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Carbohydrate Research 341 (2006) 2901–2911
Carbohydrate RESEARCH

Synthesis of *Staphylococcus aureus* lipoteichoic acid derivatives for determining the minimal structural requirements for cytokine induction

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Received 24 August 2006; accepted 3 October 2006
Available online 10 October 2006

Abstract—For the investigation of the minimal structural requirements for cytokine induction, *Staphylococcus aureus* lipoteichoic acid derivatives with two, three, four, and five glycerophosphate backbone moieties, carrying each a D-alanyl residue, were needed. Based on two different glycerophosphate building blocks and 6b-*O*-phosphitylated gentiobiosyl diacylglycerol the desired target molecules (compounds **1**–**4**) could be readily obtained and provided for biological studies.

Keywords: Bacteria; Gram-positive; Lipoteichoic acid; Synthesis; Modifications

1. Introduction

During infection, the recognition of conserved bacterial structures called pathogen-associated molecular patterns occurs via receptor recognition on immune cells and leads to the activation of the innate immune system resulting in the release of a variety of cytokines. Lipopolysaccharides (LPS) have been known as the most important conserved bacterial structures of Gram-negative bacteria inducing cytokine release for more than 50 years.[1] Immune recognition takes place by the binding of LPS to the toll-like receptor 4 (TLR4) involving also other co-factors.[2–4]

The immunostimulatory component of Gram-positive bacteria was not clear for a long time, although a structural counterpart of LPS called lipoteichoic acid (LTA) was found in the bacterial membrane; this LTA is also an amphiphilic molecule with a lipid anchor and a generally negatively charged glycerophosphate backbone. An improved preparation procedure applied to the isolation of LTA from *Staphylococcus aureus* led to biologically active LTA,[5–7] whose structure could be assigned by NMR and MS data.[5,8] The receptor for LTA recognition is TLR2[9] accompanied by the co-factors TLR6,[7] CD14,[10,11] and CD36.[12]

Modifications of the LTA structure gave information on the prerequisites for the induction of cytokine release. For instance, a complete deacylation led to less active material and selective removal of the D-alanyl residues from the glycerophosphate backbone which strongly reduced the immunostimulatory potency.[8] This result indicated that the lipid anchor and also the D-alanyl residues are essential for the immunostimulatory potency. The chemical synthesis of a truncated LTA, based on the native LTA structure of *S. aureus*, confirmed these results.[13,14] To determine the key components for immune cell activation, several LTA derivatives were synthesized starting from a molecule with two lipid anchors (=two gentiobiosyl-diacylglycerol anchors) and a backbone with six glycerophosphate units substituted

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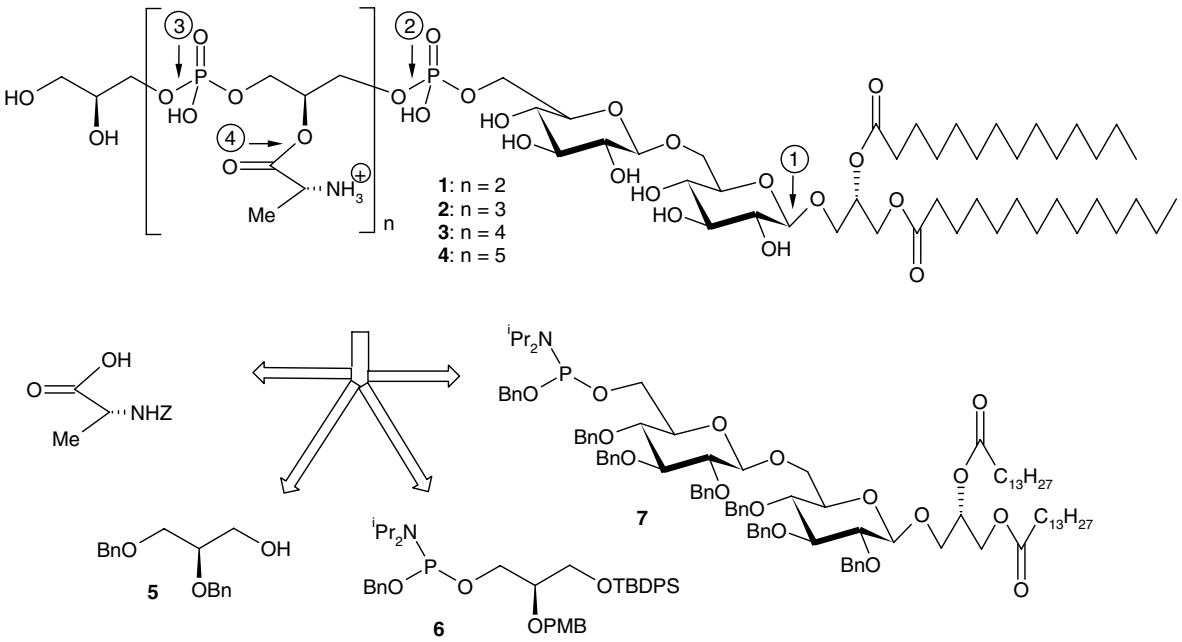
doi:10.1016/j.carres.2006.10.003



Scheme 1. Retrosynthetic scheme for target molecules **1**–**4**.

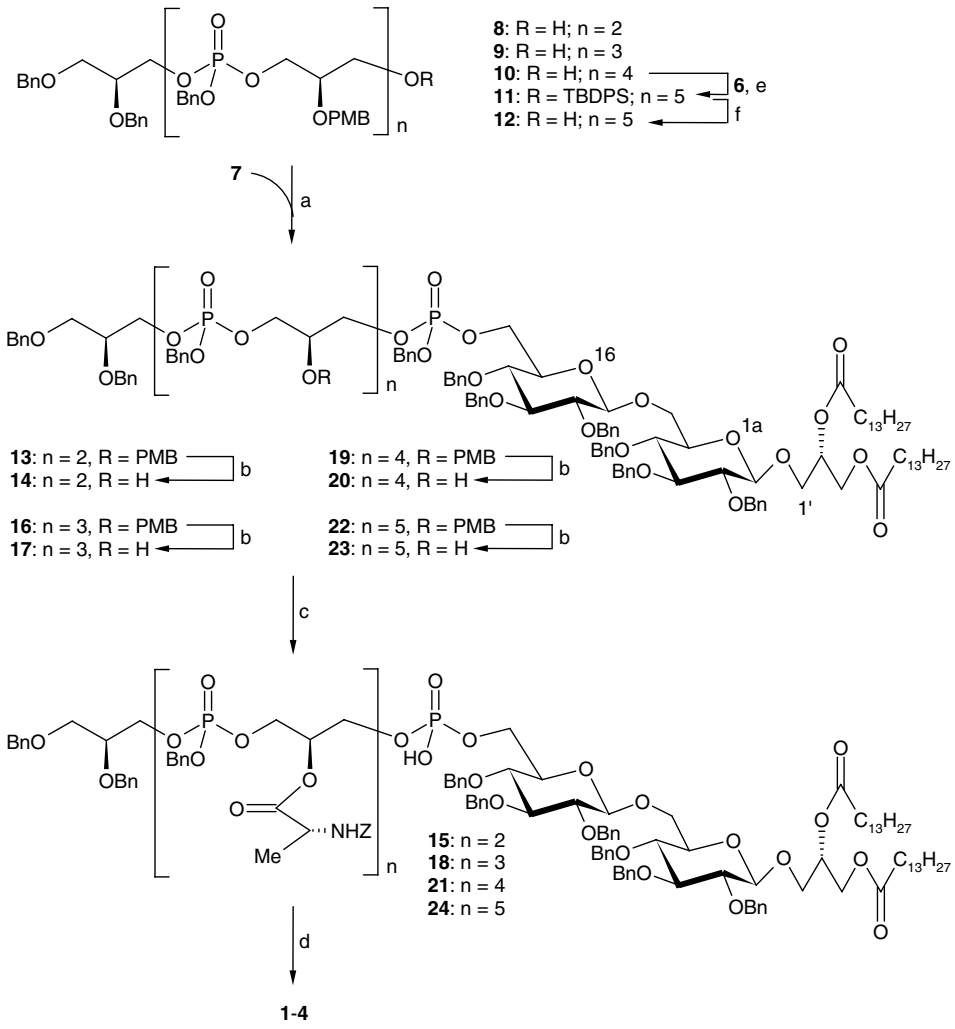
by four D-alanine residues and one *N*-acetyl-D-glucosamine residue which was more potent than natural LTA.[15] Neither the absence of the gentiobiose residue nor the loss of *N*-acetyl-D-glucosamine altered the ability of LTA with one lipid anchor to induce cytokine release; only replacement of D-alanine by L-alanine blunted the cytokine-inducing potency.[16,17] These results approximated the crucial pattern required for the immune recognition of LTA and prompted us to synthesize further LTA derivatives with a reduced structure down to the synthetic anchor, namely gentiobiosyl-diacylglycerol, in order to determine the minimal structural requirements for cytokine induction. For these studies compounds **1**–**4** (Scheme 1) were needed. The synthesis of these compounds is described in this paper.

2. Results and discussion

The retrosynthesis of compounds **1**–**4** is shown in Scheme 1. Disintegrations ①–④ lead to building blocks **5**–**7** and to *N*-benzyloxy-carbonyl (Z)-protected D-alanine. These building blocks consider the presence of gentiobiosyl diacylglycerol, *O*-(D-alanyl)-glycerol, and 2-*O*-nonsubstituted glycerol residues, respectively, and their sequence specific linkage via mixed phosphorous diester bonds. Also the most important aspect, the hydrolytic lability of the D-alanyl residues, which are readily cleaved at pH 8.5,[13] is taken into account: as temporary protecting group for building block **6**, the *p*-methoxylbenzyl (PMB) group is chosen which can be selectively cleaved after completion of the backbone synthesis. Following the attachment of D-alanyl residues with Z-protected D-alanine and then a complete O-debenzylation will provide the target molecules.

The synthesis of the previously designed building blocks **5**–**7** and also the sequence specific synthesis of oligomers **8**–**10** (Scheme 2) has been already reported.[14,15,17] From gentiobiose building block **7**[15] and glycerophosphate oligomer **8**,[17] possessing the required protecting group for regioselective chain extension and following D-alanyl residue attachment, the synthesis of target molecule **1** could be readily accomplished. Ligation of **7** and **8** in the presence of tetrazole and then oxidation with *tert*-butylhydroperoxide gave phosphate linked intermediate **13**, which contains the backbone of the target molecule with $n = 2$. The treatment of **13** with ceric(IV) ammonium nitrate (CAN)[18] liberated two of the glycerol hydroxy groups affording compound **14**. Attachment of the Z-protected D-alanyl residue was performed with excess Z-protected D-alanine in the presence of (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)[19]/*N*-methylimidazole (Me–Im) as condensing agent to give the fully protected target molecule **15**. Hydrogenolysis with Pearlman's catalyst[20] in a mixture of dichloromethane/methanol/water gave the desired final product **1** with two D-alanyl residues.

Similarly, from **7** and **9**[17] or **10**[17] compounds **16** and **19**, respectively, were obtained, which were transformed into the partially deprotected compounds **18** and **21** and then into target molecules **2** and **3**, having three or four D-alanyl residues. For the synthesis of target molecule **4**, compound **10** had to be chain extended with building block **6**.[17] The activation of **6** with tetrazole led to the desired phosphite intermediate which on oxidation with



Scheme 2. Synthesis of target molecules **1**–**4**. Reagents and conditions: (a) Tetrazole, CH_2Cl_2; *t*-BuO_2H (**13**: 73%; **16**: 84%; **19**: 74%; **22**: 67%); (b) CAN, MeCN/Tol/H_2O, −10 °C→rt (**14**: 72%; **17**: 87%; **20**: 85%; **23**: 80%); (c) (*Z*)-D-Ala, PyBOP, Me–Im, CH_2Cl_2 (**15**: 92%; **18**: 56%; **21**: 69%; **24**: 58%); (d) $Pd(OH)_2$, H_2, CH_2Cl_2/MeOH/H_2O (**1**: 32%; **2**: 41%; **3**: 26%; **4**: 28%); (e) tetrazole, CH_2Cl_2; *t*-BuO_2H (83%); (f) TBAF, THF (80%).

tert-butyl hydroperoxide furnished the phosphate **11** having six glycerol and five phosphate residues. Desilylation with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran led to intermediate **12**; further chain extension with gentiobiosyl diacylglycerol derivative **7** furnished compound **22**. Cleavage of the PMB groups (→**23**), then the attachment of five Z-protected D-alanyl residues (→**24**), and final deprotection afforded target molecule **4**.

In summary, the previously designed building blocks for the synthesis of *S. aureus* LTA proved to be highly versatile, thus permitting the preparation of variously modified LTAs. This cassette containing about six building blocks laid the basis for successful structure-activity relationship studies.[21]

The biological studies with compounds **1**–**4** and some previously prepared *S. aureus* LTA modifications revealed the following:[21] The synthetic lipid anchor alone was not sufficient to induce cytokine release, however, the addition of three unsubstituted glycerophosphate backbone units exhibits this activity. The TLR2-dependent activation was amplified about 10-fold by substitution with at least two D-alanyl residues (compound **2**)—but not by L-alanyl residues. Hence, with compounds **1**–**4** the minimal structural requirement for LTA pattern recognition by immune cells could be defined.

3. Experimental

3.1. General methods

Solvents were dried according to standard procedures. NMR spectroscopic measurements were performed at 22 °C with a Bruker DRX600 and Bruker AC250

instruments. TMS or the resonances of the deuterated solvents were used as an internal standard. $CDCl_3$ ($\delta = 7.24$ ppm) was used as an external standard; 85% of phosphoric acid was used as an external standard for ^{31}P spectra. MALDI mass spectra were recorded with a Kratos Kompact Maldi II spectrometer; 2,5-dihydroxybenzoic acid (DHB) or *p*-nitroaniline and NaI were used as matrices for positive measurements, and trihydroxyacetophenone (THAP) was used as the matrix for negative mode measurements. Optical rotations were measured with a Perkin–Elmer polarimeter 241/MS in a 1-dm cell at 22 °C. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 F_{254} plastic plates. The compounds were visualized by a treatment with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (20 g) and $Ce(SO_4)_2$ (0.4 g) in 10% H_2SO_4 (400 mL). Flash chromatography was performed on J.T. Baker Silica Gel 60 (0.040–0.063 mm) at a pressure of 0.3 bar. Target molecules were purified by hydrophobic interaction chromatography on octyl-sepharose as the stationary phase and as the elution phase was used as a gradient of propanol (15–60%) in 0.1 M ammonium acetate buffer (pH = 4.8).

3.2. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-1-*O*-*tert*-butyldiphenylsilyl-*sn*-glycerol) (11)

A mixture of the glycerol moiety **10**[17] (1.63 g, 0.94 mmol) and phosphite amide **6**[17] (0.78 g, 1.2 equiv) was co-evaporated with dry CH_2Cl_2 and dried for 1 h in high vacuum. The reaction mixture was dissolved in CH_2Cl_2 (50 mL), and tetrazole was added (132 mg, 1.88 mmol, dried previously 1 h in a high vacuum). The reaction mixture was stirred for 1.5 h at rt (TLC petroleum ether/EtOAc 2:1, 1% NEt_3, R_f 0.74) and *t*-BuOOH (1.5 mL) was added. After 20 min, the reaction mixture was diluted with CH_2Cl_2 and washed with satd $NaHCO_3$ solution, the organic phase was dried over $MgSO_4$ and the solvent was evaporated under diminished pressure. Flash chromatography (2:1 toluene–acetone) yielded phosphate **11** (1.82 g, 83%) as colorless oil. TLC (1:1 toluene–acetone): R_f 0.64, 0.59. $[\alpha]_D$ −3.4 (*c* 1, $CHCl_3$). ^1H NMR (250 MHz, $CDCl_3$): δ 1.02 (s, 9H, *t*-Bu), 3.48–3.58 (m, 2H, 18-H), 3.59–3.81 (m, 23H, 1-H, 2-H, 5-H, 8-H, 11-H, 14-H, 17-H, OMe), 3.90–4.35 (m, 20H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H, 13-H, 15-H, 16-H), 4.39–4.52 (m, 12H, CH_2–Ph), 4.58–4.66 (m, 2H, CH_2–Ph), 4.91–5.06 (m, 10H, POCH_2–Ph), 6.71–6.85 (m, 10H, Ph_{PMB}), 7.10–7.47, 7.59–7.69 (m, 55H, Ph). MALDIMS: *m*/*z* 2355.8 $[M+Na]^+$. Anal. Calcd for $C_{123}H_{143}O_{33}P_5Si$ (2332.4): C, 63.34; H, 6.18. Found: C, 63.17; H, 6.28.

3.3. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycerol) (12)

Phosphate **11** (1.8 g, 0.77 mmol) was dissolved in THF (40 mL) and treated with 1 M solution of TBAF (0.93 mL, 1.2 equiv), the reaction mixture was stirred for 30 min at rt. The reaction mixture was diluted with EtOAc and washed with satd NH_4Cl solution and water, the organic phase was dried over $MgSO_4$ and the solvent was evaporated under diminished pressure. Purification by flash silica gel (1:1 toluene–acetone) yielded compound **12** (1.3 g, 80%) as a colorless syrup. TLC (1:1 toluene–acetone): R_f 0.36. $[\alpha]_D$ −1.2 (*c* 1, $CHCl_3$). ^1H NMR (250 MHz, $CDCl_3$): δ 2.83–3.01 (br s, 1H, OH), 3.49–3.84 (m, 25H, 1-H, 2-H, 5-H, 8-H, 11-H, 14-H, 17-H, 18-H, OMe), 3.91–4.26 (m, 20 H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H, 13-H, 15-H, 16-H), 4.40–4.65 (m, 14H, CH_2–Ph), 4.92–5.09 (m, 10H, POCH_2–Ph), 6.72–6.89 (m, 10H, Ph_{PMB}), 7.13–7.44 (m, 45H, Ph). MALDIMS: *m*/*z* 2117.6 $[M+Na]^+$. Anal. Calcd for $C_{107}H_{125}O_{33}P_5{\cdot}1/2H_2O$ (2103.0): C, 61.11; H, 6.04. Found: C, 61.00; H, 6.16.

3.4. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (13)

Compound **8**[17] (250 mg, 0.250 mmol) and phosphite amide **7**[15] (403 mg, 1.3 equiv) were co-evaporated each with 10 mL of dry CH_2Cl_2 and dried 1h in high vacuum. Phosphite amide **7** was dissolved in 10 mL of dry CH_2Cl_2 and was added, under argon atmosphere, to compound **8**; tetrazole (36 mg, 2 equiv, dried previously 1 h in high vacuum) was also added. The reaction mixture was stirred at rt under argon atmosphere. After 70 min were added dropwise 0.7 mL of *t*-BuOOH, and the reaction mixture was stirred for another 35 min. CH_2Cl_2 was added to dilute and the mixture was washed with satd $NaHCO_3$ solution, the organic phase was dried over $MgSO_4$ and the solvent removed under diminished pressure. Purification by flash chromatography (petroleum ether/EtOAc 2:1) gave compound **13** (464 mg, 73%) as a colorless syrup, which was stored at −20 °C. TLC (petroleum ether/EtOAc 1:1): R_f 0.53 and 0.63. $[\alpha]_D$ +3.4 (*c* 1, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, 6H, Me), 1.08–1.34 (m, 40H, CH_2-

chain), 1.46–1.59 (m, 4H, $COCH_2CH_2R$), 2.17–2.26 (m, 4H, $COCH_2CH_2R$), 3.35 (m, 2H, 2a-H, 5a/b-H), 3,38 (m, 1H, 1′-H), 3.39 (m, 2H, 2b-H, 5a/b-H), 3.47–3.49 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 9-H), 3.60 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.66 (m, 2H, 2-H, 5-H), 3.69 (m, 6H, OMe), 3.73 (m, 1H, 8-H), 3.86 (m, 1H, 1′-H), 3.97–4.05 (m, 8H, 1-H, 3-H, 4-H, 6-H), 4.08 (m, 2H, 7-H, 3′-H), 4.14 (m, 1H, 6a-H), 4.16 (m, 1H, 3′-H), 4.18 (m, 2H, 7-H, 6b-H), 4.23 (m, 1H, 1a-H), 4.27 (m, 1H, 6b-H), 4.43 (m, 1H, 1b-H), 4.44–4.93 (m, 20H, CH_2Ph), 4.95–5.04 (m, 6H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 6.70–6.83 (m, 4H, Ph_{MPM}), 7.08–7.37 (m, 59H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 55.1 (3C, OMe), 62.9 (1C, C-3′), 65.8 (4C, C-1, C-3, C-4, C-6), 66.5 (1C, C-6b), 67.0 (1C, C-8), 68.1 (1C, C-1′), 68.7 (1C, C-6a), 69.2 (1C, C-9), 69.5 (3C, $POCH_2Ph$), 69.8 (1C, C-2′), 71.5–76.5 (10 C, CH_2Ph), 73.6 (1C, C-5a/b), 75.2 (1C, C-4a/b), 75.4 (3C, C-2, C-5), 76.7 (1C, C-7), 77.1 (1C, C-4a/b), 78.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 104.0 (1C, C-1b). MALDIMS: m/z 2552.3 $[M+Na]^+$. Anal. Calcd for $C_{145}H_{183}O_{32}P_4$ (2530.9): C, 68.81; H, 7.29. Found: C, 69.12; H, 7.53.

3.5. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (14)

Compound **13** (450mg, 0.178 mmol) was dissolved in acetonitrile/toluene/water (60:3:4, 30 mL) and cooled to −10 °C. $Ce(NH_4)_2(NO_3)_6$ (0.776 g, 8 equiv) was added portionwise and the reaction mixture stirred for 20 min at −10 °C, the cooling bath was removed and the reaction mixture was stirred for another 30–40 min (TLC-monitoring). After this time the reaction mixture was diluted with EtOAc and washed with satd $NaHCO_3$ solution, the organic phase was dried over $MgSO_4$ and was evaporated under diminished pressure. A fast purification in silica gel (2.5:1 toluene–acetone) yielded compound **14** (294 mg, 72%) as a colorless syrup, which was stored at −20 °C. TLC (2:1 toluene–acetone): R_f 0.43, R_f 0.52. $[\alpha]_D$ +3.2 (c 0.6, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.78–0.94 (t, 6H, Me), 1.08–1.38 (m, 40H, CH_2-chain), 1.43–1.60 (m, 4H, $COCH_2CH_2R$), 2.08–2.26 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.36 (m, 1H, 5a/b-H), 3,39 (m, 3H, 5a/b, 1′-H, 2b-H), 3.48 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 9-H), 3.59 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.72 (m, 1H, 8-H), 3.85 (m, 1H, 1′-H), 3.96, 4.06 (m, 8H, CH_{2Glyc}), 4.07 (m, 1H, 3′-H), 4.09 (m, 1H, 7-H), 4.12 (m, 1H, 6a-H), 4.16 (m, 1H, 7-H), 4.17 (m, 1H, 6b-H), 4.18 (m, 1H, 8-H), 4.24 (m, 1H, 1a-H), 4.30 (m, 1H, 6b-H), 4.42 (m, 1H, 1b-H), 4.46–4.95 (m, 16H, CH_2Ph), 5.02 (m, 6H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 7.08–7.44 (m, 55H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 24.8 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.1, 34.2 (2C, $COCH_2R$), 63.8 (1C, C-3′), 66.5 (1C, C-6b), 67.2 (1C, C-11), 68.1 (C-1′, C–CH_{2Glyc}), 68.6 (1C, C-6a), 68.9 (1C, C-9), 69.7 (3C, $POCH_2Ph$), 69.8 (1C, C-2′), 72.2–76.0 (8C, CH_2Ph), 73.5 (1C, C-5a/b), 75.0 (1C, C-4a/b), 76.4 (1C, C-8), 76.9 (1C, C-4a/b), 77.8 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 127.6–138.5 (C–Ph), 172.9/173.3 (2C, COOR). MALDIMS (positive Mode, Matrix p-Nitroaniline + NaI, THF): $[M+Na]^+$, m/z 2212.1; found: m/z 2212.1. $C_{129}H_{167}O_{30}P_3$ (2290.6): calcd: C, 67.64; H, 7.35. Found: C, 67.37; H, 7.10.

3.6. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (15)

Compound **14** (279 mg, 0.122 mmol), PyBOP (507 mg, 8 equiv) and *Z*-D-Ala triethylammonium salt (217 mg, 8 equiv) were dried separately 3 h in a high vacuum. After this time compound **14** was dissolved in 10 mL of dry CH_2Cl_2, *Z*-D-Ala triethylammonium salt and PyBOP were added. *N*-Methylimidazol (155 μL, 16 equiv) was added dropwise and the reaction mixture stirred for 2.5–3 h at rt under an argon atmosphere. The reaction mixture was diluted with CH_2Cl_2 and washed with satd NH_4Cl solution. The organic phase was dried over $MgSO_4$ and the solvent was removed under diminished pressure. Purification by flash chromatography (3:1 toluene–acetone) and (second column: 3:1 toluene–acetone) and (second column: 3:1 toluene–acetone) yielded diastereomer **15** (315 mg, 92%) as a colorless syrup, which was stored at −20 °C. TLC (3:1 toluene–acetone): R_f 0.25, R_f 0.33. $[\alpha]_D$ −8.1 (c 0.8, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.79–0.94 (t, 6H, Me), 1.09–1.38 (m, 40H, CH_2-chain), 1.46–1.61 (m, 4H, $COCH_2CH_2R$), 2.12–2.27 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.35 (m, 1H, 5a/b-H), 3,37 (m, 2H, 5a/b-H, 1′-H), 3.39 (m, 1H, 2b-H), 3.46 (m, 2H, 4a-H, 4b-H), 3.51 (m, 2H, 9-H), 3.58 (m, 2H, 3a-H, 3b-H), 3.60 (m, 1H, 6a-H), 3.71 (m, 1H, 8-H), 3.84 (m, 1H, 1′-H), 4.00 (m, 8H, 1-H, 3-H, 4-H, 6-H), 4.06 (m, 1H, 7-H), 4.07 (m, 1H, 3′-H), 4.12 (m, 1H, 6a-H), 4.14 (m, 1H, 3′-H), 4.17 (m, 1H, 7-H), 4.22 (m, 1H, 1a-H), 4.23 (m, 1H, 6b-H), 4.32 (m, 2H, CHNHCbz), 4.42 (m, 1H, 1b-H), 4.45–4.84 (m, 16H, CH_2Ph), 4.97 (m, 2H, CH_2Cbz), 4.99 (m, 6H, $POCH_2Ph$), 5.04 (m, 2H, CH_2Cbz), 5.08 (m, 1H, 2′-H), 5.09 (m, 2H, CH-Ala), 5.58–6.15 (br s, NH), 7.07–7.46 (m, 75H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 18.03 (2C, Ala-Me), 24.8/24.9 (2C, $COCH_2CH_2R$),

22.7/29.7/31.9 (20C, CH_2-chain), 34.0, 34.2 (2C, $COCH_2R$), 49.7 (2C, $CHNHCbz$), 62.8 (1C, C-3′), 65.0 (4C, C-1, C-3, C-4, C-6), 66.5 (1C, C-6b), 66.8 (2C, CH_2Cbz), 67.3 (1C, C-7), 68.0 (1C, C-1′), 68.6 (1C, C-6a), 68.8 (1C, C-6a), 68.9 (1C, C-9), 69.8 (1C, C-2′), 70.0 (3C, $POCH_2Ph$), 70.8 (2C, CH-Ala), 72.2–75.5 (8C, CH_2Ph), 73.6 (1C, C-5a/b), 75.1 (1C, C-4a/b), 76.4 (1C, C-8), 76.9 (1C, C-4a/b), 77.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.5 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 125.3–138.5 (C–Ph), 155.9 (2C, $COCbz$), 172.0 (3C, CO_{Ala}), 172.9/173.2 (2C, COOR). MALDIMS: m/z 2721.3 $[(M-H)+Na]^+$.

3.7. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (16)

Compound **16** was synthesized following the procedure described for compound **13**. Compound **9**[17] (200 mg, 0.146 mmol), tetrazole (21.6mg, 0.292 mmol), compound **7**[15] (347 mg, 1.5 equiv) in dry CH_2Cl_2 (7 mL). Oxidation with *t*-BuOOH (0.48 mL). Purification in flash chromatography (2:1 toluene–acetone) yielded **16** (354 mg, 84%) as a colorless oil. TLC (1:1 toluene–acetone): R_f 0.55. $[\alpha]_D$ +3.8 (*c* 1, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, 6H, Me), 1.08–1.34 (m, 40H, CH_2-chain), 1.46–1.59 (m, 4H, $COCH_2CH_2R$), 2.17–2.26 (m, 4H, $COCH_2CH_2R$), 3.35 (m, 2H, 2a-H, 5a/b-H), 3,38 (m, 1H, 1′-H), 3.39 (m, 2H, 2b-H, 5a/b-H), 3.47–3.49 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 12-H), 3.60 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.66 (m, 3H, 2-H, 5-H, 8-H), 3.69 (m, 9H, OMe), 3.73 (m, 1H, 11-H), 3.86 (m, 1H, 1′-H), 3.97–4.05 (m, 12H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H), 4.08 (m, 2H, 11-H, 3′-H), 4.14 (m, 1H, 6a-H), 4.16 (m, 1H, 3′-H), 4.18 (m, 2H, 11-H, 6b-H), 4.23 (m, 1H, 1a-H), 4.27 (m, 1H, 6b-H), 4.43 (m, 1H, 1b-H), 4.44–4.93 (m, 26H, CH_2Ph), 4.95–5.04 (m, 8H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 6.70–6.83 (m, 6H, Ph_{MPM}), 7.08–7.37 (m, 66H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 55.1 (3C, OMe), 62.9 (1C, C-3′), 65.8 (6C, C-1, C-3, C-4, C-6, C-7, C-9), 66.5 (1C, C-6b), 67.0 (1C, C-11), 68.1 (1C, C-1′), 68.7 (1C, C-6a), 69.2 (1C, C-12), 69.5 (4C, $POCH_2Ph$), 69.8 (1C, C-2′), 71.5–76.5 (13C, CH_2Ph), 73.6 (1C, C-5a/b), 75.2 (1C, C-4a/b), 75.4 (3C, C-2, C-5, C-8), 76.7 (1C, C-11), 77.1 (1C, C-4a/b), 78.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 104.0 (1C, C-1b). MALDIMS: m/z 2916.2 $[M+Na]^+$. Anal. Calcd for $C_{163}H_{204}O_{38}P_4$ (2893.3): C, 67.62; H, 7.10. Found: C, 67.38; H, 7.31.

3.8. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (17)

Compound **17** was synthesized following the same procedure as described for compound **14**. Compound **16** (354 mg, 0.122 mmol); acetonitrile/toluene/water (60:3:4, 10 mL); $Ce(NH_4)_2(NO_3)_6$ (0.796 g, 12 equiv). After flash chromatography (1:1 toluene–acetone), compound **17** was obtained (271 mg, 87%). TLC (1:1.5 toluene–acetone): R_f 0.53, R_f 0.62. $[\alpha]_D$ +3.2 (*c* 0.6, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.78–0.94 (t, 6H, Me), 1.08–1.38 (m, 40H, CH_2-chain), 1.43–1.60 (m, 4H, $COCH_2CH_2R$), 2.08–2.26 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.36 (m, 1H, 5a/b-H), 3,39 (m, 3H, 5a/b, 1′-H, 2b-H), 3.48 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 12-H), 3.59 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.72 (m, 1H, 11-H), 3.85 (m, 1H, 1′-H), 3.96, 4.06 (m, 12H, CH_{2Glyc}), 4.07 (m, 1H, 3′-H), 4.09 (m, 1H, 10-H), 4.12 (m, 1H, 6a-H), 4.16 (m, 1H, 10-H), 4.17 (m, 1H, 6b-H), 4.18 (m, 1H, 11-H), 4.24 (m, 1H, 1a-H), 4.30 (m, 1H, 6b-H), 4.42 (m, 1H, 1b-H), 4.46–4.95 (m, 16H, CH_2Ph), 5.02 (m, 8H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 7.08–7.44 (m, 60H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 24.8 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.1, 34.2 (2C, $COCH_2R$), 63.8 (1C, C-3′), 66.5 (1C, C-6b), 67.2 (1C, C-11), 68.1 (C-1′, C–CH_{2Glyc}), 68.6 (1C, C-6a), 68.9 (1C, C-12), 69.7 (4C, $POCH_2Ph$), 69.8 (1C, C-2′), 72.2–76.0 (8C, CH_2Ph), 73.5 (1C, C-5a/b), 75.0 (1C, C-4a/b), 76.4 (1C, C-11), 76.9 (1C, C-4a/b), 77.8 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 127.6–138.5 (C–Ph), 172.9/173.3 (2C, COOR). MALDIMS: m/z 2556.2 $[M+Na]^+$. Anal. Calcd for $C_{139}H_{180}O_{35}P_4$ (2533.1): C, 65.86; H, 7.16. Found: C, 66.11; H, 6.97.

3.9. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (18)

Compound **18** was synthesized following the same procedure as described for compound **15**. Compound **17** (261 mg, 0.103 mmol), PyBOP (804 mg, 15 equiv) and *Z*-D-Ala triethylammonium salt (370 mg, 15 equiv), *N*-methylimidazol (330 μL, 24 equiv). After purification, compound **18** was obtained (176 mg,

56%). TLC (1:1.5 toluene–acetone): R_f 0.77, R_f 0.81. $[\alpha]_D$ −8.9 (*c* 0.46, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.79–0.94 (t, 6H, Me), 1.09–1.38 (m, 40H, CH_2-chain), 1.46–1.61 (m, 4H, $COCH_2CH_2R$), 2.12–2.27 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.35 (m, 1H, 5a/b-H), 3,37 (m, 2H, 5a/b-H, 1′-H), 3.39 (m, 1H, 2b-H), 3.46 (m, 2H, 4a-H, 4b-H), 3.51 (m, 2H, 12-H), 3.58 (m, 2H, 3a-H, 3b-H), 3.60 (m, 1H, 6a-H), 3.71 (m, 1H, 11-H), 3.84 (m, 1H, 1′-H), 4.00 (m, 12H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H), 4.06 (m, 1H, 10-H), 4.07 (m, 1H, 3′-H), 4.12 (m, 1H, 6a-H), 4.14 (m, 1H, 3′-H), 4.17 (m, 1H, 10-H), 4.22 (m, 1H, 1a-H), 4.23 (m, 1H, 6b-H), 4.32 (m, 3H, C*H*NHCbz), 4.42 (m, 1H, 1b-H), 4.45–4.84 (m, 16H, CH_2Ph), 4.97 (m, 3H, CH_2Cbz), 4.99 (m, 8H, $POCH_2$Ph), 5.04 (m, 3H, CH_2Cbz), 5.08 (m, 1H, 2′-H), 5.09 (m, 3H, C*H*-Ala), 5.58–6.15 (br s, NH), 7.07–7.46 (m, 75H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 18.03 (3C, Ala-Me), 24.8/24.9 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.0, 34.2 (2C, $COCH_2R$), 49.7 (3C, CHNHCbz), 62.8 (1C, C-3′), 65.0 (6C, C-1, C-3, C-4, C-6, C-7, C-9), 66.5 (1C, C-6b), 66.8 (3C, CH_2Cbz), 67.3 (1C, C-10), 68.0 (1C, C-1′), 68.6 (1C, C-6a), 68.8 (1C, C-6a), 68.9 (1C, C-12), 69.8 (1C, C-2′), 70.0 (4C, $POCH_2$Ph), 70.8 (3C, CH-Ala), 72.2–75.5 (8C, CH_2Ph), 73.6 (1C, C-5a/b), 75.1 (1C, C-4a/b), 76.4 (1C, C-11), 76.9 (1C, C-4a/b), 77.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.5 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 125.3–138.5 (C–Ph), 155.9 (3C, COCbz), 172.0 (3C, CO_{Ala}), 172.9/173.2 (2C, COOR). MALDIMS (positive Mode, Matrix *p*-Nitroaniline + NaI, THF): $[(M–H) + 2Na]^+$, *m*/*z* 3148.4; found: *m*/*z* 3148.4.

3.10. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (19)

Compound **19** was synthesized following the procedure described for compound **13**. Compound **10**[17] (200 mg, 0.116 mmol), tetrazole (24.3 mg, 0.35 mmol), compound **7**[15] (280 mg, 1.5 equiv) in dry CH_2Cl_2 (5.5 mL). Oxidation with *t*-BuOOH (0.38 mL). Purification in flash chromatography (2:1 toluene–acetone) yielded **19** (275 mg, 74%) as a colorless oil. TLC (1:1 toluene–acetone): R_f 0.55. $[\alpha]_D$ +3.6 (*c* 1, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, 6H, Me), 1.08–1.34 (m, 40H, CH_2-chain), 1.46–1.59 (m, 4H, $COCH_2CH_2R$), 2.17–2.26 (m, 4H, $COCH_2CH_2R$), 3.35 (m, 2H, 2a-H, 5a/b-H), 3,38 (m, 1H, 1′-H), 3.39 (m, 2H, 2b-H, 5a/b-H), 3.47–3.49 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 15-H), 3.60 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.66 (m, 4H, 2-H, 5-H, 8-H, 11-H), 3.69 (m, 12H, OMe), 3.73 (m, 1H, 14-H), 3.86 (m, 1H, 1′-H), 3.97–4.05 (m, 16H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H), 4.08 (m, 2H, 13-H, 3′-H), 4.14 (m, 1H, 6a-H), 4.16 (m, 1H, 3′-H), 4.18 (m, 2H, 16-H, 6b-H), 4.23 (m, 1H, 1a-H), 4.27 (m, 1H, 6b-H), 4.43 (m, 1H, 1b-H), 4.44–4.93 (m, 24H, CH_2Ph), 4.95–5.04 (m, 10H, $POCH_2$Ph), 5.09 (m, 1H, 2′-H), 6.70–6.83 (m, 8H, Ph_{MPM}), 7.08–7.37 (m, 75H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 55.1 (4C, OMe), 62.9 (1C, C-3′), 65.8 (8C, C-1, C-3, C-4, C-6, C-7, C-9, C-10, C-12), 66.5 (1C, C-6b), 67.0 (1C, C-13), 68.1 (1C, C-1′), 68.7 (1C, C-6a), 69.2 (1C, c-18), 69.5 (5C, $POCH_2$Ph), 69.8 (1C, C-2′), 71.5–76.5 (12C, CH_2Ph), 73.6 (1C, C-5a/b), 75.2 (1C, C-4a/b), 75.4 (4C, C-2, C-5, C-8, C-11), 76.7 (1C, C-14), 77.1 (1C, C-4a/b), 78.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 104.0 (1C, C-1b). MALDIMS: *m*/*z* 3257.7 $[M+Na]^+$. Anal. Calcd for $C_{181}H_{225}O_{44}P_5$ (3259.6): C, 66.69; H, 6.96. Found: C, 66.92; H, 7.17.

3.11. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (20)

Compound **20** was synthesized following the same procedure as described for compound **14**. Compound **19** (269 mg, 0.082 mmol); acetonitrile/toluene/water (60:3:4, 16 mL); $Ce(NH_4)_2(NO_3)_6$ (720 mg, 16 equiv). After purification, **20** was obtained (196 mg, 85%). TLC (1:1.5 toluene–acetone): R_f 0.53, R_f 0.62. $[\alpha]_D$ +3.8 (*c* 0.6, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.78–0.94 (t, 6H, Me), 1.08–1.38 (m, 40H, CH_2-chain), 1.43–1.60 (m, 4H, $COCH_2CH_2R$), 2.08–2.26 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.36 (m, 1H, 5a/b-H), 3,39 (m, 3H, 5a/b, 1′-H, 2b-H), 3.48 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 15-H), 3.59 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.72 (m, 1H, 14-H), 3.85 (m, 1H, 1′-H), 3.96, 4.06 (m, 16H, CH_{2Glyc}), 4.07 (m, 1H, 3′-H), 4.12 (m, 1H, 6a-H), 4.16 (m, 1H, 13-H), 4.17 (m, 1H, 6b-H), 4.18 (m, 1H, 13-H), 4.24 (m, 1H, 1a-H), 4.30 (m, 1H, 6b-H), 4.42 (m, 1H, 1b-H), 4.46–4.95 (m, 16H, CH_2Ph), 5.02 (m, 10H, $POCH_2$Ph), 5.09 (m, 1H, 2′-H), 7.08–7.44 (m, 60H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 24.8 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.1, 34.2 (2C, $COCH_2R$), 63.8 (1C, C-3′), 66.5 (1C, C-6b), 67.2 (1C, C-16), 68.1 (C-1′, C–CH_{2Glyc}), 68.6 (1C, C-6a), 68.9 (1C, C-18), 69.7 (5C, $POCH_2$Ph), 69.8 (1C, C-2′), 72.2–76.0 (8C,

CH_2Ph), 73.5 (1C, C-5a/b), 75.0 (1C, C-4a/b), 76.4 (1C, C-14), 76.9 (1C, C-4a/b), 77.8 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 127.6–138.5 (C–Ph), 172.9/173.3 (2C, *C*OOR). MALDIMS: *m*/*z* 2800.2 $[M+Na]^+$. Anal. Calcd for $C_{149}H_{193}O_{40}P_5$ (2779.0): C, 64.4; H, 7.00. Found: C, 64.18; H, 7.23.

3.12. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (21)

Compound **21** was synthesized following the same procedure as described for compound **15**. Compound **20** (196 mg, 0.071 mmol), PyBOP (734 mg, 20 equiv) and *Z*-D-Ala triethylammonium salt (338 mg, 20 equiv), *N*-methylimidazol (230 μL, 40 equiv). Compound **21** (174 mg, 69%). TLC (1:1.5 toluene–acetone): R_f 0.77, R_f 0.81. $[\alpha]_D$ −10 (*c* 0.46, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.79–0.94 (t, 6H, Me), 1.09–1.38 (m, 40H, CH_2-chain), 1.46–1.61 (m, 4H, $COCH_2CH_2R$), 2.12–2.27 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.35 (m, 1H, 5a/b-H), 3,37 (m, 2H, 5a/b-H, 1′-H), 3.39 (m, 1H, 2b-H), 3.46 (m, 2H, 4a-H, 4b-H), 3.51 (m, 2H, 15-H), 3.58 (m, 2H, 3a-H, 3b-H), 3.60 (m, 1H, 6a-H), 3.71 (m, 1H, 14-H), 3.84 (m, 1H, 1′-H), 4.00 (m, 16H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H), 4.06 (m, 1H, 13-H), 4.07 (m, 1H, 3′-H), 4.12 (m, 1H, 6a-H), 4.14 (m, 1H, 3′-H), 4.17 (m, 1H, 13-H), 4.22 (m, 1H, 1a-H), 4.23 (m, 1H, 6b-H), 4.32 (m, 4H, C*H*NHCbz), 4.42 (m, 1H, 1b-H), 4.45–4.84 (m, 16H, CH_2Ph), 4.97 (m, 4H, CH_2Cbz), 4.99 (m, 10H, $POCH_2Ph$), 5.04 (m, 4H, CH_2Cbz), 5.08 (m, 1H, 2′-H), 5.09 (m, 4H, C*H*-Ala), 5.58–6.15 (br s, NH), 7.07–7.46 (m, 85H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 18.03 (4C, Ala-Me), 24.8/24.9 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.0, 34.2 (2C, $COCH_2R$), 49.7 (4C, *C*HNHCbz), 62.8 (1C, C-3′), 65.0 (8C, C-1, C-3, C-4, C-6, C-7, C-9, C-10, C-12), 66.5 (1C, C-6b), 66.8 (4C, CH_2Cbz), 67.3 (1C, C-13), 68.0 (1C, C-1′), 68.6 (1C, C-6a), 68.8 (1C, C-6a), 68.9 (1C, C-15), 69.8 (1C, C-2′), 70.0 (5C, $POCH_2Ph$), 70.8 (4C, *C*H-Ala), 72.2–75.5 (8C, CH_2Ph), 73.6 (1C, C-5a/b), 75.1 (1C, C-4a/b), 76.4 (1C, C-14), 76.9 (1C, C-4a/b), 77.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.5 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 125.3–138.5 (C–Ph), 155.9 (4C, *C*OCbz), 172.0 (4C, CO_{Ala}), 172.9/173.2 (2C, *C*OOR). MALDIMS: *m*/*z* 3642.3 $[(M-H)+2Na]^+$.

3.13. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(4-methoxybenzyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (22)

Compound **22** was synthesized following the procedure described for compound **13**. Compound **12** (870 mg, 0.416 mmol), tetrazole (58 mg, 2 equiv), compound **7**[15] (818 mg, 1.3 equiv) in dry CH_2Cl_2 (15 mL). Oxidation with *t*-BuOOH (1.2 mL). Purification in flash chromatography (2:1 toluene–acetone) yielded **22** (1 g, 67%) as a colorless oil. TLC (1:1 toluene–acetone): R_f 0.55. $[\alpha]_D$ +3.3 (*c* 1, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, 6H, Me), 1.08–1.34 (m, 40H, CH_2-chain), 1.46–1.59 (m, 4H, $COCH_2CH_2R$), 2.17–2.26 (m, 4H, $COCH_2CH_2R$), 3.35 (m, 2H, 2a-H, 5a/b-H), 3,38 (m, 1H, 1′-H), 3.39 (m, 2H, 2b-H, 5a/b-H), 3.47–3.49 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 18-H), 3.60 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.66 (m, 5H, 2-H, 5-H, 8-H, 11-H, 14-H), 3.69 (m, 15H, OMe), 3.73 (m, 1H, 17-H), 3.86 (m, 1H, 1′-H), 3.97–4.05 (m, 20H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H, 13-H, 15-H), 4.08 (m, 2H, 16-H, 3′-H), 4.14 (m, 1H, 6a-H), 4.16 (m, 1H, 3′-H), 4.18 (m, 2H, 16-H, 6b-H), 4.23 (m, 1H, 1a-H), 4.27 (m, 1H, 6b-H), 4.43 (m, 1H, 1b-H), 4.44–4.93 (m, 26H, CH_2Ph), 4.95–5.04 (m, 12H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 6.70–6.83 (m, 10H, Ph_{MPM}), 7.08–7.37 (m, 80H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 55.1 (5C, OMe), 62.9 (1C, C-3′), 65.8 (10 C, C-1, C-3, C-4, C-6, C-7, C-9, C-10, C-12, C-13, C-15), 66.5 (1C, C-6b), 67.0 (1C, C-16), 68.1 (1C, C-1′), 68.7 (1C, C-6a), 69.2 (1C, c-18), 69.5 (6C, $POCH_2Ph$), 69.8 (1C, C-2′), 71.5–76.5 (13C, CH_2Ph), 73.6 (1C, C-5a/b), 75.2 (1C, C-4a/b), 75.4 (5C, C-2, C-5, C-8, C-11, C-14), 76.7 (1C, C-17), 77.1 (1C, C-4a/b), 78.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 104.0 (1C, C-1b). MALDIMS: *m*/*z* 3647.7 $[M+Na]^+$. Anal. Calcd for $C_{199}H_{247}O_{50}P_6$ (3624.9): C, 65.94; H, 6.87. Found: C, 65.78; H, 7.04.

3.14. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-(*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (23)

Compound **23** was synthesized following the same procedure as described for compound **14**. Compound **22** (810 mg, 0.224 mmol); acetonitrile/toluene/water (60:

3:4, 37 mL); $Ce(NH_4)_2(NO_3)_6$ (3.07 g, 25 equiv). After purification (1:1→1:3 toluene–acetone), **23** was obtained (538 mg, 80%). TLC (1:1.5 toluene–acetone): R_f 0.53, R_f 0.62. $[\alpha]_D$ +4.2 (*c* 0.6, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.78–0.94 (t, 6H, Me), 1.08–1.38 (m, 40H, CH_2-chain), 1.43–1.60 (m, 4H, $COCH_2CH_2R$), 2.08–2.26 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.36 (m, 1H, 5a/b-H), 3,39 (m, 3H, 5a/b, 1′-H, 2b-H), 3.48 (m, 2H, 4a-H, 4b-H), 3.52 (m, 2H, 18-H), 3.59 (m, 2H, 3a-H, 3b-H), 3.61 (m, 1H, 6a-H), 3.72 (m, 1H, 17-H), 3.85 (m, 1H, 1′-H), 3.96, 4.06 (m, 20H, CH_{2Glyc}), 4.07 (m, 1H, 3′-H), 4.09 (m, 1H, 16-H), 4.12 (m, 1H, 6a-H), 4.16 (m, 1H, 16-H), 4.17 (m, 1H, 6b-H), 4.18 (m, 1H, 16-H), 4.24 (m, 1H, 1a-H), 4.30 (m, 1H, 6b-H), 4.42 (m, 1H, 1b-H), 4.46-4.95 (m, 16H, CH_2Ph), 5.02 (m, 12H, $POCH_2Ph$), 5.09 (m, 1H, 2′-H), 7.08–7.44 (m, 70H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 24.8 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.1, 34.2 (2C, $COCH_2R$), 63.8 (1C, C-3′), 66.5 (1C, C-6b), 67.2 (1C, C-16), 68.1 (C-1′, C–CH_{2Glyc}), 68.6 (1C, C-6a), 68.9 (1C, C-18), 69.7 (6C, $POCH_2Ph$), 69.8 (1C, C-2′), 72.2–76.0 (8C, CH_2Ph), 73.5 (1C, C-5a/b), 75.0 (1C, C-4a/b), 76.4 (1C, C-17), 76.9 (1C, C-4a/b), 77.8 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.4 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 127.6–138.5 (C–Ph), 172.9/173.3 (2C, COOR). MALDIMS: *m*/*z* 3047.6 $[M+Na]^+$. Anal. Calcd for $C_{159}H_{206}O_{45}P_6$ (3023.2): C, 63.17; H, 6.87. Found: C, 63.21; H, 7.17.

3.15. (2,3-Di-*O*-benzyl-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-(2-*O*-(*N*-benzyloxycarbonyl-D-alanyl)-*sn*-glycero)-benzyloxyphosphoryl-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-6-*O*-(2,3,4-tri-*O*-benzyl-β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (24)

Compound **24** was synthesized following the same procedure as described for compound **15**. Compound **23** (310 mg, 0.103 mmol), PyBOP (1.34 g, 25 equiv) and *Z*-D-Ala triethylammonium salt (839 mg, 25 equiv), *N*-methylimidazol (414 μL, 40 equiv). Compound **24** (240 mg, 58%). TLC (1:1.5 toluene–acetone): R_f 0.77, R_f 0.81. $[\alpha]_D$ −11 (*c* 0.46, $CHCl_3$). ^1H NMR (600 MHz, $CDCl_3$): δ 0.79–0.94 (t, 6H, Me), 1.09–1.38 (m, 40H, CH_2-chain), 1.46–1.61 (m, 4H, $COCH_2CH_2R$), 2.12–2.27 (m, 4H, $COCH_2CH_2R$), 3.34 (m, 1H, 2a-H), 3.35 (m, 1H, 5a/b-H), 3,37 (m, 2H, 5a/b-H, 1′-H), 3.39 (m, 1H, 2b-H), 3.46 (m, 2H, 4a-H, 4b-H), 3.51 (m, 2H, 18-H), 3.58 (m, 2H, 3a-H, 3b-H), 3.60 (m, 1H, 6a-H), 3.71 (m, 1H, 17-H), 3.84 (m, 1H, 1′-H), 4.00 (m, 20H, 1-H, 3-H, 4-H, 6-H, 7-H, 9-H, 10-H, 12-H, 13-H, 15-H), 4.06 (m, 1H, 16-H), 4.07 (m, 1H, 3′-H), 4.12 (m, 1H, 6a-H), 4.14 (m, 1H, 3′-H), 4.17 (m, 1H, 16-H), 4.22 (m, 1H, 1a-H), 4.23 (m, 1H, 6b-H), 4.32 (m, 5H, CHNHCbz), 4.42 (m, 1H, 1b-H), 4.45–4.84 (m, 16H, CH_2Ph), 4.97 (m, 5H, CH_2Cbz), 4.99 (m, 12H, $POCH_2Ph$), 5.04 (m, 5H, CH_2Cbz), 5.08 (m, 1H, 2′-H), 5.09 (m, 5H, CH-Ala), 5.58–6.15 (br s, NH), 7.07–7.46 (m, 95H, Ph). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 14.1 (2C, Me), 18.03 (5C, Ala-Me), 24.8/24.9 (2C, $COCH_2CH_2R$), 22.7/29.7/31.9 (20C, CH_2-chain), 34.0, 34.2 (2C, $COCH_2R$), 49.7 (5C, CHNHCbz), 62.8 (1C, C-3′), 65.0 (10 C, C-1, C-3, C-4, C-6, C-7, C-9, C-10, C-12, C-13, C-15), 66.5 (1C, C-6b), 66.8 (5C, CH_2Cbz), 67.3 (1C, C-16), 68.0 (1C, C-1′), 68.6 (1C, C-6a), 68.8 (1C, C-6a), 68.9 (1C, C-18), 69.8 (1C, C-2′), 70.0 (6C, $POCH_2Ph$), 70.8 (5C, CH-Ala), 72.2–75.5 (8C, CH_2Ph), 73.6 (1C, C-5a/b), 75.1 (1C, C-4a/b), 76.4 (1C, C-17), 76.9 (1C, C-4a/b), 77.0 (1C, C-5a/b), 81.9 (2C, C-2a, C-2b), 84.5 (2C, C-3a, C-3b), 103.6 (1C, C-1a), 103.9 (1C, C-1b), 125.3–138.5 (C–Ph), 155.9 (5C, COCbz), 172.0 (5C, CO_{Ala}), 172.9/173.2 (2C, COOR). MALDIMS: *m*/*z* 4069.8 $[(M-H)+2Na]^+$.

3.16. *sn*-Glycero-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-6-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (1)

The diastereomers **15** (180 mg, 0.067 mmol) were dissolved in CH_2Cl_2/MeOH/H_2O (7.5:7.5:1.5, 5 mL), treated with Pearlman's catalyst (10% in weight) and under hydrogen atmosphere, with a H_2-filled balloon, was stirred overnight at rt. The reaction was filtrated through Celite, washed with CH_2Cl_2/MeOH/H_2O (7.5:7.5:1.5, 4 mL) and the filtrate was diluted with 0.1 M NH_4OAc–buffer (pH 4.8). The solvent was lyophilized and purified using hydrophobic interactions HPLC. After lyophilization, compound **1** (29.5 mg, 32%) was obtained as white powder. ^1H NMR (600 MHz, D_2O): δ 0.77–0.93 (m, 6H, Me), 1.12–1.43 (m, 40H, CH_2-chain), 1.51–1.71 (m, 13H, Ala-Me, $COCH_2CH_2R$), 2.23–2.48 (m, 4H, $COCH_2CH_2R$), 3.23–4.62 (m, 28H), 5.28–5.45 (m, 3H, CH-Ala, 2′-H). ^{13}C NMR (150.9 MHz, D_2O): δ 16.6 (2C, Me), 18.1 (2C, Ala-Me), 24.0 (2C, $COCH_2CH_2R$), 25.7/33.0/35.1 (20C, CH_2-chain), 52.0 (2C, $CHNH_3^+$), 65.1 (1C, C-9), 66.8 (C–CH_{2Glyc}), 69.8 (1C, C-7), 73.9 (1C, C-8), 77.0 (3C, CH-Ala, C-2′). MALDIMS (negative mode): *m*/*z* 1439.6 $[M-H]^-$, 1369.5 $[(M-Ala)-H]^-$.

3.17. *sn*-Glycero-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-6-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (2)

Compound **2** was synthesized following the same procedure as described for compound **1**. Compound **18**

(174 mg, 0.021 mmol); CH_2Cl_2/MeOH/H_2O (7.5:7.5:1.5, 6 mL). Compound **2** (38 mg, 41%) was obtained as a white powder. ^{1}H NMR (600 MHz, D_2O): δ 0.77–0.93 (m, 6H, Me), 1.12–1.43 (m, 40H, CH_2-chain), 1.51–1.71 (m, 13H, Ala-Me, $COCH_2CH_2R$), 2.23–2.48 (m, 4H, $COCH_2CH_2R$), 3.23–4.62 (m, 38H), 5.28–5.45 (m, 4H, CH-Ala, 2′-H). ^{13}C NMR (150.9 MHz, D_2O): δ 16.6 (2C, Me), 18.1 (3C, Ala-Me), 24.0 (2C, $COCH_2CH_2R$), 25.7/33.0/35.1 (20C, CH_2-chain), 52.0 (3C, $CHNH_3^+$), 65.1 (1C, C-18), 66.8 (C–CH_{2Glyc}), 69.8 (1C, C-10), 73.9 (1C, C-11), 77.0 (4C, CH-Ala, C-2′). MALDIMS (negative mode): m/z 1664.8 $[M-H]^-$, 1595.2 $[(M-Ala)-H]^-$.

3.18. *sn*-Glycero-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-6-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (3)

Compound **3** was synthesized following the same procedure as described for compound **1**. Compound **21** (174 mg, 0.048 mmol); CH_2Cl_2/MeOH/H_2O (7.5:7.5:1.5, 6 mL). Compound **3** (24 mg, 26%) was obtained as a white powder. ^{1}H NMR (600 MHz, D_2O): δ 0.77–0.93 (m, 6H, Me), 1.12–1.43 (m, 40H, CH_2-chain), 1.51–1.71 (m, 16H, Ala-Me, $COCH_2CH_2R$), 2.23–2.48 (m, 4H, $COCH_2CH_2R$), 3.23–4.62 (m, 48H), 5.28–5.45 (m, 5H, CH-Ala, 2′-H). ^{13}C NMR (150.9 MHz, D_2O): δ 16.6 (2C, Me), 18.1 (4C, Ala-Me), 24.0 (2C, $COCH_2CH_2R$), 25.7/33.0/35.1 (20C, CH_2-chain), 52.0 (4C, $CHNH_3^+$), 65.1 (1C, C-18), 66.8 (C–CH_{2Glyc}), 69.8 (1C, C-16), 73.9 (1C, C-17), 77.0 (5C, CH-Ala, C-2′). MALDIMS (negative mode): m/z 1890.4 $[M-H]^-$.

3.19. *sn*-Glycero-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-(2-*O*-D-alanyl-*sn*-glycero)-hydroxyphosphoryl-6-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)-1,2-di-*O*-myristoyl-*sn*-glycerol (4)

Compound **4** was synthesized following the same procedure as described for compound **1**. Compound **24** (83 mg, 0.021 mmol); CH_2Cl_2/MeOH/H_2O (7.5:7.5:1.5, 6 mL). Compound **4** (12 mg, 28%) was obtained as a white powder. ^{1}H NMR (600 MHz, D_2O): δ 0.77–0.93 (m, 6H, Me), 1.12–1.43 (m, 40H, CH_2-chain), 1.51–1.71 (m, 19H, Ala-Me, $COCH_2CH_2R$), 2.23–2.48 (m, 4H, $COCH_2CH_2R$), 3.23–4.62 (m, 48H), 5.28–5.45 (m, 6H, CH-Ala, 2′-H). ^{13}C NMR (150.9 MHz, D_2O): δ 16.6 (2C, Me), 18.1 (5C, Ala-Me), 24.0 (2C, $COCH_2CH_2R$), 25.7/33.0/35.1 (20C, CH_2-chain), 52.0 (5C, $CHNH_3^+$), 65.1 (1C, C-18), 66.8 (C–CH_{2Glyc}), 69.8 (1C, C-16), 73.9 (1C, C-17), 77.0 (6C, CH-Ala, C-2′). MALDIMS (negative mode): m/z 2114.5 $[M-H]^-$, 2041.9 $[(M-Ala)-H]^-$.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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