), 4.09 (dd, *J* 11.0, 2.0 Hz, 1H, H-6b), 4.27 (dd, *J* 11.0, 4.5 Hz, 1H, H-6′b), 4.58–4.88 (m, 7H, $3CH_2$Ph, H-1′), 4.82 (d, *J* 7.5 Hz, 1H, H-1), 5.00–5.09 (m, 2H, 2 lipid-3-H), 5.16 (m, 1H, lipid-3-H), 5.26 (dd, *J* 10.0, 10.0 Hz, 1H, H-3′), 5.47 (s, 1H, CHPh), 5.93 (d, *J* 8.5 Hz, 1H, NH), 6.06 (d, *J* 8.0 Hz, 1H, NH), 7.25–7.45 (m, 20H, Ar–H). Anal. Calcd for $C_{124}H_{202}N_2O_{18}\cdot 0.5H_2O$ (2008.96): C, 73.80; H, 10.14; N, 1.39. Found: C, 73.64; H, 9.88; N, 1.41.

3.1.19. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (27). In a similar way as described for the preparation of **26**, compound **23** (350 mg, 0.206 mmol) was converted to free amine (314 mg, 100%), which was coupled with **18** (191 mg, 0.42 mmol) in the presence of DCC (130 mg, 0.62 mmol) to give **27** (226 mg, 56%). R_f 0.25 (5% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −16.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J* 6.5 Hz, 18H, $6CH_3$), 1.23 (br s, 114H, $57CH_2$), 1.55 (m, 12H, $6CH_2$), 2.13–2.48 (m, 10H, $5CH_2$), 2.50 (dd, *J* 15.0, 5.5 Hz, 1H, CH*H*), 2.59 (dd, *J* 15.0, 7.5 Hz, 1H, CH*H*), 3.38–4.24 (m, 12H, H-2, H-3, H-4, H-5, 2H-6, H-2′, H-4′, H-5′, H-6′a, $CH_2CH{=}CH_2$), 4.30 (dd, *J* 10.5, 5.5 Hz, 1H, H-6′b), 4.58 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.59 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.73 (d, *J* 8.5 Hz, 1H, H-1′), 4.77 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.83 (d, *J* 8.0 Hz, 1H, H-1), 4.85 (d, *J* 11.0 Hz, 1H, CH*H*Ph), 4.99–5.30 (m, 6H, H-3′, $CH_2{=}CH$, 3 lipid-3-H), 5.48 (s, 1H, CHPh), 5.87 (m, 1H, $CH_2{=}CH$), 5.91 (d, *J* 8.5 Hz, 1H, NH), 6.07 (d, *J* 8.0 Hz, 1H, NH), 7.35 (m, 15H, Ar–H). Anal. Calcd for $C_{120}H_{200}N_2O_{18}$ (1958.90): C, 73.58; H, 10.30; N, 1.43. Found: C, 73.40; H, 10.70; N, 1.39.

3.1.20. Benzyl 4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-deoxy-6-*O*-{2-deoxy-4,6-di-*O*-benzylidene-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (28). In a similar way as described for the preparation of **26**, compound **25** (550 mg, 0.279 mmol) was converted to free amine (500 mg, 100%). The free amine (270 mg, 0.15 mmol) was coupled with **18** (205 mg, 0.45 mmol) in the presence of DCC (139 mg, 0.68 mmol) to give **28** (240 mg, 72%). R_f 0.36 (5% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −19.6 (*c* 0.7, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J* 6.5 Hz, 21H, $7CH_3$), 1.25 (br s, 132H, $66CH_2$), 1.50 (m, 14H, $7CH_2$), 2.14–2.65 (m, 14H, $7CH_2$), 3.40–4.50 (m, 10H, H-2, H-4, H-5, H-6a/b, H-2′, H-4′, H-5′, H-6′a, lipid-3-H), 4.31 (dd, J_{gem} 10.5, $J_{6'b,5'}$ 4.5 Hz, 1H, H-6′b), 4.44 (d, J_{gem} 11.0 Hz, 1H, CH*H*Ph), 4.50 (d, J_{gem} 11.0 Hz, 2H, 2CH*H*Ph), 4.58 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 4.59 (d, 1H, CH_2Ph), 4.63 (d, J_{gem} 12.0 Hz, 1H, CH*H*Ph), 4.75 (d, $J_{1',2'}$ 8.5 Hz, 1H, H-1′), 4.85 (d, 1H, CH*H*Ph), 5.04 (m, 2H, 2 lipid-3-H), 5.15 (m, 2H, H-3, lipid-3-H), 5.27 (dd, $J_{3'2'} = J_{3',4'}$ 10.0 Hz, 1H, H-3′), 5.48 (s, 1H, CHPh), 5.80 (d, *J* 9.0 Hz, 1H, NH), 5.93 (d, *J* 8.5 Hz, 1H, NH), 7.20–7.45 (m, 20H, Ar–H). Anal. Calcd for $C_{138}H_{228}N_2O_{20}$ (2235.32): C, 74.15; H, 10.28; N, 1.25. Found: C, 74.00; H, 10.63; N, 1.40.

3.1.21. Benzyl 2-deoxy-6-*O*-{6-*O*-benzyl-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-3,4-di-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (29). To the soln of **26** (135 mg, 0.067 mmol) in dry tetrahydrofuran (8.0 mL), molecular sieves (4 Å, 0.5 g) were added and the mixture was stirred at room temperature for 20 min. Sodium cyanoborohydride (211 mg, 3.36 mmol) was added and the mixture was cooled to 0 °C and HCl(g)–Et_2O soln was added dropwise till no gas was evolved. Additional sodium cyanoborohydride (211 mg, 3.36 mmol) was added, followed by slow addition of HCl(g)–Et_2O until no gas was formed. The mixture was poured into satd aq $NaHCO_3$ soln (50 mL) and extracted with EtOAc (100 mL × 3). The organic layer was washed with satd sodium chloride soln (50 mL), dried over sodium sulfate, and concentrated. The residue was purified by flash chromatography (2–5% acetone in $CHCl_3$) to afford **29** (112 mg, 83%). R_f 0.20 (2% MeOH in CH_2Cl_2); $[\alpha]_D^{22}$ −13.5 (*c* 0.6, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J*

6.5 Hz, 18H, $6CH_3$), 1.25 (m, 114H, $57CH_2$), 1.50 (m, 12H, $6CH_2$), 2.14 (t, *J* 7.0 Hz, 2H, CH_2), 2.23–2.60 (m, 10H, $5CH_2$), 3.33 (d, *J* 3.3 Hz, 1H, OH), 3.44–3.96 (m, 10H, H-2, H-3, H-4, H-5, H-6a, H-2′, H-4′, H-5′, 2H-6′), 4.09 (dd, *J* 10.0, 2.0 Hz, 1H, H-6b), 4.49–4.86 (m, 9H, 4C*H*$_2$Ph, H-1′), 4.80 (d, *J* 7.5 Hz, 1H, H-1), 4.92–5.18 (m, 4H, H-3′, 3 lipid-3-H), 5.80 (d, *J* 9.0 Hz, 1H, NH), 5.95 (d, *J* 8.5 Hz, 1H, NH), 7.30 (m, 20H, Ar–H). Anal. Calcd for $C_{124}H_{204}N_2O_{18}\cdot H_2O$ (2010.98): C, 73.40; H, 10.23; N, 1.38. Found: C, 73.40; H, 10.04; N, 1.38.

3.1.22. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-6-*O*-{2-deoxy-6-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (30). In a similar way as described for the preparation of **29**, compound **27** (194 mg, 0.10 mmol) was converted to **30** (130 mg, 67%). R_f 0.22 (8% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −9.6 (*c* 0.5, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.89 (t, *J* 7.0 Hz, 18H, $6CH_3$), 1.25 (br s, 114H, $57CH_2$), 1.49–1.58 (m, 12H, $6CH_2$), 2.21 (t, *J* 8.0 Hz, 2H, CH_2), 2.26 (m, 3H, CH_2, CH*H*), 2.27 (t, *J* 8.0 Hz, 2H, CH_2), 2.33 (dd, *J* 14.0, 6.0 Hz, 1H, CH*H*), 2.36 (dd, *J* 15.0, 6.5 Hz, 1H, CH*H*), 2.43 (dd, *J* 15.0, 6.5 Hz, 1H, CH*H*), 2.50 (dd, *J* 16.5, 6.50 Hz, 1H, CH*H*), 2.53 (dd, *J* 16.5, 8.0 Hz, 1H, CH*H*), 3.28 (d, *J* 3.0 Hz, 1H, OH), 3.43 (m, 2H, H-4, H-5′), 3.54 (m, 2H, H-2, H-6a), 3.62 (ddd, *J* 10.0, 9.0, 3.0 Hz, 1H, H-4′), 3.71 (m, 3H, H-5, 2H-6′), 3.84 (m, 2H, H-3, H-2′), 4.06 (dd, *J* 11.0, 2.5 Hz, 1H, H-6b), 4.10–4.20 (m, 2H, C*H*$_2$CH=CH$_2$), 4.52 (d, *J* 12.0 Hz, 1H, CH*H*Ph), 4.58 (m, 4H, H-1′, 3CH*H*Ph), 4.74 (d, *J* 10.5 Hz, 1H, CH*H*Ph), 4.80 (d, *J* 8.0 Hz, 1H, H-1), 4.83 (d, *J* 11.5 Hz, 1H, CH*H*Ph), 4.95 (dd, *J* 10.0, 9.0 Hz, 1H, H-3′), 5.02 (m, 1H, lipid-3-H), 5.10 (m, 3H, 2 lipid-3-H, CH*H*=CH), 5.22 (m, 1H, CH*H*=CH), 5.77 (d, *J* 9.0 Hz, 1H, NH), 5.85 (m, 1H, CH$_2$=C*H*), 5.98 (d, *J* 8.0 Hz, 1H, NH), 7.30 (m, 15H, Ar–H). Anal. Calcd for $C_{120}H_{202}N_2O_{18}$ (1960.92): C, 73.50; H, 10.38; N, 1.43. Found: C, 73.25; H, 10.95; N, 1.60.

3.1.23. Benzyl 4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-6-*O*-{6-*O*-benzyl-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(3*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (31). In a similar way as described for the preparation of **29**, compound **28** (233 mg, 0.104 mmol) was converted to **31** (166 mg, 71%). R_f 0.32 (8% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −14.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J* 6.5 Hz, 21H, $7CH_3$), 1.24 (br s, 132H, $66CH_2$), 1.57 (m, 14H, $7CH_2$), 2.18–2.62 (m, 14H, $7CH_2$), 3.32 (d, $J_{4',OH}$ 3.0 Hz, 1H, OH), 3.44–4.05 (m, 11H, H-2, H-4, H-5, H-6a/b, H-2′, H-4′, H-5′, H-6′a/b, lipid-3-H), 4.42 (d, J_{gem} 12.0 Hz, 1H, CH*H*Ph), 4.47–4.61 (m, 8H, H-1, H-1′, 6CH*H*PH), 4.83 (d, J_{gem} 12.0 Hz, 1H, CH*H*Ph), 4.93–5.18 (m, 5H, H-3, H-3′, 3 lipid-3-H), 5.72 (d, *J* 9.5 Hz, 1H, NH), 5.81 (d, *J* 9.0 Hz, 1H, NH), 7.30 (m, 20H, Ar–H). Anal. Calcd for $C_{138}H_{230}N_2O_{20}$ (2237.34): C, 74.08; H, 10.37; N, 1.25. Found: C73.75; H, 10.41; N, 1.41.

3.1.24. Benzyl 2-deoxy-6-*O*-{6-*O*-benzyl-4-*O*-(di-*O*-benzyl-phosphono)-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-3,4-di-*O*-benzyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (32). To the soln of **29** (61 mg, 0.030 mmol) in dry CH_2Cl_2 (3 mL) were added 1*H*-tetrazole (12.6 mg, 0.18 mmol) and dibenzyl diisopropylphosphoramidite (42 mg, 0.041 mL, 0.12 mmol). The mixture was stirred at room temperature for 30 min and then cooled to 0 °C. *m*-Chloroperbenzoic acid (*m*-CPBA, 75 mg, 55%, 0.24 mmol) was added and the mixture was stirred for 30 min at 0 °C. The mixture was then poured into 10% aq $NaHSO_3$ soln (10 mL) and extracted with CH_2Cl_2 (10 mL × 3). The combined organic layer was washed with satd aq $NaHCO_3$ soln (10 mL), dried with sodium sulfate and concentrated. The residue was purified by repeated flash chromatography (1–5% acetone in $CHCl_3$ and then toluene–acetone, from 18:1 to 12:1) to afford **32** (58 mg, 85%). R_f 0.17 (1% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −3.1 (*c* 0.35, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (t, *J* 6.5 Hz, 18H, $6CH_3$), 1.24 (m, 114H, $57CH_2$), 1.40–1.57 (m, 12H, $6CH_2$), 2.11–2.50 (m, 12H, $6CH_2$), 3.52–3.94 (m, 9H, H-2, H-3, H-4, H-5, H-6a, H-2′, H-5′, 2H-6′), 4.09 (dd, *J* 11.0, 2.0 Hz, 1H, H-6b), 4.44 (m, 3H, C*H*$_2$Ph, H-4′), 4.56–4.90 (m, 12H, 6C*H*$_2$Ph), 4.78 (d, *J* 8.0 Hz, 1H, H-1′), 4.98 (d, *J* 8.0 Hz, 1H, H-1), 5.05 (m, 2H, 2 lipid-3-H), 5.16 (m, 1H, lipid-3-H), 5.39 (dd, *J* 10.0, 9.0 Hz, 1H, H-3′), 5.88 (d, *J* 8.5 Hz, 1H, NH), 6.08 (d, *J* 8.0 Hz, 1H, NH), 7.25 (m, 30H, Ar–H). Anal. Calcd for $C_{138}H_{217}N_2O_{21}P\cdot 0.5H_2O$ (2271.21): C, 72.69; H, 9.63; N, 1.22. Found: C, 72.45; H, 9.32; N, 1.19.

3.1.25. Benzyl 3-*O*-allyl-4-*O*-benzyl-2-deoxy-6-*O*-{2-deoxy-6-*O*-benzyl-4-*O*-(di-*O*-benzyl-phosphono)-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (33). In a similar way as described for the preparation of **32**, compound **30** (117 mg, 0.060 mmol) was converted to **33** (81 mg, 61%) and purified by repeated flash chromatography (1–3% acetone in $CHCl_3$; toluene–acetone, from 15:1 and 12:1; hexane–acetone, 6:1 and 5:1). R_f 0.46 (9% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −4.8 (*c* 0.33, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J* 6.5 Hz, 18H, $6CH_3$), 1.25 (br s, 114H, $57CH_2$), 1.45–1.55 (m, 12H, $6CH_2$), 2.19–2.51 (m, 12H, $6CH_2$), 3.45–4.23 (m, 12H, H-2, H-3, H-4, H-5, 2H-6, H-2′, H-5′, 2H-6′,

CH_2CH=CH_2), 4.50 (m, 3H, H-4′, CH_2Ph), 4.58 (d, J 12.5 Hz, 2H, 2CHHPh), 4.75 (d, J 11.0 Hz, 1H, CHHPh), 4.80 (d, J 8.0 Hz, 1H, H-1), 4.88 (m, 5H, 5CHHPh), 4.99 (d, J 8.0 Hz, 1H, H-1′), 5.05–5.26 (m, 5H, 3 lipid-3-H, CH_2=CH), 5.41 (dd, J 10.0, 9.0 Hz, 1H, H-3′), 5.86 (m, 1H, CH$_2$=CH), 5.93 (d, J 8.0 Hz, 1H, NH), 6.09 (d, J 7.5 Hz, 1H, NH), 7.30 (m, 25H, Ar–H). Anal. Calcd for $C_{134}H_{215}N_2O_{21}P$ (2221.15): C, 72.46; H, 9.76; N, 1.26. Found: C, 72.21; H, 9.92; N, 1.27.

3.1.26. Benzyl 4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-6-*O*-{6-*O*-benzyl-4-*O*-(di-*O*-benzyl-phosphono)-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside (34). In a similar way as described for the preparation of **32**, compound **31** (150 mg, 0.067 mmol) was converted to **34** (98 mg, 59%) after repeated purification by flash chromatography ($CHCl_3$–acetone, 100:3; toluene–acetone, 16:1 then 12:1; hexane–acetone, 8:1 then 6:1). R_f 0.27 (5% acetone in $CHCl_3$); $[\alpha]_D^{22}$ −8.5 (c 0.33, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J 6.5 Hz, 21H, 7CH_3), 1.24 (br s, 132H, 66CH_2), 1.54 (m, 14H, 7CH_2), 2.16–2.55 (m, 14H, 7CH_2), 3.52–4.06 (m, 10H, H-2, H-4, H-5, H-6a/b, H-2′, H-5′, H-6′a/b, lipid-3-H), 4.38–4.91 (m, 14H, H-1, H-4′, 6CH_2Ph), 5.00 (d, $J_{1',2'}$ 8.0 Hz, 1H, H-1′), 5.03–5.22 (m, 4H, H-3, 3 lipid-3-H), 5.38 (dd, $J_{3',2'}$ 10.0, $J_{3',4'}$ 9.0 Hz, 1H, H-3′), 5.66 (d, J 9.0 Hz, 1H, NH), 6.07 (d, J 8.0 Hz, 1H, NH), 7.30 (m, 30H, Ar–H). Anal. Calcd for $C_{152}H_{243}N_2O_{23}P$ (2497.57): C, 73.09; H, 9.81; N, 1.12. Found: C, 72.83; H, 9.96; N, 1.13.

3.1.27. 2-Deoxy-6-*O*-{2-deoxy-4-*O*-phosphono-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-α/β-D-glucopyranose (1). To the soln of **32** (64 mg, 0.028 mmol) in THF–HOAc (10:1, 60 mL) was added palladium on charcoal (5%, 60 mg). The mixture was stirred at room temperature under hydrogen atmosphere for 24 h. The solid was filtered off and the filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography ($CHCl_3$–MeOH–water, 4:1:0 and then 3:1:0.1) to give compound **1** (30 mg, 62%). R_f 0.35 ($CHCl_3$–MeOH–water, 3:1:0.1); $[\alpha]_D^{22}$ −10.0 (c 0.1, $CHCl_3$–MeOH, 4:1); ESIMS Calcd for $C_{96}H_{181}N_2O_{21}P$: 1729.3. Found (negative mode): 1728.3 [M−H$^-$].

3.1.28. 2-Deoxy-6-*O*-{2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-4-*O*-phosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-3-*O*-propyl-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-α/β-D-glucopyranose (2). In a similar way as described for the preparation of **1**, compound **33** (73 mg, 0.035 mmol) was converted to **2** (55 mg, 95%). R_f 0.35 ($CHCl_3$–MeOH–water, 3:1:0.1); $[\alpha]_D^{22}$ +6.0 (c 0.1, $CHCl_3$–MeOH, 4:1); ESIMS Calcd for $C_{99}H_{187}N_2O_{21}P$: 1771.3. Found (negative mode): 1770.3 [M−H$^-$], 1771.3 ([M−H$^-$], M+1 isotope peak).

3.1.29. 2-Deoxy-6-*O*-{2-deoxy-4-*O*-phosphono-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-β-D-glucopyranosyl}-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-α/β-D-glucopyranose (3). In a similar way as described for the preparation of **1**, compound **34** (85 mg, 0.034 mmol) was converted to **3** (50 mg, 75%). R_f 0.46 ($CHCl_3$–MeOH–water, 3:1:1); $[\alpha]_D^{20}$ −6.6 (c 0.1, $CHCl_3$–MeOH, 4:1); ESIMS Calcd for $C_{110}H_{207}N_2O_{23}P$: 1955.5. Found (negative mode): 1954.5 [M−H$^-$].

3.2. Biological evaluation

3.2.1. General method for preparation of liposomal vaccine formulation. Typically, the liposomal formulation is composed of MUC1 mucin-derived 25-mer lipopeptide BLP25,[18] monophosphoryl lipid A analogue or R595 lipid A, and lipids such as cholesterol, dimyristoyl phosphatidylglycerol (DMPC), and dipalmitoyl phosphatidylcholine (DPPC) in saline (0.9% NaCl soln). The liposomal construct is formulated by first dissolving the phospholipids, cholesterol and lipid A analogue in *tert*-butanol at 50–60 °C. Lipopeptide and water (5%, v/v) are then added to the *tert*-butanol soln. The resulting *tert*-butanol soln is injected into about 4 vol of rapidly stirred water at 50–55 °C, using a glass syringe with an 18-gauge needle. The small unilamellar vesicles (SUV) formed in this process are cooled, sterilized by filtration through a 0.22 μm membrane filter, filled into vials and lyophilized. The dry powder is re-hydrated with sterile saline before injection, resulting in the formation of multi-lamellar large vesicles (MLV). The so formed BLP25 liposomal vaccine formulation is used to immunize mice (injection dose, 100 μL).

3.2.2. Immunization of mice with liposomal vaccines. Groups of C57Bl/6 mice are immunized intradermally with BLP25 liposomal vaccine containing 40 μg of MUC1 mucin-based 25-mer lipopeptide as an antigen and 20 μg of one monophosphoryl lipid A analogue (**1**–**3**) 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 to determine the immune responses by measuring the antigen specific T-cell proliferation in vitro.

3.2.3. 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 antigen presenting cells (APCs). To each well 20 μg of MUC1-based 25-mer peptide[18] 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 (1410 LC Counter, Wallac, Turku, Finland). T-Cell proliferation results corresponding to various liposomal vaccines adjuvanted with compounds **1**, **2**, **3** or the reference natural R595 lipid A are shown in Figure 1.

3.2.4. Measurement of interferon-gamma (IFN-γ) cytokine. Interferon-gamma (IFN-γ) levels are determined in the cell culture supernatants using enzyme-linked immunoabsorbent assay (ELISA) as previously described.[36] Briefly, 96-Well plates are coated with 50 μL of catcher MAbs in 50 μL of R4.6AZ at 37 °C for 30 min. The plates are then washed and incubated with test samples for 45 min. After two washes, the second biotinylated antibody, XMG1.2, is added. After washing, peroxidase-conjugated streptavidin is added and incubated again for 30 min. Finally, 100 μL of horseradish peroxidase (HRPO) substrate soln is added. The optical density is measured with a Thermomax ELISA reader at 405 nm wavelength in kinetic mode for 10 min. Cytokine levels in the test samples are determined by comparison with reference standard. IFN-γ data reported in Figure 1 are averaged from sextet experimental measurement.

3.2.5. Measurement of lethal toxicity in mice. Actinomycin D-enhanced lethal toxicity of monophosphoryl lipid A analogue **3** and the natural R595 lipid A in C57 Black mice is determined according to the method described by Rose and Bradley.[33] Freshly prepared lipid A soln at 1 mg/1 mL in 20% DMSO/saline (v/v) is diluted with saline to the desired concentration (50, 10 and 2 μg doses in 500 μL). Groups of three mice are intraperitoneally (i.p.) injected with various doses of lipid A soln. Twenty minutes later, all groups of mice are injected with 500 μL of actinomycin D soln, which is prepared by dissolving 5 mg of actinomycin D in 1 mL of ethanol and diluting with saline to give a dose of 550 μg in 500 μL. Mice are then observed for mortality and any other symptoms of toxicity within 24 h of injection. No further death is observed after 24 h and the experiment is terminated 4 days later. The control group is injected with saline.

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