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Modulation of Innate Immune Responses with Synthetic Lipid A Derivatives

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Abstract: The lipid A moiety of lipopolysaccharides (LPS) initiates innate immune responses by interacting with Toll-like receptor 4 (TLR4), which results in the production of a wide range of cytokines. Derivatives of lipid A show potential for use as immuno-modulators for the treatment of a wide range of diseases and as adjuvants for vaccinations. Development to these ends requires a detailed knowledge of patterns of cytokines induced by a wide range of derivatives. This information is difficult to obtain by using isolated compounds due to structural heterogeneity and possible contaminations with other inflammatory components. To address this problem, we have developed a synthetic approach that provides easy access to a wide range of lipid A's by employing a common disaccharide building block functionalized with a versatile set of protecting groups. The strategy was employed for the preparation of lipid A's derived from *E. coli* and *S. typhimurium*. Mouse macrophages were exposed to the synthetic compounds and *E. coli* 055:B5 LPS, and the resulting supernatants were examined for tumor necrosis factor alpha (TNF-α), interferon beta (IFN-β), interleukin 6 (IL-6), interferon-inducible protein 10 (IP-10), RANTES, and IL-1β. It was found that for each compound, the potencies (EC_{50} values) for the various cytokines differed by as much as 100-fold. These differences did not follow a bias toward a MyD88- or TRIF-dependent response. Instead, it was established that the observed differences in potencies of secreted TNF-α and IL-1β were due to differences in the processing of respective pro-proteins. Examination of the efficacies (maximum responses) of the various cytokines showed that each synthetic compound and *E. coli* 055:B5 LPS induced similar efficacies for the production of IFN-β and IP-10. However, lipid A's **1**−**4** gave lower efficacies for the production of RANTES and IL-6 as compared to LPS. Collectively, the presented results demonstrate that cytokine secretion induced by LPS and lipid A is complex, which can be exploited for the development of immuno-modulating therapies.

Introduction

The innate immune system is an evolutionarily ancient system designed to detect the presence of microbial invaders and activate protective reactions.[1] It responds rapidly to compounds that are integral parts of pathogens that are perceived as danger signals by the host. Recognition of these molecular patterns is mediated by sets of highly conserved receptors,[2] whose activation results in acute inflammatory responses. These responses include the production of a diverse set of cytokines and chemokines, direct local attack against the invading pathogen, and initiation of responses that activate and regulate the adaptive component of the immune system.[3−8]

Evidence is emerging that innate immune responses can be exploited for therapeutic purposes such as the development of adjuvants for vaccines and the treatment of a wide range of diseases including asthma, infections, and cancer. An important concern of such therapies is, however, that over-activation of innate immunity may lead to the clinical symptoms of septic shock.[9,10] Thus, an important issue for the design of safe immune modulators is a detailed knowledge of structure−activity relationships to harness beneficial effects without causing toxicity.

Lipopolysaccharides (LPS) are structural components of the outer membrane of Gram-negative bacteria and offer great promise for the development of immuno-modulators. LPS consists of a hydrophobic domain known as lipid A, a non-repeating core oligosaccharide, and a distal polysaccharide (or O-antigen).[11,12] The lipid A moiety of *E. coli* consists of a hexaacylated bis-1,4′-phosphorylated glucosamine disaccharide, which has (*R*)-3-hydroxymyristyl residues at C-2, C-2′, C-3, and C-3′ (Figure 1). Furthermore, both of the primary (3)-hydroxyacyl chains in the distal glucosamine moiety are esterified with lauric and myristic acids, and the primary

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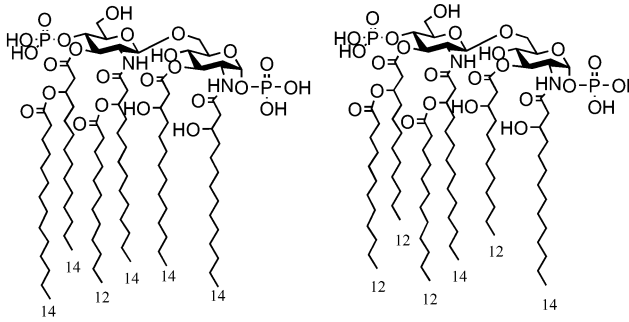
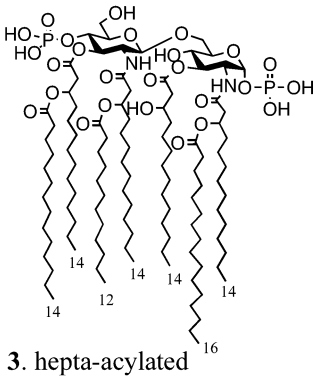
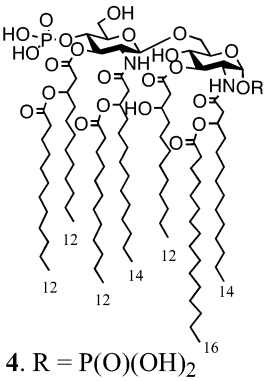


1. hexa-acylated *E. coli* lipid A

2. *E. coli* lipid A w/ shorter lipids



3. hepta-acylated *S. typhimurium* lipid A



4. R = $P(O)(OH)_2$ *S. typhimurium* lipid A w/ shorter lipids

5. R = H

Figure 1. Chemical structures of target lipid A's **1**−**5**.

hydroxyl at the C-6 position is linked to the polysaccharide through a di-KDO carbohydrate moiety. It has been demonstrated unequivocally that lipid A is the inflammation-inducing moiety of LPS.[13,14]

Lipid A triggers innate immune responses through Toll-like receptor 4 (TLR4), a member of the TLR family that participates in pathogen recognition. Immediately distal to TLR4 activation are two intracellular cascades that regulate signal transduction processes, gene expression, and production of (pro)inflammatory mediators.[7] One of these cascades requires a specific intracellular adaptor protein called MyD88, while the other cascade utilizes the TRIF adaptor protein. The MyD88-dependent pathway leads to up-regulation of cytokines and chemokines such as tumor necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β), IL-6, and monocyte chemoattractant protein 1 (MCP-1), whereas the TRIF-dependent pathway leads to the production of interferon-beta (IFN-β), which in turn activates the STAT-1 pathway, resulting in the production of mediators such as interferon-inducible protein 10 (IP-10) and nitric oxide.[15]

Recent structural studies have demonstrated that the carbohydrate backbone, degree of phosphorylation, and fatty acid acylation patterns vary considerably among bacterial species. These structural differences probably account for the highly variable in-vivo and in-vitro host responses to LPS.[11,12,16−18] There is also some indication that structurally different lipid A's may differentially induce proinflammatory responses.[19−22] For example, in one study, LPS from *E. coli* O55:B5 induced the production of mediators (TNF-α, IL-1β, MCP-1, and macrophage inflammatory protein 3alpha (MIP-3α)) arising from the MyD88-dependent pathway, but caused less production of mediators (IFN-β, nitric oxide, and IP-10) arising from the TRIF-dependent pathway. In contrast, LPS from *S. typhimurium* invoked strong production of mediators associated with the TRIF-dependent pathway, but caused only minimal production of TNF-α, IL-1β, MCP-1, and MIP-3α. Heterogeneity in the structure of lipid A within a particular bacterial strain and possible contamination with other inflammatory components of the bacterial cell-wall complicate the use of either LPS or lipid A isolated from bacteria to dissect the molecular mechanisms responsible for the biological responses to specific lipid A molecules.

Fortunately, homogeneous lipid A derivatives can be obtained by chemical synthesis.[17] The results of studies with small numbers of synthetic analogues have shown that the number of acyl chains and phosphate substitution are important for cytokine production. These studies have not examined whether particular structural modifications have different effects on the production of particular cytokines and chemokines. The development of safe immuno-modulators requires, however, such knowledge because different mediators induce different biological effects. To address this important issue, we have developed an efficient synthetic approach whereby an advanced synthetic disaccharide can easily be converted into lipid A analogues that differ in phosphorylation and acylation pattern. This strategy has been employed for the preparation of lipid A's derived from *E. coli* and *S. typhimurium*. Mouse macrophages were exposed to the synthetic compounds and *E. coli* 055:B5 LPS, and the resulting supernatants were examined for mouse TNF-α, IFN-β, IL-6, IP-10, RANTES, and IL-1β. It has been found that particular modifications had different effects on the potencies and efficacies of induction of the various cytokines. However, no bias toward a MyD88- or TRIF-dependent response was observed. Thus, for the first time, it has been shown that lipid A derivatives can modulate innate immune responses in a complex manner.

Results and Discussion

Chemical Synthesis of Lipid A's. To determine whether the structure of lipid A can modulate innate immunological responses, we have synthesized derivatives **1**−**5** (Figure 1) by a highly convergent approach. Compound **1** is a prototypical lipid A from *E. coli* and is hexa-substituted in an asymmetrical fashion. Compound **2** is derived from compound **1**, but several

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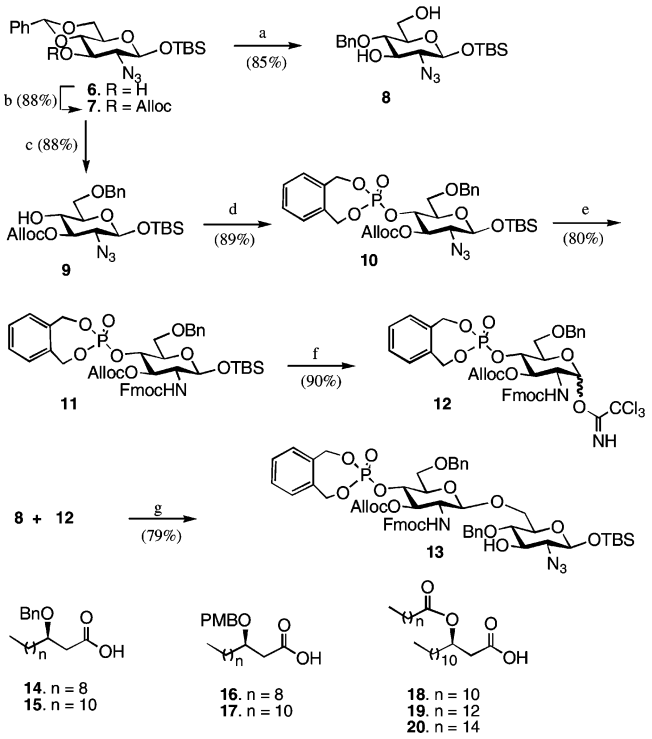
of its acyl groups have been shortened. Compounds **3**, **4**, and **5** are hepta-acylated lipid A's derived from *S. typhimurium* LPS that differ in lipid length and phosphorylation pattern.[23−26]

Previously reported approaches for lipid A synthesis employed strategies whereby monosaccharides were functionalized with lipids and phosphates, which were then used as glycosyl donors and acceptors for disaccharide synthesis, which after anomeric phosphorylation and deprotection provided target compounds.[17,27,28] Although this approach is attractive for one-compound-at-a-time synthesis, detailed structure−activity relationship studies require a synthetic approach that offers in a straightforward manner a panel of lipid A's. The strategy that we have developed employs the advanced disaccharide intermediate **13**, which can selectively be modified with any lipid at C-2, C-3, C-2′, and C-3′. A key feature of **13** is the use of the allyloxycarbonate (Alloc), the anomeric *tert*-butyldimethyl silyl ether (TBDMS), and the (9-fluorenylmethoxycarbamate (Fmoc) and azido as a set of functional groups that in a sequential manner can be deprotected or unmasked to allow selective lipid modification at each position. It was envisaged that disaccharide **13** could easily be prepared by a regio- and stereoselective glycosylation of trichloroacetimidate **12** with glycosyl acceptor **8**. In this glycosylation, the higher glycosyl accepting reactivity of the primary C-6 hydroxyl of **8** as compared to its secondary C-3 hydroxyl, and the ability of the Fmoc carbamate of **12** to control the β-anomeric configuration by neighboring group participation,[29] were exploited.

Glycosyl acceptor **8** and donor **12** could easily be prepared from common intermediate **6** (Scheme 1). Thus, a regioselective reductive opening of the benzylidene acetal of **6** using borane−THF complex in the presence of the bulky Lewis acid Bu_2BOTf gave glycosyl acceptor **8** as the only regio-isomer. Alternatively, the C-3 hydroxyl of **6**[30] could be protected by an Alloc group by treatment with Alloc chloride in the presence of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) in DCM to give **7** in a yield of 88%. Regioselective reductive opening of the benzylidene acetal of **7** using $NaCNBH_3$ and HCl in diethyl ether gave **9**,[31] which after phosphitylation with *N*,*N*-diethyl-1,5-dihydro-2,3,4,-benzodioxaphosphepin-3-amine in the presence of 1*H*-tetrazole followed by in-situ oxidation with *m*-chloroperoxybenzoic acid (mCPBA)[32] provided the phosphotriester **10**. Next, the azido function of **10** was reduced using activated Zn in a mixture of acetic acid and DCM to give an amine, which was immediately protected as an Fmoc carbamate by reaction with 9-fluorenylmethyl chloroformate (FmocCl) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in DCM to give fully protected **11**. Removal of the anomeric TBDMS ether of **11** by treatment with HF in pyridine followed by conversion of the resulting anomeric hydroxyl into a trichloroacetimidate by reaction with trichloroacetonitrile in the presence of a catalytic amount of NaH[33] afforded glycosyl donor **12** in an overall yield of 90%. A trimethylsilyl trifluoromethanesulfonate (TMSOTf)-mediated glycosylation of the trichloroacetimidate **12** with glycosyl acceptor **8** in dichloromethane gave the selectively protected disaccharide **13** in a yield of 79% as only the β-anomer. The alternative regioisomer resulting from glycosylation of the C-3 hydroxyl or the trisaccharide arising from glycosylation of both hydroxyls of **8** was not observed. The acyloxy- and acyloxyacyl lipids **14**−**20** were prepared by a reported procedure.[34]

Scheme 1 [a]



[a] Reagents and conditions: (a) BH_3·THF, Bu_2OTf, THF; (b) AllocCl, TMEDA, DCM; (c) $NaCNBH_3$, 2 M HCl in diethyl ether, DCM; (d) *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine, tetrazole, DCM; then mCPBA, −20 °C; (e) Zn/HOAc, DCM; then FmocCl, DIPEA, DCM; (f) HF/pyridine, THF; then $CNCCl_3$, NaH, THF; (g) TMSOTf, DCM, −50 °C.

Having the advanced disaccharide **13** and lipids **14**−**20** at hand, attention focused on the selective acylation of relevant hydroxyls and amines (Scheme 2). Thus, removal of the Fmoc protecting group of **13** using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DCM followed by acylation of the resulting amino group with (*R*)-3-dodecanoyl-tetradecanoic acid (**18**) using 1,3-dicyclohexylcarbodiimide (DCC) as the activation reagent gave compound **21**. Next, the C-3 hydroxyl of **21** was acylated with (*R*)-3-benzyloxy-tetradecanoic acid (**15**) using DCC and 4-dimethylaminopyridine (DMAP)[35] to give **22** in a yield of 86%.

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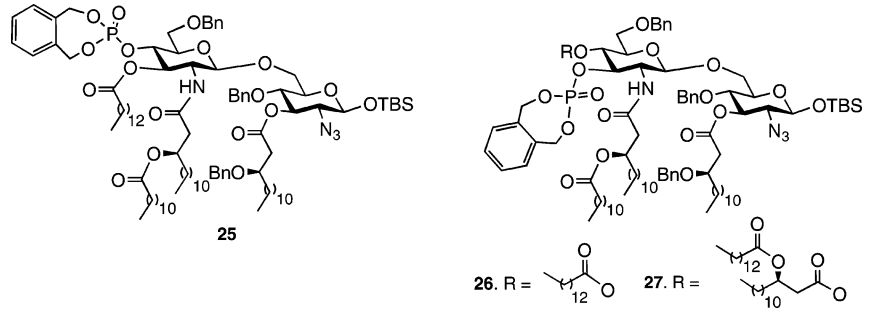
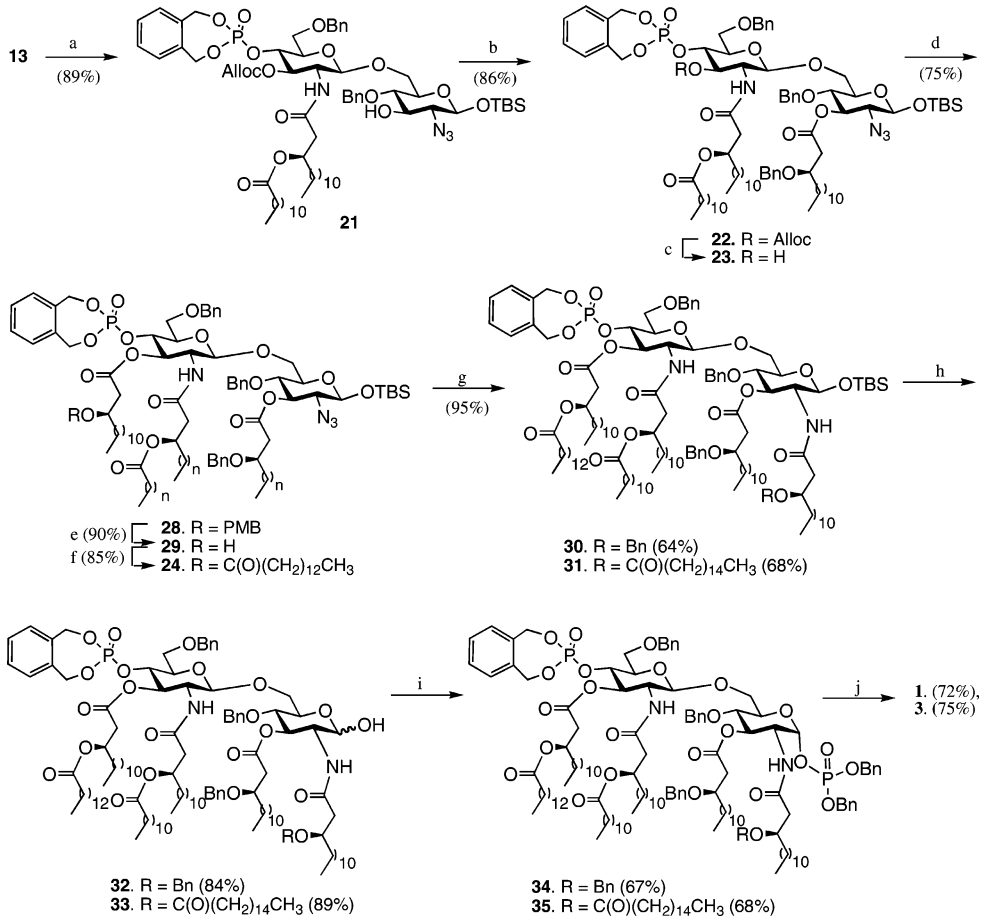


Figure 2. Chemical structures of side products **25**−**27**.

Scheme 2 [a]



[a] Reagents and conditions: (a) DBU, DCM; then (*R*)-3-dodecanoyloxy-tetradecanoic acid **18**, DCC, DCM; (b) (*R*)-3-benzyloxy-tetradecanoic acid **15**, DCC, DMAP, DCM; (c) $Pd(PPh_3)_4$, HCO_2H, *n*-$buNH_2$, THF; (d) (*R*)-3-(*p*-methoxy)benzyloxy-tetradecanoic acid **17**, DCC, DMAP, DCM; (e) DDQ, H_2O, DCM; (f) myristoyl chloride, pyridine, DMAP, DCM; (g) Zn/HOAc, DCM; then RCOOH, DCC, DCM; (h) HF/pyridine; (i) tetrabenzyl diphosphate, $LiN(TMS)_2$, THF, −78 °C; (j) H_2 (50 psi), Pd-black, THF.

The latter two reactions exploited the finding that an amine can selectively be acylated in the presence of a free hydroxyl using DCC as the activator. The addition of DMAP provides, however, a more reactive reagent and can acylate a less nucleophilic hydroxyl. The removal of the Alloc protecting group of **22** could easily be accomplished by treatment with $Pd(PPh_3)_4$;[36] however, the acylation of the resulting hydroxyl of **23** with (*R*)-tetradecanoyltetradecanoic acid (**19**) using standard conditions did not, unexpectedly, lead to the formation of **24**. Instead, compounds **25**, **26**, and **27** were identified (Figure 2). The formation of these compounds can be rationalized by migration of the phosphotriester to the C-3′ position and elimination of the acyloxy chain of (*R*)-3-tetradecanoyl-tetradecanoic acid to give tetradecanoic acid and tetradec-2-enoic acid. It is proposed that compound **25** arises from acylation of the starting material with tetradecanoic acid, whereas compounds **26** and **27** result from phosphotriester migration followed by acylation with tetradecanoic acid or **19**, respectively. To circumvent these side reactions, the (*R*)-3-tetadecanoyl-tetradecanoic ester was introduced by a three-step procedure using (*R*)-3-(*p*-methoxy)-benzyloxy-tetradecanoic acid (**17**) as the initial acylation reagent. It was reasoned that the (*p*-methoxy)benzyl (PMB) ether of **17** would be less susceptible to elimination, and hence the formation of the elimination product should be suppressed. Furthermore,

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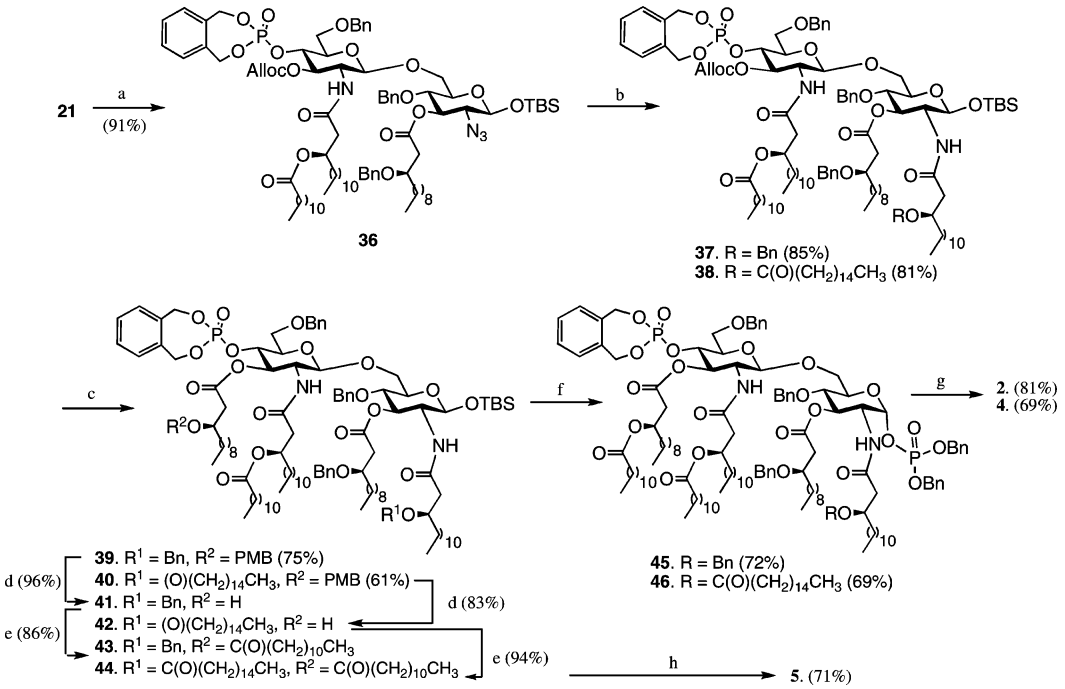
Scheme 3 [a]



[a] Reagents and conditions: (a) (*R*)-3-benzyloxy-dodecanoic acid **14**, DCC, DMAP, DCM; (b) Zn/HOAc, DCM; then RCOOH, DCC, DCM; (c) $Pd(PPh_3)_4$, HCO_2H, *n*-$BuNH_2$, THF; then (*R*)-3-(*p*-methoxy)benzyloxy-dodecanoic acid **16**, DCC, DMAP, DCM; (d) DDQ, H_2O, DCM; (e) lauroyl chloride, pyridine, DMAP, DCM; (f) HF/pyridine; then tetrabenzyl diphosphate, $LiN(TMS)_2$, THF, −78 °C; (g) H_2 (50 psi), Pd-black, THF; (h) HF/pyridine; then H_2 (50 psi), Pd-black, THF.

the higher reactivity of ether protected **17** may also suppress phosphate migration. After installment of the (*R*)-3-(*p*-methoxy)-benzyloxytetradecanoic ester and selective removal of the PMB ether, the β-hydroxy functionality can be acylated to provide the required compound. Thus, treatment of **23** with **17** in the presence of DCC and DMAP to give **28** followed by removal of the PMB ether using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in a mixture of DCM and water in the dark, and acylation of the resulting β-hydroxyl of **29** with myristoyl chloride in the presence of pyridine and DMAP, afforded **24**. Although the three-step procedure to convert **23** into **24** is more laborious than direct acylation with an acyloxyacyl acid, it offers an opportunity to devise a range of compounds that differ in β-hydroxy acylation at the C-3′ position. Next, the azido function of **24** was reduced with activated Zn in a mixture of acetic acid and DCM, and the amine of the resulting compound was reacted with **15** or **20** in the presence of DCC to give **30** and **31**, respectively. Next, attention was focused on the introduction of the anomeric phosphate and removal of the permanent protecting groups. Thus, the anomeric TBS ether of **30** and **31** was removed by treatment with HF in pyridine, conditions that do not affect the acyl and acyloxyacyl esters and the phosphodiester, to give **32** and **33**, respectively. These derivatives were phosphorylated using tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide in THF at −78 °C to give,[37] after purification using Iatro beads, **34** and **35** as only α-anomers. Global deprotection of **34** and **35** by catalytic hydrogenolysis over Pd-black gave the requisite lipid A's **1** and **3**, respectively.

Lipid A's **1** and **3** were prepared by first removal of the Alloc protecting group of **22** and acylation of the resulting hydroxyl followed by reduction of the azido moiety and modification of the corresponding amine. To study the orthogonality of the Alloc and azido function, compounds **2**, **4**, and **5** were prepared by an alternative sequence of reactions involving reduction of the azido function and modification of the C-2 amine before deprotection of Alloc group and acylation of the resulting C-3′ hydroxyl (Scheme 3). Thus, the C-3 hydroxyl of **21** was acylated with **14** using DCC and DMAP to give **36** in a yield of 91%. Next, the azido moiety of **36** was reduced with activated Zn in a mixture of acetic acid and DCM without affecting the Alloc group to provide an intermediate amine, which was immediately acylated with (*R*)-3-benzyloxy-dodecanoic acid (**14**) or (*R*)-3-tetradecanoyl-hexadecanoic acid (**20**), using DCC as the activating system, to give **37** and **38**, respectively. Next, Pd(0)-mediated removal of the Alloc group of **37** and **38**, followed by acylation of the resulting hydroxyl with (*R*)-3-(*p*-methoxy)-benzyloxy-dodecanoic acid (**16**) in the presence of DCC/DMAP, gave **39** and **40**, which after treatment with DDQ to remove the PMB ether were acylated with lauroyl chloride to give fully acylated **43** and **44**, respectively, in a good overall yield. Finally, cleavage of the anomeric TBS ether of **43** and **44** was performed under standard conditions to give intermediate lactols, which were phosphorylated using tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide to give **45** and **46**. Deprotection of the latter compounds by catalytic hydrogenolysis over Pd-black gave lipid A derivatives **2** and **4**. Monophosphoryl derivative **5** could easily be obtained by standard deprotection of the intermediate lactol.

Biological Evaluation of Lipid A's and LPS. Based on the results of recent studies,[5,7] it is clear that LPS-induced cellular activation through TLR4 is complex as many signaling elements are involved. However, it appears that there are two distinct initiation points in the signaling process, one being a specific

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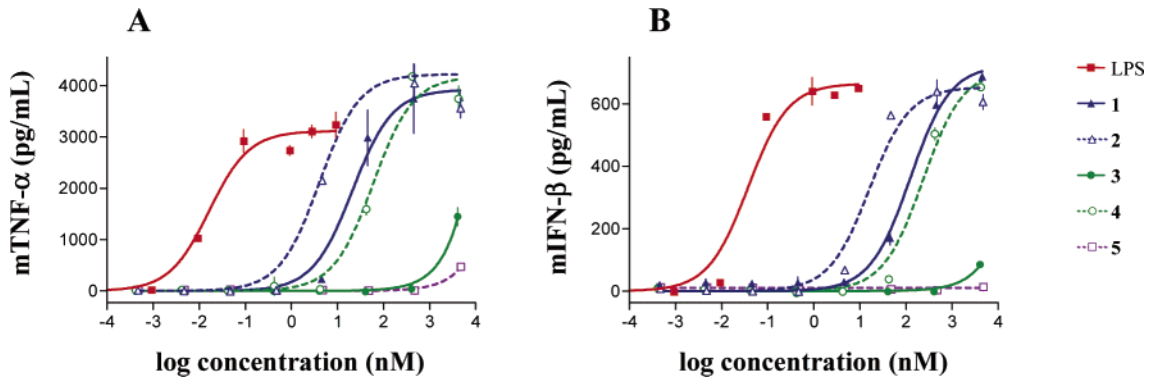

Figure 3. TNF-α and IFN-β production by murine macrophages after stimulation with LPS and lipid A derivatives. Murine RAW γNO(−) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS or lipid A derivatives **1**−**5** as indicated. TNF-α (A) and IFN-β (B) in cell supernatants were measured using ELISAs.

intracellular adaptor protein called MyD88 and the other an adaptor protein called TRIF, which operates independently of MyD88. It is well-established that TNF-α secretion is a prototypical measure for activation of the MyD88-dependent pathway, whereas secretion of IFN-β is commonly used as an indicator of TRIF-dependent cellular activation. There are some indications that structurally different lipid A's can differentially utilize signal transduction pathways leading to complex patterns of proinflammatory responses. Heterogeneity in lipid A of particular bacterial strains as well as possible contamination with other inflammatory components of the bacterial cell-wall complicates the use of either LPS or lipid A isolated from bacteria to dissect the molecular mechanisms responsible for the biological responses to specific lipid A's. To address these issues, we have examined the well-defined compounds **1**−**5** and *E. coli* LPS for the ability to initiate production of a wide range of cytokines, including TNF-α, INF-β, IL-6, IP-10, RANTES, and IL-1β. It was anticipated that analysis of potencies and efficacies of the mediators would establish whether structural differences in lipid A can modulate inflammatory responses.

Mouse macrophages (RAW 264.7 γNO(−) cells) were exposed over a wide range of concentrations to compounds **1**−**5** and *E. coli* 055:B5 LPS. After 5.5 h, the supernatants were harvested and examined for mouse TNF-α and IFN-β using a commercial and in-house developed capture ELISA assay, respectively. Potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose−response curves to a logistic equation using PRISM software. As can be seen in Figure 3, the lipid A's and *E. coli* 055:B5 displayed large differences in potencies. Thus, lipid A's **1**, **2**, and **4** and *E. coli* 055:B5 LPS yielded clear dose response curves. *S. typhimurium* lipid A **3** gave only a partial response at the highest concentration tested, whereas monophosphate **5** was inactive. Furthermore, the EC_{50} values for *E. coli* 055:B5 LPS were significantly smaller than those of *E. coli* lipid **1** and **2** (Table 1). Probably, the higher potency of LPS is due to its di-KDO moiety, which is attached to the C-6′ position of lipid A. In this respect, recent studies[38] have shown that meningococcal lipid A expressed by a strain defect in KDO biosynthesis has significantly reduced bioactivity as compared to KDO containing *Meningococcal* lipooligosaccharides. It has also been shown that removal of the KDO moieties by mild acidic treatment reduces cellular responses.[35]

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Table 1. EC_{50} Values[a] (nM) of *E. coli* LPS and Lipid A Derivatives **1**, **2**, and **4**

	E. coli LPS	lipid A **1**	lipid A **2**	lipid A **4**
TNF-α	0.016 (0.012−0.022)	21 (16−28)	4.1 (2.5−6.7)	60 (44−81)
IFN-β	0.038 (0.025−0.056)	124 (105−147)	16 (12−23)	234 (180−306)
IL-6	0.063 (0.044−0.091)	157 (91−271)	14 (6−33)	462 (383−559)
IP-10	0.030 (0.019−0.046)	44 (37−52)	12 (10−16)	156 (120−204)
RANTES	0.116 (0.103−0.131)	238 (201−281)	43 (36−51)	570 (478−681)
IL-1β	1.74[b] (1.59−1.91)	674 (622−728)	30 (26−35)	348 (284−428)
pro Il-1β	0.014 (0.008−0.025)	39 (32−47)	3.6 (1.4−9.4)	63 (48−84)
NF-κB	0.004 (0.003−0.005)	38 (30−48)	14 (9−20)	53 (42−67)

[a] Values of EC_{50} are reported as best-fit values and as minimum−maximum range (best-fit value ± std error). [b] Plateau not reached; EC_{50} value is best-fit value according to Prism.

Further examination of the data revealed that the hexa-acylated *E. coli* lipid A (**1**) is significantly more potent than the hepta-acylated *S. typhimurium* lipid A (**3**). Shortening of lipids, such as in compounds **2** and **4**, resulted in higher potencies (smaller EC_{50} values). In the case of the *E. coli* lipid A's (**1** vs **2**), the differences in EC_{50} values were relatively small, whereas for the *S. typhimurium* lipid A's (**3** vs **4**) an approximate 3 orders of magnitude of increase in potencies was observed. Finally, a comparison of the EC_{50} values of TNF-α and IFN-β for each compound indicated that the values of TNF-α are slightly smaller than those of IFN-β (2−6-fold), indicating a somewhat higher potency for TNF-α production. Previously, it was observed that mice exposed to *S. typhimurium* LPS provoked mainly cytokines associated with the TRIF-dependent pathway.[19] Interestingly, we have not observed such a bias. It may be possible that such a bias may be due to contaminants, or, alternatively, it may be due to lipid A derivatives that have a different acylation pattern.

Having established the EC_{50} values of TNF-α and IFN-β secretion by compounds **1**−**5** and *E. coli* 055:B5 LPS, attention was focused on IL-6, IP-10, RANTES, and IL-1β responses. Thus, the previously harvested supernatants were analyzed for these cytokines using capture ELISA assays (Figure 4, Table 1). For IL-6, IP-10, and RANTES, a short incubation time of 5.5 h was sufficient for detection. To achieve significant IL-1β secretion, the incubation had to be extended to 24 h. However,

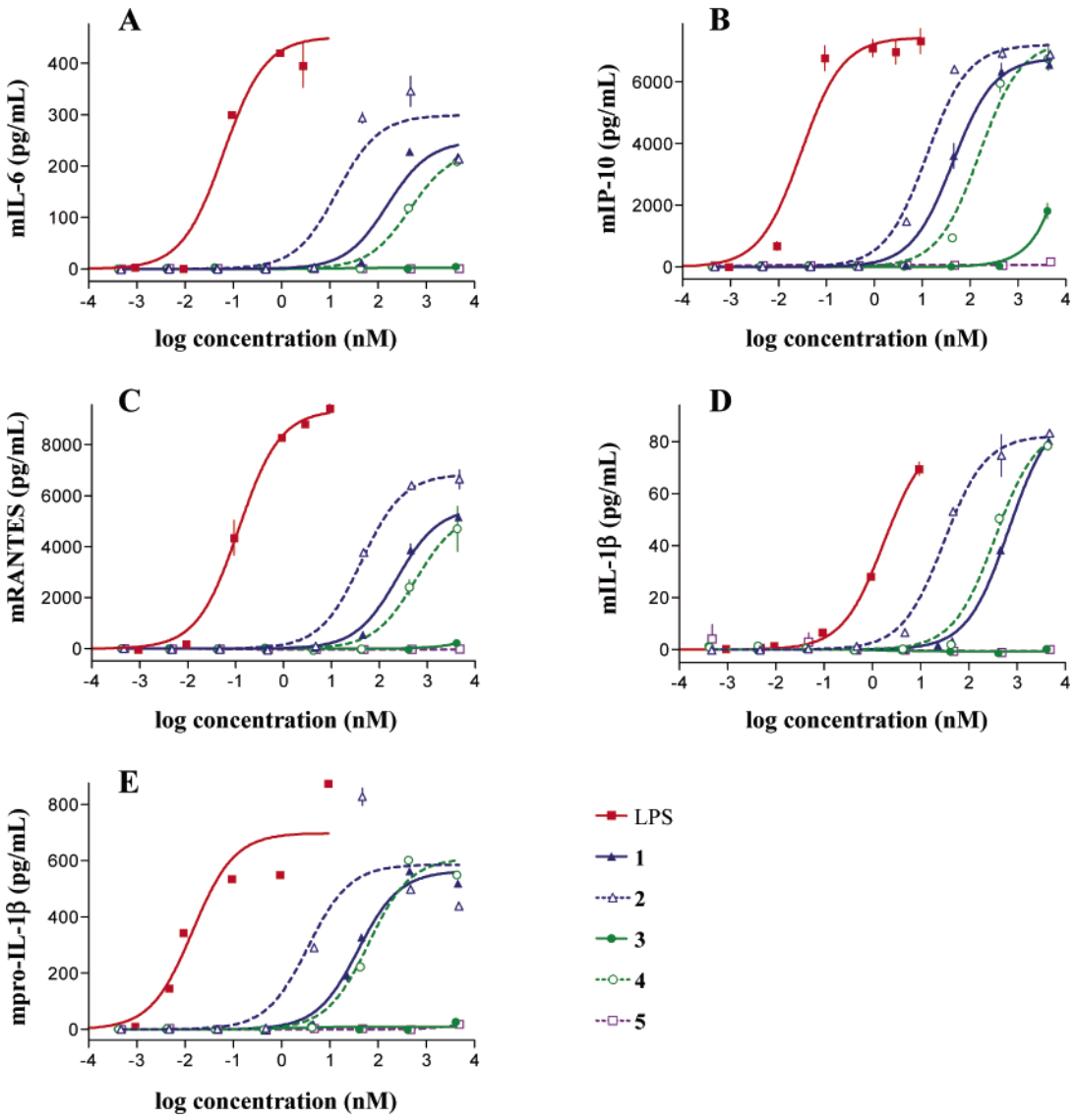

Figure 4. Cytokine production by murine macrophages after stimulation with LPS and lipid A derivatives. Murine RAW γNO(−) cells were exposed to increasing concentrations of *E. coli* LPS or lipid A derivatives **1**−**5** as indicated. Cytokine production was measured in supernatants after 5.5 h incubation for IL-6 (A), IP-10 (B), and RANTES (C) or 24 h for IL-1β (D). The cell lysates were assayed for the presence of pro-IL-1β (E) after 5.5 h incubation.

analyzing cell lysates of the activated cells showed that after 5.5 h a significant quantity of IL-1β was present intracellularly. IL-1β is expressed as a pro-protein (pro-IL-1β), which is cleaved by caspase-1 into its active form (IL-1β), which is then secreted. Indeed, analyzing IL-1β of the cell lysates by Western blotting confirmed that it was present as a pro-protein (data not shown). TNF-α is also produced as a pro-protein, which is proteolitically cleaved by tumor necrosis factor-α converting enzyme (TACE).[39,40] Interestingly, after 5.5 h, no TNF-α could be detected in the cell lysates, which indicates that proteolitic processing and secretion is not the rate-limiting step. Furthermore, for each of the synthetic compounds and LPS, EC_{50} values of secreted TNF-α and intracellular pro-IL-1β were very similar. However, EC_{50} values for secreted mature IL-1β were larger by as much as 100-fold.

TACE is constituently expressed in its active form. On the other hand, caspase-1 is present in the cytoplasm as an inactive precursor protein and must be activated by stimulation with LPS or other bacterial components.[41,42] Although the mechanism of LPS-mediated activation of caspase-1 is not well understood, it has been shown that it is independent of TLR4 associated adaptor proteins MyD88 and TRIF. Instead, experiments with macrophages obtained from $ACS^{-/-}$ mice have implicated this adaptor protein in LPS-mediated activation of caspase-1. Thus, it appears that activation of caspase-1 is dependent on ACS, whereas the expression of pro-IL-1β and pro-TNF-α is dependent on MyD88. Furthermore, it has been suggested that ACS-promoted caspase-1 activation constitutes the rate-limiting step for IL-1β secretion. On the other hand, our results show that processing of pro-TNF-α by TACE and subsequent secretion are not rate-limiting steps. Thus, our results indicate that much higher concentrations of lipid A or LPS are required for caspase-1 activation than for pro-IL-1β expression.

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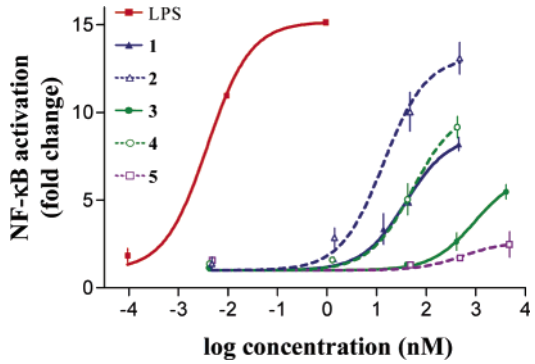

Figure 5. Response of HEK 293T cells expressing murine TLR4, MD2, and CD14 to LPS and lipid A derivatives. Induction of NF-κB activation was determined in triplicate cultures of HEK 293T cells stably transfected with murine TLR4, MD2, and CD14 and transiently transfected with pELAM-Luc, pRL-TK, and pcDNA3 plasmids. Forty-four hours post-transfection, cells were treated with *E. coli* LPS or lipid A derivatives **1**−**5** at the indicated concentrations or were left untreated (control). Forty-eight hours post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. In the transfection experiment shown, human TNF-α (10 ng/mL) induced 12.1 ± 0.3-fold activation of NF-κB.

To obtain further support that the EC_{50} values of secreted TNF-α protein are not affected by transcriptional, translational, or protein processing processes, dose response curves for the activation of the transcription factor NF-κB were determined for each compound, and the results were compared to similar data for secretion of TNF-α protein. Thus, compounds **1**−**5**, and *E. coli* LPS, were exposed at a range of concentrations to HEK 293T cells stably transfected with human TLR4/MD2/CD14 and transiently transfected with a plasmid containing the reporter gene pELAM-Luc (NF-κB-dependent firefly luciferase reporter vector) and a plasmid containing the control gene pRL-TK (*Renilla* luciferase control reporter vector). As a negative control, wild-type HEK 293T cells transiently transfected with plasmids containing the reporter gene pELAM-Luc and control gene pRL-TK were used. After an incubation time of 4 h, the activity was measured using a commercial dual-luciferase assay. As can be seen in Figure 5 and Table 1, the EC_{50} values for NF-κB activation for each compound are very similar to those of TNF-α protein production, demonstrating that transcription, translation, and protein processing do not impact the dose responses. However, the EC_{50} values for secreted IL-1β protein are at least 2 orders of magnitude larger, demonstrating that down-stream processes control the dose response of this cytokine. Collectively, our data indicate that a difference in the processing of pro-TNF-α and pro-IL-1β is responsible for the observed differences in EC_{50} values, which represents a novel mechanism for modulating innate immune responses.

Differences in EC_{50} values were observed for the other cytokines. For example, for each compound, the EC_{50} value for RANTES secretion was approximately 10-fold larger than that of TNF-α. Differential responses were also observed for *S. typhimurium* lipid **3**, which at the highest concentration tested induced the production of TNF-α, IFN-β, and IP-10, whereas no formation of IL-6, RANTES, and IL-1β could be measured.

Examination of the efficacies (maximum responses) of the various cytokines also provided unexpected structure−activity relationships (Table 2). For example, each synthetic compound and *E. coli* 055:B5 LPS induced similar efficacies for the production of IFN-β and IP-10. However, lipid A's **1**−**4** gave lower efficacies for the production of RANTES and IL-6 as compared to LPS.

Table 2. Cytokine Top Values[a] (pg/mL) of Dose−Response Curves of *E. coli* LPS, **1**, **2**, and **4**

	E. coli LPS	lipid A **1**	lipid A **2**	lipid A **4**
TNF-α	3118 ± 120	3924 ± 179	4223 ± 329	4178 ± 250
IFN-β	665 ± 38	724 ± 25	654 ± 37	710 ± 44
IL-6	451 ± 25	249 ± 29	299 ± 42	233 ± 12
IP-10	7439 ± 440	6778 ± 214	7207 ± 277	7320 ± 415
RANTES	9367 ± 188	5531 ± 214	6851 ± 216	5360 ± 263
IL-1β	82[b] ± 2	92 ± 2	82 ± 2	86 ± 4
pro Il-1β	699 ± 73	565 ± 25	587 ± 87	610 ± 35

[a] Top values are reported as best-fit values ± std error. [b] Plateau not reached; top value is best-fit value according to Prism.

Our results show that the relative quantities of secreted cytokines depend on the nature and concentration of the employed lipid A. This information is of critical importance for the development of lipid A's as immune modulators. For example, at a relative low dose of LPS or lipid A no IL-1β will be produced. This cytokine is important for the induction of IFN-γ, which in turn is important for biasing an adaptive immune response toward a T helper-1 (Th1) phenotype.

Conclusions

The results of previous studies have shown that the number of acyl chains and phosphates of lipid A are important determinants for potencies of cytokine production. These reports, however, have described the inductions of only one mediator such as TNF-α or IL-6 protein. We have determined, for the first time, the potencies and efficacies of a wide range of (pro)-inflammatory mediators induced by a number of well-defined lipid As. This undertaking required the development of a new synthetic approach that allowed for the convenient synthesis of a panel of lipid A's. The synthetic approach uses a highly functionalized disaccharide building block that is selectively protected with an Alloc, Fmoc, and anomeric TBDMS group and an azido function, which in a sequential manner can be deprotected or unmasked allowing selective lipid modifications at each position of the disaccharide backbone. The strategy was employed for the preparation of lipid A's derived from *E. coli* and *S. typhimurium*. Cellular activation studies with the synthetic compounds and LPS revealed a number of novel structure−activity relationships. For example, it was found that hepta-acylated *S. typhimurium* lipid A gave much lower activities than hexa-acylated *E. coli* lipid A. Furthermore, shortening of lipids, such as in compounds **2** and **4**, resulted in higher potencies. In the case of the *E. coli* lipid A's (**1** vs **2**), the differences in EC_{50} values were relatively small, whereas for the *S. typhimurium* lipid A's (**3** vs **4**), an approximately 3 orders of magnitude increase in potencies was observed. LPS gave much higher potencies than the synthetic lipid A's, which is probably due to its di-KDO moiety. It has been shown, for the first time, that cellular activation with a particular compound can give EC_{50} values for various mediators that differ as much as 100-fold. The differences in responses did not follow a bias toward a MyD88- or TRIF-dependent response. For example, for each compound, potencies and efficacies for the induction of TNF-α and IFN-β, which are the prototypical cytokines for the MyD88- or TRIF-dependent pathway, respectively, differed only marginally. On the other hand, large differences were observed between

the efficacies of secreted TNF-α and IL-1β, which both depend on the MyD88 pathway. Both cytokines are expressed as pro-proteins, which are processed to the active form by the proteases TACE and caspase-1, respectively. The rate-limiting step for the secretion of IL-1β is the activation of caspase-1, whereas for TNF-α it is the expression of the pro-protein. Surprisingly, our results indicate that LPS-mediated activation of MyD88 resulting in the production of pro-Il-1β and pro-TNF-α requires a much lower concentration of LPS or lipid A than does ACS-mediated activation of caspase-1. As a result, the EC_{50} values for secreted IL-1β and TNF-α differ significantly. Differences in potencies were also observed for the production of other cytokines. For example, *S. typhimurium* lipid **3** induced the secretion of TNF-α, IFN-β, and IP-10 at the highest concentration tested, whereas no formation of IL-6, RANTES, and IL-1β could be measured. Further studies are required to uncover the origin of the differences of these responses. Examination of the efficacies (maximum responses) of the various cytokines also provided unexpected structure−activity relationships. For example, each synthetic compound and *E. coli* 055:B5 LPS induced similar efficacies for the production of IFN-β and IP-10. However, lipid A's **1**−**4** gave lower efficacies for the production of RANTES and IL-6 as compared to LPS.

Collectively, the results presented in this paper demonstrate that cytokine secretion induced by LPS and lipid A is complex. In particular, the relative quantities of secreted cytokines depend on the nature of the compounds and employed concentration of initiator. This information is critical for the development of lipid A's as immune modulators. Future examination of the utilization of signaling transduction and processing pathways of pro-proteins to the active form by different compounds at different concentrations may provide further insight into the underlying mechanism of immune modulation.

Experimental Section

General Synthetic Methods. Column chromatography was performed on silica gel 60 (EM Science, 70−230 mesh). Reactions were monitored by thin-layer chromatography (TLC) on Kieselgel 60 F_{254} (EM Science), and the compounds were detected by examination under UV light and by charring with 10% sulfuric acid in MeOH. Solvents were removed under reduced pressure at <40 °C. CH_2Cl_2 was distilled from NaH and stored over molecular sieves (3 Å). THF was distilled from sodium directly prior to the application. MeOH was dried by refluxing with magnesium methoxide and then was distilled and stored under argon. Pyridine was dried by refluxing with CaH_2 and then was distilled and stored over molecular sieves (3 Å). Molecular sieves (3 and 4 Å), used for reactions, were crushed and activated in vacuo at 390 °C during 8 h in the first instance and then for 2−3 h at 390 °C directly prior to application. Optical rotations were measured with a Jasco model P-1020 polarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded with Varian spectrometers (models Inova500 and Inova600) equipped with Sun workstations. ^{1}H NMR spectra were recorded in $CDCl_3$ and referenced to residual $CHCl_3$ at 7.24 ppm, and ^{13}C NMR spectra were referenced to the central peak of $CDCl_3$ at 77.0 ppm. Assignments were made by standard gCOSY and gHSQC. High-resolution mass spectra were obtained on a Bruker model Ultraflex MALDI-TOF mass spectrometer. Signals marked with a subscript L symbol belong to the biantennary lipids, whereas signals marked with a subscript L′ symbol belong to their side chain. Signals marked with a subscript S symbol belong to the monoantennary lipids.

***tert*-Butyldimethylsilyl 3-*O*-Allyloxycarbonyl-2-azido-4,6-*O*-benzyldidine-2-deoxy-β-D-glucopyranoside (7).** To a cooled (0 °C) solution of compound **6** (3.0 g, 7.37 mmol) and TMEDA (666 μL, 4.42 mmol) in DCM (30 mL) was added dropwise allyl chloroformate (1.00 mL, 8.85 mmol). The reaction mixture was stirred at room temperature for 10 h, and then diluted with DCM (50 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 100 mL) and brine (2 × 50 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 25/1, v/v) to give **7** as a colorless oil (3.20 g, 88%). R_f = 0.57 (hexane/ethyl acetate, 5/1, v/v). $[\alpha]^{25}_D$ = −36.8° (*c* = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39−7.33 (m, 5H, aromatic), 5.97−5.88 (m, 1H, OCH_2CH═CH_2), 5.47 (s, 1H, >C*H*Ph), 5.33 (d, *J* = 17.4 Hz, OCH_2CH═CH_2), 5.22 (d, *J* = 17.4 Hz, OCH_2CH═CH_2), 4.81 (t, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 4.71 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.65 (d, 2H, *J* = 5.4 Hz, OCH_2CH═CH_2), 4.28 (d, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.77 (dd, 1H, $J_{5,6b} = J_{6a,6b}$ = 10.5 Hz, H-6b), 3.67 (d, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.50−3.40 (m, 2H, H-3, H-5), 0.92 (s, 9H, SiC(CH_3)$_3$), 0.16 (s, 3H, Si(CH_3)), 0.14 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.10 (*C*═O), 136.72−126.15 (aromatic), 131.13 (OCH_2CH═CH_2), 119.03 (OCH_2CH═CH_2), 101.51 (>*C*HPh), 97.69 (C-1), 78.55 (C-4), 75.18 (C-3), 68.90 (OCH_2CH═CH_2), 68.41 (C-6), 66.95 (C-5), 66.33 (C-2), 25.48 (SiC(CH_3)$_3$), 17.86 (SiC(CH_3)$_3$), −4.46 (Si(CH_3)$_2$), −5.23 (Si-(CH_3)$_2$). HR MS (*m*/*z*) calcd for $C_{23}H_{33}N_3O_7Si$ [M + Na]$^+$, 514.1985; found, 514.1907.

***tert*-Butyldimethylsilyl 2-Azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (8).** Compound **6** (1.32 g, 3.49 mmol) was dissolved in a solution of BH_3 (1 M) in THF (17.5 mL). After the mixture was stirred at 0 °C for 5 min, dibutylboron triflate (1 M in DCM, 3.49 mL) was added dropwise, and the reaction mixture was stirred at 0 °C for another 1 h. Subsequently, triethylamine (0.5 mL) and methanol (∼0.5 mL) were added until the evolution of H_2 gas had ceased. The solvents were evaporated in vacuo, and the residue was coevaporated with methanol (3 × 50 mL). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 8/1, v/v) to give **8** as a colorless oil (1.21 g, 85%). R_f = 0.40 (hexane/ethyl acetate, 3/1, v/v). $[\alpha]^{25}_D$ = +0.9° (*c* = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32−7.31 (m, 5H, aromatic), 4.81 (d, 1H, J_2 = 11.4 Hz, CH_{2a}Ph), 4.70 (d, 1H, J_2 = 11.4 Hz, CH_{2b}Ph), 4.55 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 3.84 (dd, 1H, $J_{5,6a}$ = 2.4 Hz, $J_{6a,6b}$ = 12.0 Hz, H-6a), 3.70 (dd, 1H, $J_{5,6b}$ = 1.5 Hz, $J_{6a,6b}$ = 12.0 Hz, H-6b), 3.49−3.43 (m, 2H, H-3, H-4), 3.33 (broad, 1H, H-5), 3.22−3.17 (m, 1H, H-2), 0.92 (s, 9H, SiC(CH_3)$_3$), 0.14 (s, 6H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 137.89−128.11 (aromatic), 96.98 (C-1), 77.17 (C-3 or C-4), 75.22 (C-5), 74.88 (C-3 or C-4), 74.75 (CH$_2$Ph), 68.69 (C-2), 61.97 (C-6), 25.56 (SiC-(CH_3)$_3$), 17.91 (SiC(CH_3)$_3$), −4.27 (Si(CH_3)$_2$), −5.16 (Si(CH_3)$_2$). HR MS (*m*/*z*) calcd for $C_{19}H_{31}N_3O_5Si$ [M + Na]$^+$, 432.1931; found, 432.1988.

***tert*-Butyldimethylsilyl 3-*O*-Allyloxycarbonyl-2-azido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (9).** A suspension of compound **7** (3.20 g, 6.52 mmol) and molecular sieves (4 Å, 500 mg) in THF (50 mL) was stirred at room temperature for 1 h, and then $NaCNBH_3$ (2.46 g, 39.0 mmol) was added. A solution of HCl (2 M in diethyl ether) was added dropwise to this reaction mixture until the mixture became acidic (∼5 mL, pH = 5). After being stirred another 0.5 h, the reaction mixture was quenched with solid $NaHCO_3$, diluted with diethyl ether (100 mL), and washed with saturated aqueous $NaHCO_3$ (2 × 100 mL) and brine (2 × 50 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 7/1, v/v) to give **9** as a colorless oil (3.20 g, 88%). R_f = 0.42 (hexane/ethyl acetate, 4/1, v/v). $[\alpha]^{25}_D$ = −6.2° (*c* = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39−7.34 (m, 5H, aromatic), 6.02−5.89 (m, 1H, OCH_2C*H*═CH_2), 5.39 (d, 1H, *J* = 17.4 Hz, OCH_2CH═CH_2), 5.30 (d, 1H, *J* = 10.5 Hz, OCH_2CH═CH_2), 4.70−4.58 (m, 5H, H-1, H-3, OCH_2CH═CH_2, CH_2Ph), 3.79−3.70 (m, 3H, H-4, H-6a, H-6b), 3.52−3.46 (m, 1H, H-5), 3.37 (dd, 1H, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 0.94 (s, 9H,

$SiC(CH_3)_3$), 0.17 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.81 (*C*=O), 137.59−127.32 (aromatic), 131.07 ($OCH_2CH{=}CH_2$), 118.97 ($OCH_2CH{=}CH_2$), 96.92 (C-1), 78.79 (C-3), 74.25 (C-5), 73.44 (CH_2Ph), 69.89, 69.55, 68.84 (C-4, C-6, $OCH_2CH{=}CH_2$), 65.84 (C-2), 25.42 ($SiC(CH_3)_3$), 17.75 ($SiC(CH_3)_3$), −4.50 ($Si(CH_3)_2$), −5.40 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{23}H_{35}N_3O_7Si$ $[M + Na]^+$, 516.2142; found, 516.2197.

***tert*-Butyldimethylsilyl 3-*O*-Allyloxycarbonyl-2-azido-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-β-D-glucopyranoside (10).** To a solution of compound **9** (1.30 g, 2.50 mmol) and 1*H*-tetrazole (3 wt %, 10.0 mmol) in DCM (30 mL) was added *N*,*N*-diethyl-1,5-dihydro-3*H*-2,4,3-benzodioxaphosphepin-3-amine (1.20 g, 1.05 mmol). After the reaction mixture was stirred at room temperature for 15 min, it was cooled (−20 °C), stirred for another 10 min, and then mCPBA (3.40 g, 50−55 wt %, 10.0 mmol) was added. The reaction mixture was stirred at −20 °C for 20 min, and then quenched by the addition of saturated aqueous $NaHCO_3$ (40 mL) and diluted with DCM (30 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 60 mL) and brine (2 × 40 mL), dried ($MgSO_4$), and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 5/1−3/1, v/v) to give **10** as a pale yellow oil (1.48 g, 89%). R_f = 0.40 (hexane/ethyl acetate, 1/1, v/v). $[\alpha]^{25}_D = -10.3°$ (*c* = 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 7.35−7.15 (m, 9H, aromatic), 5.98−5.85 (m, 1H, $OCH_2CH{=}CH_2$), 5.65 (d, 1H, *J* = 1.2 Hz, *J* = 17.4 Hz, $OCH_2CH{=}CH_2$), 5.50 (d, 1H, *J* = 1.2 Hz, *J* = 10.5 Hz, $OCH_2CH{=}CH_2$), 5.18−5.01 (m, 4H, $C_6H_4(CH_2O)P$), 3.81 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 4.64−4.52 (m, 6H, H-1, H-4, $OCH_2CH{=}CH_2$, CH_2Ph), 3.82 (d, 1H, $J_{6a,6b}$ = 9.0 Hz, H-6a), 3.72−3.61 (m, 2H, H-5, H-6b), 3.41 (dd, 1H, $J_{1,2}$ = 7.4 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 0.92 (s, 9H, $SiC(CH_3)_3$), 0.16 (s, 3H, $Si(CH_3)$), 0.15 (s, 3H, $Si(CH_3)$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.38 (C=O), 138.02−127.56 (aromatic), 131.33 ($OCH_2CH{=}CH_2$), 118.99 ($OCH_2CH{=}CH_2$), 97.13 (C-1), 76.77 (C-3), 74.27 (C-4), 74.08 (C-5), 73.50 (CH_2Ph), 69.06 ($OCH_2CH{=}CH_2$), 68.74 (C-6), 68.55 ($OC_6H_4(CH_2O)P$), 68.50 ($OC_6H_4(CH_2O)P$), 65.97 (C-2), 25.53 ($SiC(CH_3)_3$), 17.92 ($SiC(CH_3)_3$), −4.35 ($Si(CH_3)_2$), −5.28 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{31}H_{42}N_3O_{10}PSi$ $[M + Na]^+$, 698.2275; found, 698.2315.

***tert*-Butyldimethylsilyl 3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-(9-fluorenylmethoxycarbonylamino)-β-D-glucopyranoside (11).** Acetic acid (300 μL, 5.20 mmol) was added dropwise to a stirred suspension of **10** (1.40 g, 2.08 mmol) and zinc powder (676 mg, 10.4 mmol) in DCM (15 mL). The reaction mixture was stirred at room temperature for 2 h, after which it was diluted with ethyl acetate (50 mL). The solids were removed by filtration and washed with ethyl acetate (2 × 10 mL). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 × 40 mL) and brine (2 × 40 mL). The organic phase was dried ($MgSO_4$), filtered, and the filtrate was concentrated in vacuo to afford a crude amine as a pale yellow oil. R_f = 0.21 (hexane/ethyl acetate, 1/1, v/v). FmocCl (645 mg, 2.50 mmol) was added to a stirred solution of the crude amine and DIPEA (435 μL, 2.50 mmol) in DCM (15 mL) at 0 °C. The reaction mixture was stirred at room temperature for 5 h, after which it was diluted with DCM (40 mL) and washed with brine (2 × 50 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1−2/1, v/v) to afford **11** as a colorless solid (1.45 g, 80% over two steps). R_f = 0.54 (hexane/ethyl acetate, 1/1, v/v). $[\alpha]^{25}_D = -3.9°$ (*c* = 1.0, $CDCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 7.78−7.20 (m, 17H, aromatic), 5.92−5.82 (m, 1H, $OCH_2CH{=}CH_2$), 5.42 (broad, 1H, H-3), 5.31 (d, 1H, *J* = 17.6 Hz, $OCH_2CH{=}CH_2$), 5.20−5.07 (m, 6H, H-1, $OCH_2CH{=}CH_2$, $C_6H_4(CH_2O)P$), 4.67−4.56 (m, 5H, H-4, $OCH_2CH{=}CH_2$, CH_2Ph), 4.41−4.23 (m, 3H, $COOCH_2$, Fmoc, COOCH2CH, Fmoc), 3.89−3.87 (broad, 1H, H-6a), 3.76−3.74 (broad, 2H, H-5, H-6b), 3.49−3.47 (m, 1H, H-2), 0.88 (s, 4H, $SiC(CH_3)_3$), 0.14 (s, 3H, $Si(CH_3)_2$), 0.10 (s, 3H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.52 (*C*=O), 154.80 (*C*=O), 143.71−119.94 (aromatic), 131.34 ($OCH_2CH{=}CH_2$), 118.85 ($OCH_2CH{=}CH_2$), 95.41 (C-1), 74.77 (C-4), 73.85 (C-5), 73.47 (CH_2Ph), 68.94, 68.57, 68.50, 68.42 (C-3, C-6, $OCH_2CH{=}CH_2$, $OC_6H_4(CH_2O)P$), 68.50 ($OC_6H_4(CH_2O)P$), 67.12 (CO_2CH_2CH, Fmoc), 58.69 (C-2), 47.04 (CO_2CH_2CH, Fmoc), 25.52 ($SiC(CH_3)_3$), 17.88 ($SiC(CH_3)_3$), −4.26 ($Si(CH_3)_2$), −5.38 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{46}H_{54}NO_{12}PSi$ $[M + Na]^+$, 894.3051; found, 894.3937.

3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-(9-fluorenylmethoxycarbonylamino)-D-glucopyranosyl Trichloroacetimidate (12). HF/pyridine (1 mL) was added dropwise to a stirred solution of **11** (1.37 g, 1.58 mmol) in THF (10 mL). The reaction mixture was stirred at room temperature for 12 h, after which it was diluted with ethyl acetate (40 mL), and then washed with saturated aqueous $NaHCO_3$ (2 × 40 mL) and brine (2 × 40 mL), successively. The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 3/2, v/v) to give a lactol as a pale yellow oil (1.02 g, 98%). HR MS (*m*/*z*) calcd for $C_{40}H_{40}NO_{12}P$ $[M + Na]^+$, 780.2186; found, 780.2379. The lactol (1.02 g, 1.35 mmol) thus obtained was dissolved in DCM (20 mL), and trichloroacetonitrile (10 mL) and NaH (5 mg) were added, successively. The reaction mixture was stirred at room temperature for 30 min, after which another portion of NaH (5 mg) was added. After the suspension was stirred for another 20 min, the solids were removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 1/1, v/v) to give **12** as a colorless solid (1.14 g, 92%).

***tert*-Butyldimethylsilyl 6-*O*-[3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-(9-fluorenylmethoxycarbonylamino)-β-D-glucopyranosyl]-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (13).** A suspension of trichloroacetimidate **12** (1.04 g, 1.21 mmol), acceptor **8** (740 mg, 1.82 mmol), and molecular sieves (4 Å, 500 mg) in DCM (20 mL) was stirred at room temperature for 1 h. The mixture was cooled (−60 °C), and then TMSOTf (18 μL, 0.09 mmol) was added. After the reaction mixture was stirred for 15 min, it was quenched with solid $NaHCO_3$. The solids were removed by filtration, and the filtrate was washed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and brine (2 × 40 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 2/1, v/v) to give **13** as a colorless solid (1.09 g, 79%). R_f = 0.37 (DCM/methanol, 50/1, v/v). $[\alpha]^{26}_D = -3.8$ (*c* = 1.0, $CHCl_3$). ^1H NMR (600 MHz, CD_3COCD_3): δ 7.84−7.20 (m, 22H, aromatic), 6.98 (d, 1H, $J_{NH',2'}$ = 9.0 Hz, N*H*′), 5.83 (m, 1H, $OCH_2CH{=}CH_2$), 5.41 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9,6 Hz, H-3′), 5.29−5.21 (m, 3H, $OCH_2CH{=}CH_2$, $C_6H_4(CH_2O)_2P$), 5.13−5.03 (m, 3H, H-1′, $OCH_2CH{=}CH_2$, $C_6H_4(CH_2O)_2P$), 4.96−4.91 (m, 2H, $CH_{2a}Ph$, $C_6H_4(CH_2O)_2P$), 4.73−4.45 (m, 7H, H-1, H-4′, $CH_{2b}Ph$, CH_2Ph, $OCH_2CH{=}CH_2$), 4.24−4.13 (m, 4H, H-6, CO_2CH_2, Fmoc, CO_2CH_2CH, Fmoc), 3.93−3.79 (m, 4H, H-5′, H-6a, H-6′a, H-6′b), 3.69 (m, 1H, H-2′), 3.54 (broad, 3H, H-3, H-4, H-5), 3.19 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 9,0 Hz, H-2), 0.92 (s, 9H, $SiC(CH_3)_3$), 0.17 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 156.39 (*C*=O), 155.28 (*C*=O), 144.96−120.56 (aromatic, $OCH_2CH{=}CH_2$), 118.41 ($OCH_2CH{=}CH_2$), 101.14 (C-1′), 97.33 (C-1), 78.54, 77.87, 75.72, 75.25−74.42 (m), 73.83, 70.35, 69.52, 69.04−68.73 (m), 67.91, 67.12, 57.03 (C-2′), 47.64 (CO_2CH_2, Fmoc), 25.83 ($SiC(CH_3)_3$), 18.27 ($SiC(CH_3)_3$), −3.85 ($Si(CH_3)_2$), −5.21 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{59}H_{69}N_4O_{16}PSi$ $[M + Na]^+$, 1171.4113; found, 1171.4256.

***tert*-Butyldimethylsilyl 6-*O*-{3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (21).** DBU

(200 μL) was added dropwise to a solution of **13** (730 mg, 0.637 mmol) in DCM (10 mL). The reaction mixture was stirred at room temperature for 1 h, after which it was concentrated in vacuo. The residue was purified by silica gel column chromatography (DCM/methanol, 100/1−100/3, v/v) to afford the free amine as a colorless syrup (567 mg, 96%). R_f = 0.32 (DCM/methanol, 50/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−7.18 (m, 14H, aromatic), 5.96−5.88 (m, 1H, $OCH_2CH{=}CH_2$), 5.38 (d, 1H, J = 17.0 Hz, $OCH_2CH{=}CH_2$), 5.25 (d, 1H, J = 11.0 Hz, $OCH_2CH{=}CH_2$), 5.21−5.06 (m, 4H, $C_6H_4(CH_2O)_2P$), 4.85 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 4.79 (d, 1H, J = 11.0 Hz, $CH_{2a}Ph$), 4.67 (d, 1H, J = 11.0 Hz, $CH_{2b}Ph$), 4.63−4.55 (m, 5H, H-4′, 2 × CH_2Ph, $OCH_2CH{=}CH_2$), 4.52 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.22 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.14 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.87 (d, 1H, $J_{6'a,6'b}$ = 10.5 Hz, H-6′a), 3.73−3.69 (m, 1H, H-6′b), 3.67−3.65 (m, 1H, H-5′), 3.62−3.59 (m, 1H, H-6b), 3.55−3.52 (m, 1H, H-5), 3.46 (t, 1H, $J_{2,3} = J_{3,4}$ = 9,5 Hz, H-3), 3.32 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.22 (t, 1H, $J_{1,2} = J_{2,3}$ = 9.0 Hz, H-2), 2.93 (t, 1H, $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 10.0 Hz, H-2′), 0.94 (s, 9H, $SiC(CH_3)_3$), 0.19 (s, 6H, $Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{44}H_{59}N_4O_{14}PSi$ $[M + Na]^+$, 949.3432; found, 949.4922. DCC (202 mg, 0.979 mmol) was added to a stirred solution of (*R*)-3-dodecanoyl-tetradecanoic acid **18** (313 mg, 0.734 mmol) in DCM (10 mL). After the reaction mixture was stirred for 10 min, the amine (567 mg, 0.612 mmol) in DCM (4 mL) was added, and stirring was continued for another 12 h. The insoluble materials were removed by filtration, and the residue was washed with DCM (2 × 2 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 2/1, v/v) to give **21** as a white solid (760 mg, 93%). R_f = 0.68 (hexane/ethyl acetate, 1/1, v/v). $[\alpha]^{27}_D$ = −3.0° (c = 1.0, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.33−7.14 (m, 14H, aromatic), 5.92 (d, 1H, $J_{NH',2'}$ = 7.8 Hz, N*H*′), 5.91−5.85 (m, 1H, $OCH_2CH{=}CH_2$), 5.46 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3′), 5.34 (d, 1H, J = 16.8 Hz, $OCH_2CH{=}CH_2$), 5.21 (d, 1H, J = 10.2 Hz, $OCH_2CH{=}CH_2$), 5.09−5.04 (m, 4H, $C_6H_4(CH_2O)_2P$), 4.99 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1′), 5.00−4.96 (m, 1H, H-3_L), 4.73 (d, 1H, J = 12.0 Hz, $CH_{2a}Ph$), 4.63 (d, 1H, J = 12.0 Hz, $CH_{2b}Ph$), 4.59−4.48 (m, 6H, H-1, H-4′, CH_2Ph, $OCH_2CH{=}CH_2$), 4.00 (d, 1H, $J_{6'a,6'b}$ = 10.2 Hz, H-6′a), 3.82 (d, 1H, $J_{6a,6b}$ = 10.2 Hz, H-6a), 3.73−3.67 (m, 3H, H-5′, H-6b, H-6′b), 3.49−3.36 (m, 4H, H-2′, H-3, H-4, H-5), 3.18 (t, 1H, $J_{1,2} = J_{2,3}$ = 8.4 Hz, H-2), 2.33 (s, 1H, O*H*), 2.37 (dd, 1H, $J_{2La,2Lb}$ = 14.4 Hz, $J_{2La,3L}$ = 6.0 Hz, H-2_{La}), 2.29−2.22 (m, 3H, H-$2_{L'}$, H-2_{Lb}), 1.61−1.53 (m, 4H, H-4_L, H-$3_{L'}$), 1.23 (broad, 34H, 17 × CH_2, lipid), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.85−0.78 (m, 6H, 2 × CH_3, lipid), 0.13 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.70 (*C*═O), 170.00 (*C*═O), 154.59 (*C*═O), 138.08−127.57 (aromatic, $OCH_2CH{=}CH_2$), 118.84 ($OCH_2CH{=}CH_2$), 99.30 (C-1′), 96.95 (C-1), 77.65, 77.21, 76.05, 75.04, 74.41, 74.32, 73.78, 71.13, 68.95−67.93 (m), 55.91 (C-2′), −4.02 ($Si(CH_3)_2$), −5.26 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{70}H_{107}N_4O_{17}PSi$ $[M + Na]^+$, 1357.7036; found, 1357.8037.

***tert*-Butyldimethylsilyl 6-*O*-{3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-2-azido-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-β-D-glucopyranoside (22).** A reaction mixture of (*R*)-3-benzyloxy-tetradecanoic acid **15** (100 mg, 0.293 mmol) and DCC (93 mg, 0.450 mmol) in DCM (5 mL) was stirred at room temperature for 10 min, and then disaccharide **21** (300 mg, 0.225 mmol) in DCM (3 mL) and DMAP (11 mg, 0.090 mmol) were added. The reaction mixture was stirred at room temperature for 14 h, after which the solids were removed by filtration, and the residue was washed with DCM (2 × 1 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to give **22** as a white solid (319 mg, 86%). R_f = 0.41 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D$ = −2.8° (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33−7.15 (m, 19H, aromatic), 5.94−5.85 (m, 2H, N*H*, $OCH_2CH{=}CH_2$), 5.45 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 5.34 (d, 1H, J = 17.5 Hz, $OCH_2CH{=}CH_2$), 5.22 (d, 1H, J = 10.0 Hz, $OCH_2CH{=}CH_2$), 5.08−4.95 (m, 7H, H-1, H-3, H-3_L, $C_6H_4(CH_2O)_2P$), 4.61−4.44 (m, 10H, H-1, H-4′, 3 × CH_2Ph, $OCH_2CH{=}CH_2$), 3.96 (d, 1H, $J_{6'a,6'b}$ = 10.5 Hz, H-6′a), 3.88−3.85 (m, 1H, H-3_S), 3.80 (d, 1H, $J_{6a,6b}$ = 9.5 Hz, H-6a), 3.72−3.66 (m, 3H, H-5′, H-6b, H-6′b), 3.55−3.52 (m, 2H, H-4, H-5), 3.47−3.41 (m, 1H, H-2′), 3.27 (dd, 1H, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 2.56 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sa,3S}$ = 7.0 Hz, H-2_{Sa}), 2.43 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sb,3S}$ = 7.0 Hz, H-2_{Sb}), 2.35 (dd, 1H, $J_{2La,2Lb}$ = 15.0 Hz, $J_{2La,3L}$ = 6.0 Hz, H-2_{La}), 2.30−2.20 (m, 3H, H-$2_{L'}$, H-$2_{L'b}$), 1.59−1.52 (m, 6H, H-4_L, H-4_S, H-$3_{L'}$), 1.23 (broad, 52H, 26 × CH_2, lipid), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.88−0.84 (m, 9H, 3 × CH_3, lipid), 0.12 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.40 (*C*═O), 170.55 (*C*═O), 169.94 (*C*═O), 154.42 (*C*═O), 138.46−127.35 (aromatic, $OCH_2CH{=}CH_2$), 118.74 ($OCH_2CH{=}CH_2$), 99.29 (C-1′), 96.96 (C-1), 75.89, 75.62, 74.75, 74.28, 74.02, 73.67, 73.41, 71.34, 70.89, 68.87−67.85 (m), 66.48, −4.18 ($Si(CH_3)_2$), −5.38 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{91}H_{139}N_4O_{19}PSi$ $[M + Na]^+$, 1673.9438; found, 1674.1754.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-(*p*-methoxy)benzyloxy-tetradecanoyl]-β-D-glucopyranosyl}-2-azido-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-β-D-glucopyranoside (28).** Tetrakis(triphenylphosphine)palladium (29.0 mg, 0.0255 mmol) was added to a solution of **22** (210 mg, 0.127 mmol), *n*-$BuNH_2$ (25.0 μL, 0.255 mmol), and HCOOH (10.0 μL, 0.255 mmol) in THF (5 mL). After the reaction mixture was stirred at room temperature for 20 min, it was diluted with DCM (20 mL), and washed successively with water (20 mL), saturated aqueous $NaHCO_3$ (2 × 20 mL), and brine (2 × 20 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/3, v/v) to give compound **23**. A solution of (*R*)-3-(*p*-methoxy)benzyloxy-tetradecanoic acid **17** (69 mg, 0.191 mmol) and DCC (52 mg, 0.254 mmol) in DCM (4 mL) was stirred at room temperature for 10 min, and then the intermediate **23** in DCM (1 mL) and DMAP (7 mg, 0.060 mmol) were added. The reaction mixture was stirred for another 10 h, after which the solids were removed by filtration and washed with DCM (2 × 2 mL). The combined filtrates were concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to afford **28** as a white solid (182 mg, 75%). R_f = 0.46 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D$ = −2.8° (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38−6.79 (m, 23H, aromatic), 5.73 (d, 1H, $J_{NH',\ 2'}$ = 7.5 Hz, N*H*′), 5.57 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 5.07−4.87 (m, 6H, H-1, H-3, $C_6H_4(CH_2O)_2P$), 4.66−4.47 (m, 11H, H-1, H-4′, H-3_L, 3 × CH_2Ph, CH_2PhOCH_3), 3.98 (d, 1H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 3.91−3.69 (m, 9H, H-5′, H-6b, H-6′a, H-6′b, 2 × H-3_S, CH_3OPh), 3.55−3.52 (m, 2H, H-4, 5), 3.47−3.41 (m, 1H, H-2′), 3.38−3.31 (m, 2H, H-2, H-2′), 2.67−2.07 (m, 8H, H-2_L, 2 × H-2_S, H-$2_{L'}$), 1.62−1.59 (m, 8H, H-4_L, 2 × H-4_S, H-$3_{L'}$), 1.27 (broad, 70H, 35 × CH_2, lipid), 0.93 (s, 9H, $SiC(CH_3)_3$), 0.92−0.87 (m, 12H, 4 × CH_3, lipid), 0.16 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.65 (*C*═O), 171.18 (*C*═O), 170.63 (*C*═O), 169.87 (*C*═O), 159.17−113.78 (aromatic), 99.77 (C-1′), 97.06 (C-1), 75.95, 75.71, 75.26, 74.89, 74.43, 74.09, 73.97, 73.75, 73.53, 72.07, 71.48, 71.07, 70.66, 68.90−68.13 (m), 66.54 (C-2), 56.22 (C-2′), 55.17 ($CH_3OC_6H_5$), −4.08 ($Si(CH_3)_2$), −5.31 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{109}H_{169}N_4O_{20}PSi$ $[M + Na]^+$, 1936.1735; found, 1936.2613.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl}-2-azido-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-β-D-glucopyranoside (24).** DDQ (36 mg, 0.158 mmol) was added to a stirred solution of **15** (200 mg, 0.105 mmol) in a mixture of DCM and H_2O (4 mL, 10/1, v/v). The reaction mixture was stirred at room temperature for 1 h, after which

it was diluted with DCM. The mixture was washed with brine (20 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 3/1, v/v) to give the alcohol **29** as a colorless syrup (170 mg, 90%). $R_f = 0.50$ (hexane/ethyl acetate, 5/3, v/v). HR MS (*m*/*z*) calcd for $C_{101}H_{161}N_4O_{19}PSi$ $[M + Na]^+$, 1816.1160; found, 1816.3214. Lauroyl chloride (128 μL, 0.475 mmol) was added to a solution of the alcohol **29** (170 mg, 0.095 mmol), pyridine (60 μL, 0.760 mmol), and DMAP (12 mg, 0.095 mmol) in DCM (4 mL). After the reaction mixture was stirred at room temperature for 12 h, it was diluted with DCM and washed with saturated aqueous $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to afford **24** as a white solid (162 mg, 85%). $R_f = 0.46$ (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{26}_D = -2.8°$ (*c* = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−7.22 (m, 19H, aromatic), 6.26 (d, 1H, $J_{NH',2'}$ =7.5 Hz, N*H*′), 5.58 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.32−5.27 (m, 1H, H-3_L), 5.16−4.99 (m, 7H, H-1′, 3, H-3_L, $C_6H_4(CH_2O)_2P$), 4.66−4.49 (m, 8H, H-1, 4′, 3 × CH_2Ph), 4.03 (d, 1H, $J_{6a,6b}$ =10.5 Hz, H-6a), 3.93−3.88 (m, 1H, H-3_S), 3.82−3.74 (m, 3H, H-5′, H-6b, H-6′a), 3.70 (dd, 1H, $J_{5',6'b}$ = 5.0 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6′b), 3.62−3.55 (m, 2H, H-4, H-5), 3.48 (m, 1H, H-2′), 3.33 (dd, 1H, $J_{1,2} = 8.0$ Hz, $J_{2,3} = 10.5$ Hz, H-2), 2.70−2.22 (m, 10H, 2 × H-2_L, H-2_S, 2 × H-$2_{L'}$), 1.61−1.51 (m, 10H, 2 × H-4_L, H-4_S, 2 × H-$3_{L'}$), 1.26 (broad, 108H, 54 × CH_2, lipid), 0.95 (s, 9H, SiC(CH_3)$_3$), 0.92−0.90 (m, 15H, 5 × CH_3, lipid), 0.19 (s, 3H, SiCH_3), 0.18 (s, 3H, SiCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.65 (*C*═O), 173.60 (*C*═O), 170.62 (*C*═O), 170.14 (*C*═O), 170.10 (*C*═O), 138.53−127.41 (aromatic), 99.64 (C-1′), 97.05 (C-1), 75.93, 75.70, 75.43, 74.06, 73.73, 73.50, 72.60, 71.46, 70.52, 70.29, 68.82−68.24 (m), 66.54 (C-2), 56.34 (C-2′), −4.12 (Si(CH_3)$_2$), −5.32 (Si(CH_3)$_2$). HR MS (*m*/*z*) calcd for $C_{115}H_{187}N_4O_{20}PSi$ $[M + Na]^+$, 2026.3143; found, 2026.6381.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoylamino]-2-deoxy-β-D-glucopyranoside (30).** A suspension of **16** (100 mg, 0.05 mmol), zinc (33.0 mg, 0.50 mmol), and acetic acid (18 μL, 0.30 mmol) in DCM (4 mL) was stirred at room temperature for 12 h, after which it was diluted with ethyl acetate (25 mL). The solids were removed by filtration and washed with ethyl acetate (2 × 3 mL), and the combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 2.5/1, v/v) to afford the amine as a pale yellow syrup (94 mg, 95%). $R_f = 0.29$ (hexane/ethyl acetate, 5/2, v/v). HR MS (*m*/*z*) calcd for $C_{115}H_{189}N_2O_{20}PSi$ $[M + Na]^+$, 2000.3238; found, 2000.6035. DCC (12 mg, 0.06 mmol) was added to a stirred solution of (*R*)-3-benzyloxy-tetradecanoic acid **15** (10.0 mg, 0.03 mmol) in DCM (1.5 mL). After the reaction mixture was stirred for 10 min, the amine (30.0 mg, 0.015 mmol) in DCM (1 mL) and DMAP (1.0 mg, 0.0075 mmol) were added, and stirring was continued for another 12 h. The insoluble materials were removed by filtration, and the residue was washed with DCM (2 × 1 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by preparative silica gel TLC chromatography (hexane/ethyl acetate, 3.5/1, v/v) to give **30** as a white solid (22.0 mg, 64%). $R_f = 0.54$ (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D = -2.6°$ (*c* = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38−7.19 (m, 24H, aromatic), 6.21 (d, 1H, $J_{NH',2'} = 7.0$ Hz, N*H*′), 6.15 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 5.59 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.31−5.26 (m, 1H, H-3_L), 5.15−4.97 (m, 7H, H-1′, H-3, H-3_L, $C_6H_4(CH_2O)_2P$), 4.65−4.44 (m, 10H, H-1, H-4′, 4 × CH_2Ph), 4.01 (d, 1H, $J_{6a,6b} = 9.5$ Hz, H-6a), 3.90−3.82 (m, 3H, H-2, H-6′a, H-3_S), 3.76−3.69 (m, 4H, H-5′, H-6b, H-6′b, H-3_S), 3.57 (t, 1H, $J_{3,4} = J_{4,5} = 9.0$ Hz, H-4), 3.53−3.50 (m, 1H, H-5), 3.43−3.38 (m, 1H, H-2′), 2.66−2.22 (m, 12H, 2 × H-2_L, 2 × H-2_S, 2 × H-$2_{L'}$), 1.71−1.45 (m, 12H, 2 × H-4_L, 2 × H-4_S, 2 × H-$3_{L'}$), 1.26 (broad, 108H, 54 × CH_2, lipid), 0.91−0.88 (m, 18H, 6 × CH_3, lipid), 0.86 (s, 9H, SiC(CH_3)$_3$), 0.10 (s, 3H, SiCH_3), 0.05 (s, 3H, SiCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.19 (*C*═O), 173.68 (*C*═O), 173.55 (*C*═O), 171.45 (*C*═O), 170.87 (*C*═O), 170.10 (*C*═O), 138.62−127.42 (aromatic), 99.48 (C-1′), 96.25 (C-1), 76.13, 75.85, 75.44, 74.76, 74.38, 74.10, 72.61, 71.34, 70.62, 70.53, 70.29, 68.94, 68.88−68.22 (m), 56.48 (C-2), 56.04 (C-2′), −3.72 (Si(CH_3)$_2$), −5.05 (Si(CH_3)$_2$). HR MS (*m*/*z*) calcd for $C_{136}H_{221}N_2O_{22}PSi$ $[M + Na]^+$, 2316.5641; found, 2316.9641.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoylamino]-β-D-glucopyranoside (31).** The free amine obtained above (56.0 mg, 0.028 mmol) was acylated in a manner similar to the synthesis of **30** with (*R*)-3-(hexadecanoyl)oxy-tetradecanoic acid **20** (27 mg, 0.057 mmol) to yield **31** as a white solid (47 mg, 68%). $R_f = 0.48$ (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{25}_D = -0.87°$ (*c* = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−7.21 (m, 19H, aromatic), 6.20 (d, 1H, $J_{NH',2'} = 7.5$ Hz, N*H*′), 5.76 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.58 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.29−5.26 (m, 1H, H-3_L), 5.15−4.97 (m, 8H, H-1′, H-3, 2 × H-3_L, $C_6H_4(CH_2O)_2P$), 4.72 (d, 1H, $J_{1,2} = 8.0$ Hz, H-1), 4.64−4.44 (m, 7H, H-4, H-3 × CH_2Ph), 4.02 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.87−3.81 (m, 3H, H-2, H-6′a, H-3_S), 3.74−3.69 (m, 3H, H-5′, H-6′b, H-6b), 3.59−3.58 (m, 2H, H-4, H-5), 3.44−3.39 (m, 1H, H-2), 2.64−2.22 (m, 14H, 3 × H-2_L, H-2_S, 3 × H-$2_{L'}$), 1.60 (broad, 14H, 3 × H-4_L, H-4_S, 3 × H-3_L), 1.26 (broad, 132H, 66 × CH_2, lipid), 0.90−0.87 (m, 30H, 7 × CH_3, lipid, SiC(CH_3)$_3$), 0.12 (s, 3H, SiCH_3), 0.10 (s, 3H, SiCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.68 (*C*═O), 173.63 (*C*═O), 173.57 (*C*═O), 171.54 (*C*═O), 170.15 (*C*═O), 170.10 (*C*═O), 169.17 (*C*═O), 138.52−127.46 (aromatic), 99.45 (C-1′), 96.16 (C-1), 76.00, 75.40, 74.92, 74.45, 74.14, 73.50, 72.58, 71.26, 70.84, 70.53, 70.28, 68.89−68.33 (m), 56.40 (C-2 or 2′), 56.35 (C-2 or 2′), −3.83 (Si(CH_3)$_2$), −5.13 (Si(CH_3)$_2$). HR MS (*m*/*z*) calcd for $C_{145}H_{245}N_2O_{23}PSi$ $[M + Na]^+$, 2464.7468; found, 2465.0632.

6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoylamino]-2-deoxy-α-D-glucopyranose (32). HF/pyridine (50 μL) was added dropwise to a stirred solution of **30** (20.0 mg, 0.0087 mmol) in THF (3 mL). The reaction mixture was stirred at room temperature for 5 h, after which it was diluted with ethyl acetate (15 mL), and washed with saturated aqueous $NaHCO_3$ (2 × 25 mL) and brine (2 × 20 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 3/1−4/3, v/v) to give **32** as a white solid (16.0 mg, 84%). $R_f = 0.38$ (hexane/ethyl acetate, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−7.19 (m, 24H, aromatic), 6.36 (d, 1H, $J_{NH',2'} = 7.0$ Hz, N*H*′), 6.28 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 5.52 (d, 1H, $J_{1',2'} = 9.0$ Hz, H-1′), 5.51 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.41 (t, 1H, $J_{2,3} = J_{3,4} = 10.0$ Hz, H-3), 5.27−5.25 (m, 1H, H-3_L), 5.15−4.96 (m, 6H, H-1, H-3_L, $C_6H_4(CH_2O)_2P$), 4.64−4.43 (m, 9H, H-4′, 4 × CH_2Ph), 4.23−4.19 (m, 1H, H-2), 4.13−4.09 (m, 1H, H-5), 3.94−3.82 (m, 4H, H-6a, H-6′a, 2 × H-3_S), 3.76−3.69 (m, 3H, H-5′, H-6′a, H-6b), 3.36−3.33 (m, 2H, H-2′, H-4), 2.69−2.27 (m, 12H, 2 × H-2_L, 2 × H-2_S, 2 × H-$2_{L'}$), 1.58 (broad, 12H, 2 × H-4_L, 2 × H-4_S, 2 × H-$3_{L'}$), 1.26 (broad, 108H, 54 × CH_2, lipid), 0.91−0.81 (m, 18H, 6 × CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{130}H_{207}N_2O_{22}PSi$ $[M + Na]^+$, 2202.4776; found, 2202.8279.

6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoy-

lamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoylamino]-α-D-glucopyranose (33). **31** (39.0 mg, 0.016 mmol) was deprotected in a manner similar to the synthesis of **32** with HF/pyridine (100 mL) in THF (5 mL) to yield **33** as a white solid (33.0 mg, 89%). $R_f = 0.52$ (hexane/ethyl acetate, 4/3, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40−7.17 (m, 19H, aromatic), 6.41 (d, 1H, $J_{NH',2'} = 6.5$ Hz, N*H*′), 5.95 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.56 (d, 1H, $J_{1',2'} = 8.5$ Hz, H-1′), 5.51 (t, 1H, $J_{2',3'} = J_{3',4'} = 10.0$ Hz, H-3′), 5.39 (t, 1H, $J_{2,3} = J_{3,4} = 10.0$ Hz, H-3), 5.29−5.26 (m, 1H, H-3_L), 5.15−4.95 (m, 7H, H-1, 2 × H-3_L, $C_6H_4(CH_2O)_2P$), 4.65−4.42 (m, 7H, H-4′, 3 × CH_2Ph), 4.17−4.08 (m, 2H, H-2, H-5), 3.92 (d, 1H, $J_{6a,6b} = 12.0$ Hz, H-6a), 3.91−3.82 (m, 2H, H-6′a, H-3_S), 3.76−3.69 (m, 3H, H-5′, H-6b, H-6′b), 3.36−3.30 (m, 2H, H-2′, H-4), 2.69−2.27 (m, 14H, 3 × H-2_L, H-2_S, 3 × H-$2_{L'}$), 1.59 (broad, 14H, 3 × H-4_L, H-4_S × 2, 3 × H-3_L), 1.26 (broad, 132H, 66 × CH_2, lipid), 0.90−0.88 (m, 21H, 7 × CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{139}H_{231}N_2O_{23}PSi$ $[M + Na]^+$, 2350.6603; found, 2350.8623.

Bis(benzyloxy)phosphoryl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoylamino]-2-deoxy-α-D-glucopyranose (34). To a cooled (−78 °C) solution of **32** (16.0 mg, 0.0073 mmol) and tetrabenzyl diphosphate (16.0 mg, 0.029 mmol) in THF (4 mL) was added dropwise lithium bis(trimethylsilyl)amide in THF (1.0 M, 30 μL, 0.03 mmol). The reaction mixture was stirred for 1 h, and then allowed to warm to −20 °C. After the reaction mixture was stirred at −20 °C for 1 h, it was quenched with saturated aqueous $NaHCO_3$ (10 mL) and extracted with ethyl acetate (15 mL). The organic phase was washed with brine (2 × 15 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by Iatro beads column chromatography (hexane/ethyl acetate, 5/1−3/1−4/3, v/v) to give **34** as a pale yellow oil (12.0 mg, 67%).

Bis(benzyloxy)phosphoryl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoylamino]-α-D-glucopyranose (35). The phosphorylation of **33** (12 mg, 0.0052 mmol) was performed in a manner similar to that for **34** to give **35** as a white solid (9.0 mg, 68%).

6-*O*-{2-Deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-*β*-D-glucopyranosyl}-2-deoxy-3-*O*-[(*R*)-3-hydroxy-tetradecanoyl]-2-[(*R*)-3-hydroxy-tetradecanoylamino]-α-D-glucopyranose 1,4′-Bisphosphate (1). A mixture of **34** (12.0 mg, 0.0049 mmol) and Pd-black (15.0 mg) in anhydrous THF (5 mL) was shaken under an atmosphere of H_2 (50 psi) at room temperature for 30 h, after which it was neutralized with triethylamine (10 μL), the catalyst was removed by filtration, and the residue was washed with THF (2 × 1 mL). The combined filtrates were concentrated in vacuo to afford **1** as a colorless film (6.3 mg, 72%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.19 (broad, 1H, H-1), 4.87−4.83 (m, 4H, H-3, H-3′, 2 × H-3_L), 4.43 (d, 1H, $J_{1',2'} = 8.4$ Hz, H-1′), 3.93−3.89 (m, 1H, H-4′), 3.87−3.85 (m, 1H, H-2), 3.74 (broad, 1H, H-5), 3.70 (d, 1H, $J_{6a,6b}$ or $J_{6'a,6'b} = 11.4$ Hz, H-6a or 6′a), 3.65 (broad, 1-H, H-3_S), 3.57−3.48 (m, 4H, H-6a or 6′a, 6b, 6′b, H-3_S), 3.21 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, H-4), 3.14−3.11 (m, 1H, H-5′), 2.37−1.96 (m, 12H, 2 × H-2_L, 2 × H-2_S, 2 × H-$2_{L'}$), 1.27 (broad, 12H, 2 × H-4_L, 2 × H-4_S, 2 × H-3_L), 0.94 (broad, 108H, 54 × CH_2, lipid), 0.56−0.54 (m, 18H, 6 × CH_3, lipid). HR MS (*m*/*z*) (negative) calcd for $C_{94}H_{178}N_2O_{25}P_2$, 1797.2194; found, 1796.5488 [M − H], 1797.5510 [M].

6-*O*-{2-Deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-tetradecanoyloxy-tetradecanoyl]-*β*-D-glucopyranosyl}-2-deoxy-2-[(*R*)-3-hexadecanoyl-tetradecanoylamino]-3-*O*-[(*R*)-3-hydroxy-tetradecanoyl]-α-D-glucopyranose 1,4′-Bisphosphate (3). Compound **35** (9.0 mg, 0.0035 mmol) was deprotected in a manner similar to the synthesis of **1** to provide **3** as a colorless film (5.4 mg, 75%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.11 (broad, 1H, H-1), 4.87−4.82 (m, 5H, H-3, H-3′, 3 × H-3_L), 4.40 (d, 1H, $J_{1',2'} = 8.4$ Hz, H-1′), 3.92−3.88 (m, 1H, H-4′), 3.85−2.83 (m, 1H, H-2), 3.77 (broad, 1H, H-5), 3.71−3.62 (m, 3H, H-3_S), 3.53−3.43 (m, 3H, H-2′), 3.18 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, H-4), 3.10−3.07 (m, 1H, H-5′), 2.34−1.96 (m, 14H, 3 × H-2_L, H-2_S, 3 × H-$2_{L'}$), 1.23 (broad, 14H, 3 × H-4_L, H-4_S, 3 × H-3_L), 0.99 (broad, 132H, 66 × CH_2, lipid), 0.57−0.55 (m, 21H, 7 × CH_3, lipid). HR MS (*m*/*z*) (negative) calcd for $C_{110}H_{208}N_2O_{26}P_2$, 2035.4491; found, 2034.4668 [M − H], 2035.4692 [M].

***tert*-Butyldimethylsilyl 6-*O*-{3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-2-azido-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-*β*-D-glucopyranoside (36).** A solution of (*R*)-3-benzyloxy-dodecanoic acid **14** (86 mg, 0.281 mmol) and DCC (78 mg, 0.376 mmol) in DCM (5 mL) was stirred at room temperature for 10 min, and then disaccharide **21** (250 mg, 0.188 mmol) in DCM (2 mL) and DMAP (11 mg, 0.094 mmol) were added. The reaction mixture was stirred for another 14 h, after which the solids were removed by filtration, and the residue was washed with DCM (2 × 1 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to give **36** as a white solid (277 mg, 91%). $R_f = 0.41$ (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D = -3.0°$ ($c = 1.0$, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.34−7.15 (m, 19H, aromatic), 6.01 (d, 1H, $J_{NH',2} = 7.2$ Hz, N*H*′), 5.92−6.86 (m, 1H, $OCH_2CH{=}CH_2$), 5.46 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, H-3′), 5.34 (d, 1H, $J = 16.8$ Hz, $OCH_2CH{=}CH_2$), 5.22 (d, 1H, $J = 10.8$ Hz, $OCH_2CH{=}CH_2$), 5.08−4.97 (m, 7H, H-1′, H-3, H-3_L, $C_6H_4(CH_2O)_2P$), 4.61−4.45 (m, 10H, H-1, H-4′, 3 × CH_2Ph, OCH_2CH=CH_2), 3.97 (d, 1H, $J_{6'a,6'b} = 10.5$ Hz, H-6′a), 3.88−3.86 (m, 1H, H-3_S), 3.81 (d, 1H, $J_{6a,6b} = 9.5$ Hz, H-6a), 3.73−3.68 (m, 3H, H-5′, H-6b, H-6′b), 3.56−3.46 (m, 3H, H-2′, H-4, H-5), 3.28 (dd, 1H, $J_{1,2} = 7.8$ Hz, $J_{2,3} = 10.2$ Hz, H-2), 2.56 (dd, 1H, $J_{2Sa,2Sb} = 15.6$ Hz, $J_{2Sa,3S} = 7.2$ Hz, H-2_{Sa}), 2.44 (dd, 1H, $J_{2Sa,2Sb} = 15.6$ Hz, $J_{2Sb,\ S3} = 6.0$ Hz, H-2_{Sb}), 2.36 (dd, 1H, $J_{2La,2Lb} = 15.0$ Hz, $J_{2La,3\ L} = 6.0$ Hz, H-2_{La}), 2.30−2.21 (m, 3H, H-$2_{L'}$, H-$2_{L'b}$), 1.57−1.53 (m, 6H, H-4_L, H-4_S, H-$3_{L'}$), 1.24 (broad, 48H, 24 × CH_2, lipid), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.89−0.85 (m, 9H, 3 × CH_3, lipid), 0.13 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.54 (*C*=O), 170.64 (*C*=O), 170.03 (*C*=O), 154.49 (*C*=O), 138.52−127.43 (aromatic, $OCH_2CH{=}CH_2$), 118.84 (OCH_2-$CH{=}CH_2$), 99.34 (C-1′), 97.02 (C-1), 76.00, 75.70, 74.33, 74.08, 73.73, 73.48, 71.46, 71.01, 68.95−67.99 (m), 66.55, −4.13 ($Si(CH_3)_2$), −5.32 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{89}H_{135}N_4O_{19}PSi$ [M + Na], 1645.9125; found, 1646.2435.

***tert*-Butyldimethylsilyl 6-*O*-{3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-*β*-D-glucopyranoside (37).** A suspension of **36** (180 mg, 0.111 mmol), zinc (72 mg, 1.11 mmol), and acetic acid (25 μL, 0.444 mmol) in DCM (5 mL) was stirred at room temperature for 12 h, after which it was diluted with ethyl acetate, the solids were removed by filtration, and the residue was washed with ethyl acetate (2 × 2 mL). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 × 15 mL) and brine (2 × 15 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 2.5/1, v/v) to afford the amine as a pale yellow syrup (160 mg, 90%). $R_f = 0.35$ (hexane/ethyl acetate, 2/1, v/v). HR MS (*m*/*z*) calcd for $C_{89}H_{137}N_2O_{19}PSi$ $[M + Na]^+$, 1619.9220; found, 1620.1069. DCC (34 mg, 0.169 mmol) was added to a stirred solution of (*R*)-3-benzyloxy-tetradecanoic acid **15** (47 mg, 0.141 mmol) in DCM (1.5 mL). After the mixture was stirred for 10 min, the amine (150 mg, 0.094 mmol) in DCM (1 mL) was added.

The reaction mixture was stirred at room temperature for 10 h, after which the insoluble materials were removed by filtration, and the residue was washed with DCM (2 × 1 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by preparative silica gel TLC chromatography (hexane/ethyl acetate, 5/1, v/v) to give **37** as a white solid (153 mg, 85%). R_f = 0.34 (hexane/ethyl acetate, 3/2, v/v). $[\alpha]^{26}_D = -2.3°$ (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38−7.19 (m, 24H, aromatic), 6.15 (d, 1H, $J_{NH,\ 2}$ = 9.0 Hz, N*H*), 5.97−5.89 (m, 2H, N*H*′, OCH_2CH═CH_2), 5.57 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 5.38 (d, 1H, J = 17.5 Hz, OCH_2CH═CH_2), 5.26 (d, 1H, J = 10.5 Hz, OCH_2CH═CH_2), 5.15−5.02 (m, 7H, H-1′, H-3, H-3_L, $C_6H_4(CH_2O)_2P$), 4.67−4.44 (m, 10H, H-1, H-4′, 4 × CH_2Ph), 4.01 (d, 1H, $J_{6a,6b}$ = 11.5 Hz, H-6a), 3.90−3.81 (m, 3H, H-2, H-6′a, H-3_S), 3.76−3.67 (m, 4H, H-5′, H-6_b, H-6′$_b$, H-3_S), 3.57 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.53−3.50 (m, 1H, H-5), 3.45−3.40 (m, 1H, H-2′), 2.61−2.25 (m, 8H, H-2_L, 2 × H-2_S, H-$2_{L'}$), 1.61−1.44 (m, 8H, H-4_L, 2 × H-4_S, H-$3_{L'}$), 1.27 (broad, 66H, 33 × CH_2, lipid), 0.91−0.86 (m, 21H, 4 × CH_3, lipid, $SiC(CH_3)_3$), 0.09 (s, 3H, $SiCH_3$), 0.04 (s, 3H, $SiCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.49 (*C*═O), 171.43 (*C*═O), 170.82 (*C*═O), 170.10 (*C*═O), 154.45 (*C*═O), 138.54−127.44 (aromatic, OCH_2CH═CH_2), 118.79 (OCH_2CH═CH_2), 98.94 (C-1′), 96.27 (C-1), 76.07, 75.89, 75.77, 75.41, 74.89, 74.63, 74.18, 73.78, 73.66, 71.32, 70.95, 70.56, 68.93−68.24 (m), 56.18 (C-2 or 2′), 55.96 (C-2 or 2′), −3.74 ($Si(CH_3)_2$), −5.11 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{110}H_{169}N_2O_{21}PSi$ $[M + Na]^+$, 1936.1622; found, 1936.2714.

***tert*-Butyldimethylsilyl 6-*O*-{3-*O*-Allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyl-tetradecanoyl]-*β*-D-glucopyranoside (38).** In a manner similar to the synthesis of **37**, the free amine (99 mg, 0.062 mmol) synthesized by reduction of **36** was acylated with (*R*)-3-hexadecanoyl-tetradecanoic acid **20** (45 mg, 0.093 mmol), using DCC (26 mg, 0.124 mmol) as activating agents, to yield **38** as a white solid (103 mg, 81%). R_f = 0.52 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D = -5.3°$ (c = 1.0, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.36−7.17 (m, 19H, aromatic), 5.98 (d, 1H, $J_{NH',2'}$ = 7.2 Hz, N*H*′), 5.93−5.87 (m, 1H, OCH_2CH═CH_2), 5.76 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.56 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.0 Hz, H-3′), 5.36 (d, 1H, J = 17.4 Hz, OCH_2CH═CH_2), 5.23 (d, 1H, J = 10.2 Hz, OCH_2CH═CH_2), 5.14−4.99 (m, 8H, H-1′, 3, 2 × H-3_L, $C_6H_4(CH_2O)_2P$), 4.70 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.62−4.42 (m, 7H, H-4′, 3 × CH_2Ph), 3.99 (d, 1H, $J_{6a,6b}$ = 11.4 Hz, H-6a), 3.84−3.78 (m, 3H, H-2, H-6′a, H-3_S), 3.74−3.67 (m, 3H, H-5, H-5′, H-6′$_b$), 3.58−3.55 (m, 2H, H-4, H-6b), 3.41−3.37 (m, 1H, H-2′), 2.54−2.19 (m, 10H, 2 × H-2_L, H-2_S, 2 × H-$2_{L'}$), 1.59−1.50 (m, 10H, 2 × H-4_L, H-4_S, 2 × H-$3_{L'}$), 1.23 (broad, 90H, 45 × CH_2, lipid), 0.88−0.84 (m, 24H, 5 × CH_3, lipid, $SiC(CH_3)_3$), 0.09 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.60 (*C*═O), 173.43 (*C*═O), 171.57 (*C*═O), 170.04 (*C*═O), 169.14 (*C*═O), 154.42 (*C*═O), 138.47−127.43 (aromatic, OCH_2CH═CH_2), 118.72 (OCH_2CH═CH_2), 99.09 (C-1′), 96.05 (C-1), 75.96, 75.36, 74.95, 74.80, 74.26, 74.10, 73.77, 73.69, 71.21, 70.91, 70.76, 68.87−67.98 (m), 56.32, 56.03 (C-2′), −3.91 ($Si(CH_3)_2$), −5.20 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{110}H_{169}N_2O_{21}PSi$ $[M + Na]^+$, 2084.3450; found, 2084.6633.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-(*p*-methoxy)benzyloxy-dodecanoylamino]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-*β*-D-glucopyranoside (39).** Tetrakis(triphenylphosphine)palladium (6.6 mg, 0.006 mmol) was added to a solution of **37** (55 mg, 0.029 mmol), *n*-$BuNH_2$ (5.7 μL, 0.058 mmol), and HCOOH (2.2 μL, 0.058 mmol) in THF (5 mL). After the reaction mixture was stirred at room temperature for 20 min, it was diluted with DCM (15 mL), and washed with water (10 mL), saturated aqueous $NaHCO_3$ (2 × 10 mL), and brine (2 × 10 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/3, v/v) to give the alcohol intermediate. A solution of (*R*)-3-(*p*-methoxy)-benzyloxy-dodecanoic acid **16** (16.5 mg, 0.049 mmol) and DCC (13.6 mg, 0.066 mmol) in DCM (5 mL) was stirred at room temperature for 10 min, after which the alcohol intermediate in DCM (1 mL) and DMAP (7 mg, 0.060 mmol) were added. The reaction mixture was stirred at room temperature for 5 h, after which the solids were removed by filtration and washed with DCM (2 × 2 mL). The combined filtrates were concentrated in vacuo, and the residue was purified by preparative silica gel TLC (hexane/ethyl acetate, 3/1, v/v) to afford **39** as a white solid (47 mg, 75%). R_f = 0.29 (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{26}_D = -4.5°$ (c = 1.0, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38−6.72 (m, 28H, aromatic), 6.11 (d, 1H, $J_{NH,\ 2}$ = 9.0 Hz, N*H*), 5.74 (d, 1H, $J_{NH',\ 2'}$ = 7.8 Hz, N*H*′), 5.59 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3′), 5.10−5.06 (m, 2H, H-1′, H-3), 5.00−4.85 (m, 5H, H-3_L, $C_6H_4(CH_2O)_2P$), 4.61 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.0 Hz, H-4′), 4.57−4.41 (m, 11H, H-1, 4 × CH_2Ph, CH_2PhOCH_3), 3.97 (d, 1H, $J_{6a,6b}$ = 10.8 Hz, H-6a), 3.88−3.81 (m, 4H, H-2, H-6′a, 2 × H-3_S), 3.71−3.68 (m, 7H, H-5′, H-6b, H-6′b, H-3_S, CH_3OPh), 3.55 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.47 (broad, 1H, H-5), 3.30−3.26 (m, 1H, H-2′), 2.64−1.69 (m, 10H, H-2_L, 3 × H-2_S, H-$2_{L'}$), 1.67−1.41 (m, 10H, H-4_L, 3 × H-4_S, H-$3_{L'}$), 1.24 (broad, 80H, 40 × CH_2, lipid), 0.87−0.81 (m, 24H, 5 × CH_3, lipid, $SiC(CH_3)_3$), 0.06 (s, 3H, $SiCH_3$), 0.01 (s, 3H, $SiCH_3$). HR MS (*m*/*z*) calcd for $C_{126}H_{195}N_2O_{22}PSi$ $[M + Na]^+$, 2170.3606; found, 2170.4929.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-3-*O*-[(*R*)-3-(*p*-methoxy)benzyloxy-dodecanoylamino]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoyl]-*β*-D-glucopyranoside (40).** In a manner similar to that described for the synthesis of **39**, the Alloc group of **38** (72 mg, 0.035 mmol) in THF (6 mL) was removed with tetrakis(triphenylphosphine)-palladium (12 mg, 0.011 mmol) in the presence of *n*-$BuNH_2$ (6.9 μL, 0.07 mmol) and HCOOH (2.6 μL, 0.07 mmol). After purification by silica gel column chromatography (hexane/ethyl acetate, 4/3, v/v), the resulting intermediate was acylated with (*R*)-3-(*p*-methoxy)benzyloxy-docanoic acid **16** (18 mg, 0.052 mmol) in DCM (5 mL), using DCC (15 mg, 0.07 mmol) and DMAP (2.5 mg, 0.02 mmol) as activating agents. Purification by preparative silica gel TLC (hexane/ethyl acetate, 3/1, v/v) afforded **40** as a white solid (49 mg, 61%). R_f = 0.30 (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{25}_D = -6.0°$ (c 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−6.73 (m, 23H, aromatic), 5.80−5.79 (broad, 2H, N*H*, N*H*′), 5.64 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 5.16−5.11 (m, 2H, H-1′, H-3), 5.06−4.84 (m, 6H, 2 × H-3_L, $C_6H_4(CH_2O)_2P$), 4.71 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.67−4.44 (m, 9H, H-4′, 4 × CH_2-Ph), 4.01 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.88−3.79 (m, 4H, H-2, 6′a, 2 × H-3_S), 3.74−3.69 (6, 3H, H-5′, H-6b, H-6′b, CH_3OPh), 3.61−3.58 (m, 2H, H-4, H-5), 3.30−3.25 (m, 1H, H-2′), 2.65−2.01 (m, 12H, 2 × H-2_L, 2 × H-2_S, 2 × H-$2_{L'}$), 1.61−1.50 (m, 12H, 2 × H-4_L, 2 × H-4_S, 2 × H-$3_{L'}$), 1.25 (broad, 102H, 51 × CH_2, lipid), 0.88−0.84 (m, 27H, 6 × CH_3, lipid, $SiC(CH_3)_3$), 0.08 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.63 (*C*═O), 171.62 (*C*═O), 171.14 (*C*═O), 169.88 (*C*═O), 169.14 (*C*═O), 159.20−113.77 (aromatic), 99.61 (C-1′), 96.17 (C-1), 75.98, 75.39, 75.23, 74.91, 74.42, 74.15, 73.92, 73.51, 72.02, 71.26, 71.05, 70.81, 70.63, 68.95, 68.52−68.18 (m), 56.32 (C-2 or 2′), 55.17 (CH_3OPh), −3.83 ($Si(CH_3)_2$), −5.13 ($Si(CH_3)_2$). HR MS (*m*/*z*) calcd for $C_{135}H_{219}N_2O_{23}PSi$ $[M + Na]$, 2318.5433; found, 2318.7700.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoylamino]-2-deoxy-*β*-D-glucopyranoside (43).** DDQ (5 mg, 0.0223 mmol) was added to a

stirred solution of **39** (32 mg, 0.0149 mmol) in a mixture of DCM and H_2O (3 mL, 10/1, v/v). After the reaction mixture was stirred at room temperature for 1 h, it was diluted with DCM (10 mL) and washed with brine (10 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 3/1, v/v) to give free alcohol **41** as a colorless syrup (29 mg, 96%). $R_f = 0.36$ (hexane/ethyl acetate, 2/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39−7.18 (m, 24H, aromatic), 6.30 (d, 1H, $J_{NH',\,2'} = 7.5$ Hz, N*H*′), 6.16 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.60 (t, 1H, $J_{2',3'} = J_{3',4'} = 10.0$ Hz, H-3′), 5.15−4.98 (m, 7H, H-1′, H-3, H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.68−4.63 (m, 1H, H-4′), 4.58−4.44 (m, 9H, H-1, 4 × C*H*$_2$Ph), 4.07 (broad, 1H, H-3$_S$), 4.01 (d, 1H, $J_{6a,6b} = 10.0$ Hz, H-6a), 3.87−3.82 (m, 3H, H-2, H-6′a, H-3$_S$), 3.73−3.71 (m, 4H, H-5′, H-6b, H-6′b, H-3$_S$), 3.59 (t, 1H, $J_{3,4} = J_{4,5} = 9.0$ Hz, H-4), 3.53−3.50 (m, 1H, H-2′, H-5), 2.64−2.23 (m, 10H, H-2$_L$, 3 × H-2$_S$, H-2$_{L'}$), 1.69−1.46 (m, 10H, H-4$_L$, 3 × H-4$_S$, H-3$_{L'}$), 1.26 (broad, 80H, 40 × C*H*$_2$, lipid), 0.91−0.84 (m, 24H, 5 × C*H*$_3$, lipid, SiC(C*H*$_3$)$_3$), 0.09 (s, 3H, SiC*H*$_3$), 0.04 (s, 3H, SiC*H*$_3$). HR MS (*m*/*z*) calcd for $C_{118}H_{187}N_2O_{21}PSi$ [M + Na]$^+$, 2050.3031; found, 2050.5063. Lauroyl chloride (50 μL) was added to a solution of alcohol **41** (27 mg, 0.0133 mmol), pyridine (100 μL), and DMAP (1.2 mg, 0.01 mmol) in DCM (2 mL). After the reaction mixture was stirred at room temperature for 12 h, it was diluted with DCM (15 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL). The organic phase was dried ($MgSO_4$) and filtered. Next, the filtrate was concentrated in vacuo. The residue was purified by preparative silica gel TLC (toluene/ethyl acetate, 5/1, v/v) to afford **43** as a white solid (25 mg, 86%). $R_f = 0.56$ (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D = -2.9°$ ($c = 1.0$, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38−7.21 (m, 24H, aromatic), 6.19 (d, 1H, $J_{NH',\,2'} = 7.5$ Hz, N*H*′), 6.17 (d, 1H, $J_{NH,\,2} = 9.0$ Hz, N*H*), 5.59 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.30−5.27 (m, 1H, H-3$_L$), 5.15−4.98 (m, 7H, H-1′, H-3, H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.65−4.42 (m, 10H, H-1, H-4′, 4 × C*H*$_2$Ph), 4.01 (d, 1H, $J_{6a,6b} = 9.5$ Hz, H-6a), 3.91−3.82 (m, 3H, H-2, H-6′a, H-3$_S$), 3.75−3.69 (m, 4H, H-5′, H-6b, H-6′b, H-3$_S$), 3.58 (t, 1H, $J_{3,4} = J_{4,5} = 9.0$ Hz, H-4), 3.53−3.50 (m, 1H, H-5), 3.43−3.38 (m, 1H, H-2′), 2.65−2.22 (m, 12H, 2 × H-2$_L$, 2 × H-2$_S$, 2 × H-2$_{L'}$), 1.66−1.52 (m, 12H, 2 × H-4$_L$, 2 × H-4$_S$, 2 × H-3$_{L'}$), 1.27 (broad, 96H, 48 × C*H*$_2$, lipid), 0.91−0.88 (m, 18H, 6 × C*H*$_3$, lipid), 0.86 (s, 9H, SiC(C*H*$_3$)$_3$), 0.09 (s, 3H, SiC*H*$_3$), 0.05 (s, 3H, SiC*H*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.18 (*C*═O), 173.66 (*C*═O), 173.54 (*C*═O), 171.45 (*C*═O), 170.89 (*C*═O), 170.12 (*C*═O), 138.64−127.45 (aromatic), 99.52 (C-1′), 96.26 (C-1), 76.15, 75.88, 75.44, 74.78, 74.39, 74.10, 72.65, 71.36, 70.62, 70.54, 70.29, 68.96, 68.89−68.22 (m), 56.50 (C-2), 56.06 (C-2′), −3.77 (Si(*C*H$_3$)$_2$), −5.09 (Si(*C*H$_3$)$_2$). HR MS (*m*/*z*) calcd for $C_{130}H_{209}N_2O_{22}PSi$ [M + Na]$^+$, 2232.4702; found, 2232.8787.

***tert*-Butyldimethylsilyl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoylamino]-β-D-glucopyranoside (44).** The PMB group of **40** (41 mg, 0.018 mmol) was removed in a manner similar to the synthesis of **41** with DDQ (6.1 mg, 0.158 mmol) in a mixture of DCM and H_2O (5 mL, 10/1, v/v). Purification by silica gel column chromatography (hexane/ethyl acetate, 3/1, v/v) gave free alcohol **42** as a colorless syrup (32 mg, 83%). $R_f = 0.39$ (hexane/ethyl acetate, 2/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.34−7.15 (m, 24H, aromatic), 6.26 (d, 1H, $J_{NH',2'} = 7.2$ Hz, N*H*), 5.71 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.55 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, H-3′), 5.13−4.95 (m, 8H, H-1′, H-3, 2 × H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.71 (d, 1H, $J_{1,2} = 7.8$ Hz), 4.65−4.59 (m, 1H, H-4′), 4.55−4.10 (m, 6H, 3 × C*H*$_2$Ph), 4.04 (broad, 1H, H-3$_S$), 3.99 (d, 1H, $J_{6a,6b} = 10.2$ Hz, H-6a), 3.82−3.76 (m, 3H, H-2, H-6′a, H-3$_S$), 3.73−3.68 (m, 3H, H-5′, H-6b, H-6′b), 3.60−3.54 (m, 2H, H-4, H-5), 3.51−3.47 (m, 1H, H-2′), 2.61−2.18 (m, 12H, 2 × H-2$_L$, 2 × H-2$_S$, 2 × H-2$_{L'}$), 1.74−1.41 (m, 12H, 2 × H-4$_L$, 2 × H-4$_S$, 2 × H-3$_{L'}$), 1.24 (broad, 104H, 52 × C*H*$_2$, lipid), 0.87−0.84 (m, 27H, 6 × C*H*$_3$, lipid, SiC(C*H*$_3$)$_3$), 0.08 (s, 3H, SiC*H*$_3$), 0.06 (s, 3H, SiC*H*$_3$). HR MS (*m*/*z*) calcd for $C_{127}H_{211}N_2O_{22}PSi$ [M + Na]$^+$, 2198.4858; found, 2198.7722. In a manner similar to the synthesis of **43**, alcohol **42** (28 mg, 0.013 mmol) was acylated with lauroyl chloride (50 μL) in the presence of pyridine (100 μL) and DMAP (1.6 mg, 0.013 mmol) in DCM (2 mL). Purification by silica gel column chromatography (toluene/ethyl acetate, 10/1−6/1, v/v) afforded **44** as a pale yellow oil (28.5 mg, 94%). $R_f = 0.52$ (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D = -1.7°$ ($c = 1.0$, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.34−7.16 (m, 19H, aromatic), 6.14 (d, 1H, $J_{NH',2'} = 8.0$ Hz, N*H*′), 5.73 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 5.57 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.29−5.27 (m, 1H, H-3$_L$), 5.15−4.99 (m, 8H, H-1′, 3, 2 × H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.73 (d, 1H, $J_{1,2} = 7.5$ Hz, H-1), 4.65−4.40 (m, 7H, H-4′, 3 × C*H*$_2$Ph), 4.02 (d, 1H, $J_{6a,6b} = 10.5$ Hz, H-6a), 3.88−3.79 (m, 3H, H-2, H-6′a, H-3$_S$), 3.75−3.69 (m, 3H, H-5′, H-6′b, H-6b), 3.62−3.59 (m, 2H, H-4, H-5), 3.46−3.41 (m, 1H, H-2), 2.68−2.23 (m, 14H, 3 × H-2$_L$, H-2$_S$, 3 × H-2$_{L'}$), 1.63−1.61 (m, 14H, 3 × H-4$_L$, H-4$_S$, 3 × H-3$_L$), 1.27 (broad, 120H, 60 × C*H*$_2$, lipid), 0.91−0.88 (m, 30H, 7 × C*H*$_3$, lipid, SiC(C*H*$_3$)$_3$), 0.13 (s, 3H, SiC*H*$_3$), 0.10 (s, 3H, SiC*H*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.67 (*C*═O), 173.62 (*C*═O), 173.55 (*C*═O), 171.62 (*C*═O), 170.13 (*C*═O), 170.10 (*C*═O), 169.15 (*C*═O), 138.52−127.48 (aromatic), 99.57 (C-1′), 96.15 (C-1), 76.00, 75.40, 74.91, 74.45, 74.14, 73.50, 72.56, 71.26, 70.83, 70.54, 70.27, 68.89−68.33 (m), 56.36 (C-2 or 2′), −3.84 (Si(*C*H$_3$)$_2$), −5.13 (Si(*C*H$_3$)$_2$). HR MS (*m*/*z*) calcd for $C_{139}H_{233}N_2O_{23}PSi$ [M + Na]$^+$, 2380.6529; found, 2380.8301.

Bis(benzyloxy)phosphoryl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-[(*R*)-3-benzyloxy-tetradecanoylamino]-2-deoxy-α-D-glucopyranose (45). Compound **43** (16 mg, 0.72 μmol) was deprotected in a manner similar to the synthesis of **32** with HF/pyridine (50 μL) in THF (3 mL) to yield the intermediate lactol as a white solid (13 mg, 86%). $R_f = 0.35$ (hexane/ethyl acetate, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40−7.18 (m, 24H, aromatic), 6.37 (d, 1H, $J_{NH',\,2'} = 7.5$ Hz, N*H*′), 6.26 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 5.55 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 5.52 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 5.42 (t, 1H, $J_{2,3} = J_{3,4} = 10.0$ Hz, H-3), 5.28−5.24 (m, 1H, H-3$_L$), 5.15−4.96 (m, 6H, H-1, H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.65−4.43 (m, 9H, H-4′, 4 × C*H*$_2$Ph), 4.24−4.19 (m, 1H, H-2), 4.13−4.09 (m, 1H, H-5), 3.94−3.82 (m, 4H, H-6a, H-6′a, 2 × H-3$_S$), 3.77−3.68 (m, 3H, H-5′, H-6b, H-6′b), 3.37−3.31 (m, 2H, H-2′, H-4), 2.69−2.27 (m, 12H, 2 × H-2$_L$, 2 × H-2$_S$, 2 × H-2$_{L'}$), 1.59 (broad, 12H, 2 × H-4$_L$, 2 × H-4$_S$, 2 × H-3$_{L'}$), 1.26 (broad, 80H, 40 × C*H*$_2$, lipid), 0.91−0.88 (m, 18H, 6 × C*H*$_3$, lipid). HR MS (*m*/*z*) calcd for $C_{124}H_{195}N_2O_{22}PSi$ [M + Na]$^+$, 2118.3837; found, 2118.6284. The anomeric hydroxyl of the resulting lactol (16.0 mg, 0.0073 mmol) was phosphorylated in a manner similar to the synthesis of **34** to afford **45** as a white solid (11.0 mg, 72%).

Bis(benzyloxy)phosphoryl 6-*O*-{6-*O*-Benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetradecanoylamino]-α-D-glucopyranose (46). Compound **44** (24 mg, 0.010 mmol) was deprotected in a manner similar to the synthesis of **32** with HF/pyridine (100 μL) in THF (3 mL) to yield the intermediate lactol as a white solid (22 mg, 97%). $R_f = 0.52$ (hexane/ethyl acetate, 1/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.39−7.15 (m, 19H, aromatic), 6.33 (d, 1H, $J_{NH',2'} = 7.2$ Hz, N*H*′), 5.89 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.55 (d, 1H, $J_{1',2'} = 8.4$ Hz, H-1′), 5.48 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, H-3′), 5.36 (t, 1H, $J_{2,3} = J_{3,4} = 9.6$ Hz, H-3), 5.26−5.22 (m, 1H, H-3$_L$), 5.11−4.88 (m, 7H, H-1, 2 × H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.62−4.40 (m, 7H, H-4′, 3 × C*H*$_2$Ph), 4.14−4.05 (m, 2H, H-2, H-5), 3.89 (d, 1H, $J_{6a,6b} = 12.6$ Hz, H-6a), 3.84−3.79 (m, 2H, H-6′a, H-3$_S$), 3.74−3.67 (m, 3H, H-5′, H-6b, H6′b), 3.31−3.28 (m, 2H, H-2′, H-4), 2.66−2.23 (m, 14H, 3 × H-2$_L$,

H-2_S, 3 × H-$2_{L'}$), 1.62−1.53 (broad, 14H, 3 × H-4_L, H-4_S × 2, 3 × H-3_L), 1.3 (broad, 120H, 60 × CH_2, lipid), 0.87−0.85 (m, 21H, 7 × CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{133}H_{219}N_2O_{23}PSi$ $[M + Na]^+$, 2266.5664; found, 2266.8252. The anomeric hydroxyl of the resulting lactol (12.0 mg, 0.0053 mmol) was phosphorylated in a manner similar to the synthesis of **34** to afford **46** as a white solid (9.2 mg, 69%).

6-*O*-{2-Deoxy-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-2-deoxy-3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-2-[(*R*)-3-hydroxy-tetredecanoylamino]-α-D-glucopyranose 1,4′-Bisphosphate (2). Compound **45** (8.0 mg, 0.0034 mmol) was deprotected in a manner similar to the synthesis of **1** to provide **2** as a colorless film (4.7 mg, 81%). ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ 5.08 (broad, 1H, H-1), 4.79−4.76 (m, 4H, H-3, H-3′, 2 × H-3_L), 4.35 (d, 1H, $J_{1',2'} = 7.8$ Hz, H-1′), 3.82 (broad, 1H, H-4′), 3.77−3.75 (m, 1H, H-2), 3.67 (broad, 1H, H-5), 3.61 (d, $J_{6a,6b}$ or $J_{6'a,6'b} = 11.4$ Hz, H-6a or 6′a), 3.56 (m, 1H, H-3_S), 3.49−3.40 (m, 5H, H-2′, H-6a or H-6′a, H-6b, H-6′b, H-3_S), 3.12 (t, 1H, $J_{3,4} = J_{4,5} = 9.0$ Hz, H-4), 3.02 (broad, 1H, H-5′), 2.29−1.84 (m, 12H, 2 × H-2_L, 2 × H-2_S, 2 × H-$2_{L'}$), 1.18 (broad, 12H, 2 × H-4_L, 2 × H-4_S, 2 × H-3_L), 0.85 (broad, 80H, 40 × CH_2, lipid), 0.47−0.45 (m, 18H, 6 × CH_3, lipid). HR MS (*m*/*z*) (negative) calcd for $C_{88}H_{166}N_2O_{25}P_2$, 1713.1255; found, 1712.0845 [M − H], 1713.0880 [M].

6-*O*-{2-Deoxy-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetredecanoylamino]-3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-α-D-glucopyranose 1,4′-Bisphosphate (4). Compound **46** (9.2 mg, 0.0041 mmol) was deprotected in a manner similar to the synthesis of **1** to provide **4** as a colorless film (5.5 mg, 69%). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ 5.33 (broad, 1H, H-1), 5.11−5.03 (m, 5H, H-3, H-3′, 3 × H-3_L), 4.61 (d, 1H, $J_{1',2'} = 8.5$ Hz, H-1′), 4.16−3.10 (m, 1H, H-4′), 4.09−4.07 (m, 1H, H-2), 4.04 (broad, 1H, H-5), 3.94−3.89 (m, H-6a or H-6′a, H-3_S), 3.75−3.67 (m, H-2′), 3.39 (dd, $J = 8.5$ Hz, $J = 9.5$ Hz, H-4), 3.31−3.29 (m, 1H, H-5′), 2.63−2.19 (m, 14H, 3 × H-2_L, H-2_S, 3 × H-$2_{L'}$), 1.52 (broad, 14H, 3 × H-4_L, H-4_S, 3 × H-3_L), 1.18 (broad, 120H, 60 × CH_2, lipid), 0.81−0.78 (m, 21H, 7 × CH_3, lipid). HR MS (*m*/*z*) (negative) calcd for $C_{104}H_{196}N_2O_{26}P_2$, 1951.3552; found, 1950.4846 [M − H], 1951.4910 [M].

6-*O*-{2-Deoxy-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-*β*-D-glucopyranosyl}-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-tetredecanoylamino]-3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-α-D-glucopyranose (5). The resulting lactol in the synthesis of **46** (8.5 mg, 0.0038 mmol) was deprotected in a manner similar to the synthesis of **1** to provide **5** as a colorless film (5.1 mg, 71%). ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ 5.01−4.91 (m, 5H, H-3, H-3′, 3 × H-3_L), 4.89 (broad, 1H, H-1), 4.48 (d, 1H, $J_{1',2'} = 8.4$ Hz, H-1′), 4.06 (broad, 1H, H-4′), 3.90−3.85 (m, 3H, H-2, H-5, H-6a or H-6′a), 3.75 (broad, H-3_S), 3.70 (broad, 1H, H-6a or H-6′a), 3.67−3.62 (m, 2H, H-2′, H-6b or H-6′b), 3.58 (broad, 1H, H-6b or 6′b), 3.28−3.20 (m, 2H, H-4, H-5′), 2.61 (m, 1H, H-2_{Sa}), 2.53 (m, 1H, H-2_{Sb}), 2.40−2.12 (m, 6H, 3 × H-2_L), 2.11−2.08 (m, 6H, 3 × H-$2_{L'}$), 1.45 (broad, 14H, 3 × H-4_L, H-4_S, 3 × H-3_L), 1.12 (broad, 120H, 60 × CH_2, lipid), 0.76−0.83 (m, 21H, 7 × CH_3, lipid). HR MS (*m*/*z*) (negative) calcd for $C_{104}H_{195}N_2O_{23}P$, 1871.3888; found, 1870.4127 [M − H], 1871.4128 [M].

Reagents for Biological Experiments. *E. coli* 055:B5 LPS was obtained from List Biologicals. All data presented in this study were generated using the same batch of *E. coli* 055:B5 LPS. Synthetic lipid A's were reconstituted in PBS with DMSO (10%) and stored at −80 °C.

Cell Maintenance. RAW 264.7 γNO(−) cells, derived from the RAW 264.7 mouse monocyte/macrophage cell line, were obtained from ATCC. The cells were maintained in RPMI 1640 medium (ATCC) with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), HEPES (10 mM), and sodium pyruvate (1.0 mM), and supplemented with penicillin (100 u/mL)/streptomycin (100 μg/mL; Mediatech) and fetal bovine serum (FBS, 10%; Hyclone). Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g/L), and sodium bicarbonate (1.5 g/L), supplemented with penicillin (100 u/mL)/streptomycin (100 μg/mL), Normocin (100 μg/mL), and FBS (10%). Stably transfected HEK 293T cells with murine TLR4, MD2, and CD14 (InvivoGen) were obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with the selective agents HygroGold (50 μg/mL; InvivoGen) and blasticidin (10 μg/mL; InvivoGen). All cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

Cytokine Induction and ELISAs. RAW 264.7 γNO(−) cells were plated on the day of the exposure assay as 2 × 10^5 cells/well in 96-well tissue culture plates (Nunc). Cells were incubated with different stimuli for 5.5 and 24 h in replicates of five. Culture supernatants were then collected, pooled, and stored frozen (−80 °C) until assayed for cytokine production. After removal of the supernatant, cells were lysed by adding PBS containing Tween 20 (0.01%) and BSA (1%) in the same volume as that of the supernatant and sonicating for 5 min. The cell lysates were pooled and stored frozen (−80 °C) until assayed for cytokine production.

All cytokine ELISAs were performed in 96-well MaxiSorp plates (Nunc). Cytokine DuoSet ELISA Development Kits (R&D Systems) were used for the cytokine quantification of mouse TNF-α, IL-6, IP-10, RANTES, and IL-1β according to the manufacturer's instructions. The absorbance was measured at 450 nm with wavelength correction set to 540 nm using a microplate reader (BMG Labtech). Concentrations of IFN-β in culture supernatants were determined as follows. ELISA MaxiSorp plates were coated with rabbit polyclonal antibody against mouse IFN-β (PBL Biomedical Laboratories). IFN-β in standards and samples was allowed to bind to the immobilized antibody. Rat anti-mouse IFN-β antibody (USBiological) was then added, producing an antibody−antigen−antibody "sandwich". Next, horseradish peroxidase (HRP) conjugated goat anti-rat IgG (H+L) antibody (Pierce) and a chromogenic substrate for HRP 3,3′,5,5′-tetramethylbenzidine (TMB; Pierce) were added. After the reaction was stopped, the absorbance was measured at 450 nm with wavelength correction set to 540 nm. All cytokine values are presented as the mean ± SD of triplicate measurements, with each experiment being repeated three times.

Transfection and NF-κB Activation Assay. The day before transfection, HEK 293T wild-type cells and HEK 293T cells stably transfected with murine TLR4/MD2/CD14 were plated in 96-well tissue culture plates (16 000 cells/well). The next day, cells were transiently transfected using PolyFect Transfection Reagent (Qiagen) with expression plasmids pELAM-Luc (NF-κB-dependent firefly luciferase reporter plasmid, 50 ng/well)[43] and pRL-TK (*Renilla* luciferase control reporter vector, 1 ng/well; Promega) as an internal control to normalize experimental variations. The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng/well). Forty-four hours post-transfection, cells were exposed to the stimuli at the indicated concentrations for 4 h, after which cell extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and the Fluoroskan Accent FL combination luminometer/fluorometer (Thermo Electron Corp.). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data are reported as the means ± SD of triplicate treatments. The transfection experiments were repeated at least twice.

(43) Chow, J. C.; Young, D. W.; Golenbock, D. T.; Christ, W. J.; Gusovsky, F. *J. Biol. Chem.* **1999**, *274*, 10689−10692.

Data Analysis. Concentration−response data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). These data were fit with the following four-parameter logistic equation: $Y = E_{max}/(1 + (EC_{50}/X)^{Hill\ slope})$, where Y is the cytokine response, X is logarithm of the concentration of the stimulus, E_{max} is the maximum response, and EC_{50} is the concentration of the stimulus producing 50% stimulation. The Hill slope was set at 1 to be able to compare the EC_{50} values of the different inducers.

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Supporting Information Available: Copies of NMR spectra of synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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