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Synthesis of a Monophosphoryl Derivative of *Escherichia coli* Lipid A and Its Efficient Coupling to a Tumor-Associated Carbohydrate Antigen

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Abstract: Monophosphoryl lipid A is a safe and potent immunostimulant and vaccine adjuvant, which is potentially useful for the development of effective carbohydrate-based conjugate vaccines. This paper presents a convergent and efficient synthesis of a monophosphoryl derivative of *E. coli* lipid A that has an alkyne functionality at the reducing end, which is suitable for coupling with various molecules. The coupling of this derivative to an N-modified analogue of tumor-associated antigen GM3 through click chemistry is also presented.

Keywords: cancer · carbohydrates · glycoconjugates · glycolipids · lipids

Introduction

Lipopolysaccharides (LPS), which constitute the major components on the cell surface of Gram-negative bacteria,[1] are particularly endotoxic and cause septicemia.[2] It has been further demonstrated that the LPS anchor part, namely, lipid A, is primarily responsible for the endotoxicity of LPS. Lipid A binds to Toll-like receptor 4 (TLR4) to activate a cascade of immunological responses, including the production of a number of cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interferon-β (IFN-β).[3] Thus, lipid A has become a valuable molecular template in the discovery of new immunostimulants,[4,5] for example, vaccine adjuvants, and in the design and development of novel conjugate vaccines.[6]

Lipid A is a highly hydrophobic glycolipid, consisting of a β-1,6-linked diglucosamine with two phosphate or pyrophosphate groups and four to eight long lipid chains attached to the 1,4′-O- and 2,2′-*N*-3,3′-O-positions, respectively. The structures of lipid A derived from different bacteria can vary significantly in terms of the number, structures, and locations of their lipids.[1] To understand and eventually mitigate the endotoxicity of lipid A for the development of useful immunostimulants, numerous lipid A derivatives have been designed, synthesized, and biologically assayed.[7–10] The structure–activity relationship studies of natural lipid A and various synthetic analogues have shown that both the number and lengths of acyl chains and the phosphorylation state of lipid A have a significant impact on its endotoxicity. It appears that the diphosphorylated hexaacyl form of lipid A, such as *Escherichia coli* lipid A (Scheme 1), is optimally recognized by TLR4 to exhibit the full spectrum of endotoxicity.[1] Most importantly, it was observed that the endotoxic activity of lipid A could be significantly reduced after the removal of its anomeric phosphate group,[1] whereas its immunostimulatory properties remained unaffected. For example, although *Salmonella minnesota* lipid A (Scheme 1) is a potent endotoxin, the 4′-O-monophosphorylated form is essentially nontoxic.[11,12] Monophosphoryl lipid A (MPLA) is clinically safe as a vaccine adjuvant[13] and has even been explored as a potential vaccine against bacterial infections and cancer.[6]

The special carbohydrates expressed by bacterial and cancer cells are important targets for the design and development of bacterial and cancer vaccines.[14,15] However, a major problem for carbohydrate antigens, especially the tumor-associated carbohydrate antigens (TACAs), is that they are typically poorly immunogenic.[14] To overcome this problem, much recent effort has been focused on synthetic multicomponent glycoconjugate vaccines, which consist of a carbohydrate antigen, an immunostimulant, and/or other functional epitopes.[16–22] It has been demonstrated that covalently coupling an immunostimulant to a carbohydrate antigen can remarkably improve the immunogenicity of the latter and generate potent glycoconjugate vaccines. In this

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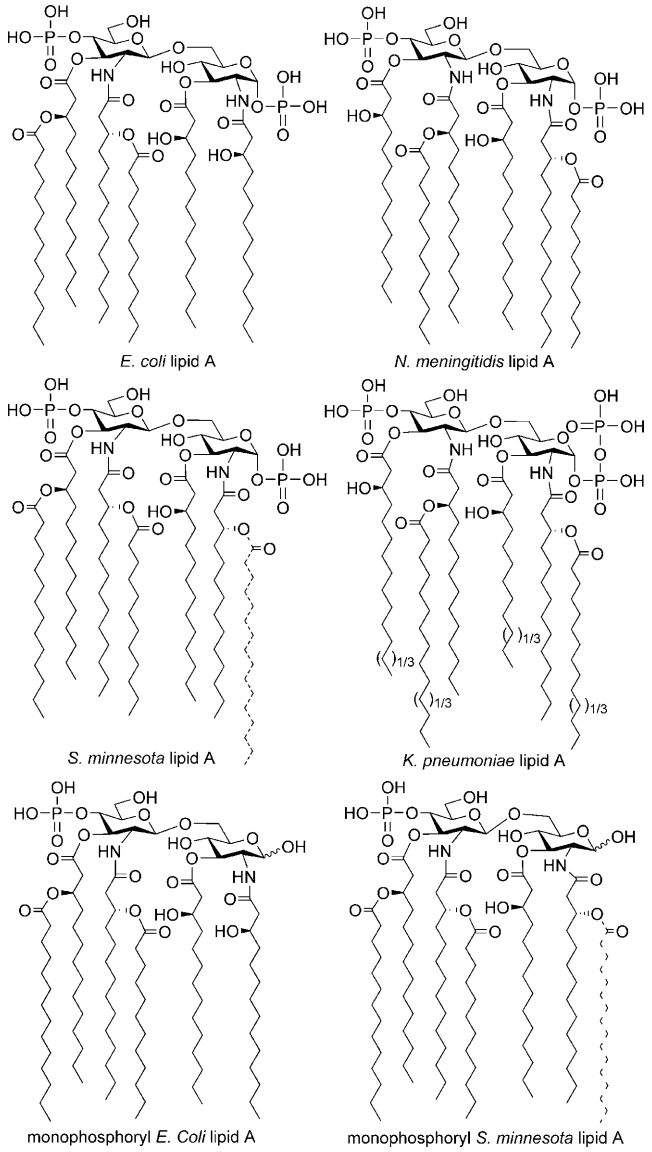
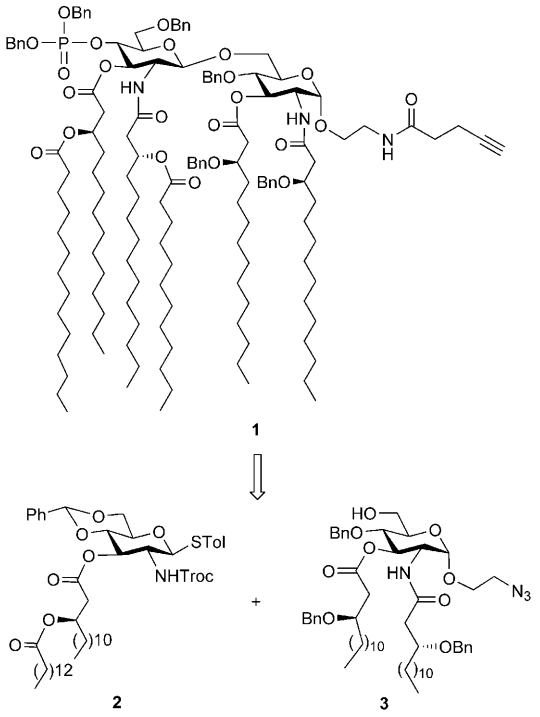


Scheme 1. Selected lipid A structures derived from different Gram-negative bacteria. Dashed lines indicate partial structures, which result from incomplete biosynthesis, and the numbers in parentheses indicate the potential variation of chain lengths.



Scheme 2. Structure of the target MPLA derivative and the synthetic plan. Bn = benzyl, Ph = phenyl, Tol = tolyl.

regard, MPLA can be particularly useful as an extremely potent and nontoxic immunostimulant.

To exploit the immunostimulatory or adjuvant activity of MPLA for the development of functional conjugate vaccines, we designed a monophosphoryl derivative **1** (Scheme 2) of *E. coli* lipid A,[1] which is suitable for coupling with peptides/proteins and carbohydrates for the assembly of multicomponent glycoconjugates. Although several syntheses of lipid A and MPLA have already been reported,[23–37] compound **1** is unique in that it contains an alkyne functionality, enabling its coupling with other molecules through click chemistry. In addition, an alkyne functionality can be readily transformed into a carboxyl or carbonyl group to facilitate other coupling methods, such as those based on carboxylic acid–amine condensation reactions or on reductive amination reactions. This report describes the chemical synthesis of **1** and its coupling with a TACA by means of click chemistry.

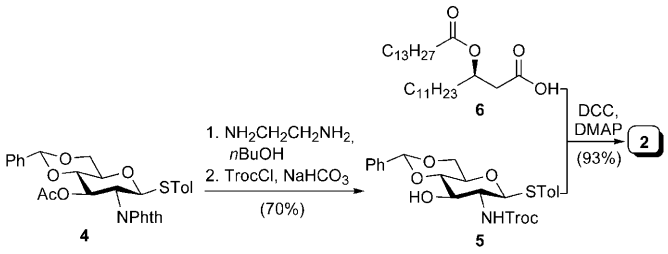
In **1**, the functionality that can be utilized to couple MPLA with other epitopes is located at the disaccharide reducing end. Structure–activity relationship studies of MPLA have revealed that modifying the reducing end of MPLA has little influence on its binding to the lipid A receptor and that MPLA derivatives with spacers attached to the reducing end had activities similar to that of MPLA.[1,38] Thus, we anticipated that glycoconjugates derived from **1**, which will have both the carbohydrate antigen and other molecular epitopes linked to the MPLA reducing end, will retain the immunostimulatory activities of MPLA and be highly immunogenic.

Results and Discussion

Depending on the structures and the locations of the acyl chains in the target molecule, there are two general strategies for the synthesis of lipid A and MPLA. One strategy is to assemble the disaccharide backbone first and then to introduce individual lipid chains at the correct positions.[23–29] In this case, various positions of the disaccharide unit have to be differentially protected by orthogonal protecting groups, especially when different lipid chains are present in the synthetic target. The second strategy is to build the lipi-

dated monosaccharides first and then to couple them together through a glycosylation reaction.[30–37] In this case, the glycosylation reaction can be challenging because of increased steric hindrance. Our target molecule **1** has three different types of lipids attached to 2,2′-*N*, 3,3′-*O* positions and we chose to adopt the second synthetic strategy (Scheme 2). For the monosaccharide **2**, we planned to introduce the lipid moiety at the 3′-*O*-position and to protect the 2′-*N*-position with a trichloroethyloxycarbonyl (Troc) group to minimize the steric hindrance of the glycosyl donor. The Troc group can be readily removed later for the introduction of the lipid moiety and the acylation of a free amino group should be relatively easy. For the monosaccharide block **3**, we planned to introduce both lipid chains at the initial stage because they were expected to have little influence on the relatively reactive primary alcohol as a glycosyl acceptor. Compound **3** was also designed to include an azidoethyl group linked to the anomeric position, which can be used as a molecular handle to perform various chemical transformations.

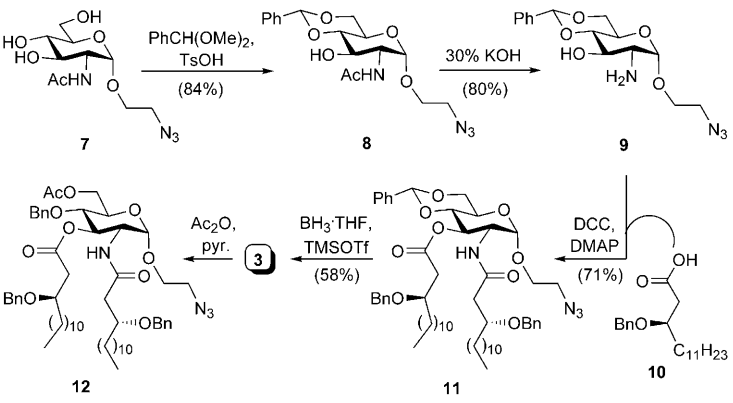
The synthesis of **2** is outlined in Scheme 3. Compound **4** was prepared from glucosamine after a series of established transformations.[29,39] The phthaloyl and acetyl groups pro-



Scheme 3. Synthesis of monosaccharide building block **2**. DCC = *N,N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, Phth = phthalyl.

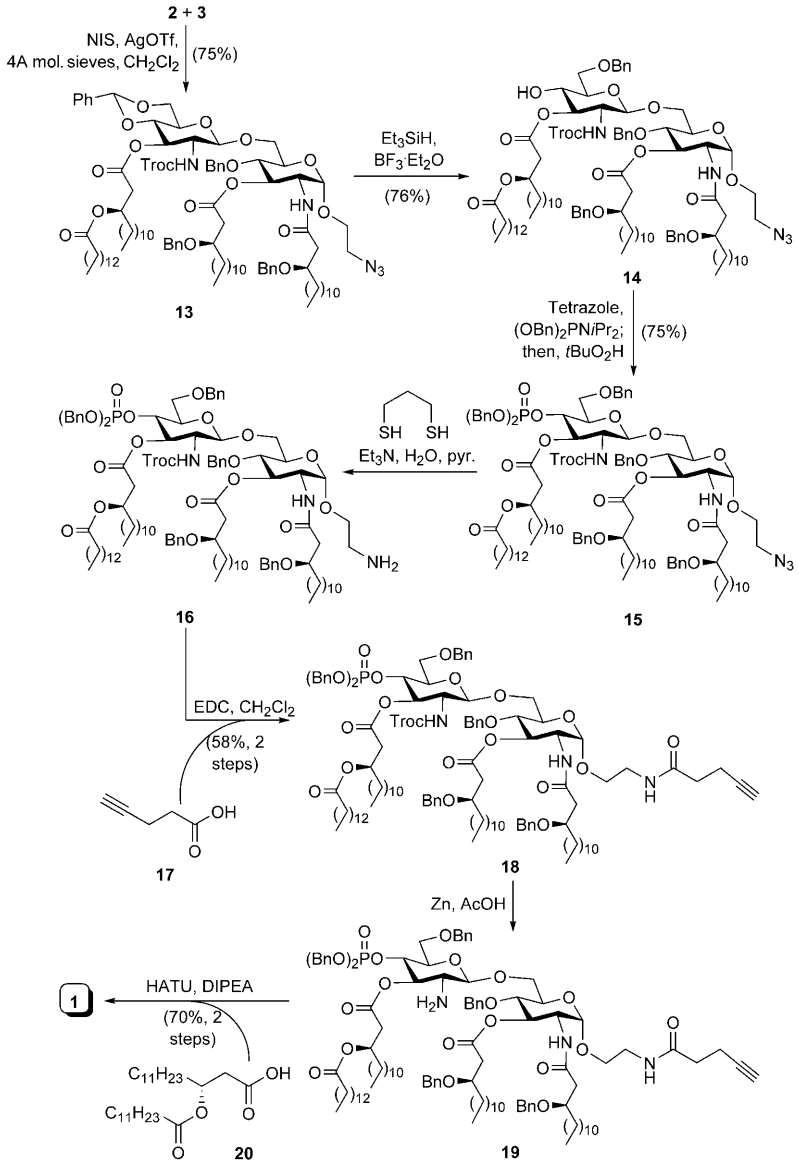
tecting the 2-*N*- and 3-*O*-positions, respectively, were removed by treatment with ethylenediamine, then regioselective protection of the free amino group with a Troc group was carried out to give **5**. The lipidation of **5** by means of coupling with (*R*)-3-tetradecanoyloxytetradecanoic acid (**6**) was accomplished by using *N,N*′-dicyclohexylcarbodiimide (DCC) as the coupling reagent to afford building block **2** in an excellent yield.

The synthesis of **3** (Scheme 4) started with the preparation of **7** by following a reported procedure.[40] The 4,6-*O*-positions in **7** were then selectively protected with a benzylidene group, followed by removal of the acetyl group under basic conditions. The amino and hydroxyl groups of **9** were simultaneously acylated by using (*R*)-3-benzyloxytetradecanoic acid (**10**), with DCC as a coupling reagent, to yield **11**. The benzylidene acetal ring was then opened regioselectively by using BH_3·THF as the reducing reagent and trimethylsilyl triflate (TMSOTf) as the catalyst to afford building block **3**. The regiochemistry of **3** was confirmed by the acetylation of **3**, which resulted in the signals in the ^{1}H NMR spectrum for protons 6a and 6b shifting downfield significantly.



Scheme 4. Synthesis of monosaccharide building block **3**. TsOH = 4-methyl benzenesulfonic acid, TMSOTf = trimethylsilyl triflate, Pyr. = pyridine.

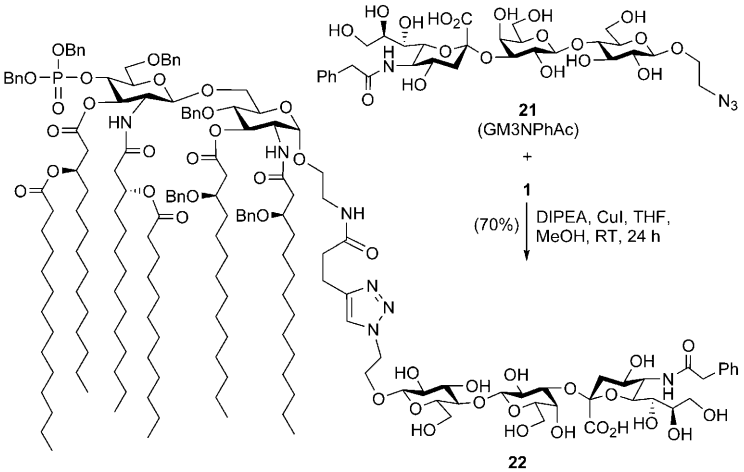
For the assembly of the synthetic target **1** (Scheme 5), **3** was glycosylated by **2** using *N*-iodosuccinimide (NIS) and silver triflate (AgOTf) as the promoters. This reaction was stereoselective and afforded only the desired β anomer (J-(1,2) = 3.2 Hz, J(1′,2′) = 8.0 Hz), probably owing to neighboring group participation, and **13** was obtained in a 75% isolated yield. For the regioselective opening of the benzylidene acetal ring of **13**, we were surprised to observe that **13** did not react with sodium cyanoborohydride ($NaCNBH_3$)/HCl (g) and the starting material was recovered completely. After exploring a number of reactions and conditions, we eventually found that the regioselective ring opening could be accomplished smoothly by using Et_3SiH and BF_3·Et_2O to expose the C-4′ hydroxyl group. The regiochemistry of product **14** was also confirmed by an acetylation experiment, which resulted in a significant downfield shift of the signal of H-4′ in the ^{1}H NMR spectrum. Next, compound **14** was phosphorylated by means of a one-pot protocol, using dibenzyl phosphoramidite and tBuO_2H as the phosphorylating and oxidizing reagents, respectively, to furnish **15**. We decided to introduce the alkyne functionality at this stage. Thus, the azido group of **15** was reduced with propane-1,3-dithiol and the free amine **16** was acylated with 4-pentynoic acid (**17**) by using *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) hydrochloride as the coupling reagent. It is worth noting that functionalities other than a carbon–carbon triple bond can also be introduced at this stage to facilitate alternative methods for the coupling of MPLA to various structures later on. Moreover, we found that other traditional methods employed to reduce azides, such as those using phosphines or the Lindlar catalyst/H_2, did not work for **15**. The former reaction gave a good yield of an iminophosphorane derivative that was difficult to hydrolyze and the latter reaction was slow and inefficient. Finally, the Troc group of **18** was removed by treatment with Zn in AcOH, and the resulting amine was coupled with (*R*)-3-dodecanoyloxy-tetradecanoic acid (**20**) by using (7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate (HATU)/*N,N*-diisopropylethylamine (DIPEA) to afford the synthetic target **1** in a



Scheme 5. Synthesis of the target MPLA derivative **1**. NIS = *N*-iodosuccinimide, EDC = *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride, HATU = (7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate, DIPEA = *N*,*N*-diisopropylethylamine.

70% yield. We observed that using DCC or EDC for the final condensation reaction only provided a low yield of the desired product. Compound **1** was characterized by ^{1}H NMR spectroscopy and high-resolution mass spectrometry.

To illustrate that **1** can be conveniently and efficiently coupled to suitably modified carbohydrates for investigating multicomponent conjugate vaccines, we then examined the reaction between **1** and an azido derivative **21** of *N*-phenylacetyl GM3 (GM3NPhAc) by means of a click reaction[41] (Scheme 6). GM3NPhAc, an unnatural analogue of GM3,[42] can form promising vaccines that are presently being investigated in our laboratory for cancer immunotherapy.[43,44] To our satisfaction, the reaction between **1** and **21** in the presence of CuI/DIPEA proceeded smoothly in a MeOH/THF mixture to give a very good yield of the MPLA–GM3NPhAc conjugate **22** (70% isolated yield after silica gel column chromatography).



Scheme 6. Coupling of **1** and GM3NPhAc through click chemistry.

Conclusion

A highly efficient synthesis of a monophosphoryl derivative **1** of *E. coli* lipid A has been developed. This synthesis is based upon the convergent assembly of the MPLA framework through the coupling of two lipidated monosaccharide building blocks **2** and **3**. The synthetic method should be applicable to similar MPLA structures. The synthetic derivatives of MPLA are useful for the exploration of novel MPLA-derived immunostimulants or immunoadjuvants. Furthermore, these MPLA derivatives can be readily coupled with various structures, as illustrated by the coupling of **1** and TACA **21** to form a MPLA–carbohydrate conjugate **22**, for the exploration of effective conjugate vaccines. Finally, glycoconjugate **22** was readily deprotected through Pd-catalyzed hydrogenolysis and both **22** and its deprotected product are being evaluated as cancer vaccines in our laboratory.

Experimental Section

General methods: NMR spectra were recorded on a 400 or 500 MHz Varian spectrometer with the chemical shifts reported in ppm (δ) in reference to tetramethylsilane (TMS), unless otherwise specified. Coupling constants (J) are reported in hertz (Hz). Optical rotations were obtained with an Autopol III polarimeter. High-resolution electrospray-ionization mass spectra (HR-ESIMS) were recorded by using a Waters Micromass-LCTPremier-XE mass spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Ultraflex mass spectrometer. TLC was performed on silica gel GF254 plates and visualized by staining with phosphomolybdic acid or 1% H_2SO_4 in EtOH. Molecular sieves were dried under high vacuum at 170–180 °C for 6–10 h before use. Commercial anhydrous solvents and other reagents were used without further purification.

Compound 2: DCC (184 mg, 0.90 mmol) and DMAP (28 mg, 0.23 mmol) were added to a stirred solution of **6** (200 mg, 0.44 mmol) in CH_2Cl_2 (6.0 mL) at RT. After stirring at RT for 10 min, a solution of **5** (200 mg, 0.36 mmol) in CH_2Cl_2 (1.5 mL) was added. The reaction mixture was stirred for 16 h at RT, then the solid materials were removed by filtration, and the residue was washed with CH_2Cl_2 (10 mL). The filtrate was concentrated under vacuum and the residue was purified by silica gel column chromatography to afford **2** as a white solid (330 mg, 93%). $[\alpha]_D = +14.1$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.42$–7.26 (m, 7H; Ar-H), 7.12 (d, $J = 7.2$ Hz, 2H; Ar-H), 5.51 (d, $J = 9.6$ Hz, 1H; NH), 5.48 (s, 1H; PhCH), 5.38 (t, $J = 9.6$ Hz, 1H; H-3), 5.20–5.14 (m, 1H; lipid), 4.90 (d, $J = 10.4$ Hz, 1H; H-1), 4.76 (d, $J = 4$ Hz, 2H; CH_2CCl_3), 4.34 (dd, $J = 4.8$, 10.4 Hz, 1H; H-6a), 3.78 (t, $J = 10.0$ Hz, 1H; H-6b), 3.68–3.60 (m, 2H; H-4, H-2), 3.56–3.50 (m, 1H; H-5), 2.62–2.48 (m, 2H; CH_2), 2.35 (s, 3H; CH_3), 2.17 (t, $J = 7.2$ Hz, 2H; CH_2), 1.55–1.52 (m, 4H; CH_2), 1.32–1.20 (m, 40H; lipid), 0.88 ppm (t, $J = 7.6$ Hz, 6H; lipid-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 173.7$, 170.2, 154.3, 138.8, 137.0, 133.7, 130.1, 129.4, 128.5, 126.4, 101.7, 88.1, 78.8, 74.8, 72.5, 70.7, 70.2, 68.7, 56.0, 39.5, 34.6, 34.1, 32.1, 29.9, 29.8, 29.59, 29.56, 29.4, 25.3, 25.2, 22.9, 21.4, 14.4 ppm; HRMS (ESI): m/z calcd for $C_{51}H_{76}Cl_3NO_9SNa$ $[M+Na]^+$: 1006.4204; found: 1006.4201.

Compound 11: DCC (0.64 g, 3.2 mmol) and DMAP (0.10 g, 0.08 mmol) were added to a stirred solution of **10** (0.70 g, 2.1 mmol) in CH_2Cl_2 (10 mL) at RT. After stirring for 10 min at RT, a solution of **9** (0.27 g, 0.81 mmol) in CH_2Cl_2 (1.5 mL) was added and the reaction mixture was stirred for 16 h at RT. The solid materials were removed by filtration and the residue was washed with CH_2Cl_2 (10 mL). The filtrate was concentrated under vacuum and the residue was purified by silica gel column chromatography to afford **11** as a white solid (0.56 g, 71%). $[\alpha]_D = +26.8$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.40$–7.21 (m, 15H; Ar-H), 6.20 (d, $J = 9.6$ Hz, 1H; NH), 5.46 (s, 1H; PhC*H*), 5.38 (t, $J = 9.6$ Hz, 1H; H-3), 4.73 (d, $J = 3.2$ Hz, 1H; H-1), 4.56–4.35 (m, 5H; 2PhCH_2, H-2), 4.25 (dd, $J = 9.6$, 4.4 Hz, 1H; H-5), 3.92–3.68 (m, 6H; H-6a, H-6b, H-4, 2 BnOC*H*, OCH_2CH$_2$N$_3$), 3.25 (dd, $J = 7.6$, 16.4 Hz, 2H; OCH_2CH_2N$_3$), 3.15–3.09 (m, 1H; CH_2N$_3$), 2.66 (dd, $J = 6.8$, 10.8 Hz, 1H; CH*H*), 2.44–2.36 (m, 1H; CH*H*), 2.33–2.26 (m, 2H; CH_2), 1.47–1.43 (m, 4H; CH_2), 1.30–1.19 (m, 36H; lipid), 0.89–0.86 ppm (t, $J = 6.4$ Hz, 6H; lipid); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 172.0$ 171.6, 138.9, 138.8, 137.1, 129.3, 128.6, 128.5, 128.4, 128.0, 127.9, 127.8, 127.6, 126.3, 101.8, 98.5, 79.4, 76.7, 75.7, 71.6, 71.3, 70.0, 69.0, 67.3, 63.4, 52.3, 50.4, 42.0, 40.0, 34.8, 34.1, 32.2, 29.92, 29.89, 29.8, 29.6, 25.5, 25.4, 22.9, 14.4 ppm; HRMS (ESI): m/z: calcd for $C_{57}H_{84}N_4O_9Na$ $[M+Na]^+$: 991.6136; found: 991.6152.

Compound 3: TMSOTf (0.08 mL, 0.46 mmol) was added dropwise to a stirred solution of compound **11** (365 mg, 0.38 mmol) in BH_3·THF/THF(2 mL, 2.0 mmol) at 0 °C. After stirring for 1 h, TLC analysis showed that the reaction was complete. The reaction was then quenched with triethylamine (0.5 mL) and MeOH (0.5 mL), the mixture was concentrated, and the residue was purified by silica gel column chromatography to afford **3** as a white solid (213 mg, 58%). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 7.33–7.20 (m, 15H; Ar-H), 6.13 (d, $J = 8.8$ Hz, 1H; NH), 5.39–5.34 (m, 1H; H-3), 4.71 (d, $J = 4.0$ Hz, 1H; H-1), 4.65–4.43 (m, 6H; 3PhCH_2), 4.28–4.22 (m, 1H; H-2), 3.85–3.62 (m, 7H; H-6b, H-4, H-5, H-6a, 2BnOC*H*, OCH_2CH$_2$N$_3$), 3.47–3.38 (m, 2H; OCH_2CH_2N$_3$), 3.25–3.19 (m, 2H; OCH_2CH_2N$_3$), 3.12–3.07 (m, 1H; CH_2N$_3$), 2.56 (dd, $J = 7.2$, 16.0 Hz, 1H; CH*H*), 2.39 (dd, $J = 5.6$, 16.0 Hz, 1H; CH*H*), 2.29–2.27 (m, 2H; CH_2), 1.66–1.43 (m, 4H; CH_2), 1.37–1.16 (m, 36H; lipid), 0.89–0.86 ppm (t, $J = 6.4$ Hz, 6H; lipid-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 172.3$, 171.6, 139.0, 138.8, 137.9, 128.7, 128.6, 128.5, 128.2, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 97.9, 76.7, 75.7, 75.3, 74.9, 73.3, 71.7, 71.6, 71.5, 70.8, 67.2, 61.7, 52.4, 50.4, 42.1, 40.0, 34.5, 34.2, 34.1, 32.2, 30.0, 29.93, 29.89, 29.8, 29.6, 26.7, 26.6, 25.5, 25.4, 25.2, 22.9, 14.4 ppm; HRMS (ESI): m/z: calcd for $C_{57}H_{86}N_4O_9Na$ $[M+Na]^+$: 993.6293; found: 993.6284.

Compound 13: A mixture of **2** (162 mg, 0.165 mmol), **3** (150 mg, 0.155 mmol), and 4 Å molecular sieves (0.5 g) was stirred in anhydrous CH_2Cl_2 (2 mL) at RT for 0.5 h. The reaction mixture was then cooled to −50 °C, and NIS (85 mg, 10 mmol) was added. The reaction mixture was allowed to warm to −30 °C and stirred for 1 h, then a catalytic amount of AgOTf was added. When TLC analysis showed that the glycosyl donor **2** was completely consumed, the reaction mixture was quenched with the addition of triethylamine (0.5 mL). The mixture was filtered through a pad of Celite and the filtrate was washed with water (1.0 mL), dried, and evaporated under vacuum. The residue was purified by silica gel column chromatography to give **13** as a white solid (212 mg, 75%). $[\alpha]_D = +11.2$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.42$–7.20 (m, 20H; Ar-H), 6.12 (d, $J = 9.6$ Hz, 1H; NH), 5.49 (s, 1H; PhC*H*), 5.36 (t, $J =$ 9.2 Hz, 1H; H-3), 5.29–5.25 (m, 1H; H-3′), 5.20–5.17 (m, 1H; lipid), 4.72 (d, $J = 3.2$ Hz, 1H; H-1), 4.68–4.46 (m, 8H; Troc, 3PhCH_2), 4.42 (d, $J =$ 8.0 Hz, 1H; H-1′), 4.33 (dd, $J = 4.8$, 10.4 Hz, 1H; H-6a′), 4.29–4.23 (m, 1H; H-2), 4.01 (br d, $J = 10.4$ Hz, 1H; H-6b), 3.87–3.75 (m, 4H; 2BnOC*H*, H-5, H-6b′), 3.69–3.63 (m, 5H; H-2′, H-4, H-4′, H-6a, OCH_2CH$_2$N$_3$), 3.47–3.40 (m, 2H; H-5′, lipid), 3.25–3.19 (m, 2H; OCH_2CH_2N$_3$), 3.12–3.07 (m, 1H; CH_2N$_3$), 2.62–2.53 (m, 3H; CH_2), 2.41 (dd, $J = 4.8$, 16.0 Hz, 1H; CH*H*), 2.28 (d, $J = 6.0$ Hz, 2H; CH_2), 2.19 (t, $J = 7.2$ Hz, 2H; CH_2), 1.62–1.44 (m, 8H; CH_2), 1.38–1.25 (m, 86H; lipid), 0.94–0.88 ppm (m, 12H; lipid-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 173.7, 172.2, 171,4, 170.3, 154.3, 138.9, 138.8, 138.2, 137.1, 129.4, 128.8, 128.6, 128.5, 128.2, 128.1, 128.0, 127.7, 126.4, 101.7, 97.8, 95,6, 78.9, 76.7, 75.7, 74.8, 74.5, 73.7, 71.6, 71.3, 70.8, 70.4, 70.2, 68.7, 67.2, 66.6, 57.0, 52.1, 50.4, 42.1, 40.0, 39.5, 34.6, 34.4, 34.3, 34.1, 32.2, 29.9, 29.8, 29.6, 29.4, 26.7, 25.4, 25.2, 22.9, 14.4 ppm; HRMS (ESI): m/z: calcd for $C_{101}H_{154}Cl_3N_5O_{18}Na$ $[M+Na]^+$: 1853.0252; found: 1853.0221.

Compound 14: Et_3SiH (15 equiv, 0.10 mL) and BF_3·Et_2O (2 equiv, 10 μL) were added to a stirred solution of **13** (70 mg, 0.038 mmol) in CH_2Cl_2 (2.0 mL) at 0 °C. The mixture was warmed to 25 °C over a period of 2 h, then diluted with CH_2Cl_2 (30 mL), washed with an aqueous solution of $NaHCO_3$ (5 mL), dried, and concentrated. The residue was purified by silica gel column chromatography to provide **14** (53 mg, 76%). $[\alpha]_D = +$ 17.4 ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.36$–7.17 (m, 20H; Ar-H), 6.10 (d, $J = 9.6$ Hz, 1H; NH), 5.31 (dd, $J = 8.8$, 10.4 Hz, 1H; H-3), 5.12–5.10 (m, 1H; lipid), 5.09 (d, $J = 8.4$ Hz, 1H; NH′), 4.91 (t, $J =$ 9.6 Hz, 1H; H-3′), 4.70 (d, $J = 4.0$ Hz, 1H; H-1), 4.63–4.42 (m, 10H; Troc, 4PhCH_2), 4.28–4.20 (m, 2H; H-2, H-1′), 4.06–4.02 (m, 1H; H-6b), 3.84–3.72 (m, 5H; 2BnOC*H*, H-5, H-6a′), 3.67–3.60 (m, 5H; H-2′, H-4, H-4′, H-6a, H-6b′), 3.48–3.38 (m, 2H; H-5′, OCH_2CH$_2$N$_3$), 3.24–3.17 (m, 2H; OCH_2CH_2N$_3$), 3.11–3.05 (m, 1H; CH_2N$_3$), 2.60–2.51 (m, 3H; CH_2), 2.41 (dd, $J = 4.8$, 15.2 Hz, 1H; CH_2), 2.30–2.26 (m, 4H; CH_2), 1.62–1.44 (m, 8H; CH_2), 1.38–1.25 (m, 86H; lipid), 0.94–0.88 ppm (m, 12H; lipid-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 174.6$, 172.3, 171,7, 171.5, 154.3, 138.9, 138.7, 138.3, 138.0, 128.7, 128.5, 128.2, 128.0, 127.9, 127.5, 101.3, 97.7, 95,7, 76.7, 75.7, 75.5, 75.4, 74.7, 73.9, 71.6, 71.2, 70.8, 70.5, 70.2, 67.7, 67.2, 55.8, 52.1, 50.4, 42.1, 40.3, 40.1, 36.9, 34.9, 34.7, 34.4, 34.3, 32.4, 32.2, 30.1, 29.9, 29.7, 29.6, 26.7, 25.4, 25.2, 24.9, 23.6, 22.9, 14.4 ppm; HRMS (ESI): m/z: calcd for $C_{101}H_{159}Cl_3N_5O_{18}Na$ $[M+Na]^+$: 1855.0409; found: 1855.0339.

Compound 15: 1*H*-tetrazole (0.45 M, 0.5 mL) and dibenzyl diisopropylphosphoramidite (0.05 mL, 0.15 mmol) were added to a stirred solution of **14** (50 mg, 0.027 mmol) in dry CH_2Cl_2 (5 mL). The mixture was stirred for 2 h at RT, then cooled to −30 °C, and *t*BuOOH (0.4 mmol) was added. The mixture was stirred for another 0.5 h at 0 °C. The resulting solution was washed sequentially with a saturated aqueous solution of $NaHCO_3$, then the organic phase was dried and concentrated. The resi-

due was purified by silica gel column chromatography to give **15** as an oil (43 mg, 75%). $[\alpha]_D = +5.2$ ($c = 0.5$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.36–7.22$ (m, 30H; Ar-H), 6.10 (d, $J = 9.6$ Hz, 1H; NH), 5.45 (d, $J = 8.4$ Hz, 1H; NH′), 5.38–5.32 (m, 2H; H-3, H-3′), 5.18 (m, 1H; lipid), 4.90–4.80 (m, 4H; 2 PhCH_2), 4.71(d, J=3.2 Hz, 1H; H-1), 4.65–4.58 (m, 3H; H-1′, PhCH_2), 4.54–4.38 (m, 8H; H-4′, Troc, PhCH_2), 4.29–4.24 (m, 1H; H-2), 4.06–4.03 (m, 1H; H-6b), 3.85–3.77 (m, 4H; 2 BnOCH, H-5, H-6a′), 3.69–3.56 (m, 5H; H-4, H-5′, H-6a), 3.54–3.45 (m, 1H; H-2′), 3.44–3.38 (m, 1H; OCH_2CH$_2$N$_3$), 3.26–3.17 (m, 2H; OCH_2CH_2N$_3$), 3.10–3.04 (m, 1H; CH_2N$_3$), 2.55 (dd, $J = 7.2$, 16.0 Hz, 1H; CHH), 2.47–2.33 (m, 1H; CHH), 2.29–2.21 (m, 5H; CH$_2$), 1.94–1.87 (m, 1H; CH$_2$), 1.62–1.44 (m, 8H; CH$_2$), 1.38–1.25 (m, 86H; lipid), 0.94–0.88 ppm (m, 12H; lipid-CH$_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 173.9$, 172.2, 171.4, 170.5, 154.2, 138.9, 138.8, 138.3, 138.2, 135.7, 129.7, 128.8, 128.6, 128.3, 128.1, 127.8, 127.6, 100.6, 97.8, 95.5, 76.6, 75.7, 74.7, 74.6, 74.4, 73.6, 72.5, 71.6, 70.8, 70.5, 70.3, 69.8, 68.8, 68.1, 67.6, 67.1, 56.7, 52.1, 50.4, 42.1, 40.0, 39.8, 36.9, 34.7, 34.4, 34.3, 32.2, 29.9, 29.6, 29.4, 26.7, 25.4, 24.9, 23.7, 23.2, 22.9, 14.3 ppm; ^{31}P NMR ($CDCl_3$, 162 MHz): $\delta = -0.92$ ppm; HRMS (ESI): m/z: calcd for $C_{115}H_{169}Cl_3N_5O_{21}PNa$ $[M+Na]^+$: 2115.1011; found: 2115.0942.

Compound 18: Triethylamine (0.2 mL) was added to a stirred solution of **15** (50 mg, 0.024 mmol) and 1,3-propanedithiol (0.15 mL) in pyridine (4 mL) and H_2O (0.2 mL). The mixture was stirred at 0°C until the starting material disappeared (monitored by TLC). The solvent was then evaporated under vacuum and the residue was coevaporated with toluene (2×10 mL) and ethanol (2×10 mL). The residue was purified by silica gel column chromatography to give **16**, which was then dissolved in CH_2Cl_2 (1.5 mL). EDC (9 mg, 0.048 mmol) and a solution of **17** (7 mg, 0.072 mmol) in CH_2Cl_2 (3 mL) was added to this solution. The reaction mixture was stirred for 2 h at RT. The mixture was diluted with CH_2Cl_2 and washed with H_2O and brine. The organic phase was dried, concentrated, and the residue was purified by silica gel column chromatography to afford **18** (30 mg, 58%). $[\alpha]_D = +9.2$ ($c = 0.65$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.32–7.20$ (m, 30H; Ar-H), 6.28 (d, $J = 9.6$ Hz, 1H; NH), 5.94 (s, 1H; NH), 5.68 (d, $J = 7.6$ Hz, 1H; NH′), 5.42 (dd, J= 9.6 Hz, 8.4 Hz, 1H; H-3′), 5.28 (dd, $J = 11.2$, 8.4 Hz, 1H; H-3), 5.17 (m, 1H; lipid), 4.91–4.88 (m, 4H; PhCH_2), 4.67–4.39 (m, 13H; H-1, H-1′, H-4′, Troc, PhCH_2), 4.27–4.21 (m, 1H; H-2), 4.06–4.04 (m, 1H; H-6b), 3.91–3.76 (m, 4H; 2BnOCH, H-5, H-6a′), 3.63–3.53 (m, 5H; H-4, H-5′, H-6a, OCH_2CH$_2$NHCO), 3.45–3.41 (m, 1H; H-2′), 3.14–3.08 (m, 2H; OCH_2CH_2NHCO), 3.00 (m, 1H, OCH$_2$CH_2NHCO), 2.58–2.20 (m, 12H; CH$_2$), 1.65–1.38 (m, 10H; CH$_2$), 1.38–1.25 (m, 90H; lipid), 0.87 ppm (t, $J = 6.4$ Hz, 12H; lipid-CH$_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 173.8$, 172.3, 171.5, 170.4, 154.3, 138.8, 138.4, 138.3, 137.9, 135.7, 128.8, 128.6, 128.3, 127.9, 127.8, 127.7, 127.6, 127.0, 100.4, 97.2, 95.5, 83.5, 77.1, 73.6, 72.3, 71.4, 71.2, 70.5, 70.3, 69.8, 68.8, 68.3, 67.6, 66.8, 56.7, 52.1,41.9, 39.9, 39.6, 39.0, 35.2, 34.7, 34.4, 34.1, 32.2, 29.9, 29.8, 29.6, 29.4 , 25.4, 25.2, 22.9, 15.0, 14.3 ppm; ^{31}P NMR ($CDCl_3$, 162 MHz): $\delta = -0.91$; HRMS (ESI): m/z: calcd for $C_{120}H_{175}Cl_3N_5O_{22}PNa$ $[M+Na]^+$: 2169.1368; found: 2169.1301.

Compound 1: Zn powder (100 mg) was added to a stirred solution of **18** (52 mg, 0.024 mmol) in acetic acid (5 mL) and the resulting mixture was stirred at RT for 24 h. The solid materials were removed by filtration through a pad of Celite. The filtrate was concentrated and coevaporated with toluene three times. The residue was dissolved in CH_2Cl_2 (40 mL) and washed sequentially with a saturated aqueous solution of $NaHCO_3$ (20 mL), H_2O (10 mL), and brine (10 mL). The organic phase was dried and concentrated. The residue was purified by silica gel column chromatography to afford **19** as a foamy solid.

A solution of HATU (50 mg, 0.132 mmol) in DMF (0.2 mL), was added to a stirred solution of **20** (50 mg, 0.121 mmol) and DIPEA (0.02 mL) in DMF (0.5 mL) under nitrogen. The resulting solution was stirred for 30 min, then added to a stirred solution of **19** in DMF (0.5 mL), and the resulting solution was stirred for 3.5 h at 60°C. The mixture was diluted with CH_2Cl_2 (50 mL) and washed sequentially with a saturated aqueous solution of $NaHCO_3$ (10 mL), H_2O (10 mL) and brine (3×20 mL). The organic phase was dried, concentrated, and the residue was purified by flash silica gel column chromatography to afford **1** as a white solid (39 mg, 70%). $[\alpha]_D = +16.4$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.31–7.19$ (m, 30H; Ar-H), 6.31 (d, $J = 9.2$ Hz, 1H; NH), 6.25 (d, $J = 7.2$ Hz, 1H; NH′), 5.41 (t, $J = 9.6$ Hz, 1H; H-3′), 5.27 (t, J= 9.6 Hz, 1H; H-3), 5.16 (m, 1H; lipid), 5.07 (m, 1H; lipid), 4.90–4.88 (m, 4H; PhCH_2), 4.64 (d, $J = 4.0$ Hz, 1H; H-1), 4.56–4.41 (m, 10H; H-1′, H-4′, 4PhCH_2), 4.28–4.22 (m, 1H; H-2), 4.06–4.03 (m, 1H; H-6b), 3.88–3.75 (m, 4H; 2ROCH, H-5, H-6a′), 3.66–3.48 (m, 6H; H-4, H-5′, H-6a, H-2′, OCH_2CH$_2$NHCO), 3.22–3.10 (m, 3H; OCH_2CH_2NHCO), 2.56–2.19 (m, 15H), 2.13 (d, $J = 2.0$ Hz, 1H; CH$_2$), 1.55–1.42 (m, 12H; CH$_2$), 1.24–1.03 (m, 116H; lipid), 0.98–0.88 ppm (m, 18H; lipid-CH$_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 173.8$, 172.1, 171.5, 171.3, 170.5, 138.8, 138.5, 138.3, 137.7, 135.8, 128.8, 128.7, 128.5, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 100.5, 97.3, 95.5, 83.7, 76.9, 76.1, 75.6, 74.7, 74.4, 73.8, 73.5, 72.7, 71.4, 71.2, 70.7, 70.4, 70.0, 69.8, 68.8, 62.0, 57.9, 52.2, 50.7, 41.9, 39.9, 35.2, 34.7, 34.5, 34.2, 32.2, 29.9, 29.6, 29.5, 29.5, 25.5, 25.4, 25.3, 22.9, 15.1, 14.4 ppm; ^{31}P NMR ($CDCl_3$, 162 MHz): $\delta = -0.91$ ppm; HRMS (ESI): m/z: calcd for $C_{143}H_{222}N_3O_{23}PNa$ $[M+Na]^+$: 2403.5930; found: 2403.5903.

Compound 22: DIPEA (10 equiv) was added to a stirred solution of **1** (10 mg, 0.004 mmol), **21** (4 mg, 0.005 mmol), and CuI (10 equiv) in MeOH/THF (2:1, 0.6 mL). The resulting mixture was stirred for 24 h at RT, then filtered through a Celite pad, and the solvent was evaporated under high vacuum. The crude product was purified by flash silica gel column chromatography (eluent: from AcOEt/hexane (4:1) to AcOEt and then to MeOH/CH_2Cl_2 1:5) to give **22** as a white solid (9 mg, 70%). $[\alpha]_D = 4.4$ ($c = 0.45$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$/CD_3OD (3:1), 500 MHz): $\delta = 7.45$ (s, 1H; triazole-H), 7.19–6.90 (m, 35H; Ar-H), 5.24–5.21 (m, 1H; H-3′), 5.15–5.08 (m, 1H; H-3), 4.98 (m, 1H; lipid), 4.88 (m, 1H; lipid), 4.72–4.65 (m, 4H; PhCH_2), 4.43 (d, $J = 3.5$ Hz, 1H; H-1), 4.39–4.42 (m, 12H; H-1′, H-4′, PhCH_2), 4.09 (d, $J = 7.0$ Hz, 1H; H-sugar), 4.28–4.22 (m, 1H; H-2), 3.96 (m, 1H; H-6b), 3.78 (m, 1H; H-sugar), 3.68–3.35 (m, 4H; 2ROCH, H-5, H-6a′), 2.98–2.92 (m, 2H; CH$_2$), 2.75 (m, 2H; CH$_2$), 2.35–1.99 (m, 12H; CH$_2$), 1.86–1.80 (m, 3H; CH$_2$), 1.55–1.42 (m, 12H; CH$_2$), 1.24–1.03 (m, 116H; lipid), 0.98–0.88 ppm (m, 18H; lipid); ^{13}C NMR ($CDCl_3$/CD_3OD (3:1), 125 MHz): $\delta = 129.0$, 128.7, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 127.0, 123.2, 103.0, 100.2, 97.4, 77.6, 76.7, 75.9, 75.4, 75.0, 74.6, 74.5, 74.0, 73.4, 73.3, 73.1, 72.6, 71.5, 71.2, 70.6, 70.0, 69.8, 68.5, 68.0, 62.0, 57.9, 52.2, 51.7, 50.2, 49.3, 49.1, 49.0, 48.8, 48.6, 41.4, 39.6, 38.8, 35.1, 34.5, 34.2, 31.9, 29.6, 29.3, 29.2, 25.4, 25.1, 25.0, 22.7, 21.2, 13.9 ppm; ^{31}P NMR ($CDCl_3$/CD_3OD 3:1, 162 MHz): $\delta = -1.50$ ppm; MALDI-TOF MS: m/z: calcd for $C_{174}H_{268}N_7O_{42}P$: 3158.9 $[M]^+$; found: 3181.8 $[M+Na]^+$, 3203.8 $[M-H+2Na]^+$.

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