CHEMICAL SYNTHESIS OF LIPID A DERIVATIVES TO STUDY THEIR IMMUNOLOGICAL ACTIVITIES

by

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(Under the Direction of Geert-Jan Boons)

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. The structures of lipid As vary considerably among bacterial species, which likely accounts for the highly variable *in-vivo* and *in-vitro* host responses to LPS.

To develop lipid A as immuno-modulators requires a detailed knowledge of patterns of cytokines induced by a wide range of derivatives. To achieve this end, we synthesized several lipid A derivatives by a convergent approach. The synthetic compounds and *E. coli* 055:B5 LPS were examined for a variety of cytokines. Significant differences in potency and efficacy were observed in the induction of cytokines by the different lipid As, which can be exploited for the development of immune-modulating therapies.

All of the above synthetic lipid A derivatives have lower efficacies than *E. coli* 055:B5 LPS for all the tested cytokines. To exploit the specific structural features responsible for this difference, we synthesized lipid A from *Neisseria meningitidis* and its derivative containing 3-Deoxy-D-manno-octulosonic acid (Kdo). Examination of the biological results showed that the

lipid A derivative containing Kdo was slightly less active than its parent LPS, which indicates that one Kdo moiety is sufficient for the restoration of biological activity.

The lipid A from *R. sin*-1 can antagonize TNF-α production by human monocytes induced by *E. coli* LPS. To establish the relevance of its unusual long chain 27-hydroxyoctacosanoic acid for antagonistic properties, a highly convergent strategy for the synthesis of several derivatives of the lipid A of *Rhizobium sin*-1 has been developed. Cellular activation studies with a human monocytic cell line have shown that the octacosanoic acid is important, but not necessary for antagonistic properties.

There is some indication that *P. gingivalis* LPS initiates innate immune responses through TLR2. To identify specific structural features that are responsible for this unusual activation pathway, we have synthesized two lipid As of *P. gingivalis* LPS by a highly convergent approach, which provides easy access to a wide range of lipid As.

INDEX WORDS: Carbohydrate, Glycolipid, LPS, Lipid A, Immune modulators, Cytokines, Toll-like receptor, Kdo, Sepsis, Antagonists.

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ABBREVIATIONS

Å.....Angstrom

Ac.....Acetyl

All ..... Allyl

Alloc.....Allyloxycarbonate

ASC..... Apoptosis Associated Speck-like Protein

BINAP.....2,2'-bis(diphenylphosphino)-1,1'-binaphthyl

Bn..... Benzyl

Bu.....Butyl

CD14..... Cluster Differentiation 14

COSY.....Correlation Spectroscopy

mCPBA.....m-Chloroperoxybenzoic acid

DAST ..... (Diethylamino)sulfur trifluoride

DBU .....1, 8-Diazabicyclo[5.4.0]undec-7-ene

DCC ..... *N*, *N*′-Dicyclohexylcarbodiimide

DCM .....Dichloromethane

DCHA ..... Dicyclohexylamine

DDQ.....2, 3-Dichloro-5, 6-dicyano-p-benzoquinone

DIPEA.....Diisopropylethylamine

DMAP..... 4-(Dimethylamino)pyridine

DMF..... *N*, *N*-Dimethylformamide

DMSO ..... Dimethyl sulfoxide

ELISA ..... Enzyme-Linked ImmunoSorbent Assay

Et ..... Ethyl

FBS ..... Fetal Bovine Serum

Fmoc 9-Fluorenylmethoxycarbonyl

HEK ..... Human Embryonic Kidney

Hep ..... L-glycero-D-manno-heptose

hfmc ..... Tris[3-(heptafluoropropylhydroxymethymethylene)-(+)-camphorate]

HMPA ..... Hexamethylphosphoramide

HRP ..... Horseradish Peroxidase

HSQC ..... Heteronuclear Single Quantum Correlation

IFN-β ..... Interferon β

IL ..... Interleukin

KDO ..... 3-Deoxy-D-manno-octulosonic acid

Lev ..... Levulinoyl

LOS ..... Lipooligosaccharide

LPS ..... Lipopolysaccharide

MAP ..... Mitogen-activated Protein

MCP-1 ..... Monocyte Chemoattractant Protein-1

Me ..... Methyl

MIP-3α ..... Macrophage Inflammatory Protein 3α

NBS ..... *N*-Bromosuccinimide

NF κb ..... Nuclear Factor κb

NIS ..... *N*-Iodosuccinimide

NMR ..... Nuclear Magnetic Resonance

PCC ..... Pyridinium Chlorochromate

Ph ..... Phenyl

Phth ..... Phthalimido

PMB ..... p-Methoxybenzyl

psi ..... Pounds per Square Inch

Py ..... Pyridine

RANTES ..... Regulated upon Activation, Normal T-cell Expressed, and Secreted

SAR ..... Structure-activity Relationship

TACE ..... Tumor Necrosis Factor-α Converting Enzyme

TBS ..... *tert*-Butyldimethylsilyl

TDS ..... Dimethylthexylsilyl

TEA ..... Triethyl amine

Tf ..... Trifluoromethanesulfonyl

TFA ..... Trifluoroacetic acid

THF ..... Tetrohydrofuran

TLC ..... Thin Layer Chromatography

TLR ..... Toll Like Receptor

TMB ..... 3,3',5,5'-Tetramethylbenzidine

TMEDA ..... *N*,*N*,*N'*,*N'*-tetramethylethylenediamine

TMS ..... Trimethylsilyl

TNF-α ..... Tumor Necrosis Factor α

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Gram-negative sepsis is associated with 100,000 human deaths annually in the United States.[1] Currently, no effective treatment exists for this life-threatening syndrome other than supportive therapy in an intensive care setting.[2,3] The development of sepsis is often linked to a systemic inflammatory resonses to endotoxin (lipopolysaccharide; LPS) in the blood of affected patients.[4-6] As an integral component the of outer membrane of Gram-negative bacteria (**Figure 1.1**), LPS are essential for bacterial growth and survival, and potentially endotoxemia critical factors in pathogenesis.[7-10]

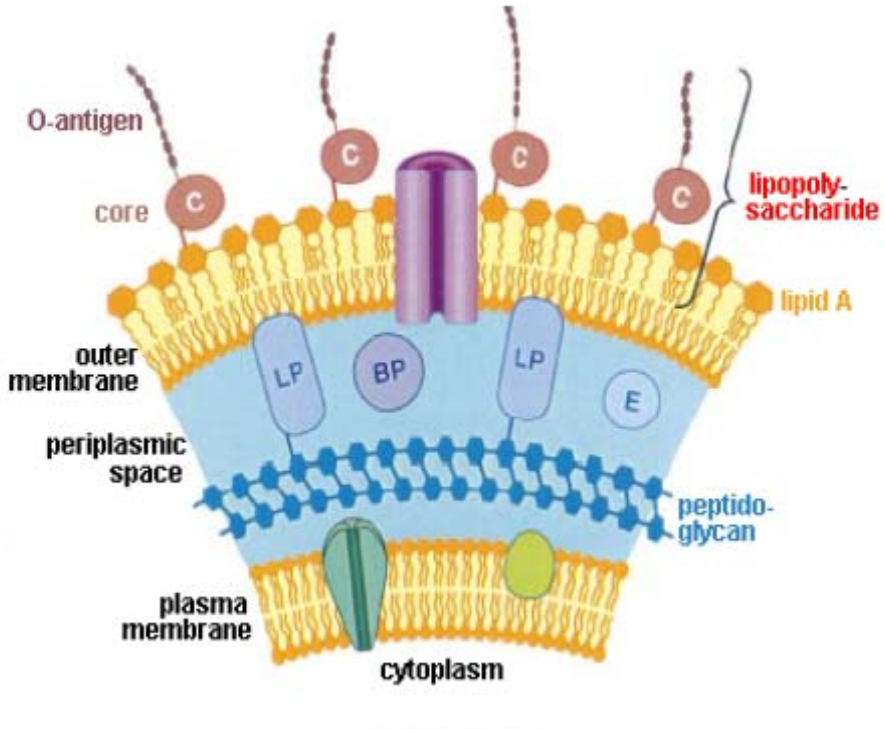


Figure 1.1 Cell envelope of a Gram-negative bacterium.[11]

General Structure of LPS

Lipopolysacchrides are complex amphiphilic compounds, which vary widely in chemical composition both between and among bacterial species. Their basic structure can be classified into three separate regions: lipid A, core oligosaccharide and *O*-polysaccharide (**Figure 1.2**).

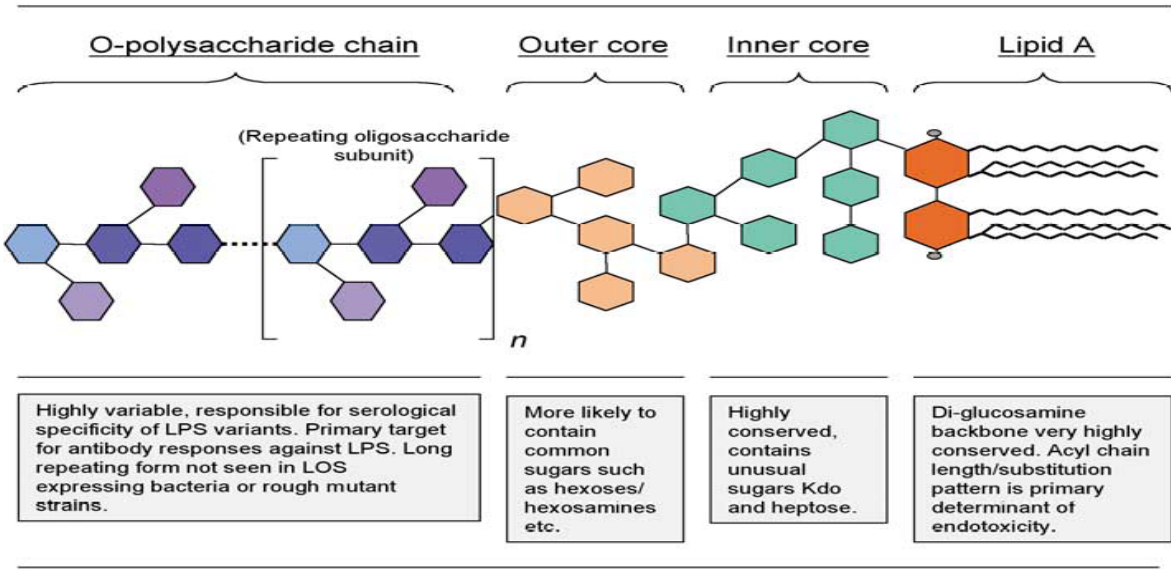


Figure 1.2 General structure of Lipopolysaccharide.[12]

Lipid A is the hydrophobic part of LPS and anchored in the outer membrane of Gram-negative bacterium cell wall. Lipid A is typically composed of a ß-D-GlcN-(1-6)-D-GlcN disacchride carrying two phosphoryl groups at positions 1 and 4' (**Figure 1.3**). Both phosphates can be further substituted with groups such as ethanolamine, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabino-pyranose and D-arabino-furanoese. A maximum of four acyl chains are attached to the disaccharide backbone by ester or amide linkages. These acyl chains can be further substituted by fatty acids to give lipid A with up to seven fatty acid chains, each species varying quite considerably in nature, number, length, order, distribution, and saturation.[12] Lipid A is the toxic principle of LPS due to its lethal toxicity, pyrogenicity, and cytokine-activating properties that are similar to native LPS.[13,14]

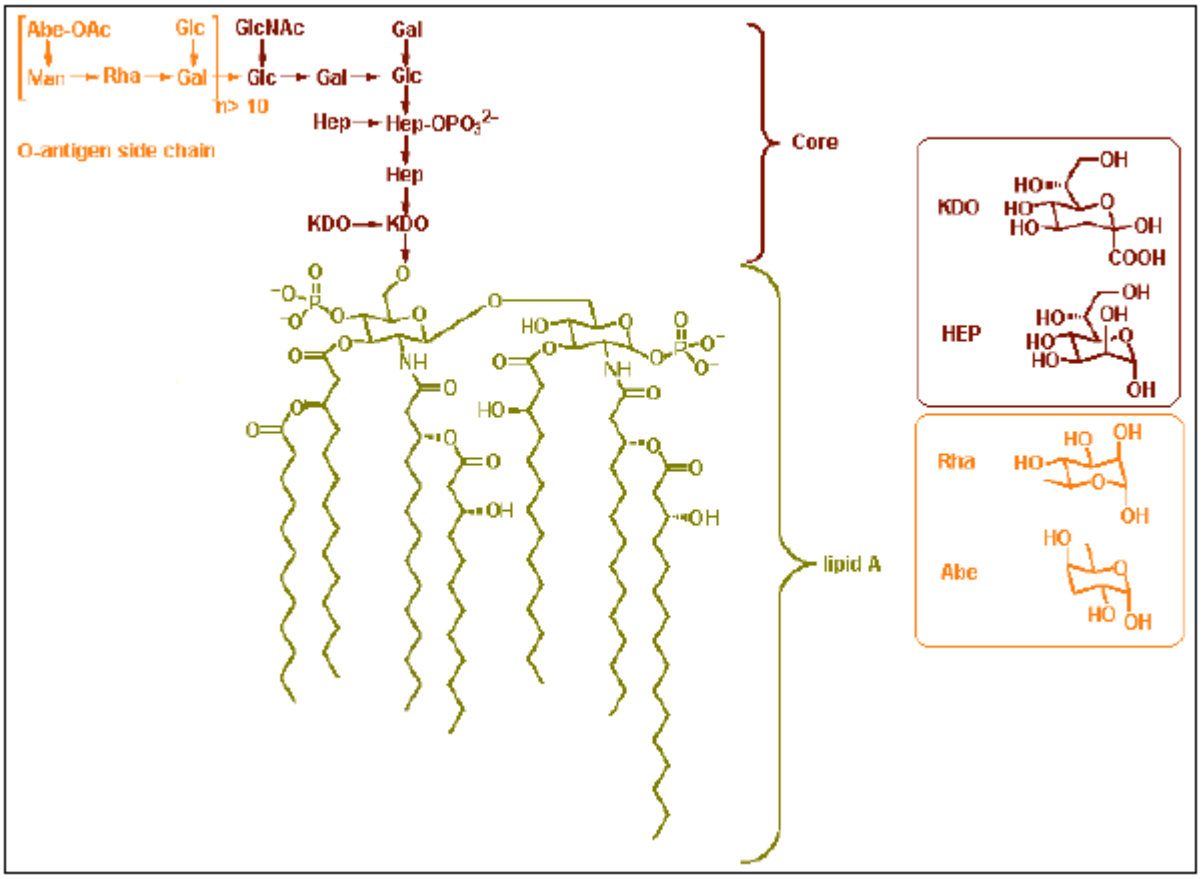


Figure 1.3 Lipopolysaccharide of *Salmonella typhimurium* (GlcNAc- N-acetylglucosamine, KDO- 3-deoxy-D- manno-octulosonic acid, Hep- L-glycero-D-manno-heptose, Rha- (L-rhamnos) 6-deoxy-L- manno-hexose, Abe- (abequose) 3,6-dideoxy-D-xylo-hexose).[15]

The core oligosaccharide is covalently attached to lipid A and can be formally divided into an inner and outer core. The inner core is proximal to lipid A and the outer core extends from the bacterial surface. The outer core typically consists of common hexose sugars such as glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalatosamine (hence often referred to as the hexose region) and is generally more variable in structure than the inner core. The inner core consists of more unusual sugars, particularly Kdo (3-deoxy-D-manno-octulosonic acid) and heptose (L-glycero-D-manno-heptose). Kdo is found in almost every LPS known to date, and is α-bound to

the carbohydrate backbone of lipid A in each case. While the *O*-polysaccharide and majority of the core can be dispensed in some viable mutants, the Kdo residue is essential for bacterial viability.[12]

The *O*-polysaccharide (O-antigen) is the outermost part of LPS and is therefore the major antigen targeted by host antibody responses. It consists of repeating oligosaccharide subunits made up of 2-8 monosaccharides. The repeating subunits differ by means of the sugars, sequence, chemical linkage, substitution, and ring forms. In addition, the number of subunits varies between 0 and ~50.[12] As a result, the structure of *O*-polysaccharide is very variable.

Initiation of Immune Responses by LPS

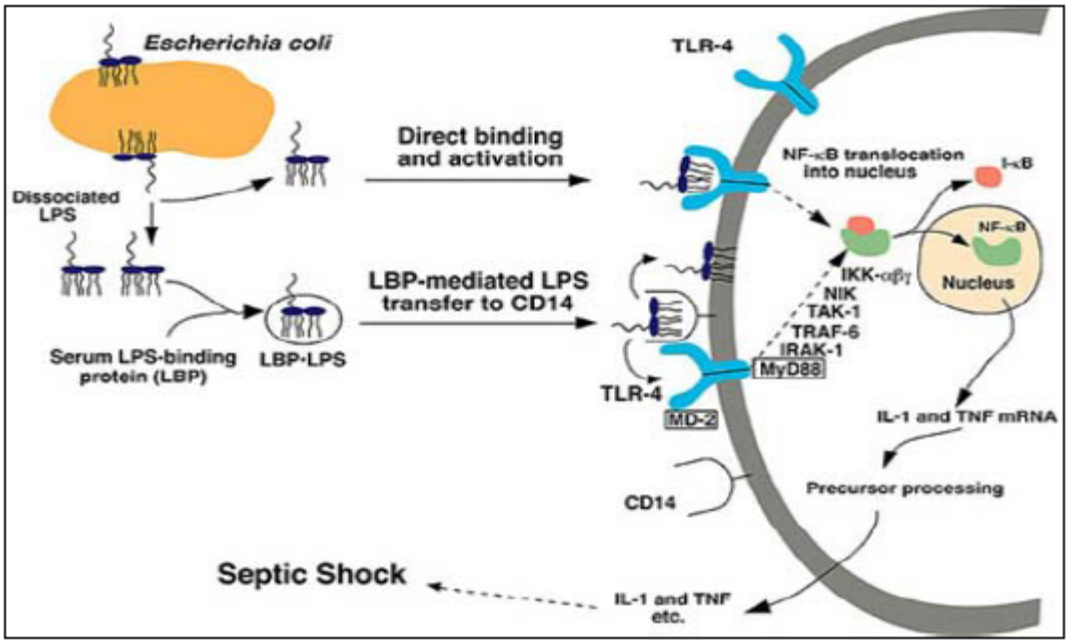


Figure 1.4 Process of septic shock caused by endotoxin.[24]

Many of the initial events in the interaction of LPS with animal and human cells have been elucidated in the past ten years.[16-19] As Figure **1.4** demonstrates, LPS is shed in small amounts throughout the life cycle of the gram-negative cell and released in large quantities upon cell death and lysis. LPS binding protein (LBP) in serum can sequester and mediate the transfer of

monomerized LPS from its aggregated form, or in some cases from intact Gram-negative bacteria, to the cluster differentiation antigen CD14.[20] CD14 is a glycosylphosphatidylinositol-anchored protein found on the surface of immune cells. It lacks transmembrane and cytoplasmic domains, and is therefore unable to transmit LPS binding signals directly to the interior of the cell[21-23]. The actual signal transduction is initiated after CD14 has transferred the LPS to the toll-like receptor 4 (TLR4), which is in turn complexed to an accessory protein MD2.[20] TLR4 is a membrane-spanning protein containing extracellular, transmembrane, and intracellular domains.

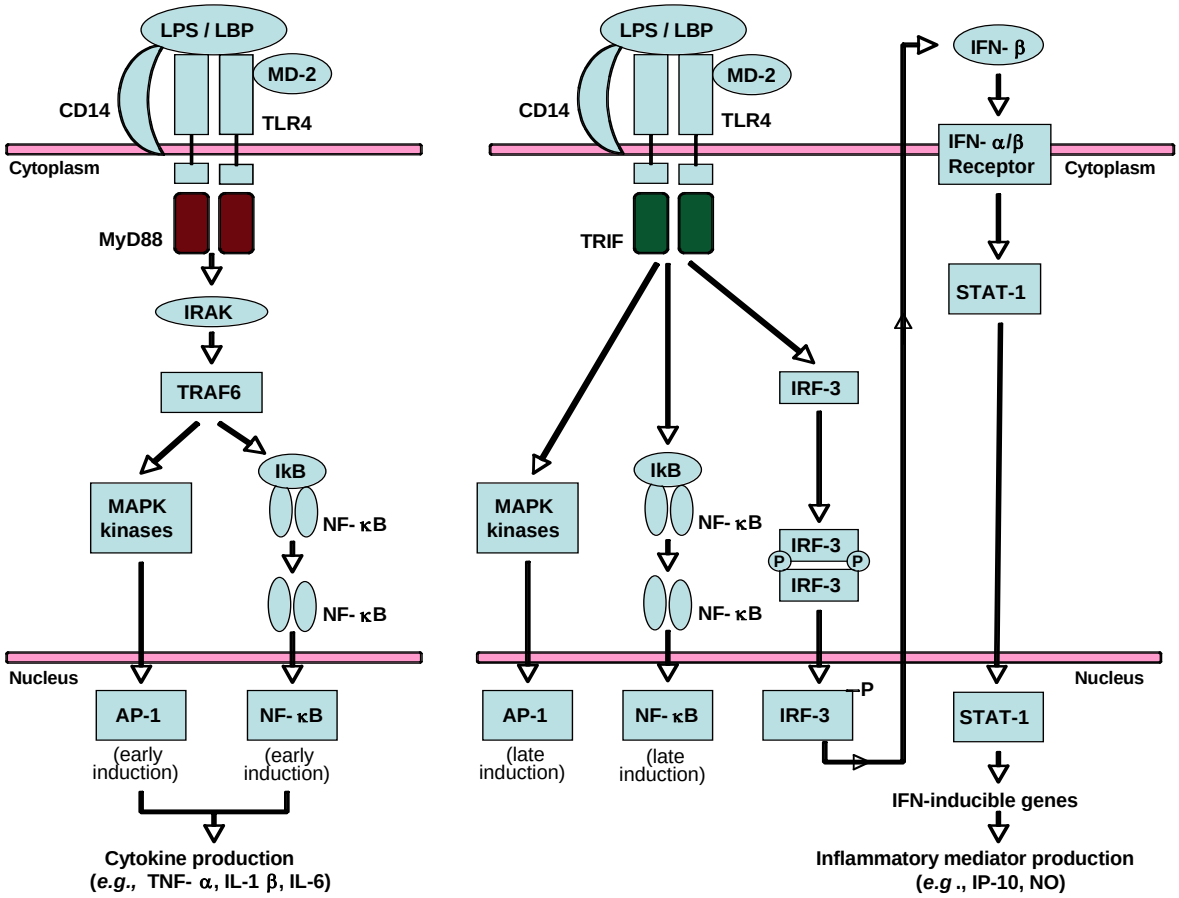


Figure 1.5 Proposed model of the signaling pathways through TLR4.

Immediately distal to TLR4 activation are two intracellular cascades that regulate signal transduction processes, gene expression, and production of (pro)inflammatory mediators (**Figure 1.5**).[25] One of these cascades requires a specific intracellular adaptor protein called Myd88, while the other cascades 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 1 beta (IL-1β), IL-6, and monocyte chemoattractant protein 1 (MCP-1), whereas the TRIF-dependent pathway leads the production of interferon-beta (IFN-β), which in turn activates the SAT-1 pathway, resulting in the production of mediators such as interferon-inducible protein 10 (IP-10) and nitric oxide.[26] The resulting mediators can eradicate the immediate infection. Unfortunately, the presence of a large amount of endotoxin in the blood can cause the overproduction of the mediators which can result in septic inflammatory response syndrome (SIRS) and include life-threatening symptoms such as vascular fluid leak, tissue damage, hypotension, shock, and organ failure.[26]

Lipid A Structure *vs*. Function

As previously mentioned, the lipid A moiety is responsible for endotoxical activity, and therefore it is important to understand the relationship between its structure and biological activity.

To date, a number of synthetic structures have been tested in biological assays and much has been learned about the contribution of individual parts of the compound towards its overall toxicity. Most work has concentrated on the role of the pattern of acyl chains attached to the lipid A and the disaccharide backbone.

It has appeared that the disaccharide backbone is required for a lipid A's activity because a variety of synthetic analogs of GlcN monosaccharide lack activity in general.[12] Much effort has

been made to investigate the influence of the phosphorylation state of the disaccharide backbone on the overall activity, and it has been found that the substitution of phosphates with phosphono-oxyethyl, or carboxyl methyl does not alter the compound's activity. More surprisingly, lipid A analogs with ß-anomeric acidic groups have comparable biological activity to the natural-type α-analogs,[28] thus indicating that although the acid functional groups are essential, their species and spatial arrangement can vary without loss of biological activity.

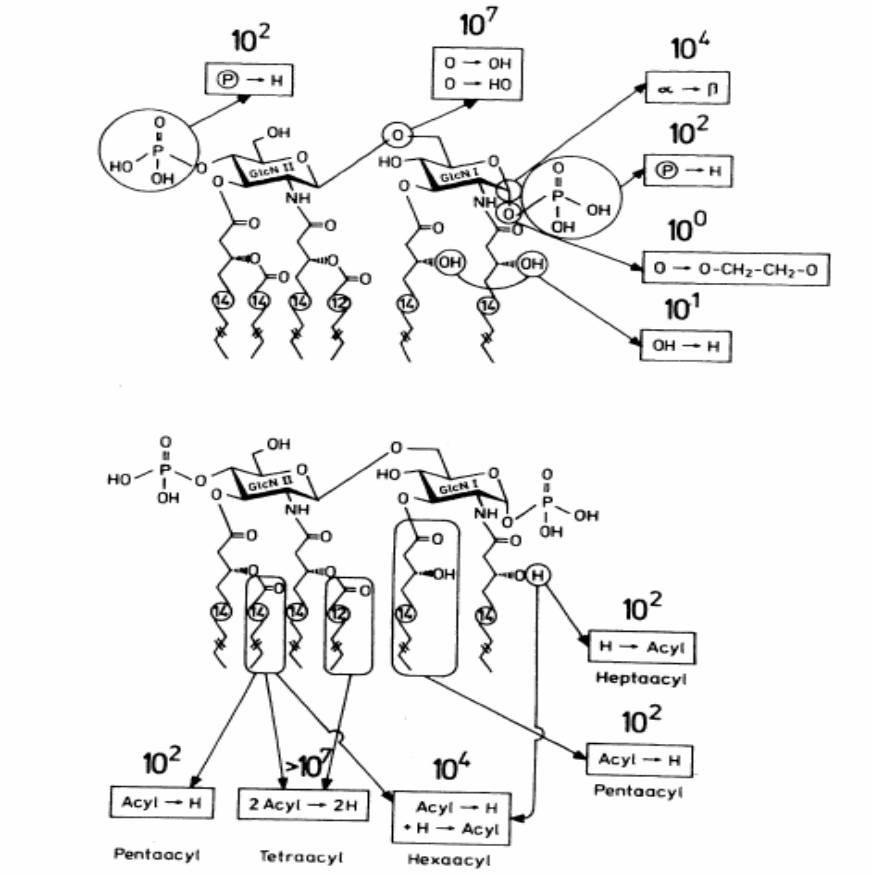


Figure 1.6. Effect of structural modifications on lipid A bioactivity. Figures shown represent factors by which modified structures are reduced in bioactivity when compared to complete *E. coli* lipid A. Top: modifications to hydrophilic section. Bottom: modifications to hydrophobic sections.[27]

While the structure of lipid A's disaccharide backbone is conserved, its acylation pattern is more variable. As such much research has focused on the manner in which the acylation pattern affects the compound's activity. **Figure 1.6** summarises the effects of structural modifications on the bioactivity of *E. coli* lipid A. As shown, the modification of the acyl chain in nature, number, length, order and saturation can all lead to a change in lipid A' s endotoxical activity.

Strategies for Drug Discovery for the Treatment of Sepsis

Based on the mechanism for the induction of sepsis by LPS, a variety of approaches have been examined for the treatment of sepsis. These approaches have focused on different points in the cascade of events that lead to severe sepsis. Although the majority of the approaches targeting the later stages of the endotoxin response have demonstrated efficacy in both *in vitro* and animal models, to date, none have proven to be effective in the treatment of human sepsis. Therefore, a more promising strategy for the treatment of sepsis may be to antagonize the interaction of LPS with its cell-surface receptor. A number of approaches based on this strategy have been studied by using various antibodies directed against parts of the LPS molecule and the molecule as a whole so that this interaction would enhance LPS clearance or neutralize the ability of LPS to activate cells.[29]

LPS is a large and complex compound, so the use of antibodies directed against the entire LPS (in general the O-antigen region) would tend to be specific to the species from which LPS was derived, and would limit its usefulness to countering infection by only a narrow range of bacterial stereotypes. In contrast, the lipid A region is more conserved. The antibodies directed against lipid A should be a good candidate for antagonizing the interaction of LPS with its cell-surface receptor and treating sepsis.[29] As is often the case, efficacious pharmacological receptor antagonists are often derived by modifying a compound that has agonist activity. As mentioned

before, lipid A is the endotoxical active part of LPS, so its structural analogs are promising candidates for actively antagonizing the effects of LPS.

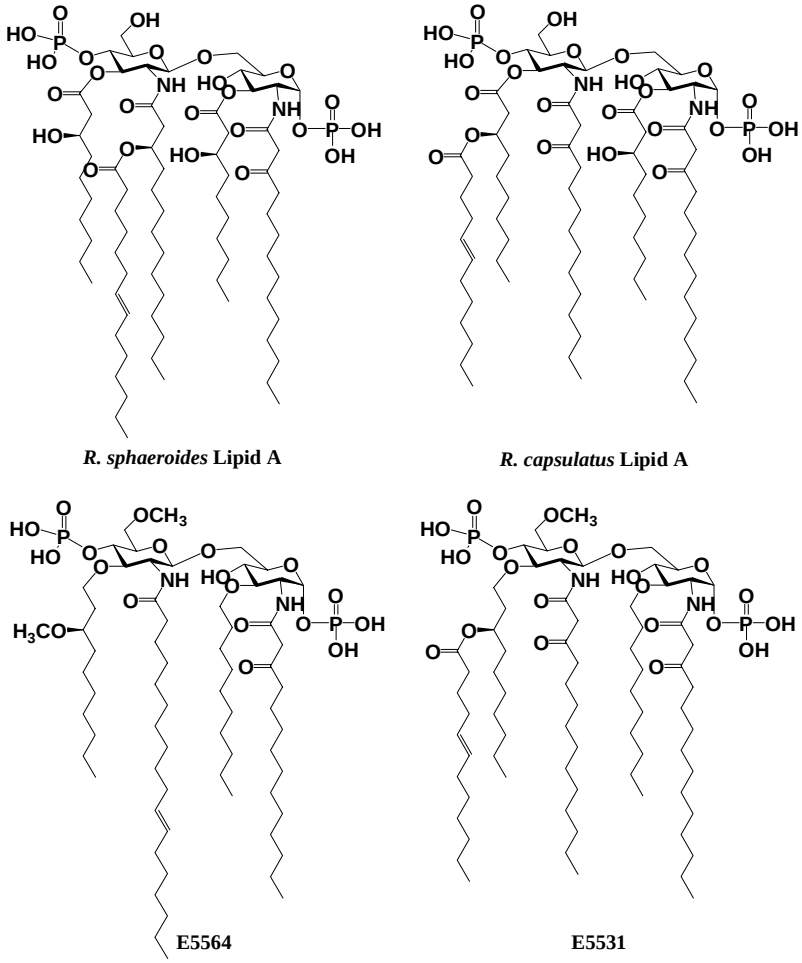


Figure 1.7 Lipid As with antagonistic activity

Certain lipid A analogs have been examined for their antagonistic properties. These analogs include naturally occurring lipid A precursors such as lipid X and lipid IV_A, as well as a number of the synthetic analogs of these precursors. The best-studied derivatives are synthetic analogs derived from the lipid A of *Rhodobacter sphaeroides* or *Rhodobacter capsulatus* (**Figure 1.7**),[30-32] which have very similar lipid A structure. It has been shown that the *R. sphaeroides/R. capsulatus* lipid A lacks toxic effects and is an antagonist of enteric endotoxin.[30,33,34] Unfortunately both natural *R. sphaeroides/R. capsulatus* lipid-A and their synthetic analogs

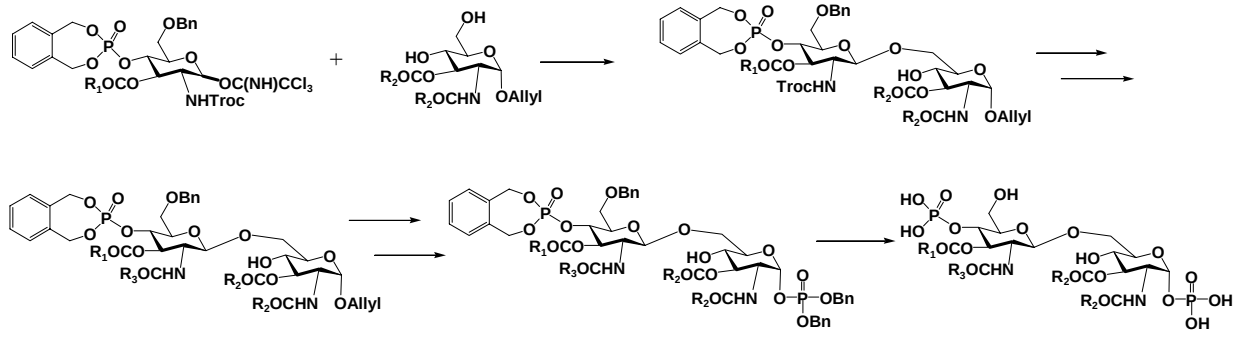
having ester-linked fatty acids to the glucosamine disaccharide backbone can undergo degradation to 2,2'-di-ß-hydroxymyristyl-1,4'-bisphosphorylated glucosamine disaccharide that has agonistic properties.[35] To overcome this problem, Christ and coworkers synthesized the analogs (E5531[32] and E5564) (**Figure 1.7**) in which the fatty acid ester linkages to the disaccharide backbone were replaced by fatty alcohol ether linkages. It was discovered that E5531[35-37] and E5564[38] prevent the pyrogenic effects of enteric LPS in rabbits, protect against LPS-induced lethality in mice, and blocks the Toll-like receptor 4-mediated NF-*k*B activation by LPS. However, the synthetic strategy is complex, specifically designed to produce this single compound, and not amenable to the production of numerous lipid-A analogs that can be examined for beneficial activities.

Synthesis of Lipid A Derivatives

Minor modification of the structure of lipid A can incite major change in its endotoxic activity. Natural lipid A preparations are often heterogeneous and contain contaminants from bacterial cells such as lipoproteins and polysaccharides, which limits the identification of the specific structural features responsible for the endotoxicity of LPS. Fortunately, chemical synthesis is a good tool for the provision of pure lipid A analogues with well-defined structures. Chemical synthesis of lipid As not only contributes to the determination of their chemical structure, but also provides enough pure material for structure-activity relationship studies. Moreover, it provides access to a wide range of unnatural lipid A analogues, aiding in the search for some structures with enhanced beneficial activities and stability, which may find application for the clinical treatment of diseases.

The synthesis of lipid A is quite challenging due to the presence of the labile anomeric phosphate and its amphiphilic structure. The first synthesis of *Escherichia coli* lipid A was

completed by Kusumoto and co-workers in 1985.[39] Since then, a variety of lipid As have been synthesized, and a number of synthetic approaches developed. With the development of various scientific fields, particularly carbohydrate chemistry, approaches are becoming more and more efficient and reliable. Based on these syntheses and the molecular structure of lipid A, several valuable strategies can be summarized, which should be kept in mind when designing a scheme for the synthesis of lipid As. The strategies are: (i) all of persistent protecting groups should be able be removed in mild conditions, normally by hydrogenation, without affecting the labile anomeric phosphate; (ii) the *N*-3-acyloxyacyl group on 2-amino sugar moieties was found to readily undergo β-elimination to form an α, β-unsaturated acyl derivative when the glycosidic position is activated for glycosylation, so the *N*-acyl group of the distal glucosamine residue should be introduced after formation of the disaccharide; and (iii) the anomeric phosphate should be introduced at the final synthetic stage just before global deprotection.[40]



R_1, R_2, R_3: faccty acid

Figure 1.8 A typical synthesis of lipid A analogues

The vast majority of current approaches for lipid A synthesis employ strategies whereby monosaccharides are functionalized with lipids and 4'-phosphates, which are then used as glycosyl donors and acceptors for disaccharide synthesis. A typical example was shown in **Figure 1.8**. These approaches are attractive for one-compound-at-a-time syntheses, but detailed

structure-activity relationship studies require a synthetic approach that offers in a straightforward manner a panel of lipid As. Although a convergent approach has been developed,[41] the employed protecting groups are not fully orthogonal, so not all hydroxyl and amino groups were differentiated. Thus, the need is great for the development of a highly convergent approach which allows easy access to a wide range of lipid As.

To identify the specific structural features responsible for the endotoxic activity of lipid A and to improve its beneficial activities, a wide range of unnatural lipid A analogues were synthesized. For example, to investigate the influence of the anomeric phosphate on overall activity, lipid A analogues with a phosphate or carboxylate group which is linked to the anomeric position through a spacer were synthesized.[42-44] Lipid A analogues with ß-anomeric acidic groups were also synthesized.[45] To study the action mechanism of lipid A, the synthesis of several lipid A analogues labeled with fluorescence, biotin, and isotope was completed.[46,47] Other unnatural lipid A analogues studied include those containing glucose instead of glucosamine[48-50], an ether chain instead of an ester chain,[51] and (*S*)-3-hydroxy fatty acids instead of (*R*)-configuration.[52]

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CHAPTER 2

MODULATION OF INNATE IMMUNE RESPONSES WITH SYNTHETIC LIPID A DERIVATIVES[#]

[#] Yanghui Zhang, Jidnyasa Gaekwad, Margreet A. Wolfert, and Geert-Jan Boons*. 2007. *Journal of the American Chemical Society.* 29:5200-5216.

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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 As by employing a common disaccharide building block functionalized with a versatile set of protecting groups. The strategy was employed for the preparation of lipid As 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 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 towards 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 As **1**-**4** gave lower efficacies for the production of RANTES and IL-6 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.

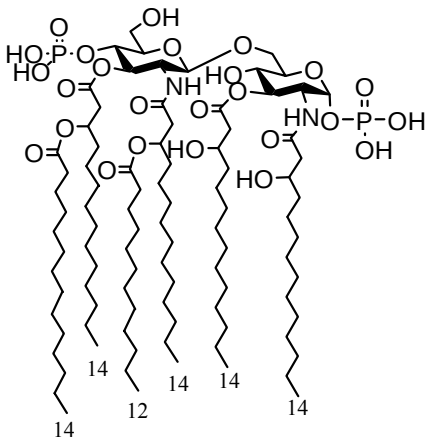
Lipopolysaccharides (LPS) are structural components of the outer membrane of Gram-negative bacteria and offer great promise for the development of immuno-modulators. LPS consists of a hydrophobic domain known as lipid A, a non-repeating core oligosaccharide and a distal polysaccharide (or O-antigen).[11,12] 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 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]

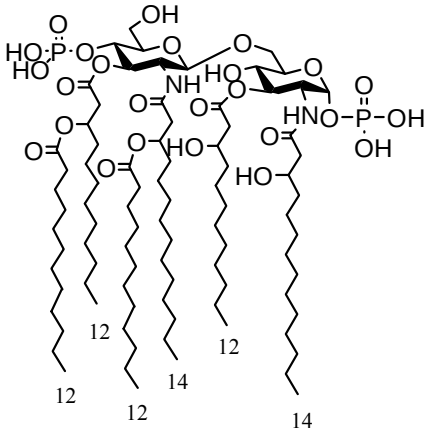
Lipid A triggers innate immune responses through Toll-like receptor 4 (TLR4), a member of the TLR family that participates in pathogen recognition. Immediately distal to TLR4 activation are *two intracellular cascades* that regulate signal transduction processes, gene expression, and production of (pro)inflammatory 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 As 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

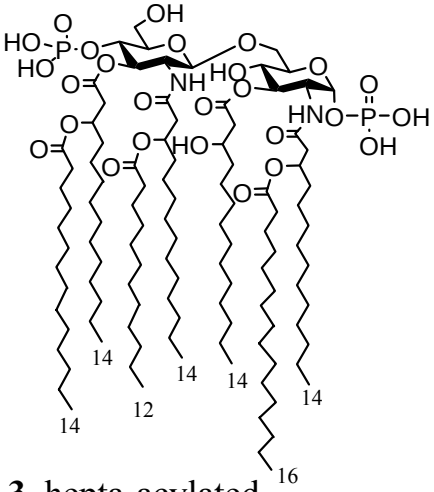
associated with the TRIF-dependent pathway, but caused only minimal production of TNF-α, IL-1β, MCP-1, and MIP-3α. Heterogeneity in the structure of lipid A within a particular bacterial strain and possible contamination with other inflammatory components of the bacterial cell-wall complicate the use of either LPS or lipid A isolated from bacteria to dissect the molecular mechanisms responsible for the biological responses to specific lipid A molecules.



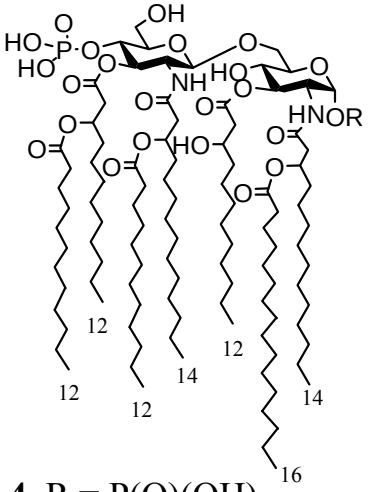
1. hexa-acylated *E. coli* lipid A



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

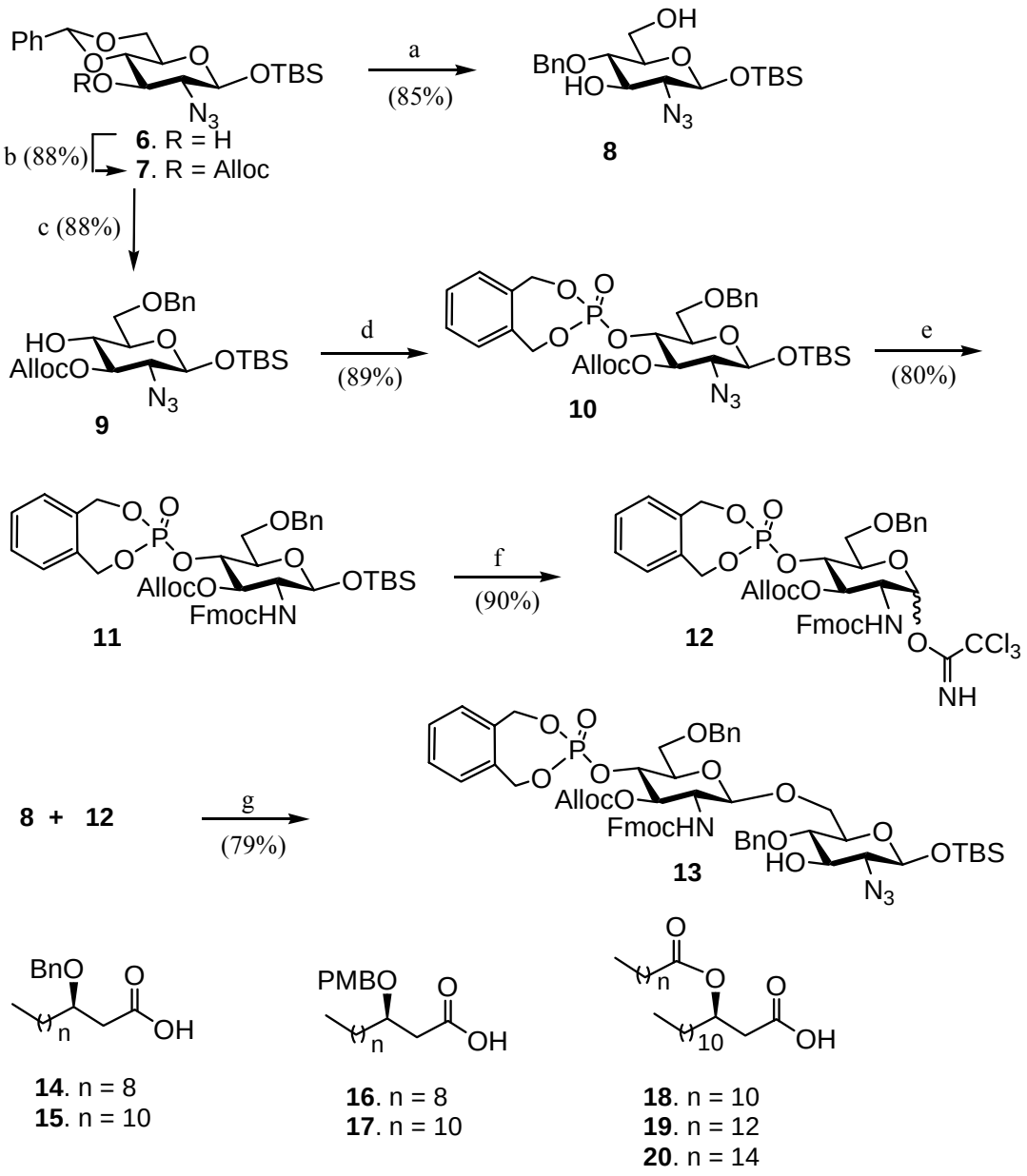
5. R = H

Figure 2.1 Chemical structures of target lipid As **1-5**

Fortunately, homogeneous lipid A derivatives can be obtained by chemical synthesis.[17] The results of studies with small numbers of synthetic analogs 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. In order to address this important issue, we have developed an efficient synthetic approach whereby an advanced synthetic disaccharide can easily be converted into lipid A analogs that differ in phosphorylation and acylation pattern. This strategy has been employed for the preparation of lipid As 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 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 towards 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 As. To determine whether the structure of lipid A can modulate innate immunological responses, we have synthesized derivatives **1-5** (**Figure 2.1**) 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 of its acyl groups have been shortened. Compounds **3, 4**, and **5** are hepta-acylated lipid As derived from *S. typhimurium* LPS that differ in lipid length and phosphorylation pattern.[23-26]



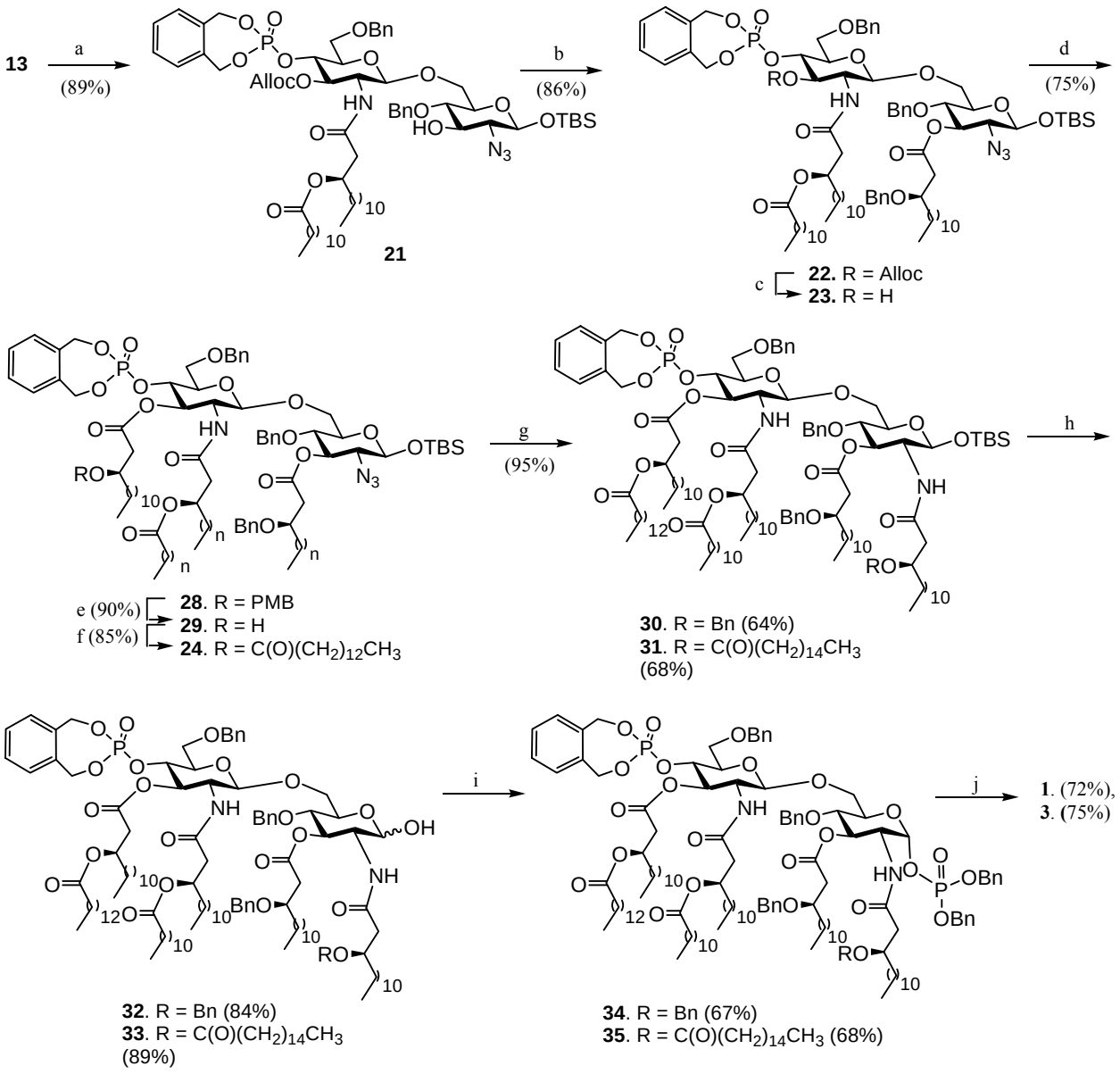
Scheme 2.1 Reagents and conditions: (a) BH_3.THF, Bu_2OTf, THF; (b) AllocCl, TMEDA, DCM; (c) $NaCNBH_3$, 2M HCl in diethyl ether, DCM; (d) *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine, tetrazole, DCM; then mCPBA, -20 ºC; (e) Zn/HOAc, DCM; then FmocCl, DIPEA, DCM; (f) HF/pyridine, THF; then $CNCCl_3$, NaH, THF; (g) TMSOTf, DCM, -50 ºC.

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 As. 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 *t*-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** 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] was 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]

Having the advanced disaccharide **13** and lipids **14-20** at hand, attention focused on the selective acylation of relevant hydroxyls and amines (**Scheme 2.2**). 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%. The latter two reactions exploited the finding that an amine can selectively be acylated in the presence of a free hydroxyl



Scheme 2.2 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 ºC; (j) H_2 (50psi), Pd-black, THF.

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 2.2**). 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

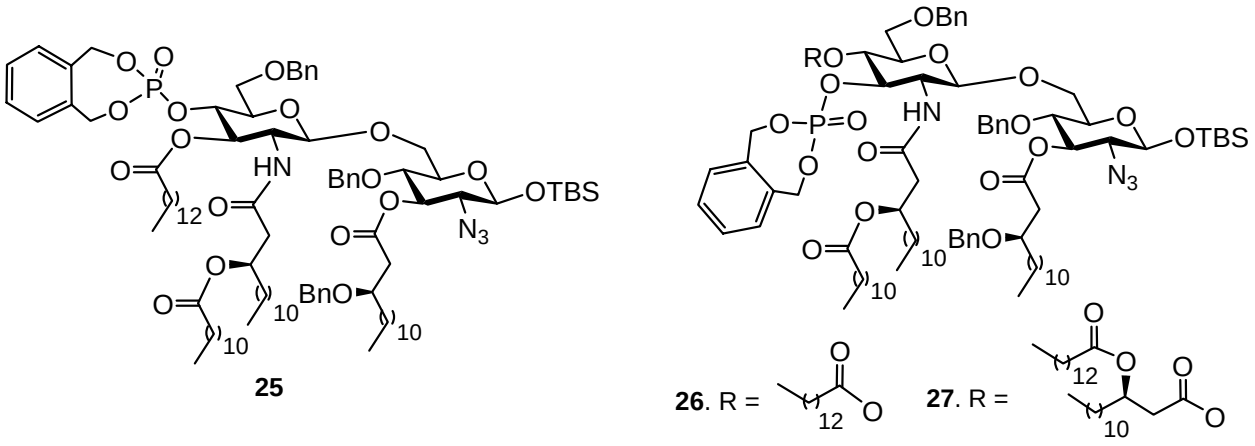
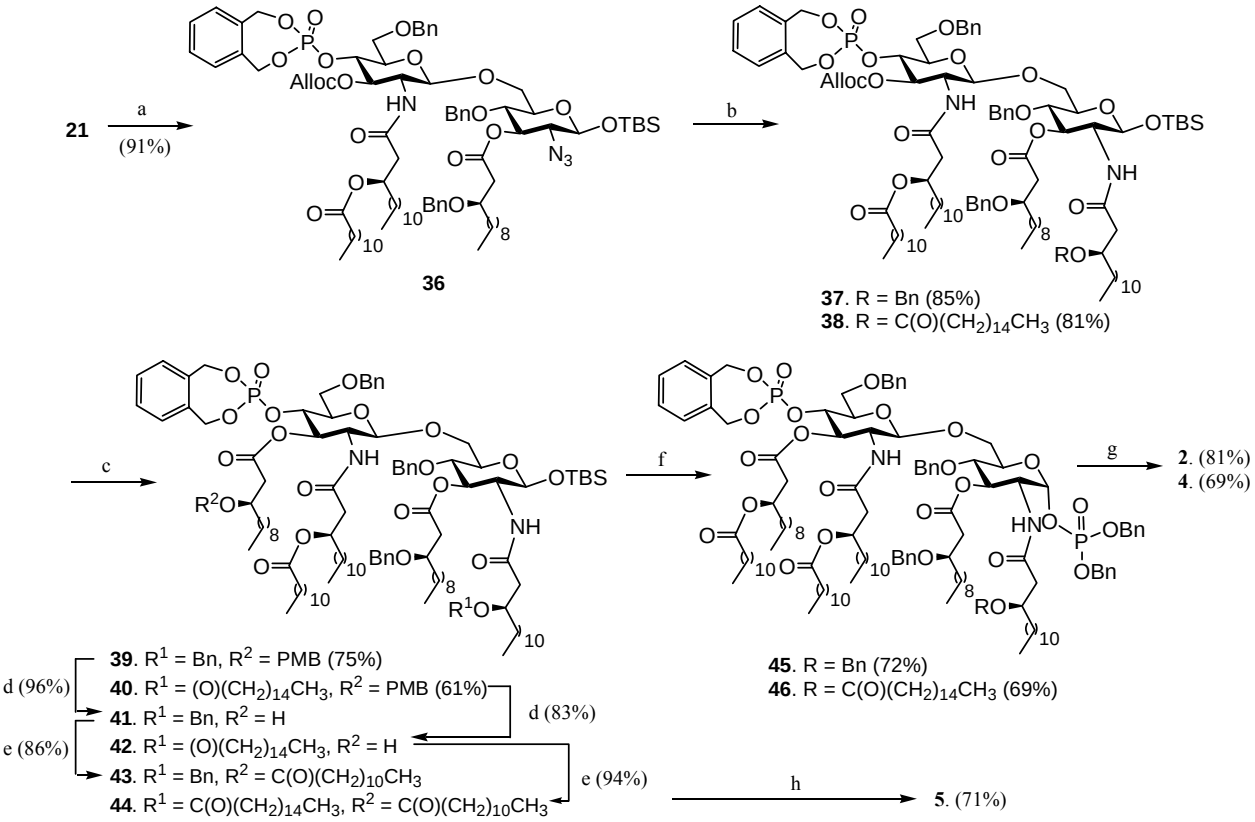


Figure 2.2 Chemical structures of side products 25-27

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, 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



Scheme 2.3 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 ºC; (g) H_2 (50psi), Pd-black, THF; (h) HF/pyridine; then H_2 (50psi), Pd-black, THF.

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. Then, 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 As **1** and **3**, respectively.

Lipid As **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 As 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 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 As 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 As. 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.

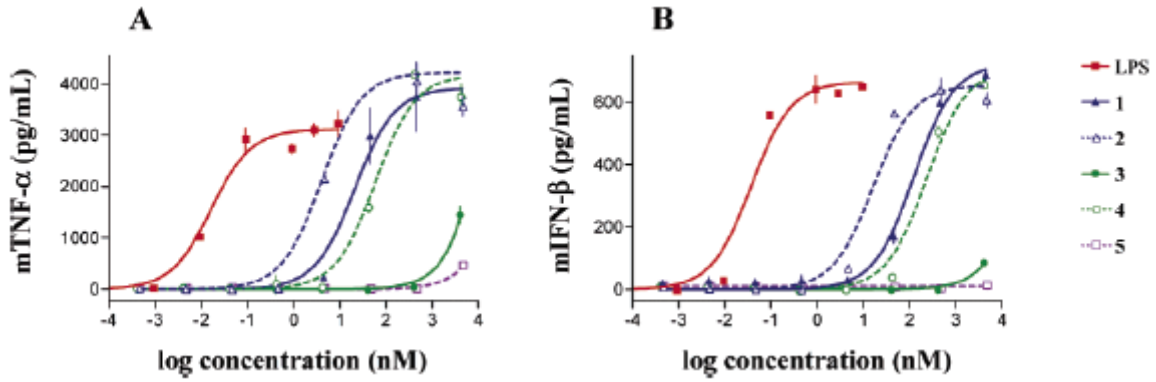


Figure 2.3 TNF-α and IFN-β production by murine macrophages after stimulation with LPS and lipid A derivatives. Murine RAW γNO(-) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS or lipid A derivatives **1-5** as indicated. TNF-α (A) and IFN-β (B) in cell supernatants were measured using ELISAs.

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 hours, 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 As and *E. coli* 055:B5 displayed large differences in potencies. Thus, lipid As **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 that 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 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]

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 As (**1** *vs.* **2**), the differences in EC_{50} values were relatively small, whereas for the *S. typhimurium* lipid As (**3** *vs.* **4**) an approximate three 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 2.4**, **Table 2.1**). For IL-6, IP-10, and RANTES, a short incubation time of 5.5 hrs was sufficient for detection. To achieve significant IL-1β secretion, the incubation had to be

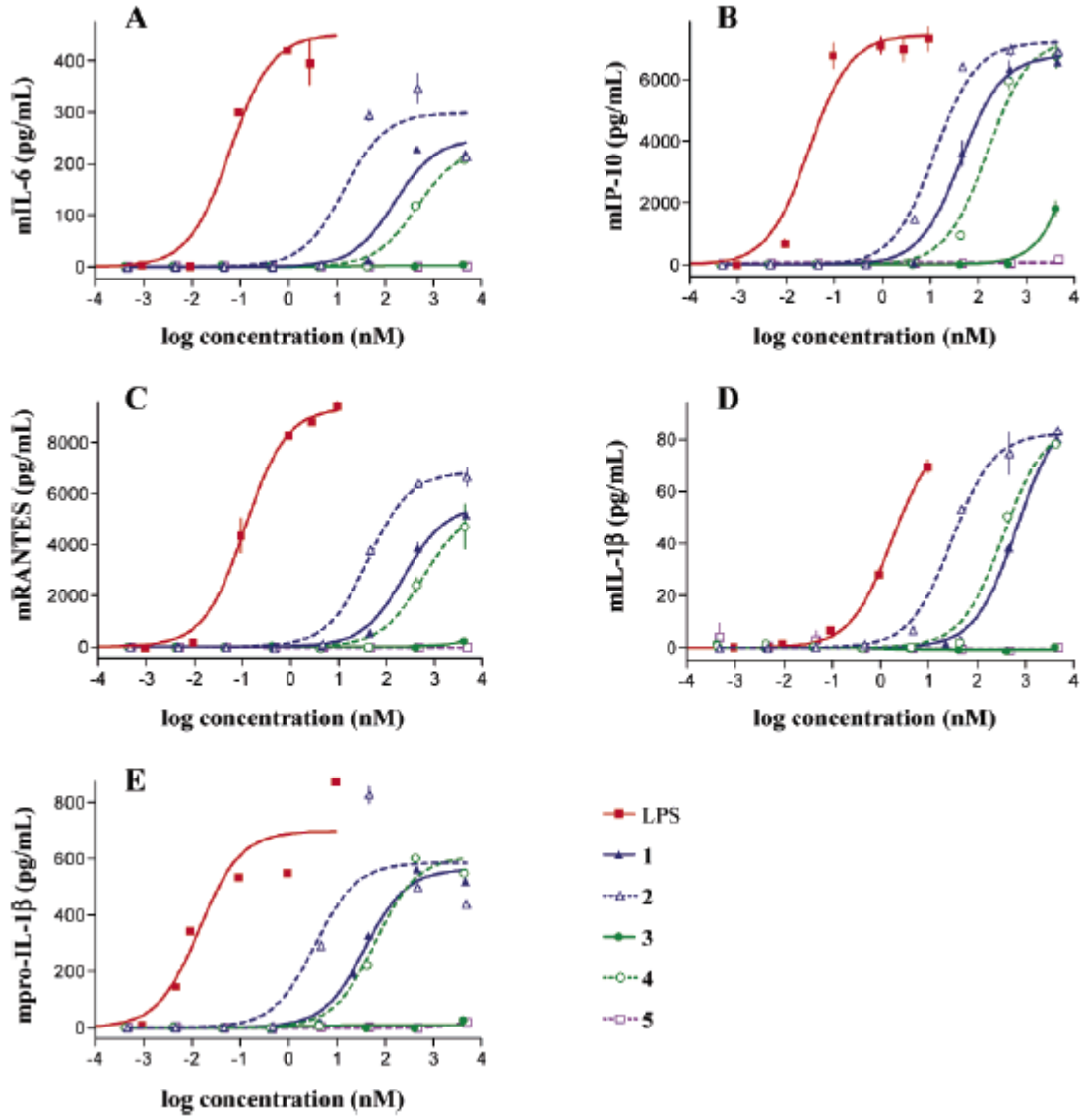


Figure 2.4 Cytokine production by murine macrophages after stimulation with LPS and lipid A derivatives. Murine RAW *γ*NO(-) cells were exposed to increasing concentrations of *E. coli* LPS or lipid A derivatives **1-5** as indicated. Cytokine production was measured in supernatants after 5.5 h incubation for IL-6 (A), IP-10 (B), and RANTES (C) or 24 h for IL-1*β* (D). The cell lysates were assayed for the presence of pro-IL-1*β* (E) after 5.5 h incubation.

extended to 24 hrs. However, analyzing cell lysates of the activated cells showed that after 5.5 hrs 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 hrs, no TNF-α could be detected in the cell lysates, which indicates that proteolitic

Table 2.1 EC_{50} values* (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	21	4.1	60
	(0.012 - 0.022)	(16 – 28)	(2.5 - 6.7)	(44 – 81)
IFN-β	0.038	124	16	234
	(0.025 - 0.056)	(105 – 147)	(12 – 23)	(180 – 306)
IL-6	0.063	157	14	462
	(0.044 - 0.091)	(91 – 271)	(6 – 33)	(383 – 559)
IP-10	0.030	44	12	156
	(0.019 - 0.046)	(37 – 52)	(10 – 16)	(120 – 204)
RANTES	0.116	238	43	570
	(0.103 - 0.131)	(201 – 281)	(36 – 51)	(478 – 681)
IL-1β	1.74[#]	674	30	348
	(1.59 - 1.91)	(622 – 728)	(26 – 35)	(284 – 428)
pro Il-1β	0.014	39	3.6	63
	(0.008 - 0.025)	(32 – 47)	(1.4 - 9.4)	(48 – 84)
NF-κB	0.004	38	14	53
	(0.003 - 0.005)	(30 – 48)	(9 – 20)	(42 – 67)

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

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 are 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.

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 compared with 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 two orders of a 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.

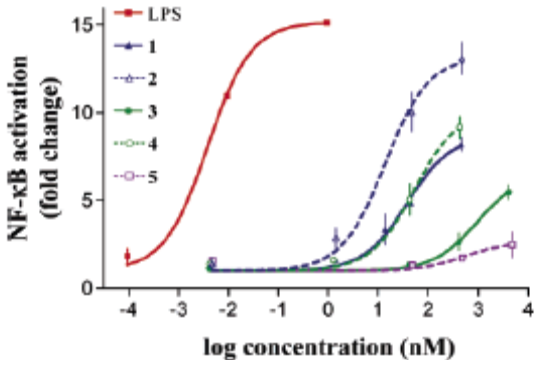


Figure 2.5 Response of HEK 293T cells expressing murine TLR4, MD2, and CD14 to LPS and lipid A derivatives. Induction of NF-κB activation was determined in triplicate cultures of HEK 293T cells stably transfected with murine TLR4, MD2, and CD14 and transiently transfected with pELAM-Luc, pRL-TK, and pcDNA3 plasmids. Forty-four h 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 h 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 $\pm$ 0.3-fold activation of NF-κB.

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.

Table 2.2 Cytokine top values* (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[#] ± 2	92 ± 2	82 ± 2	86 ± 4
pro Il-1β	699 ± 73	565 ± 25	587 ± 87	610 ± 35

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

Examination of the efficacies (maximum responses) of the various cytokines also provided unexpected structure-activity relationships (**Table 2.2**). 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 As **1**-**4** gave lower efficacies for the production of RANTES and IL-6 compared to LPS.

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 As 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 towards 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 As. 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 As 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 As (**1** *vs.* **2**), the differences in EC_{50} values were relatively small, whereas for the *S. typhimurium* lipid As (**3** *vs.* **4**) approximate three orders of magnitude increase in potencies was observed. LPS gave much higher potencies than the synthetic lipid As, 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 towards 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 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 As **1-4** gave lower efficacies for the production of RANTES and IL-6 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 As 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 in 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.

***t*-Butyldimethylsilyl 3-*O*-allyloxycarbonyl-2-azido-4,6-*O*-benzyldidine-2-deoxy-β-D-glucopyranoside** (**7**): To a cooled (0 ℃) 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 x 100 mL) and brine (2 x 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(*C*H_3)$_3$), 17.86 (Si*C*(CH_3)$_3$), -4.46 (Si(*C*H_3)$_2$), -5.23 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{23}H_{33}N_3O_7Si$ $[M+Na]^+$, 514.1985; found, 514.1907.

***t*-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 stirring at 0 ℃ for 5 min, dibutylboron triflate (1 M in DCM, 3.49 mL) was added dropwise, and the reaction mixture was stirred at 0 ℃ 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 x 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^{o} (c = 1.0, $CHCl_3$). ^{1}H NMR (300MHz, $CDCl_3$): 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, $J_{5,6a}$ = 2.4Hz, $J_{6a,6b}$ = 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 (75MHz, $CDCl_3$): 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 (*C*H_2Ph), 68.69 (C-2), 61.97 (C-6), 25.56 (SiC(*C*H_3)$_3$), 17.91 (Si*C*(CH$_3$)$_3$), -4.27 (Si(*C*H_3)$_2$), -5.16 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{19}H_{31}N_3O_5Si[M+Na]^+$, 432.1931; found, 432.1988.

***t*-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 stirring 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 x 100 mL) and brine (2 x 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_2CH=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_2*C*H=CH_2), 118.97 (OCH_2CH=*C*H_2), 96.92 (C-1), 78.79 (C-3), 74.25 (C-5), 73.44 (*C*H_2Ph), 69.89, 69.55, 68.84 (C-4, C-6, O*C*H_2CH=CH_2), 65.84 (C-2), 25.42 (SiC(*C*H_3)$_3$), 17.75 (Si*C*(CH$_3$)$_3$), -4.50 (Si(*C*H_3)$_2$), -5.40 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{23}H_{35}N_3O_7Si[M+Na]^+$, 516.2142; found, 516.2197.

***t*-Butyldimethylsilyl 3-*O*-allyloxycarbonyl-2-azido-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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-3H-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 ℃), 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 ℃ 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 x 60 mL) and brine (2 x 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, $CHCl_3$). ^{1}H 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.

***t*-Butyldimethylsilyl 3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 10 mL). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 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 ℃. 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 x 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^o (c = 1.0, $CDCl_3$); ^{1}H 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^5-3H-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 x 40 mL) and brine (2 x 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 stirring the suspension 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%).

***t*-Butyldimethylsilyl 6-*O*-[3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 ℃) and then TMSOTf (18 μL, 0.09 mmol) was added. After stirring the reaction mixture 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 x 50 mL) and brine (2 x 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, $CHCl_3$). ^{1}H NMR (600 MHz, CD_3COCD_3): δ 7.84-7.20 (m, 22H, aromatic), 6.98 (d, 1H, $J_{NH',2'}$ = 9.0Hz, 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, $J_{2,3}$ = 9,0Hz, 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.

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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

x 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 stirring the reaction mixture 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 x 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^o (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$_2$C*H*=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$_2$CH=CH_2), 5.21 (d, 1H, *J* = 10.2 Hz, OCH$_2$CH=CH_2), 5.09-5.04 (m, 4H, C_6H_4(CH_2O)$_2$P), 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_2 = 12.0 Hz, CH_{2a}Ph), 4.63 (d, 1H, J_2 = 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 x CH_2, lipid), 0.90 (s, 9H, SiC(CH_3)$_3$), 0.85-0.78 (m, 6H, 2 x 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.

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 washed with DCM (2 x 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^o (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 x 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 x CH_2, lipid), 0.90 (s, 9H, SiC(CH_3)$_3$), 0.88-0.84 (m, 9H, 3 x 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(*C*H_3)$_2$), -5.38 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{91}H_{139}N_4O_{19}Psi[M+Na]^+$,1673.9438; found, 1674.1754.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 20 mL), and brine (2 x 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 stirrd 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 x 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 x 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 x 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 x H-2_S, H-$2_{L'}$), 1.62-1.59 (m, 8H, H-4_L, 2 x H-4_S, H-$3_{L'}$), 1.27 (broad, 70H, 35 x CH_2, lipid), 0.93 (s, 9H, $SiC(CH_3)_3$), 0.92-0.87 (m, 12H, 4 x 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.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 20 mL) and brine (2 x 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 x 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 x H-2_L, H-2_S, 2 x H-$2_{L'}$), 1.61-1.51 (m, 10H, 2 x H-4_L, H-4_S, 2 x H-$3_{L'}$), 1.26 (broad, 108H, 54 x CH_2, lipid), 0.95 (s, 9H, $SiC(CH_3)_3$), 0.92-0.90 (m, 15H, 5 x 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.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 3 ml), and the combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 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 stirring the reaction mixture 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 x 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, $J_{NH',2'}$ = 7.0Hz, 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 x 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 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.71-1.45 (m, 12H, 2 x H-4_L, 2 x H-4_S, 2 x H-$3_{L'}$), 1.26 (broad, 108H, 54 x CH_2, lipid), 0.91-0.88 (m, 18H, 6 x 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.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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^o (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 x 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 x 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 x H-2$_L$, H-2$_S$, 3 x H-2$_{L'}$), 1.60 (broad, 14H, 3 x H-4$_L$, H-4$_S$, 3 x H-3$_L$), 1.26 (broad, 132H, 66 x CH_2, lipid), 0.90-0.87 (m, 30H, 7 x 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^5-3H-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 x 25 mL) and brine (2 x 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, NH'), 6.28 (d, 1H, $J_{NH,2}$ = 9.5 Hz, NH), 5.52 (d, 1H, $J_{1',2'}$ = 9.0 Hz, H-1'), 5.51 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5Hz, 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)$_2$P), 4.64-4.43 (m, 9H, H-4', 4 x 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 x 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 x H-2$_L$, 2 x H-2$_S$, 2 x H-2$_{L'}$), 1.58 (broad, 12H, 2 x H-4$_L$, 2 x H-4$_S$, 2 x H-3$_{L'}$), 1.26 (broad, 108H, 54 x CH_2, lipid), 0.91-0.81 (m, 18H, 6 x 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^5-3H-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 (**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 x H-3_L, $C_6H_4(CH_2O)_2P$), 4.65-4.42 (m, 7H, H-4', 3 x CH_2Ph), 4.17-4.08 (m, 2H, H-2, H-5), 3.92 (d, 1H, $J_{6a,6b}$ = 12.0Hz, 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 x H-2_L, H-2_S, 3 x H-$2_{L'}$), 1.59 (broad, 14H, 3 x H-4_L, H-4_S x 2, 3 x H-3_L), 1.26 (broad, 132H, 66 x CH_2, lipid), 0.90-0.88 (m, 21H, 7 x 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^5-3H-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℃) 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 1h, and then allowed to warm up to -20 ℃. After stirring the reaction mixture at -20 ℃ 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 x 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^5-3H-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 as 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), and the catalyst removed by filteration and the residue washed with THF (2 x 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 x 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 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.27 (broad, 12H, 2 x H-4_L, 2 x H-4_S, 2

x H-3_L), 0.94 (broad, 108H, 54 x CH_2, lipid), 0.56-0.54 (m, 18H, 6 x 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 x 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 x H-2_L, H-2_S, 3 x H-$2_{L'}$), 1.23 (broad, 14H, 3 x H-4_L, H-4_S, 3 x H-3_L), 0.99 (broad, 132H, 66 x CH_2, lipid), 0.57-0.55 (m, 21H, 7 x 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].

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 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). $[\alpha]^{26}_{D} = -3.0^{o}$ (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 x 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 x CH_2, lipid), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.89-0.85 (m, 9H, 3 x 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 ($OCH_2CH{=}CH_2$), 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.

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 removed by filtration and the residue washed with ethyl acetate (2 x 2 mL). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 15 mL) and brine (2 x 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 stirring the mixture 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 washed with DCM (2 x 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 x CH_2Ph), 4.01 (d, 1H, $J_{6a,6b}$ = 11.5Hz, 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 x H-2_S, H-$2_{L'}$), 1.61-1.44 (m, 8H, H-4_L, 2 x H-4_S, H-$3_{L'}$), 1.27 (broad, 66H, 33 x CH_2, lipid), 0.91-0.86 (m, 21H, 4 x 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.

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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^o (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, NH'), 5.93-5.87 (m, 1H, OCH$_2$CH=CH$_2$), 5.76 (d, 1H, $J_{NH,2}$ = 9.0 Hz, NH), 5.56 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 9.0 Hz, H-3'), 5.36 (d, 1H, J = 17.4 Hz, OCH$_2$CH=CH_2), 5.23 (d, 1H, J = 10.2 Hz, OCH$_2$CH=CH_2), 5.14-4.99 (m, 8H, H-1', 3, 2 x H-3$_L$, C$_6$H$_4$(CH_2O)$_2$P), 4.70 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.62-4.42 (m, 7H, H-4', 3 x 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 x H-2$_L$, H-2$_S$, 2 x H-2$_{L'}$), 1.59-1.50 (m, 10H, 2 x H-4$_L$, H-4$_S$, 2 x H-3$_{L'}$), 1.23 (broad, 90H, 45 x CH_2, lipid), 0.88-0.84 (m, 24H, 5 x 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$_2$*C*H=CH$_2$), 118.72 (OCH$_2$CH=*C*H$_2$), 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.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 stirring the reaction mixture at room temperature for 20 min, it was diluted with DCM (15 mL), and washed with water (10 mL), saturated aqueous $NaHCO_3$ (2 x 10 mL) and brine (2 x 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 x 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) afforded **39** as a white solid (47 mg, 75%). R_f = 0.29 (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{26}_D$ = -4.5^o (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 x 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 x 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 x H-2_S, H-$2_{L'}$), 1.67-1.41 (m, 10H, H-4_L, 3 x H-4_S, H-$3_{L'}$), 1.24 (broad, 80H, 40 x CH_2, lipid), 0.87 -0.81 (m, 24H, 5 x 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.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 as 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), 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^o (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 x 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 x CH_2Ph), 4.01 (d, 1H, $J_{6a,6b} = 10.5$ Hz, H-6a), 3.88-3.79 (m, 4H, H-2, 6'a, 2 x 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 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.61-1.50 (m, 12H, 2 x H-4_L, 2 x H-4_S, 2 x H-$3_{L'}$), 1.25 (broad, 102H,

51 x CH_2, lipid), 0.88-0.84 (m, 27H, 6 x 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 (*C*H$_3$OPh), -3.83 (Si(*C*H$_3$)$_2$), -5.13 (Si(*C*H$_3$)$_2$). HR MS (m/z) calcd for $C_{135}H_{219}N_2O_{23}PSi$[M+Na], 2318.5433; found, 2318.7700.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 stirring the reaction mixture 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)$_2$P), 4.68-4.63 (m, 1H, H-4'), 4.58-4.44 (m, 9H, H-1, 4 x CH_2Ph), 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 x H-2$_S$, H-2$_{L'}$), 1.69-1.46 (m, 10H, H-4$_L$, 3 x H-4$_S$, H-3$_{L'}$), 1.26 (broad, 80H, 40 x CH_2, lipid), 0.91-0.84 (m, 24H, 5 x CH_3,

lipid, SiC(CH_3)$_3$), 0.09 (s, 3H, SiCH_3), 0.04 (s, 3H, SiCH_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 x 10 mL) and brine (2 x 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^o (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 x CH_2Ph), 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 x H-2$_L$, 2 x H-2$_S$, 2 x H-2$_{L'}$), 1.66-1.52 (m, 12H, 2 x H-4$_L$, 2 x H-4$_S$, 2 x H-3$_{L'}$), 1.27 (broad, 96H, 48 x CH_2, lipid), 0.91-0.88 (m, 18H, 6 x CH_3, lipid), 0.86 (s, 9H, SiC(CH_3)$_3$). 0.09 (s, 3H, SiCH_3), 0.05 (s, 3H, SiCH_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(CH_3)$_2$), -5.09 (Si(CH_3)$_2$). HR MS (m/z) calcd for $C_{130}H_{209}N_2O_{22}PSi[M+Na]^+$, 2232.4702; found, 2232.8787.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-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 x 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 x CH_2Ph), 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 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.74-1.41 (m, 12H, 2 x H-4_L, 2 x H-4_S, 2 x H-$3_{L'}$), 1.24 (broad, 104H, 52 x CH_2, lipid), 0.87-0.84 (m, 27H, 6 x CH_3, lipid, SiC(CH_3)$_3$), 0.08 (s, 3H, SiCH_3), 0.06 (s, 3H, SiCH_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^o (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 x 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 x CH_2Ph), 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 x H-2_L, H-2_S, 3 x H-$2_{L'}$), 1.63-1.61 (m, 14H, 3 x H-4_L, H-4_S, 3 x H-3_L), 1.27 (broad, 120H, 60 x CH_2, lipid), 0.91-0.88 (m, 30H, 7 x CH_3, lipid, SiC(CH_3)$_3$), 0.13 (s, 3H, SiCH_3), 0.10 (s, 3H, SiCH_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^5-3H-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, $J_{2',3'} = J_{3',4'}$ = 9.5Hz, 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)$_2$P), 4.65-4.43 (m, 9H, H-4', 4 x CH_2Ph), 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 x 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 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.59 (broad, 12H, 2 x H-4_L, 2 x H-4_S, 2 x H-$3_{L'}$), 1.26 (broad, 80H, 40 x CH_2, lipid), 0.91-0.88 (m, 18H, 6 x CH_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^5-3H-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 x H-3$_L$, $C_6H_4(CH_2O)_2P$), 4.62-4.40 (m, 7H, H-4', 3 x 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 x H-2$_L$, H-2$_S$, 3 x H-2$_{L'}$), 1.62-1.53 (broad, 14H, 3 x H-4$_L$, H-4$_S$ x 2, 3 x H-3$_L$), 1.3 (broad, 120H, 60 x C*H*$_2$, lipid), 0.87-0.85 (m, 21H, 7 x C*H*$_3$, 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 x 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 $J_{6'a,6'b}$ = 11.4Hz, 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, $J_{3,4} = J_{4,5}$ = 9.0Hz, H-4), 3.02 (broad, 1H, H-5'), 2.29-1.84 (m, 12H, 2 x H-2_L, 2 x H-2_S, 2 x H-$2_{L'}$), 1.18 (broad, 12H, 2 x H-4_L, 2 x H-4_S, 2 x H-3_L), 0.85 (broad, 80H, 40 x C*H*$_2$, lipid), 0.47-0.45 (m, 18H, 6 x C*H*$_3$, 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 x 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 x H-2_L, H-2_S, 3 x H-$2_{L'}$), 1.52 (broad, 14H, 3 x H-4_L, H-4_S, 3 x H-3_L), 1.18 (broad, 120H, 60 x C*H*$_2$, lipid), 0.81-0.78 (m, 21H, 7 x C*H*$_3$, 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 x 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 x H-2_L), 2.11-2.08 (m, 6H, 3 x H-$2_{L'}$), 1.45 (broad, 14H, 3 x H-4_L, H-4_S, 3 x H-3_L), 1.12 (broad, 120H, 60 x CH_2, lipid), 0.76-0.83 (m, 21H, 7 x 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 As were reconstituted in PBS with DMSO (10%) and stored at –80 ^{o}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 ^oC.

Cytokine Induction and ELISAs. RAW 264.7 γNO(-) cells were plated on the day of the exposure assay as 2 x 10^5 cells/well in 96-well tissue culture plates (Nunc). Cells were incubated with different stimuli for 5.5 and 24 hours in replicates of five. Culture supernatants were then collected, pooled, and stored frozen (-80 ^oC) 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 ^oC) 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 means ± 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 h 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 Corporation). 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.

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 Hillslope was set at 1 to be able to compare the EC_{50} values of the different inducers.

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CHAPTER 3

SYNTHESIS AND IMMUNOLOGICAL PROPERTIES OF KDO CONTAINING LIPID A DERIVATIVES FROM *NEISSERIA MENINGITIDIS*[#]

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Abstract

3-Deoxy-D-manno-octulosonic acid (Kdo) is an integral constituent of LPS and a necessary component for Gram-negative bacterial viability. An understanding of the impacts of Kdo on the ability of LPS to initiate cytokine production is essential in order to find suitable lipid A derivatives for use as immuno-modulators for the treatment of a wide range of diseases and as adjuvants for vaccinations. It is difficult to obtain homogeneous lipid A derivatives containing Kdos from a natural source. To address this problem, we have developed a convergent synthetic approach that provides easy access to a number of analogues. This approach was employed for the synthesis of a lipid A derivative containing Kdo from *Neisseria meningitidis*. The synthetic compound was tested for the production of various cytokines.

Examination of the biological results showed that the lipid A derivative containing Kdo was much more active than the lipid A derivative without Kdo and slightly less active than its parent LPS, which indicates that one Kdo moiety is sufficient for the restoration of biological activity.

Introduction

Lipopolysacchardies (LPS) are constituents of the outer membrane of Gram-negative bacteria, and are structurally complex, amphipathic, microheterogeneous macromolecules which consists of three regions: the *O*-polysaccharide, core oligosaccharide, and lipid A.[1] The *O*-polysaccharide chains are composed of repeating oligosaccharide units and highly variable in structure and composition, giving rise to a high number of known stereotypes. The core oligosaccharide is comprised of an outer and inner core. While the outer core typically consists of common hexose sugars and is generally variable, the inner core structurally well conserved and characterized by more unusual sugars, particularly 3-deoxy-D-manno-octulosonic-acid (Kdo) and L-glycero-D-manno-heptose (Hep).[2] Lipid A consists of a diglucosamine acylated by long chain fatty acids.

As the major constituents of the outer membrane of gram-negative bacteria, LPS serve as one of the primary targets of the innate arm of the mammalian immune system.[2] LPS initiate innate immune responses by interacting with Toll-like receptor 4 (TLR4), which results in the production of a wide range of cytokines. Convincing evidence exists to show that TLR4 initiates these responses through at least two pathways: the MyD88-dependent pathway, which results in the generation of well-known inflammatory cytokines such as TNF-α and IL-1β; and the TRIF-dependent pathway, which leads to the synthesis of another family of inflammatory mediators, including IFN-β and nitric oxide.[3,4]

LPS show potential for use as immuno-modulators for the treatment of a wide range of diseases and as adjuvants for vaccinations. An important concern of such therapies is that over-activation of innate immunity may lead to the clinical symptoms of septic shock, as intact LPS could be too toxic for use as a safe immune-modulator.[5,6] The structural complexity and heterogeneity of LPS and possible contaminations with other inflammatory components makes it almost impossible to study their SAR and modulate their activities.

Lipid A is the toxic principle of LPS due to its lethal toxicity, pyrogenicity, and cytokine-activating properties that are similar to native LPS.[7,8] Because it is less toxic than LPS, lipid A is an attractive candidate for the development of immuno-modulators. Furthermore, the structure of lipid A is well conserved between species and strains of bacteria, so lipid A treatments alone have the advantage of by-passing the investigation of specific antigens, making them easier to establish on a broader range of diseases.[9] However, it has been demonstrated that lipid As are much less active than intact LPS in terms of cytokine production, or even inactive.[10]

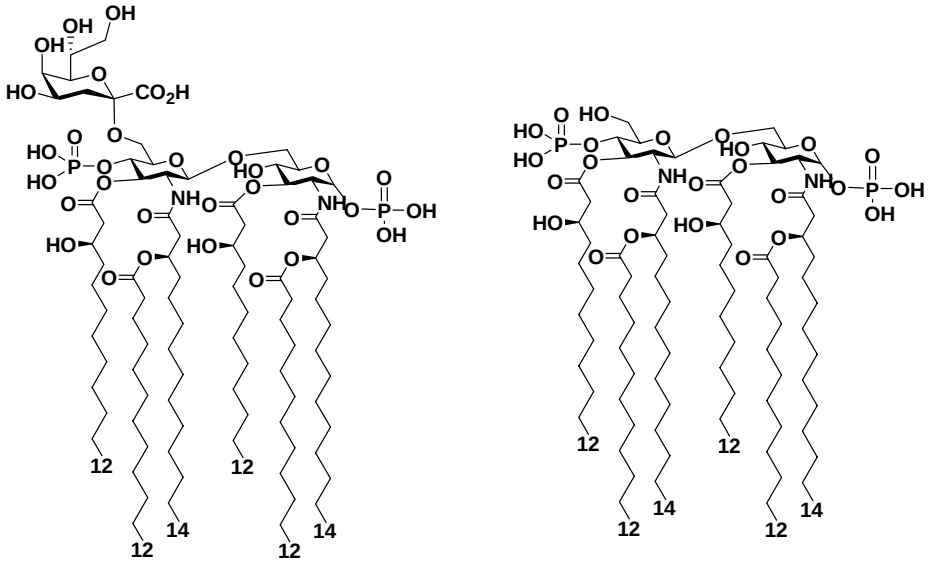
Kdo is found in almost every LPS known to date, and is α-bound to the carbohydrate backbone of lipid A in each case. While the *O*-polysaccharide and majority of the core can be

dispensed in some viable mutants, the Kdo residue is essential for bacterial viability.[2] For example, the smallest saccharide component seen in the LPS of any organism is that of a deep rough mutant of *Haemophilus influenzae*, which has only one Kdo residue attached to its lipid A.[11] Of the naturally occurring bacteria, the smallest core known to date is that of *Chlamydia* spp. It consists of only a triplet of Kdo units.[12] It is therefore essential to synthesize lipid A derivatives containing Kdos and study their abilities to induce cytokine production, so factors can be found to account for endotoxicity differences between LPS and their lipid As. More importantly, lipid A derivatives containing Kdos could be more potent immune-modulators, and the development of lipid As with Kdos as immune-modulators would not affect the advantageous inducement of an antibody of broad specificity.

Neisseria meningitidis LPS is termed lipooligosaccharide (LOS) because it lacks a long repeating *O*-polysaccharide and expresses only a core saccharide joined to a short non-repeating polysaccharide. In terms of endotoxicity, *N. meningitidis* lipid A is very active and closely resembles that of *E.coli* lipid A.[2] Hence, *N. meningitidis* LOS is an idea model to study the impacts of Kdo on its ability to induce cytokine production.

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.[7,13-15] Studying the SAR of LPS using lipid As with Kdo may reflect more completely these differences among LPS than using lipid A alone. To address this important issue, we have developed an efficient synthetic approach whereby an advanced synthetic trisaccharide can be easily converted into analogues that differ in phosphorylation and acylation pattern. This approach has been employed for the synthesis of *N. meningitidis* lipid A containing

Kdo **1** (**Figure 3.1**). The analogue was tested for the production of variuos cytokines, which is of critical importance for the development of safe immuno-modulators, because different mediators induce different biological effects. For comparison, the synthetic *N. meningitidis* lipid A **2** (**Figure 3.1**) and its parent LOS were also tested. It has been found that in all biological evaluations, the lipid A derivative without Kdo was less active than its parent LPS and the lipid A derivative with Kdo, which indicates the importance of the core region of LPS for biological activity. The lipid A derivative containing Kdo showed slightly higher EC_{50} values compared to the parent LPS, indicating that one Kdo moiety is sufficient for the restoration of biological activity.



1: *Neisseria meninitidis* lipid A containing KDO **2: *Neisseria meninitidis* lipid A**

Figure 3.1 Chemical structures of target lipid A derivatives

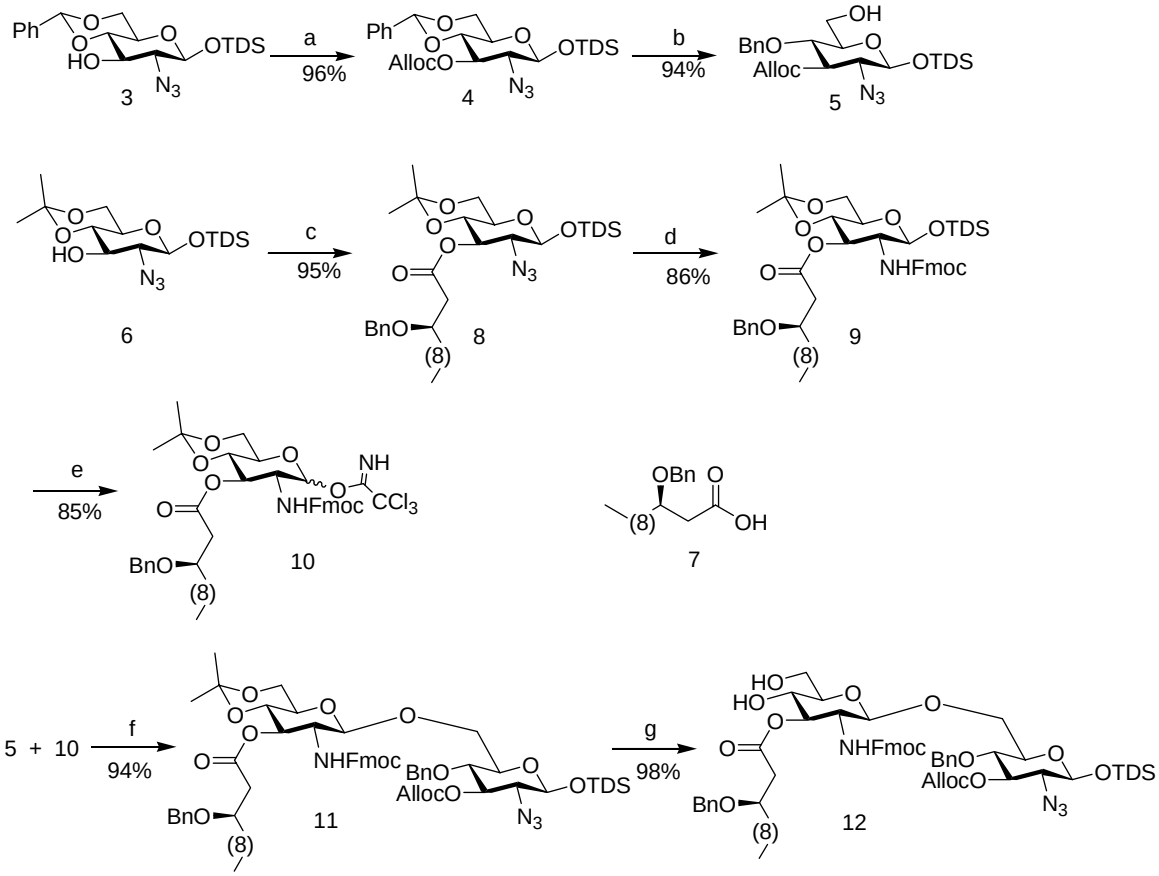
Results and Discussion

Chemical Synthesis of the Lipid A Derivatives. The total synthesis of Re LPS was not accomplished until 2001 by Kusumoto and co-workers due to its complex structure, which contains the highly acid-labile glycosyl phosphate as well as base-labile ester functional groups in the amphiphilic structure.[16] The synthesis employed strategies whereby the disaccharide was

functionalized with lipids, which was then coupled with Kdos. Although this approach is attractive for one-compound-at-a-time syntheses, detailed structure-activity relationship studies require a straightforward synthetic approach that offers a panel of lipid A analogues. Hence, the convergent approach that we have developed employs the advanced intermediate **22** with the Kdo moiety (**scheme 3.1**), which is protected with a set of orthogonal protecting groups (9-fluorenylmethoxycarbomate (Fmoc), allyloxycarbonate (Alloc) and azido and can be modified with any lipid at C-2, C-2' and C-3. C-3' was acylated at an early stage because it was found that 4'-phosphate tends to migrate to the 3'-hydroxyl.

Disaccharide **12** was synthesized by a stereoselective coupling reaction of glycosyl donor **10** and acceptor **5**. The C-3 hydroxyl of known compound **3** could be protected by an Alloc group by treatment with Alloc chloride in the presence of *N*,*N*,*N'*,*N'*-tetramethylenediamnie (TMEDA) in DCM to give **4** in a yield of 96%. Regioselective reductive opening of the benzylidene acetal of **4** proved more difficult than anticipated. Conventional procedures such as treatment with BH_3·THF/Bu_2BOTf or BH_3·THF/TMSOTf resulted in a loss of the Alloc group. Fortunately, treatment of **4** with triethyl silane and $PhBCl_2$ in the presence of molecular sieves (4Å) at -75 ºC gave **5** in an excellent regioselectivity and yield of 94%. The C-3 hydroxyl of known compound **6** was acylated by reaction with (*R*)-3-benzyloxy-dodecanoic acid **7**, using 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as the activation agents, to give **8** in an excellent yield of 95%. Next, the azido function of **8** was reduced with zinc in a mixture of acetic acid and DCM, and the resulted amine immediately protected as an Fmoc carbomate by reaction with FmocCl in the presence of diisopropylethylamine (DIPEA) to give fully protected **9**. Removal of the anomeric TDS ether of **9** 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 afforded glycosyl donor **12** as an α/β mixture (about 1:1).[17]



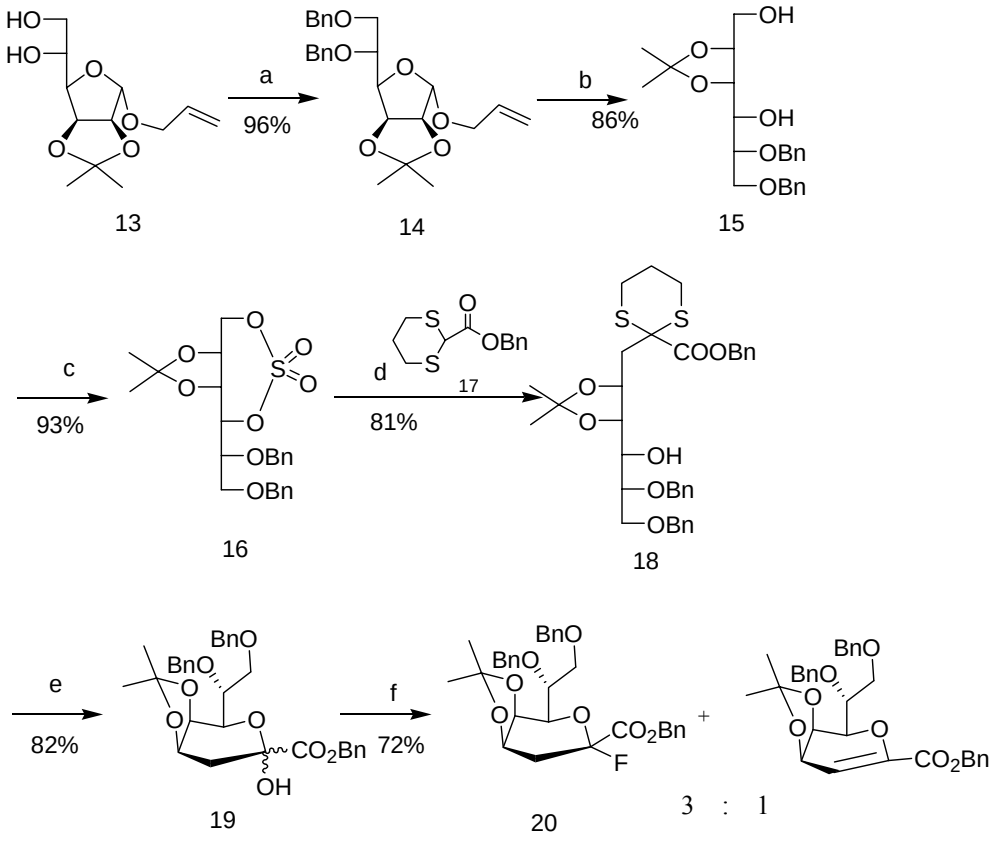
Scheme 3.1 (a) AllocCl, TMEDA, DCM; (b) $PhBCl_2$, Et_3SiH, MS 4Å, DCM, -78 ºC; (c) **7**, DCC, DMAP, DCM; (d) 1: Zn, AcOH, DCM; 2: FmocCl, DIPEA, DCM; (e) 1: Bu_4NF, AcOH, THF; 2: $CNCl_3$, Cs_2CO_3, DCM; (f) TfOH, MS 4Å, DCM, -50 ºC; (g) TFA, H_2O, DCM.

A TMSOTf-mediated glycosylation of **5** with **10** in the presence of molecular sieves (4 Å) in DCM at -40 ºC proved problematic. Only the β-anomer of glycosyl donor **10** was consumed, while the α-anomer remained intact even when the temperature or amount of TMSOTf was increased. Two factors could contribute to the unusual high-stability of this trichloroimidate. First, both the electron-withdrawing group at C-2 and C-3 and 4,6-di-O-isopropylidene

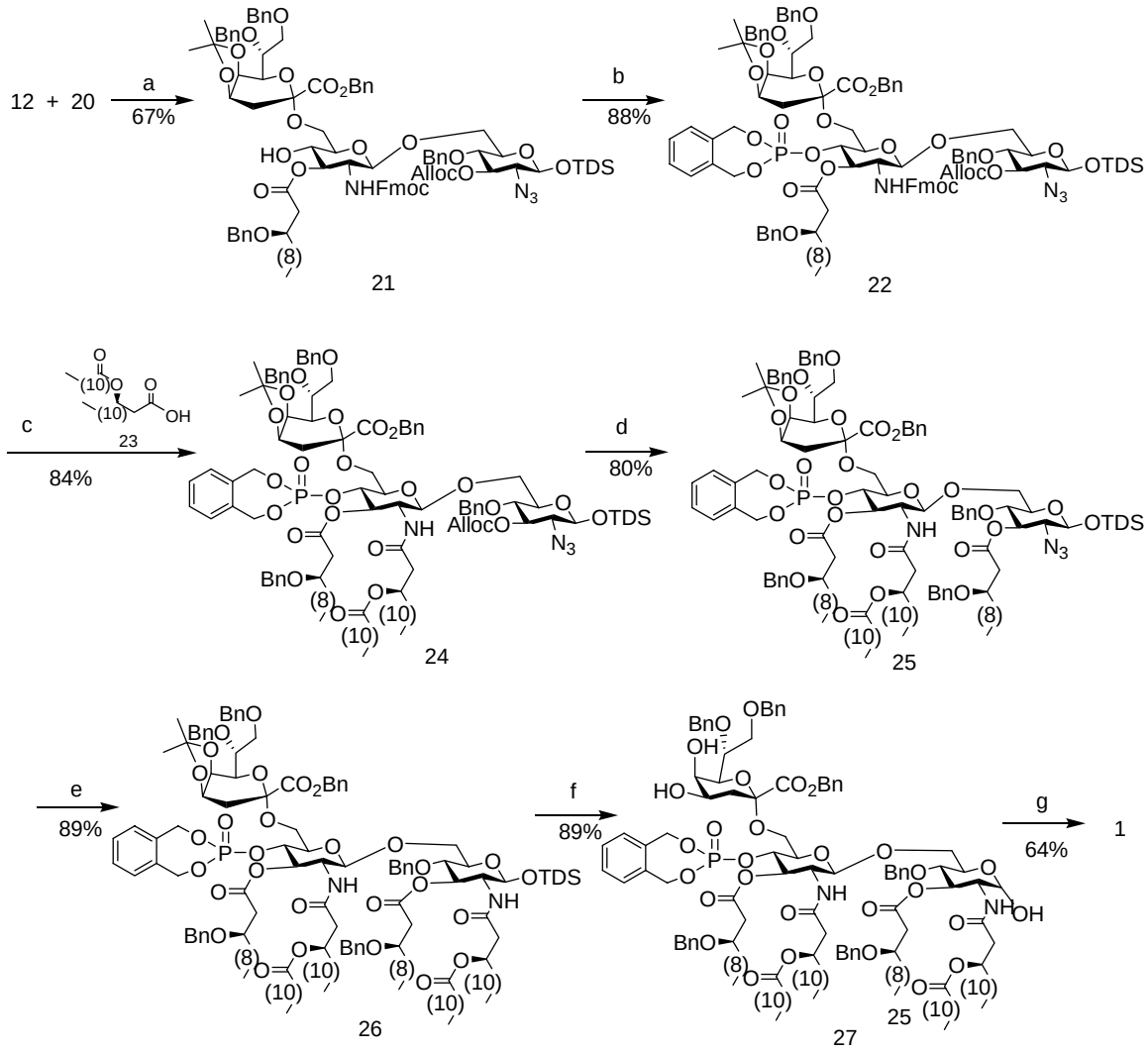
destabilize oxocarbenium which could be produced after removal of trichloroimidiate. As a consequence, activating the trichloroimidate is more difficult. Second, the bulky Fmoc at C-2 and lipid at C-3 could prevent TMSOTf access to the trichloroimidiate of **10**. The β-trichloroimidiate of **10** is more reactive than the α-anomer because the α-anomer is stabilized by anomeric effect and the β-anomer is less hindered, so it could be consumed more easily in the glycosylation reaction. An attempt to improve the ratio of β-anomer by using a weaker base (Cs_2CO_3) unexpectedly resulted in α-anomer as a dominant product. To find how to activate the α-trichloroimidiate, we employed TfOH instead of TMSOTf, because it was envisaged that the proton is a smaller and more powerful activation agent than TMS. Gratefully, TfOH could drive the glycosylation reaction to completion with both the α- and β-anomer consumed, and the reaction gave **11** in an excellent yield of 94%. Next, the isopropylidene group of **11** was removed with TFA to yield **12** in a yield of 98%.

Kdo fluoride **20** was synthesized according to the approach developed by van Boom and co-workers,[18] starting from known compound **13**, which was prepared from D-mannose in three steps **(scheme 3.2**). Thus, the 5, 6-hydroxyls of **13** were benzylated by reaction with benzyl bromide in the presence of NaH to afford **14** in a yield of 96%. An attempt to remove the allyl group of **14** with $PdCl_2$ in the presence of AcOH/NaOAc in ethyl acetate resulted in a mixture of compounds, and $Pd(Ph_3)_4$ in a mixture of acetic acid and DCM did not cause any reactions. Fortunately, the allyl group could be removed through isomerization by Pd/C in refluxing methanol followed by treatment with I_2 in the presence of pyridine. The resulting hemiacetal was reduced with $NaBH_4$ in ethanol to afford **15**. Compound **15** was reacted with thionyl chloride in the presence of Et_3N to give a cyclic sulfite, which was oxidized by a catalytic amount of $RuCl_3$ and cooxidant $NaIO_4$ to afford **16**. Next, the coupling reaction of **16** and **17** was conducted in a

mixture of HMPA and THF with the aid of BuLi. Hydrolysis of the resulting coupling product afforded **18**. The reaction of **18** with NBS in the presence of $NaHCO_3$ gave **19** as an α/β mixture. Finally, compound **19** was treated with DAST in the presence of MS 4Å at -60ºC to afford an inseparable mixture of α-glycosyl fluoride **20** and glycal (ca, 4/1).[16]



Scheme 3.2 (a) BnBr, NaH, DMF; (b) 1: Pd/C, CH_3OH, reflux; 2: I_2, pyridine, H_2O, THF; 3: $NaBH_4$, EtOH; (c) 1: $SOCl_2$, Et_3N, DCM, -15 ºC; 2: $NaIO_4$, $RuCl_3$, H_2O, CH_3CN, DCM; (d) BuLi, HMPA, THF, -40 ºC; then H_2SO_4, H_2O, THF, 50 ºC; (e) NBS, $NaHCO_3$, H_2O, acetone; (f) DAST, MS 4Å, DCM, -60 ºC – rt.



Scheme 3.3 (a) $BF_3{\cdot}Et_2O$, DCM, MS 4Å, 0 ºC; (b) *N*,*N*-diethyl-1,5-dihydro-3H-2,3,4-benzodioxaphosphepin-3-amine, 1*H*-tetrazole, DCM; then mCPBA, -20 ºC; (c) 1: DBU, DCM; 2: DCC, DCM; (d) 1: $Pd(PPh_3)_4$, HCOOH, $BuNH_2$, THF; 2: **7**, DCC, DMAP, DCM; (e) 1: Zn, HOAc, DCM; 2: **22**, DCC, DCM; (f) TFA, H_2O, DCM; (g) 1: tetrabenzyl diphosphate, $LiN(TMS)_2$, THF, - 78 ºC – 20 ºC; 2: H_2 (60 psi), Pd (black), THF.

Glycosylation of disaccharide **12** and Kdo donor **20** was carried out with the aid of $BF_3{\cdot}OEt_2$ in the presence of MS 4Å in DCM to afford an inseparable mixture of **21** and its β-anomer (**scheme 3.3**). The anomeric configurations of common sugars can be determined by calculating

the coupling constant of the anomeric proton and its vicinal proton. This method is not applicable for Kdo glycosides due to the absence of an anomeric proton. The current method to determine the anomeric configurations of Kdo glycosides is to compare the differences between the chemical shift values of C-3 methylene protons.[19] It has been found that for Kdo glycosides with a boat form, a larger difference between the chemical shift values of C-3 methylene protons is observed in α-anomers as compared to the corresponding values in β-anomers. This is a characteristic feature of boat form 4,5:7,8-di-O-isopropylidene derivatives of Kdo and can be regarded as a criterion of anomeric configurations. In the case of Kdo glycosides with a chair form, on the other hand, a similar large chemical shift difference could be observed not in α- but in β-anomers.[20]

After a careful examination of the NMR spectrum of the glycosylation product, we found that the chemical shift difference of the C-3 methylene protons of the major product was 0.59 while the corresponding difference was 0.12 for the minor product (**Table 3.1**). According to the above method, this implied that the major product was an α-anomer because the Kdos of the resulting products adopt a boat configuration when their 4, 5-hydroxyls are protected as a di-O-isopropylidene acetal, which is also demonstrated by the coupling constants of the C-3 methylene protons. To afford more evidences to determine the configuration of the major product, the isopropylidene acetal of the glycosylation product was removed by treatment with TFA. NMR experiments showed that the chemical shift difference of the C-3 methylene protons of the major product decreased to 0.08 while the corresponding difference increased to 0.39 for the minor product. This observation is a convincing evidence that the major product is an α-anmoer because when the isopropylidene acetal was removed, the Kdo existed as a chair form. Fortunately, the α/β mixture could be separated after phosphilation with *N,N*-diethyl-1,5-

Table 3.1 NMR analysis of the compounds containing Kdo

compound	δ difference	Chemical shift		Coupling constant	
21	0.59	2.56	1.97	-	15.0; 2.4
β-anomer of **21**	0.12	2.28	2.16	15.0; 4.8	15.0; 4.8
21': the derivative of **21** without isopropylidene	0.08	2.03	1.95	-	-
β-anomer of **21'**	0.39	2.39	2.00	-	-
22	0.14 (0.14*)	2.26 (2.24*)	2.12 (2.10*)	14.4; 7.2	14.4; 4.8
β-anomer of **22**	0.15	2.35	2.20	15.0; 5.0	15.0; 5.0
22': the derivative of **22** without isopropylidene	0.10 (0.16*)	2.15 (2.19*)	2.05 (2.03*)	13.0; 5.0 (12.0; 3.5*)	(12.0; 12.0*)
β-anomer of **22'**	0.37	2.49	2.12	-	-
24*	0.07	2.18	2.11	14.4; 7.2	14.4; 4.8
27*	0.22	2.18	1.96	12.6; 4.6	12.6; 12.6
1#	0.15	1.76	1.61	15.0; 5.4	15.0; 15.0

Note 1: *: $CDCl_3$; #: $CDCl_3/CD_3OD$ (1/1, v/v); for all the other NMR experiments, the solvent was CD_3COCD_3. Considering the potential influence of solvents on the chemical shifts, parallel experiments were conducted by using $CDCl_3$ or CD_3COCD_3. $CDCl_3$ was the solvent used in the above rule. As shown in the table, no big differences in the chemical shifts were observed for the two solvents.

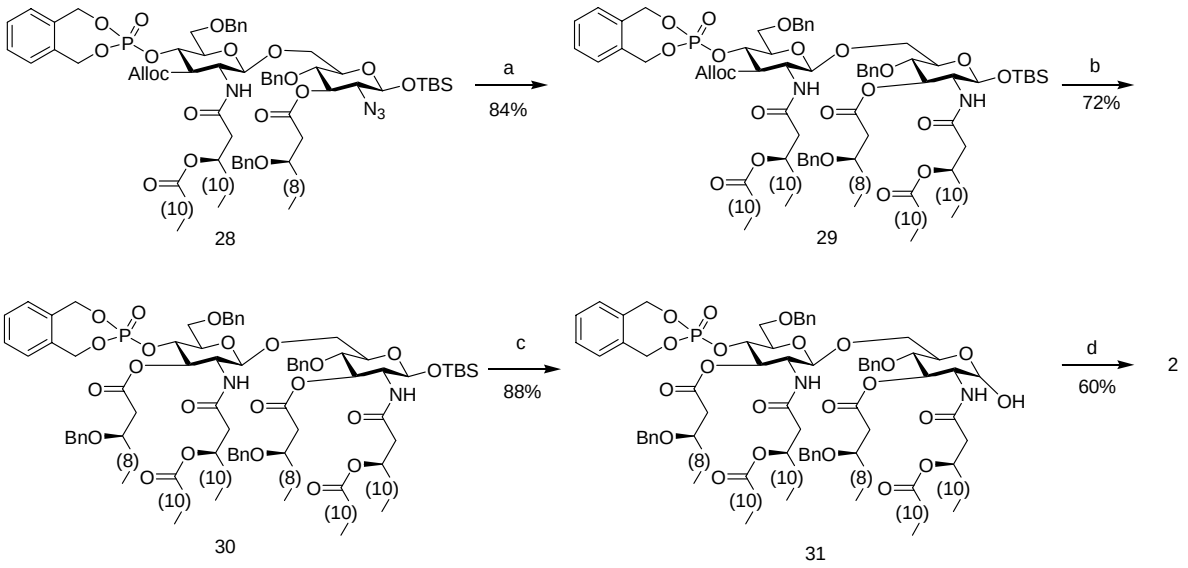
2: the coupling constants on the left (right) corresponding to the chemical shifts on the left (right).

dihydro-2,3,4-benzodioxaphosphepin-3-amine in the presence of 1H-tetrazole followed by in situ oxidation with m-chloroperoxybenzoic acid (mCPBA). The ratio of the resulting α- and β-isomer was 9 to 1.

It was interesting to find that both the chemical shift difference of compound **22** and that of its β-anomer are very small, which contradicts the above rule. This contradiction could be attributed to either the conformation change caused by the bulky phosphoryl group or the chemical shift changes caused by the phenyl group of the phosphate, when one or both of the C-3 methylene protons of Kdo happen to be located within the magnetic field of the benzene ring. Actually, the rule is not applicable for all subsequent compounds with the isopropylidene acetal. To provide additional information, the isopropylidene of **22** and its β-anomer were removed. NMR experiments showed that the chemical shift difference of the C-3 methylene protons of **22** was 0.10 while the corresponding difference increased to 0.37 for the α-anomer. This observation is an additional piece of evidence to convincingly show that the major product is an α-anmoer, because when the isopropylidene acetal was removed, the Kdo existed as a chair form. The chair form was demonstrated by the large coupling constant (12.0) between H-4 and H-3_{axe} of compound **22'**.

Having the advanced trisaccharide **21** in hand, attention focused on the selective acylation of relevant hydroxyls and amines. Thus, the Fmoc protecting group of **21** was removed using DBU in DCM, and the resulting amino group acylated with (*R*)-3-dodecanoyl-tetradecanoic acid using DCC as the activating agent to give compound **24**. Removal of the Alloc group was easily accomplished by treatment with $Pd(PPh_3)_4$ in the presence of $BuNH_2$ and HCOOH, and subsequent acylation of the resulting hydroxyl using DCC and DMAP as activating agent afforded **25**. Next, reduction of the azido function of **25** with zinc and acetic acid in DCM

followed by acylation of the resulting amine using standard conditions furnished fully acylated **26**. Then, both the isopropylidene and anomeric TDS of **26** were removed by treatment with TFA/H_2O (3/2) in DCM gave lactol **27**. The anomeric hydroxyl of **27** was phosphorylated regioselectively using tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide in THF at -78 ºC to give the phosphorylation intermediate as only an α-anomer.[21] Finally, global deprotection of the above obtained intermediate by catalytic hydrogenolysis over Pd-black gave target product **1**.



Scheme 3.4 (a) 1: Zn, HOAc, DCM; 2: **23**, DCC, DCM; (b) 1: $Pd(PPh_3)_4$, HCOOH, $BuNH_2$, THF; 2: **7**, DCC, DMAP, DCM; (c) HF/pyridine, THF; (d) 1: tetrabenzyl diphosphate, $LiN(TMS)_2$, THF, - 78 ºC – 20 ºC; 2: H_2 (60 psi), Pd (black), THF.

The other target molecule (**2**) was synthesized easily using the approach developed in our research group, starting from known conpound **28** (scheme **2.4**).[10] The azido function of **28** was reduced with activated Zn in a mixture of acetic acid and DCM and the amine of the resulting compound was reacted with (*R*)-2-dodecanoyloxy-tetradecanoic acid in the presence of DCC to

give **29**. The removal of the Alloc protecting group of **29** could easily be accomplished by treatment with $Pd(PPh_3)_4$; the resulting hydroxyl group was acylated with (*R*)-2-benzyloxy-tetradecanoic acid using DCC and DMAP as activating agents to afford fully acylated **30**. The anomeric TBS ether of **30** was removed by treatment with HF in pyridine and the resulting anomeric hydroxyl phosphorylated using tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide in THF at –78°C followed by global deprotection by catalytic hydrogenanolysis to give the requisite lipid A **2**.

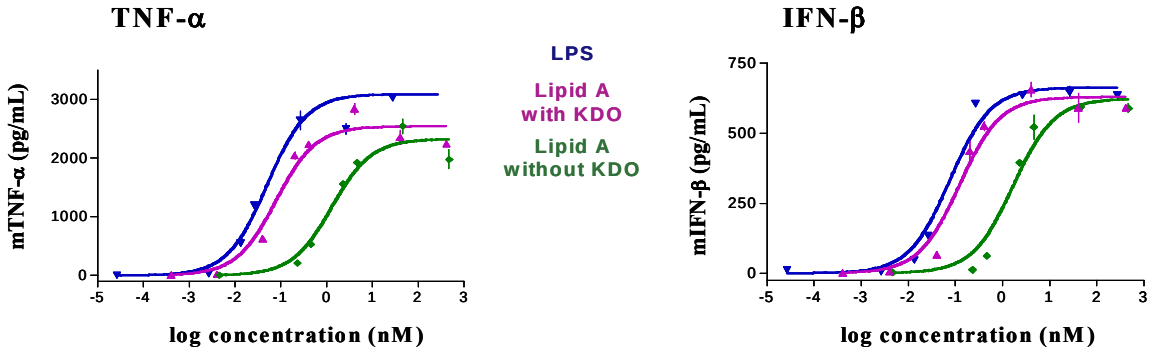


Figure 3.2 Cytokine production by murine macrophages after stimulation with *Neisseria meningitidis* LPS and lipid As. Mouse macrophages were exposed to different concentrations of *N. meningitidis* lipid As and LPS and TNF-α and IFN-β responses were measured using capture ELISAs.

Biological Evaluation of Lipid As and LPS. Mouse macrophages were exposed to different concentrations of *N. meningitidis* lipid As (compound **1** and **2**) and LPS. The supernatants were harvested and examined for mouse TNF-α and IFN-β using capture ELISA assay. 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 3.2**, in all the biological evaluations, while the EC_{50} values for the lipid A

derivative without Kdo were much smaller than those of its parent LPS, the lipid A derivative with Kdo had slightly lower EC_{50} values than natural LPS. These results indicate the importance of the core region of LPS for biological activity, and that one Kdo moiety is sufficient for the restroation of biological activity of LPS. No significant differences were observed in the potences of secreted TNF-α and IFN-β. Biological assays for other cytokines are underway.

Table 3.2 EC_{50} values* (nM) of *N.meningitidis* LPS and lipid A derivatives.

	LPS	**1: lipid A with Kdo**	**2: lipid A without Kdo**
TNF-α	**0.051**	**0.079**	**1.3**
IFN-β	**0.076**	**0.122**	**1.7**

* Values of EC_{50} are reported as best-fit values.

Conclusions

A convergent approach for the synthesis of lipid A derivatives containing Kdo has been developed, which allows for the convenient synthesis of a panel of analogues differing in fatty acid acylation patterns and degree of phosphorylation. This approach was employed for the synthesis of *N. meningitidis* lipid A containing Kdo. The compound was tested for cytokine production along with the synthetic *N. meningitidis* lipid A and its parent LPS. Examination of the biological results showed that the lipid A derivative containing Kdo was much more active than the lipid A derivative without Kdo and just slightly less active than its parent LPS, indicating that one Kdo moiety is sufficient for the restoration of biological activity.

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 F254 (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 ºC. CH_2Cl_2 was distilled from NaH and stored over molecular sieves (3 Å). Tetrahydrofuran (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 ºC during 8 h in the first instance and then for 2-3 h at 390 ºC 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.

Dimethylthexylsilyl 3-*O*-allyloxycarbonyl-2-azido-4,6-*O*-benzyldidine-2-deoxy-β-D-glucopyranoside (**4**): To a cooled (0 ºC) solution of compound **3** (1.25 g, 2.87 mmol) and *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TMEDA) (281 µL, 1.87 mmol) in DCM (10 mL) was added dropwise allyl chloroformate (366 µL, 3.44 mmol). The reaction mixture was stirred at room temperature for 3 h, and then diluted with DCM (20 mL) and washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried ($MgSO_4$) was concentrated *in vacuo.* The residue was purified by silica gel column chromatography

(hexane/ethyl acetate, 30/1, v/v) to give **4** as a colorless oil (1.43 g, 96%). R_f = 0.60 (hexane/ethyl acetate, 5/1, v/v); $[\alpha]^{25}_D$ = -34.9° (*c* = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42-7.32 (m, 5H, aromatic), 5.98-5.86 (m, 1H, $OCH_2CH{=}CH_2$), 5.47 (s, 1H, >C*H*Ph), 5.33 (d, *J* = 17.0 Hz, $OCH_2CH{=}CH_2$), 5.22 (d, *J* = 11.0 Hz, $OCH_2CH{=}CH_2$), 4.87 (t, $J_{2,3} = J_{3,4}$ = 10.0 Hz, H-3), 4.69 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.64 (d, 2H, *J* = 11.0 Hz, $OCH_2CH{=}CH_2$), 4.28 (dd, 1H, $J_{5,6a}$ = 5.0 Hz, $J_{6a,6b}$ = 10.0 Hz, H-6a), 3.76 (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), 3.48-3.40 (m, 2H, H-2, 5), 1.68-1.63 (m, 1H, $CH(CH_3)$), 0.89-0.87 (m, 12H, $SiC(CH_3)CH(CH_3)$), 0.19 (s, 3H, $Si(CH_3)$), 0.18 (s, 3H, $Si(CH_3)$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.15 (*C*=O), 136.75-126.17 (m, aromatic, $OCH_2CH{=}CH_2$), 119.07 ($OCH_2CH{=}CH_2$), 101.54 (>*C*HPh), 97.56 (C-1), 78.57 (C-4), 75.35 (C-3), 68.93 ($OCH_2CH{=}CH_2$), 68.57 (C-6), 67.12 (C-2), 66.32 (C-5), 33.82 ($SiC(CH_3)_2CH(CH_3)_2$), 24.78 ($SiC(CH_3)_2CH(CH_3)_2$), 19.88, 19.76, 18.46, 18.36, -2.21 ($SiCH_3$), -3.24 ($SiCH_3$). HR MS (m/z) calcd for $C_{25}H_{37}N_3O_7Si$ $[M+Na]^+$, 542.2298; found, 542.2475.

Dimethylthexylsilyl 3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**5**): A suspension of **4** (1.20 g, 2.31 mmol) and molecular sieve 4Å ( ) in DCM (20 mL) was stirred at room temperature for 1h. The mixture was cooled (-75 ºC) and then triethylsilane (0.55 mL, 3.47 mmol) and $PhBCl_2$ (0.52 mL, 3.93 mmol) were added dropwise. After stirring the reaction mixture for 1h, it was quenched by addition of Et_3N (1mL) and methanol (1 mL). The reaction mixture was warmed up to room temperature and then diluted with ethyl acetate (40 mL). The molecular sieve was removed by filtration, and the filtrate was washed with saturated aqueous $NaHCO_3$ (30 mL). R_f = 0.35 (hexane/ethyl acetate, 5/1, v/v); $[\alpha]^{25}_D$ = -17.1º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300MHz, $CDCl_3$): δ 7.28-7.22 (m, 5H, aromatic), 5.99-5.85 (m, 1H, $OCH_2CH{=}CH_2$), 5.37 (dd, *J* = 1.2 Hz, *J* = 15.6 Hz, $OCH_2CH{=}CH_2$), 5.27 (dd, *J* = 1.2 Hz, *J* =

11.0 Hz, $OCH_2CH=CH_2$), 4.80 (dd, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 4.70-2.59 (m, 5H, H-1, CH_2Ph, $OCH_2CH=CH_2$), 3.84 (dd, 1H, $J_{5,6a}$ = 2.4 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 3.71 (dd, 1H, $J_{5,6b}$ = 4.2 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6b), 3.65 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, H-4), 3.42-3.37 (m, 1H, H-5), 3.34 (dd, 1H, $J_{1,2}$ = 7.2 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 1.71-1.62 (m, 1H, $CH(CH_3)$), 0.90-0.88 (m, 12H, $SiC(CH_3)CH(CH_3)$), 0.20 (s, 3H, $Si(CH_3)$), 0.17 (s, 3H, $Si(CH_3)$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.24 (*C*=O), 137.26-127.89 (m, aromatic), 131.17 ($OCH_2CH=CH_2$), 119.13 ($OCH_2CH=CH_2$), 96.81 (C-1), 78.52 (C-3), 75.29 (C-4), 74.99 (C-5), 74.66 ($OCH_2CH=CH_2$), 68.82 (CH_2Ph), 66.49 (C-2), 61.49 (C-6), 33.73 ($SiC(CH_3)_2CH(CH_3)_2$), 24.69 ($SiC(CH_3)_2CH(CH_3)_2$), 19.79, 19.71, 18.35, 18.28, -2.22 ($SiCH_3$), -3.32 ($SiCH_3$). HR MS (m/z) calcd for $C_{25}H_{39}N_3O_7Si[M+Na]^+$, 544.2455; found, 544.2748.

Dimethylthexylsilyl 2-azido-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside (**8**): A reaction mixture of (*R*)-3-benyloxy-dodecanoic acid **7** (970 mg, 3.17 mmol) and DCC (949 mg, 4.60 mmol) in DCM (10 mL) was stirred at room temperature for 10 min, and then compound **6** (1.15 g, 2.88 mmol) and DMAP (35 mg, 0.29 mmol) were added. The reaction mixture was stirred at room temperature for 10 h, after which the solids were removed by filtration, and the residue was washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo*. The residue was purified by silicon gel column chromatography (eluent: hexane/ethyl acetate, 15/1, v/v) to yield **8** as a syrup (1.82 g, 94%). R_f = 0.55 (hexane/ethyl acetate, 8/1, v/v); $[\alpha]^{25}_D$ = -10.0º (*c* = 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.34-7.26 (m, 5H, aromatic), 4.91 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 4.62 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.60 (d, 1H, *J* = 11.4 Hz, CH_2Ph), 4.48 (d, 1H, *J* = 11.4 Hz, CH_2Ph), 3.89-3.83 (m, 2H, H-6a, $H\text{-}3_S$), 3.74 (t, 1H, $J_{5,6b}$ = $J_{6a,\,6b}$ = 10.5 Hz, H-6b), 3.64 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, H-4), 3.34-3.23 (m, 2H, H-2, 5), 2.71 (dd, $J_{2Sa,2Sb}$ = 15.0 Hz, $J_{2Sa,3S}$ = 6.3 Hz, $H\text{-}2_{Sa}$), 2.51

(dd, $J_{2Sa,2Sb}$ = 15.0 Hz, $J_{2Sb,3S}$ = 6.0 Hz, H-2_{Sb}), 1.69-1.47 (m, 3H, H-4_S, C*H*(CH_3)), 1.38 (s, 3H, CH_3 of isopropylidiene), 1.28 (s, 3H, CH_3 of isopropylidiene), 1.24 (bs, 14H, H-(5_S-11_S)), 0.89-0.84 (m, 15H, H-12_S, SiC(C*H*$_3$)CH(C*H*$_3$)), 0.19 (s, 3H, Si(C*H*$_3$)), 0.18 (s, 3H, Si(C*H*$_3$)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.56 (*C*=O), 138.50-127.50 (m, aromatic), 99.67 (*C*(CH_3)$_2$ of isopropylidiene), 97.43 (C-1), 75.70 (C-3_S), 71.63 (C-4), 71.46, 71.41 (C-3, *C*H_2Ph), 67.46, 67.37 (C-2, 5), 61.93 (C-6), -2.25 (Si*C*H_3), -3.32 (Si*C*H_3). HR MS (m/z) calcd for $C_{36}H_{61}N_3O_7Si[M+Na]^+$, 698.4176; found, 698.3518.

Dimethylthexylsilyl 3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-(9-fluorenylmethoxycarbonyl)-4,6-*O*-isopropylidene-β-D-glucopyranoside (**9**): A suspension of compund **8** (1.82 g, 2.70 mmol) and zinc (1.75 g, 27.0 mmol) in a mixture of acetic acid (300 µl) and DCM (15 ml) was stirred at room temperature for 5 h, after which it was diluted with ethyl acetate (40 mL). The solids were removed by filtration, and the residue was washed with ethyl acetate (2 x 3 ml). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 30 mL) and brine. The organic phase was dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* to afford the crude amine as a pale yellow oil. The resulting amine was dissolved in DCM (15 mL), and then FmocCl (767 mg, 2.97 mmol) and DIPEA (517 µl, 2.97 mmol) were added. The reaction mixture was stirred at room temperature for 2 h, after which it was diluted with DCM (20 mL) and washed with brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 10/1, v/v) to yield **9** as a colorless syrup (2.02 g, 86%, two steps). R_f = 0.55 (hexane/ethyl acetate, 5/1, v/v); $[\alpha]^{25}_D$ = -2.8º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73-7.25 (m, 13H, aromatic), 5.35 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.27 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 4.81 (d, 1H, $J_{1,2}$ = 7.2 Hz, H-1), 4.55 (d, 1H, *J* = 11.1 Hz, C*H*$_2$Ph), 4.43 (d, 1H, *J*

= 11.1 Hz, CH_2Ph), 4.27-4.13 (m, 3H, OCH_2CH of Fmoc), 3.89-3.69 (m, 5H, H-2, 4, 6a, 6b, H-3_S), 3.47 (bs, 1H, H-5), 2.70 (dd, $J_{2Sa,2Sb}$ = 14.7 Hz, $J_{2Sa,3S}$ = 5.1 Hz, H-2_{Sa}), 2.46 (dd, $J_{2Sa,2Sb}$ = 14.7 Hz, $J_{2Sb,3S}$ = 6.0 Hz, H-2_{Sb}), 1.59-1.50 (m, 3H, H-4_S, CH(CH_3)), 1.43 (s, 3H, CH_3 of isopropylidiene), 1.34 (s, 3H, CH_3 of isopropylidiene), 1.23-1.16 (m, 14H, H-(5_S-11_S)), 1.16-0.84 (m, 15H, H-12_S, SiC(CH_3)CH(CH_3)), 0.13 (s, 3H, Si(CH_3)), 0.10 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.78 (*C*=O), 155.82 (*C*=O), 143.79-119.83 (m, aromatic), 99.45 (*C*(CH_3)$_2$ of isopropylidiene), 96.93 (C-1), 75.66 (C-3_S), 72.08 (C-3), 71.83 (C-4), 71.15 (*C*H_2Ph), 67.07 (C-5, O*C*H_2 of Fmoc), 62.00(C-6), 58.55 (C-2), 46.92 (OCH_2*C*H of Fmoc), 39.76 (C-2_S), -2.00 (Si*C*H_3), -3.42 (Si*C*H_3). HR MS (m/z) calcd for $C_{51}H_{73}NO_9Si[M+Na]^+$, 894.4952; found, 894.4984.

3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-(9-fluorenylmethoxycarbonyl)-4,6-*O*-isopropylidene-β-D-glucopyranoside trichloroacetimidate (**10**): A mixture of Bu_4NF (1 M in THF, 5 mL) and acetic acid (800 μl) was added dropwise to a stirred solution of **9** (1.30 g, 1.49 mmol) in THF (15 mL). After stirring at room temperature for 36 h, the reaction mixture was diluted with DCM (20 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 25 mL) and brine (25 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/2, v/v) to afford the lactol as a pale yellow oil (978 mg, 90%). The resulting lactol (810 mg, 1.11 mmol) was dissolved in a mixture of trichloroacetonitrile (2.0 mL) and DCM (6 mL), and then Cs_2CO_3 (181 mg, 0.55 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, after which it was diluted with DCM (20 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 25 mL) and brine (2 x 25 mL). The organic phase was dried (Na_2SO_4) and concentrated *in*

vacuo. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 2/1, v/v) to yield **10** as a pale yellow foam (880 mg, 91%).

Dimethylthexylsilyl 3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-(9-fluorenylmethoxycarbonyl)-4,6-*O*-isopropylidene-β-D-glucopyranosyl-(1→6)-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (11): A suspension of trichloroacetimidate **10** (880 mg, 1.01 mmol), acceptor **5** (480 mg, 0.92 mmol) and molecular sieves (4 Å, 500 mg) in DCM (10 mL) was stirred at room temperature for 1 h. The mixture was cooled (-50 ºC) and then TfOH (4.4 µl , 0.05 mmol) was added. After stirring the reaction mixture for 30 min, it was allowed to warm up to -10 ºC in 30 min and then quenched with solid $NaHCO_3$ (50 mg) and diluted with DCM (20 mL). The solution was washed with saturated aqueous $NaHCO_3$ (2 x 25 mL) and brine (2 x 25 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 7/1-4/1, v/v) to yield disaccharide **11** as a colorless solid (1.07 g, 94%). (R_f = 0.50 (hexane/ethyl acetate, 4/1, v/v); $[\alpha]^{24}_D$ = -9.6º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.82-7.19 (m, 18H, aromatic), 6.76 (d, 1H, $J_{NH',2}$ = 9.6 Hz, N*H'*), 5.88-5.83 (m, 1H, $OCH_2CH{=}CH_2$), 5.29 (d, *J* = 16.8 Hz, $OCH_2CH{=}CH_2$), 5.23 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3'), 5.16 (d, *J* = 10.2 Hz, $OCH_2CH{=}CH_2$), 4.88 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.80-4.69 (m, 2H, H-1, 3), 4.69 (d, 1H, *J* = 10.8 Hz, CH_2Ph), 4.60-4.52 (m, 4H, CH_2Ph, $OCH_2CH{=}CH_2$), 4.39 (d, 1H, *J* = 11.4 Hz, CH_2Ph), 4.24-4.21 (m, 1H, OCH_{2a} of Fmoc), 4.12 (d, 1H, $J_{6a,6b}$ = 10.8 Hz, H-6a), 4.08-4.01 (m, 2H, OCH_{2b}, OCH_2CH of Fmoc), 3.89-3.84 (m, 2H, H-6a', 6b), 3.82-3.79 (m, 2H, H-4', 6b'), 3.78-3.72 (m, 3H, H-2', 4, 3L), 3.67-3.65 (m, 1H, H-5), 3.40-3.33 (m, 2H, H-2, 5'), 2.57 (dd, $J_{2Sa,2Sb}$ = 15.6 Hz, $J_{2Sa,3S}$ = 6.0 Hz, H-2_{Sa}), 2.33 (dd, $J_{2Sa,2Sb}$ = 15.0 Hz, $J_{2Sb,3S}$ = 6.0 Hz, H-2_{Sb}), 1.71-1.66 (m 1H, $CH(CH_3)_2$), 1.46-1.39 (m, 2H, H-4_S), 1.39-1.11 (m, 20H, CH_3 of

isopropylidiene, H-(5_S-11_S)), 0.91-0.83 (m, 15H, H-12_S, SiC(CH_3)CH(CH_3)), 0.24 (s, 3H, Si(CH_3)), 0.23 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.30 (*C*=O), 156.58 (*C*=O), 154.91 (*C*=O), 144.85-118.58(m, OCH_2CH=CH_2, aromatic), 102.42 (C-1'), 99.91 ($C(CH_3)_2$ of isopropylidiene), 97.08 (C-1), 75.66 (C-3_S), 79.23 (C-3), 76.67, 75.97 (C-4, 3_S), 75.05 (CH_2Ph), 74.51 (C-5), 72.96 (C-3'), 73.46 (C-4'), 71.29 (CH_2Ph), 68.93 (OCH_2CH=CH_2), 68.51 (C-6), 67.86, 67.25 (C-2, 5'), 67.08 (OCH_2 of Fmoc), 62.40 (C-6'), 57.27 (C-2'), 47.58 (OCH_2CH of Fmoc), 34.00 (C-3_S), -1.81 ($SiCH_3$), -3.26 ($SiCH_3$). HR MS (m/z) calcd for $C_{68}H_{92}N_4O_{15}Si[M+Na]^+$, 1255.6221; found, 1255.6068.

Dimethylthexylsilyl 3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyl-(1→6)-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (12): TFA/H_2O (3/2, v/v, 250 μl) was added dropwise to a stirred solution of **11** (960 mg, 0.78 mmol) in DCM (15 mL). The reaction mixture was stirred at room temperature for 30 min, after which it was diluted with ethyl acetate (15 mL) and then wahsed with saturated aqueous $NaHCO_3$ (2 x 15 mL) and brine (2 x 15 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 2/1, v/v) to afford **12** as pale yellow oil (882 mg, 95%). R_f = 0.35 (hexane/ethyl acetate, 1/1, v/v); $[\alpha]^{24}_D$ = -9.2º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.83-7.19 (m, 18H, aromatic), 6.64 (d, 1H, $J_{NH',2}$ = 9.5 Hz, N*H'*), 5.90-5.82 (m, 1H, OCH_2CH=CH_2), (d, *J* = Hz, OCH_2CH=CH_2), (m, 2H, H-3', OCH_2CH=CH_2), (m, 3H, H-1, 1', 3), (d, 1H, *J* = Hz, CH_2Ph), (m, 4H, CH_2Ph, OCH_2CH=CH_2), (d, 1H, *J* = Hz, CH_2Ph), (m, 1H, OCH_{2a} of Fmoc, 6a or 6a'), (m, 2H, OCH_{2b}, OCH_2CH of Fmoc), 2.61 (dd, $J_{2Sa,2Sb}$ = 15.5 Hz, $J_{2Sa,3S}$ = 6.5 Hz, H-2_{Sa}), 2.41 (dd, $J_{2Sa,2Sb}$ = 15.5 Hz, $J_{2Sb,3S}$ = 5.5 Hz, H-2_{Sb}), 1.80-1.66 (m 1H, CH(CH_3)$_2$), 1.44-1.41 (m, 2H, H-4_S), 1.25-1.08 (m, 14H, H-(5_S-11_S)), 0.91-

0.83 (m, 15H, H-12$_S$, SiC(CH_3)CH(CH_3)), 0.25 (s, 3H, Si(CH_3)), 0.24 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 171.80 (*C*=O), 156.48 (*C*=O), 154.86 (*C*=O), 144.81-120.51 (m, $OCH_2CH{=}CH_2$, aromatic), 118.51 ($OCH_2CH{=}CH_2$), 102.01 (C-1'), 97.02 (C-1), 79.21 (C-3), 71.11 (C-5), 76.72, 76.56, 76.07 (C-3', 4, 3$_S$), 74.93 (CH_2Ph), 74.66 (C-5'), 71.37 (CH_2Ph), 69.66 (C-4'), 68.86 ($OCH_2CH{=}CH_2$), 68.46 (C-6), 67.20 (C-2), 67.02 (OCH_2 of Fmoc), 62.40 (C-6'), 56.79 (C-2'), 47.59 (OCH_2CH of Fmoc), 39.8 (C-3$_S$), -1.83 (SiCH_3), -3.34 (SiCH_3). HR MS (m/z) calcd for $C_{65}H_{88}N_4O_{15}Si[M+Na]^+$, 1215.5913; found, 1215.6797.

Allyl 5,6-di-*O*-benzyl-2,3-di-*O*-isopropylidene-α-D-mannofuranoside (**14**): NaH (1.27 g, 53.0 mmol) was added portionwise to a stirred solution of **13** (2.50 g, 10.6 mmol) in dry DMF (20 mL). After stirring the reaction mixture for 30 min, it was cooled (0 ºC) and then BnBr (5.0 ml, 42.4 mmol) was added. The reaction mixture was stirred at room temperature for 10 h, after which it was quenched by addition of methanol (5 mL), diluted with ethyl acetate (50 mL), and washed with brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 10/1, v/v) to afford **14** as a colorless oil (4.29 g, 92%). R_f = 0.65 (hexane/ethyl acetate, 6/1, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39-7.20 (m, 10H, aromatic), 5.86 (m, 1H, $OCH_2CH{=}CH_2$), 5.23 (dd, 1H, *J* = 17.4 Hz, *J* = 1.5 Hz, $OCH_2CH{=}CH_{2a}$), 5.15 (dd, 1H, *J* = 17.4 Hz, 10.2 Hz, $OCH_2CH{=}CH_{2b}$), 5.00 (s, 1H, H-1), 4.84 (dd, 1H, *J* = 3.3 Hz, *J* = 5.7 Hz, H-3), 4.80 (d, 1H, *J* = 11.1 Hz, CH_2 of Bn), 4.69 (d, 1H, *J* = 11.1 Hz, CH_2 of Bn), 4.65-4.54 (m, 3H, H-2, CH_2 of Bn), 4.11-4.05 (m, 2H, H-4, $OCH_{2a}CH{=}CH_2$), 4.00-3.81 (m, 3H, H-4, H-6a, $OCH_{2b}CH{=}CH_2$), 3.65 (dd, 1H, $J_{5,6b}$ = 5.4 Hz, $J_{6a,6b}$ = 16.5 Hz, H-6b), 1.44 (s, 3H, CH_3), 1.36 (s, 3H, CH_3); HR MS (m/z) calcd for $C_{26}H_{32}O_6[M+Na]^+$, 463.2091; found, 463.2118.

5,6-di-*O*-benzyl-2,3-di-*O*-isopropylidene-D-mannitol (**15**): A suspension of **14** (3.20 g, 7.27 mmol) and Pd/C (50 mg) in methanol (70 mL) was refluxed for 16 h, after which the catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to afford the isomerization product as a pale yellow. The obtained intermediate was dissolved in a mixture of THF (50 mL), pyridine (2 mL) and H_2O (10 mL) at 0 ºC, and then I_2 (2.77 g, 10.9 mmol) was added portionwise. After stirring the reaction mixture for 30 min, it was diluted with ethyl acetate (100 mL), washed with aqueous NaS_2O_3 (2 x 50 mL, 15%), saturated aqueous $NaHCO_3$ (2 x 50 mL) and brine (2 x 50 mL), successively. The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/1-3/1, v/v) to afford the lactol as a colorless oil (2.18 g, 75%). R_f = 0.65 (hexane/ethyl acetate, 2/1, v/v); HR MS (m/z) calcd for $C_{23}H_{28}O_6[M+Na]^+$, 423.1778; found, 423.2083. The above obtained lactol (2.00 g, 5.00 mmol) was dissolved in ethanol (30 mL), and then $NaBH_4$ (285 mg, 7.50 mmol) was added portionwise. After stirring the reaction mixture for 10 h, it was cooled (0 ºC), quenched with acetic acid (15 mL), and diluted with ethyl acetate (80 mL). The solution was washed with saturated aqueous $NaHCO_3$ (2 x 50 mL) and brine (2 x 40 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/2-3/2, v/v) to afford **15** as a white solid (1.89 g, 94%). R_f = 0.45 (hexane/ethyl acetate, 3/2, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39-7.22 (m, 10H, aromatic), 4.73 (d, 1H, *J* = 11.7 Hz, C*H*$_2$ of Bn), 4.58-4.54 (m, 3H, CH_2 of Bn), 4.45 (dd, 1H, *J* = 1.5 Hz, *J* = 6.9 Hz, H-3), 4.22 (m, 1H, H-2), 3.86-3.71 (m, 5H, 2 x H-1, H-4, H-5, H-6b); 3.63 (dd, 1H, *J* = 3.9 Hz, *J* = 8.1 Hz, H-6a), 1.56 (s, 3H, C*H*$_3$), 1.38 (s, 3H, C*H*$_3$). HR MS (m/z) calcd for $C_{23}H_{30}O_6[M+Na]^+$, 425.1935; found, 425.1886.

5,6-di-*O*-benzyl-2,3-di-*O*-isopropylidene-1,4-di-*O*-sulfate-D-mannitol (**16**): To a cooled (-15 ºC) solution of **15** (1.44 g, 3.53 mmol) and Et_3N (2.0 mL, 14.2 mmol) in DCM (20 mL) was added dropwise thionyl chloride (387 µl, 5.30 mmol). After stirring the reaction mixture for 30 min, it was diluted with DCM (30 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 40 mL). The organic phase was allowed to pass through a pad of silica gel, which was then eluted with ethyl acetate (50 mL). The combined eluents were concentrated *in vacuo* to afford the crude cyclic sulfite as a slightly colored oil. The above obtained crude product was dissolved in a mixture of DCM (10 mL) and acetonitrile (10 mL), and then $RuCl_3 \cdot H_2O$ (14.7 mg, 71 µmol), $NaIO_4$ (1.13 g, 5.30 mmol) and H_2O (15 mL) were added, successively. After stirring the reaction mixture for 20 min, it was diluted with ethyl acetate (40 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 40 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 10/1, v/v) to afford **16** as a white solid (1.89 g, 94%). R_f = 0.45 (hexane/ethyl acetate, 4/1, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40-7.22 (m, 10H, aromatic), 4.99 (d, $J_{4,5}$ = 9.0 Hz, H-4), 4.77-4.72 (m, 2H, H-3, CH_2 of Bn), 4.66 (d, 1H, J = 12.0 Hz, CH_2 of Bn), 4.59 (d, 1H, J = 11.4 Hz, CH_2 of Bn), 4.53 (d, 1H, J = 12.0 Hz, CH_2 of Bn), 4.43-4.26 (m, 3H, H-1, H-2); 3.95 (ddd, 1H, J = 1.8 Hz, J = 3.9 Hz, J = 9.0 Hz, H-5), 3.82 (dd, 1H, $J_{5,6a}$ = 1.8 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.82 (dd, 1H, $J_{5,6b}$ = 3.9 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6b),1.54 (s, 3H, CH_3), 1.48 (s, 3H, CH_3). HR MS (m/z) calcd for $C_{23}H_{28}O_8S[M+Na]^+$, 487.1397; found, 487.1464.

Benzyl 2-deoxy-4,5-di-*O*-isopropylidene-7,8-di-*O*-benzyl-D-glycero-D-galacto-octulosonate 1,3-propylene dithioacetal (**18**): To a cooled solution (-45 ºC) of **17** (330 mg, 1.3 mmol) in a mixture of THF (2 mL) and HMPA (0.8 mL) was added BuLi (2.5 M in hexane, 0.56 mL, 1.4

mmol). The reaction mixture was stirred for 2 h, after which a solution of **16** (470 mg, 1.0 mmol) in THF (1 mL) was added dropwise. The stirring continued at room temperature for another 2 h till TLC analysis showed compound **16** nearly completely disappeared. Then, the reaction mixture was first neutralized with sulfuric acid (1 M in THF, 1 mL) followed by the addition of H_2O (15 μl), after which another portion of sulfuric acid (1 M in THF, 1 mL) was added till pH 3. After heating the mixture (50 ºC) for 1h, it was cooled (25 ºC), diluted with ethyl acetate (30 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: toluene/ethyl acetate, 30/1, v/v) to afford **19** as a colorless oil (510 mg, 78%). R_f = 0.55 (hexane/ethyl acetate, 3/1, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40-7.21 (m, 15H, aromatic), 5.24 (d, J = 12.6 Hz, CH_2 of COOBn), 5.14 (d, J = 12.6 Hz, CH_2 of COOBn), 4.74 (d, J = 11.4 Hz, CH_2 of Bn), 4.63-4.53 (m, 4H, H-4, CH_2 x 3 of Bn), 4.39 (dd, 1H, J = 1.2 Hz, J = 6.9 Hz, H-5), 3.85 (dd, 1H, J = 3.0 Hz, J = 10.5 Hz, H-8a), 3.75-3.67 (m, 2H, H-6, 8b), 3.60-3.54 (m, 1H, H-7), 3.25 (ddd, 1H, J = 2.7 Hz, J = 14.6 Hz, CH_{2axi} of SCH_2), 3.06 (ddd, 1H, J = 2.4 Hz, J = 14.6 Hz, CH'_{2axi} of SCH'_2), 2.75-2.60 (m, 3H, H-3a, CH_{2equo} of SCH_2, CH'_{2equo} of SCH'_2), 2.43 (dd, 1H, $J_{3a,3b}$ = 15.0 Hz, $J_{3b,4}$ = 3.0 Hz, H-3b), 2.09-2.03 (m, 1H,) 1.92-1.78 (m, 1H), 1.39 (s, 3H, CH_3 of isopropylidine), 1.29 (s, 3H, CH_3 of isopropylidine). HR MS (m/z) calcd for $C_{32}H_{42}O_7S_2[M+Na]^+$, 661.2264; found, 661.2397.

.**Benzyl 3-deoxy-4,5-di-*O*-isopropylidene-7,8-di-*O*-benzyl-α,β-D-manno-2-octulopyranosonate** (**19**): To a stirred suspension of **18** (1.06 g, 1.66 mmol) and $NaHCO_3$ (1 g, 11.9 mmol) in a mixture of CH_3COCH_3 (20 mL) and H_2O (1 mL) was added NBS (1.77 g, 9.96 mmol) at 0 ºC. After stirring the reaction mixture for 10 min, it was quenched with aqueous $Na_2S_2O_3$ (15%, 100 mL), diluted with ethyl acetate (50 mL), and then washed with saturated

aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 40 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 6/1-4/1, v/v) to afford **18** as a colorless oil (1.89 g, 94%). R_f = 0.35 (hexane/ethyl acetate, 4/1, v/v). HR MS (m/z) calcd for $C_{32}H_{36}O_8$ $[M+Na]^+$, 571.2302; found, 571.3219.

Benzyl 3-deoxy-4,5-di-*O*-isopropylidene-7,8-di-*O*-benzyl-α,β-D-manno-2-octulopyranosyl fluoride (**20**): A suspension of **19** (700 mg, 1.28 mmol) and molecular sieves (4 Å, 100 mg) in DCM (6 mL) was stirred at room temperature for 1h. The mixture was cooled (-60 ºC) and then DAST (220 µl, 1.66 mmol) was added dropwise. After stirring the reaction mixture at room temperature for 30 min, it was cooled (-30 ºC) and then quenched by stirring with acetic acid (150 µl) for 2 min. Then, the solids were removed by filtration, and the filtrate was washed with saturated aqueous $NaHCO_3$ (2 x 40 mL) and brine (2 x 40 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 6/1, v/v) to afford a mixture (631 mg) of **20** (75%) and its elimination product (15%). R_f = 0.60 (hexane/ethyl acetate, 5/1, v/v). HR MS (m/z) calcd for $C_{32}H_{35}FO_7$ $[M+Na]^+$, 573.2259; found, 573.2516.

Dimethylthexylsilyl benzyl (7,8-di-O-benzyl-3-deoxy-4,5-O-isopropylidene-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyl-(1→6)-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (21): A suspension of 12 (610 mg, 0.51 mmol), 20 (495 mg, 0.91 mmol) and molecular sieves (4 Å, 400 mg) in DCM (8 mL) was sitrred at room temperature for 1h. The mixture was cooled (0 ºC) and then $BF_3{\cdot}Et_2O$ (77 µl, 0.61 mmol) was added dropwise. After stirring the reaction mixture for 1 h, it was quenched with solid $NaHCO_3$

(100 mg) and diluted with DCM (20 mL). The solids were removed by filtration, and the filtrate was washed with saturated aqueous $NaHCO_3$ (2 x 25 mL) and brine (2 x 25 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 6/1-4/1, v/v) to afford **21** as a colorless solid (590 mg, 67%). R_f = 0.45 (hexane/ethyl acetate, 3/1, v/v); $[\alpha]^{24}_D$ = -4.9º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, CD_3COCD_3): δ 7.84-7.18 (m, 33H, aromatic), 6.62 (d, 1H, $J_{NH',2}$ = 9.0 Hz, N*H'*), 5.89-5.82 (m, 1H, OCH_2CH=CH_2), 5.30-23 (m, 2H, $COOCH_{2a}Ph$, OCH_2CH=CH_{2a}), 5.20-5.12 (m, 3H, H-3', $COOCH_{2b}Ph$, OCH_2CH=CH_{2b}), 4.84-4.49 (m, 12H, H-1, 1', 3, CH_2Ph x 7, OCH_2CH=CH_2), 4.43-4.39 (m, 3H, H-4", 5", CH_2Ph), 4.23-4.18 (m, 1H, OCH_{2a} of Fmoc), 4.17-4.10 (m, 3H, H-6a, OCH_{2b}, OCH_2CH of Fmoc), 4.00-3.91 (m, 3H, H-6", 7", 8a"), 3.84 (dd, 1H, $J_{5',6a'}$ = 5.4 Hz, $J_{6a',6b'}$ = 10.8 Hz, H-6a'), 3.78-3.77 (m, 2H, H-3_S, 8b"), 3.73-3.70 (m, 3H, H-4, 5, 6b), 3.67-3.65 (m, 2H, H-2', 6b'), 3.48 (bs, 2H, H-4', 5'), 3.81 (dd, 1H, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 2.62-2.54 (m 2H, H-3_a", 2_{Sa}), 2.39 (dd, $J_{2Sa,2Sb}$ = 15.6 Hz, $J_{2Sb,3S}$ = 5.4 Hz, H-2_{Sb}), 1.97 (dd, 1H, $J_{3a",3b"}$ = 15.0 Hz, $J_{3b", 4"}$ = 2.4 Hz, H-3b"), 1.68-1.62 (m 1H, $CH(CH_3)_2$), 1.43-1.39 (m, 2H, H-4_S), 1.31 (s, 3H, CH_3 of isopropylidiene), 1.27 (s, 3H, CH_3 of isopropylidiene), 1.16-1.08 (m, 14H, H-(5_S-11_S)), 0.88-0.83 (m, 15H, H-12_S, $SiC(CH_3)CH(CH_3)$), 0.22 (s, 3H, $Si(CH_3)$), 0.21 (s, 3H, $Si(CH_3)$). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 171.76 (*C*=O), 168.49 (*C*=O), 156.46 (*C*=O), 154.85 (*C*=O), 144.81-120.48 (m, OCH_2CH=CH_2, aromatic), 118.59 (OCH_2CH=CH_2), 109.11 (C-2"), 102.02 (C-1'), 98.34 ($C(CH_3)_2$ of isopropylidiene), 97.00 (C-1), 79.20 (C-3), 77.87 (C-7"), 76.99 (C-4), 76.48 (C-3'), 76.07 (C-3_S), 75.29 (C-5'), 74.99 (CH_2Ph), 74.85 (C-5), 73.54 (CH_2Ph), 73.47 (CH_2Ph), 72.32 (C-4"), 71.74 (C-6), 71.41(C-8", CH_2Ph), 70.71 (C-5"), 70.21 (C-6"), 69.72 (C-4'), 68.90 (OCH_2CH=CH_2), 68.73 (C-6), 67.11 (C-2, $COOCH_2Ph$), 67.00 (OCH_2 of Fmoc), 63.30 (C-6'), 56.74 (C-2'), 47.64 (OCH_2CH of Fmoc), 39.96 (C-2_S), -1.72

(Si*C*H_3), -3.29 (Si*C*H_3). HR MS (m/z) calcd for $C_{99}H_{126}N_4O_{20}Si[M+Na]^+$, 1745.8218; found, 1745.9780.

Dimethylthexylsilyl benzyl (7,8-di-O-benzyl-3-deoxy-4,5-O-isopropylidene-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-O-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-2-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyl-(1→6)-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (22): 1*H*-tetrazole (3% wt, 10.0 mmol) in DCM (2.5 ml) was added to a solution of compound **21** (480 mg, 0.28 mmol) and *N,N*-diethyl-1,5-dihydro-3H-2,4,3-benzodioxaphosphepin-3-amine (133 mg, 0.56 mmol) in DCM (8 mL). After the reaction mixture was stirred at room temperature for 40 min, it was cooled (-20 ºC), stirred for another 10 min and then 3-chloroperoxybenzoic acid (mCPBA) (500 mg, 50-55% wt, 1.12 mmol) was added. The reaction mixture was stirred for 20 min at -20 ºC, and then quenched by the addition of saturated aqueous $NaHCO_3$ (20 ml) and diluted with DCM (20 ml). The solution was washed with saturated aqueous $NaHCO_3$ (2 x 30 ml) and brine (2 x 20 ml). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/1, v/v) to give **22** as a white solid (470 mg, 88%). R_f = 0.45 (hexane/ethyl acetate, 3/1, v/v); $[\alpha]^{24}_D$ = +6.0º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, CD_3COCD_3): δ 7.85-7.20 (m, 35H, aromatic), 6.84-6.74 (m, 3H, aromatic x 2, N*H*'), 6.62 (d, 1H, $J_{NH',2}$ = 9.0 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'), 5.32-5.23 (m, 3H, $COOCH_2Ph$, OCH_2CH=CH_{2a}), 5.18(dd, 1H, *J* = 1.2 Hz, *J* = 10.8 Hz, OCH_2CH=CH_{2b}), 5.08-4.89 (m, 5H, H-1', 3, CH_2Ph x 3), 4.85-4.79 (m, 3H, H-1, CH_2Ph x 2), 4.71-4.63 (m, 4H, H-4', 4", CH_2Ph x 2), 4.61-4.56 (m, 6H, CH_2Ph x 4, OCH_2CH=CH_2), 4.46-4.43 (m, 2H, H-5", CH_2Ph), 4.20-4.16 (m, 3H, OCH_2CH of Fmoc), 4.10-4.07 (m, 2H, H-6a, 6"),

4.02-4.01 (m, 2H, H-7", 8"), 3.93 (dd, 1H, $J_{5',6a'}$ = 4.8 Hz, $J_{6a',6b'}$ = 11.4 Hz, H-6a'), 3.85 (dd, 1H, $J_{7",8a"}$ = 4.8 Hz, $J_{8a",8b"}$ = 10.8 Hz, H-8a"), 3.81-3.77 (m, 4H, H-5, 6a, 6b', 3_S), 3.67-3.60 (m, 3H, H-2', 4, 5'), 3.35 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.2 Hz, H-2), 2.70 (dd, $J_{2Sa,2Sb}$ = 16.8 Hz, $J_{2Sb,3S}$ = 6.6 Hz, H-2_{Sb}), 2.53 (dd, $J_{2Sa,2Sb}$ = 16.8 Hz, $J_{2Sb,3S}$ = 4.8 Hz, H-2_{Sb}), 2.26 (dd, 1H, $J_{3a",3b"}$ = 14.4 Hz, $J_{3a",4"}$ = 7.2 Hz, H-3a"), 2.12 (dd, 1H, $J_{3a",3b"}$ = 14.4 Hz, $J_{3b",4"}$ = 4.8 Hz, H-3b"), 168-1.63 (m 1H, CH(CH_3)$_2$), 1.47-1.43 (m, 2H, H-4_S), 1.37 (s, 3H, *CH*$_3$ of isopropylidiene), 1.31 (s, 3H, *CH*$_3$ of isopropylidiene), 1.16-1.08 (m, 14H, H-(5_S-11_S)), 0.8890.83 (m, 15H, H-12_S, SiC(CH_3)CH(CH_3)), 0.24 (s, 3H, Si(CH_3)), 0.23 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 171.78 (*C*=O), 168.35 (*C*=O), 156.47 (*C*=O), 155.03 (*C*=O), 144.93-120.62 (m, $OCH_2CH=CH_2$, aromatic), 118.71 ($OCH_2CH=CH_2$), 109.06 (C-2"), 102.03 (C-1'), 99.13 (*C*(CH_3)$_2$ of isopropylidiene), 97.02 (C-1), 79.25 (C-3), 78.01 (C-7"), 77.49 (C-4), 75.91 (C-5 or 3_S), 74.96, 74.90 (C-5 or 3_S, *C*H_2Ph), 74.68 (C-4'), 73.97 (C-3'), 73.76 (*C*H_2Ph), 73.42 (C-5', *C*H_2Ph), 71.80 (C-5"), 71.65 (*C*H_2Ph), 70.56 (C-4"), 70.35 (C-8"), 69.54 (C-6), 69.19 (C-6"), 69.03 (O*C*H_2CH=CH_2), 68.82 ((O*C*H_2)$_2$Ph x 2), 67.43(COO*C*H_2Ph), 67.34 (C-2), 67.25 (O*C*H_2 of Fmoc), 62.91 (C-6'), 57.32 (C-2'), 47.77 (O*C*H_2CH of Fmoc), 39.78 (C-2_S), 34.55 (C-3"), -1.61 (Si*C*H_3), -3.18 (Si*C*H_3). HR MS (m/z) calcd for $C_{105}H_{129}N_4O_{25}Si[M+Na]^+$, 1927.8350; found, 1927.8330.

Dimethylthexylsilyl benzyl (7,8-di-O-benzyl-3-deoxy-4,5-O-isopropylidene-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-O-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl-(1→6)-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (24): DBU (100 μl) was added dropwise to a stirred solution of **22** (300 mg, 0.16 mmol) in DCM (5 mL). The reaction mixture was stirred at room temperature for 30 min,

and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/2, v/v) to yield the amine intermediate as a pale yellow oil (250 mg, 94%). R_f = 0.25 (hexane/ethyl acetate, 5/2, v/v); A reaction mixture of **23** (95 mg, 0.22 mmol) and DCC (62 mg, 0.30 mmol) in DCM (3 mL) was stirred at room temperature for 10 min, and then the above obtained amine (250 mg, 0.15 mmol) 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 x 0.5 mL). The combine filtrates were concentrated in vacuo, and the residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/1, v/v) to yield **24** as a white solid (280 mg, 89%). R_f = 0.55 (hexane/ethyl acetate, 5/2, v/v); $[\alpha]^{25}_{D}$ = +7.1º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.36-7.07 (m, 27H, aromatic), 6.69 (d, *J* = 7.2 Hz, aromatic), 6.51 (d, *J* = 7.2 Hz, aromatic), 5.90-5.84 (m, 1H, OCH_2CH=CH_2), 5.62 (d, 1H, $J_{NH',2}$ = 7.2 Hz, N*H'*), 5.53 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 9.6 Hz, H-3'), 5.20 (d, 1H, *J* = 17.4 Hz, OCH_2CH=CH_{2a}), 5.23-5.16 (m, 3H, $COOCH_2Ph$, OCH_2CH=CH_{2b}), 5.03-5.16 (m, 3H, H-1', 3_L, CH_2Ph), 4.89-4.81 (m, 2H, H-3, CH_2Ph), 4.75-4.71 (m, 2H, H-3, CH_2Ph), 4.68-4.64 (m, 4H, H-4', CH_2Ph x 3), 4.61-4.57 (m, 4H, H-1, 4", OCH_2CH=CH_2), 4.57-4.47 (m, 6H, CH_2Ph x 6), 4.38 (bs, 1H, H-5"), 4.04 (bs, 1H, H-7"), 3.99 (d, 1H, *J* = 10.2 Hz, H-6"), 3.89-3.84 (m, 2H, H-8a", 3_S), 3.80-3.74 (m, 3H, H-6a, 6a', 8a"), 3.63 (d, 1H, $J_{6a',6b'}$ = 10.8 Hz, H-6b'), 3.59-3.54 (m, 2H, H-5, 6b), 3.80 (dd, 1H, *J* = 4.8 Hz, *J* = 9.6 Hz, H5'), 3.33 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, H-4), 3.26 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.2 Hz, H-2), 3.11 (dd, 1H, *J* = 7.8 Hz, H-2'), 2.69-2.63 (m, 2H, H-2_S), 2.32-2.20 (m, 3H, H-2_L, $2_{L'}$), 2.18 (dd, 1H, $J_{3a",3b"}$ = 14.4 Hz, $J_{3a", 4"}$ = 6.6 Hz, H-3a"), 2.10 (dd, 1H, $J_{3a",3b"}$ = 14.4 Hz, $J_{3b", 4"}$ = 5.4 Hz, H-3b"), 2.05 (dd, $J_{2La,2Lb}$ = 15.0 Hz, $J_{2Lb,3L}$ = 5.4 Hz, H-2_{Lb}), 1.66-1.46 (m, 7H, H-4_S, 4_L, $3_{L'}$, $CH(CH_3)_2$), 1.38 (s, 3H, CH_3 of isopropylidiene), 1.31 (s, 3H, CH_3 of isopropylidiene), 1.22 (bs, 48H, H-(5_S-11_S), H-(5_L-13_L), H-($4_{L'}$-$11_{L'}$)), 0.86-0.84 (m,

21H, H-12$_S$, 14$_L$, 12$_{L'}$, SiC(C*H*$_3$)$_2$CH(C*H*$_3$)$_2$), 0.16 (s, 6H, Si(C*H*$_3$)). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 173.59 (*C*=O), 171.06 (*C*=O), 169.98 (*C*=O), 167.78 (*C*=O), 154.35 (*C*=O), 138.84-127.46 (m, OCH$_2$*C*H=CH$_2$, aromatic), 119.17 (OCH$_2$CH=*C*H$_2$), 108.66 (C-2"), 99.89 (C-1'), 98.58 (*C*(CH$_3$)$_2$ of isopropylidiene), 96.48 (C-1), 78.60(C-3), 77.00 (C-7"), 76.87 (C-4), 75.54 (C-3$_S$), 74.46 (*C*H$_2$Ph), 74.12 (C-4'), 73.84 (C-5), 73.35 (*C*H$_2$Ph), 73.19 (*C*H$_2$Ph), 72.56 (C-5'), 71.72 (C-3'), 71.16 (*C*H$_2$Ph), 70.83 (C-5"), 70.74 (C-3$_L$), 69.79 (C-4", 8"), 68.83 (C-6, OCH$_2$CH=CH$_2$), 68.44 (C-6", *C*H$_2$Ph), 68.71 (*C*H$_2$Ph), 66.97 (COO*C*H$_2$Ph), 66.62 (C-2), 61.81 (C-6'), 56.76 (C-2'), 41.62 (C-2$_L$), 38.91 (C-2$_S$), -1.86 (Si*C*H$_3$), -3.41 (Si*C*H$_3$). HR MS (m/z) calcd for $C_{105}H_{129}N_4O_{25}Si[M+Na]^+$, 2114.1273; found, 2114.2964.

Dimethylthexylsilyl benzyl (7,8-di-O-benzyl-3-deoxy-4,5-O-isopropylidene-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-O-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl-(1→6)-2-azido-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-β-D-glucopyranoside (25): Tetrakis(triphenylphosphine)palladium (32.5 mg, 0.028 mmol) was added to a solution of **24** (295 mg, 0.141 mmol), n-BuNH$_2$ (28 μL, 0.28 mmol), and HCOOH (11 μL, 0.28 mmol) in THF (5 mL). After stirring the reaction mixture 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 x 20 mL) and brine (2 x 20 mL). The organic phase was dried (MgSO$_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to give the alcohol intermediate as a colorless syrup (268 mg, 95%)**.** R_f = 0.45 (eluent: hexane/ethyl acetate, 3/1, v/v); ^{1}H NMR (500 MHz, CDCl3): δ 7.43-7.14 (m, 27H, aromatic), 6.75 (d, *J* = 7.5 Hz, aromatic), 6.65 (d, *J* = 7.0 Hz, aromatic), 5.68 (d, 1H, $J_{NH',2}$ = 8.0 Hz, N*H'*), 5.53 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 10.0 Hz, H-3'), 5.23 (d, 1H, *J* = 12.5 Hz,

COOCH_{2a}Ph), 5.18 (d, 1H, J = 12.5, COOCH_{2b}Ph), 5.10-4.92 (m, 4H, H-1', 3_L, CH_2Ph x 2), 4.85-4.50 (m, 13H, H-1, 4', 4", CH_2Ph x 10), 4.41 (bs, 1H, H-5"), 4.10-4.08 (m, 1H, H-7"), 4.02 (d, 1H, J = 9.5 Hz, H-6"), 3.95-3.89 (m, 3H, H-6a', 8a", 3_S), 3.83-3.79 (m, 2H, H-6a, 8b"), 3.73 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6b), 3.67 (dd, 1H, $J_{5',6b'}$ = 5.5 Hz, $J_{a',b'}$ = 11.0 Hz, H-6b'), 3.48-3.42 (m, 3H, H-3, 5, 5'), 3.33-3.24 (m, 2H, H-2', 4), 3.15 (dd, 1H, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 2.76-2.67 (m, 2H, H-2_S), 2.37-2.27 (m, 4H, H-3a", 2_{La}, $2_{L'}$ x 2), 2.15-2.08 (2H, H-3b", 2_{Lb}), 1.68-1.53 (m, 7H, H-4_S, H-4_L, $3_{L'}$, CH(CH_3)$_2$), 1.42 (s, 3H, CH_3 of isopropylidiene), 1.36 (s, 3H, CH_3 of isopropylidiene), 1.28 (bs, 48H, H-(5_S-11_S), H-(5_L-13_L), H-($4_{L'}$-$11_{L'}$)), 0.91-0.90 (m, 21H, H-12_S, 14_L, $12_{L'}$, SiC(CH_3)$_2$CH(CH_3)$_2$), 0.20 (s, 6H, Si(CH_3)). HR MS (m/z) calcd for $C_{112}H_{163}N_4O_{24}Si[M+Na]^+$, 2030.1062; found, 2030.4662. A solution of (*R*)-3-benzyloxy-dodecanoic acid **7** (72 mg, 0.234 mmol) and DCC (72 mg, 0.351 mmol) in DCM (5 mL) was stirrd at room temperature for 10 min, and then the above obtained intermediate (235 mg, 0.117 mmol) and DMAP (7 mg, 0.06 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 x 2 mL). The combined filtrates were concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/1-4/1, v/v) to afford **25** as a white solid (228 mg, 84%). R_f = 0.60 (hexane/ethyl acetate, 3/1, v/v); $[\alpha]^{25}_D$ = +7.9º (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, CDCl3): δ 7.42-7.12 (m, 32H, aromatic), 6.74 (d, J = 7.5 Hz, aromatic), 6.56 (d, J = 7.0 Hz, aromatic), 5.65 (d, 1H, $J_{NH',2}$ = 7.5 Hz, NH'), 5.58 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 9.5 Hz, H-3'), 5.25 (s, 2H, COOCH_2Ph), 5.16 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.10-00 (m, 2H, H-3_L, CH_2Ph), 4.98 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.92 (dd, 1H, J = 11.0 Hz, J = 14.0 Hz, CH_2Ph), 4.81-4.47 (m, 15H, H-1, 4', 4", CH_2Ph x 12), 4.44 (bs, 1H, H-5"), 4.12-4.10 (m, 1H, H-7"), 4.05 (d, 1H, J = 9.5 Hz, H-6"), 3.96-3.89 (m, 3H, H-8a", 2 x 3_S), 3.85-3.79 (m, 3H, H-6a, 6a', 8b"), 3.68 (d, 1H, $J_{6a',6b'}$ =

11.0 Hz, H-6b'), 3.64-3.57 (m, 2H, H-5, 6b), 3.41 (dd, 1H, J = 3.0 Hz, J = 10.0 Hz, H-5'), 3.33 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.22 (dd, 1H, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 3.18 (dd, 1H, J = 7.5 Hz, H-2'), 2.76-2.68 (m, 2H, H-2_S), 2.63 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sa,3S}$ = 7.0 Hz, H-2_{Sa}), 2.50 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sb,3S}$ = 6.0 Hz, H-2_{Sb}), 2.38-2.26 (m, 3H, H-2_{La}, $2_{L'}$ x 2), 2.22 (dd, 1H, $J_{3a",3b"}$ = 14.0 Hz, $J_{3a", 4"}$ = 7.5 Hz, H-3a"), 2.16 (dd, 1H, $J_{3a",3b"}$ = 14.0 Hz, $J_{3b", 4"}$ = 5.5 Hz, H-3b"), 2.11 (dd, $J_{2La,2Lb}$ = 14.5 Hz, $J_{2Lb,3L}$ = 5.0 Hz, H-2_{Lb}), 1.69-1.54 (m, 9H, 2 x H-4_S, H-4_L, $3_{L'}$, CH(CH_3)$_2$), 1.44 (s, 3H, CH_3 of isopropylidiene), 1.37 (s, 3H, CH_3 of isopropylidiene), 1.22 (bs, 62H, 2 x H-(5_S-11_S), H-(5_L-13_L), H-($4_{L'}$-$11_{L'}$)), 0.92-0.90 (m, 24H, 2 x H-12_S, 14_L, $12_{L'}$, SiC(CH_3)$_2$CH(CH_3)$_2$), 0.22 (s, 6H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.56 (C=O), 171.09 (C=O), 170.58 (C=O), 169.92 (C=O), 167.77 (C=O), 138.84-127.43 (m, aromatic), 108.66 (C-2"), 99.90 (C-1'), 98.60 (C(CH_3)$_2$ of isopropylidiene), 96.40 (C-1), 77.43-76.58 (C-3, 7"), 76.58 (C-4), 75.75 (C-3_S), 75.51 (C-3_S), 74.17 (C-5), 73.94 (C-4'), 73.87 (CH$_2$Ph), 73.36 (CH$_2$Ph), 73.19 (CH$_2$Ph), 72.52 (C-5'), 71.74 (C-3'), 71.47 (CH$_2$Ph), 71.15 (CH$_2$Ph), 70.83 (C-5"), 70.75 (C-3_L), 69.80 (C-4", 8"), 68.95 (C-6), 68.40 (C-6", CH$_2$Ph), 68.09 (CH$_2$Ph), 66.97 (COOCH$_2$Ph), 66.90 (C-2), 61.79 (C-6'), 56.70 (C-2'), 41.64 (C-2_L), 39.72 (C-2_S), 38.92 (C-2_S), -1.84 (SiCH$_3$), -3.42 (SiCH$_3$). HR MS (m/z) calcd for $C_{131}H_{191}N_4O_{26}Si[M+Na]^+$, 2318.3151; found, 2318.5374.

Dimethylthexylsilyl benzyl (7,8-di-O-benzyl-3-deoxy-4,5-O-isopropylidene-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-O-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl-(1→6)-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranoside (26): A suspension of compund **25** (120 mg, 52 μmol) and zinc (210 mg, 3.2 mmol) in a mixture of acetic acid (100 μl) and DCM (5 ml)

was stirred at room temperature for 1 h, after which it was diluted with ethyl acetate (20 mL). The solids were removed by filtration, and the residue was washed with ethyl acetate (2 x 3 ml). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4/1, v/v) to afford the amine as a pale yellow syrup (108 mg, 92%). R_f = 0.45 (hexane/ethyl acetate, 5/2, v/v). A solution of (*R*)-3-dodecanoyloxy-dodecanoic acid **23** (43 mg, 100 μmol) and DCC (31 mg, 150 μmol) in DCM (5 mL) was stirrd at room temperature for 10 min, and then the above obtained intermediate (120 mg, 53 μmol) was added. The reaction mixture was stirred for another 10 h, after which the solids were removed by filtration and washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/1-4/1, v/v) to afford **26** as a white solid (126 mg, 89%).R_f = 0.65 (hexane/ethyl acetate, 5/2, v/v); $[\alpha]^{25}_D$ = +4.3º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.47-7.17 (m, 32H, aromatic), 6.76 (d, *J* = 7.8 Hz, aromatic), 6.66 (d, *J* = 6.0 Hz, aromatic), 5.71-5.69 (m, 2H, N*H*, N*H'*), 5.64 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.30 (d, 1H, *J* = 13.2 Hz, COOCH_{2a}Ph), 5.25 (d, 1H, *J* = 13.2 Hz, COOCH_{2b}Ph), 5.16 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.15 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.13-5.05 (m, 3H, 2 x H-3_L, CH_2Ph), 5.04 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.97 (dd, 1H, *J* = 11.4 Hz, *J* = 13.8 Hz, CH_2Ph), 4.87-4.75 (m, 4H, CH_2Ph x 4), 4.71 (dd, *J* = 9.0 Hz, H-4'), 4.68 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.65-4.46 (m, 10H, H-4", 5", CH_2Ph x 8), 4.15-4.13 (m, 1H, H-7"), 4.06 (d, 1H, *J* = 9.6 Hz, H-6"), 3.99-3.93 (m, 3H, H-2, 8a", 3_S), 3.89-3.84 (m, 4H, H-6a, 6a', 8b", 3_S), 3.66 (d, 1H, $J_{6a',6b'}$ = 10.8 Hz, H-6b'), 3.62-3.56 (m, 2H, H-5, 6b), 3.49 (dd, 1H, *J* = 4.2 Hz, *J* = 10.2 Hz, H-5'), 3.38 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.0 Hz, H-4), 3.19 (dd, 1H, *J* = 7.8 Hz, H-2'), 2.77 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sa,3S}$ = 7.2 Hz, H-2_{Sa}), 2.72

(dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sb,3S}$ = 4.8 Hz, H-2_{Sb}), 2.61 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sa,3S}$ = 7.2 Hz, H-2_{Sa}), 2.49 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sb,3S}$ = 5.4 Hz, H-2_{Sb}), 2.43-2.24 (m, 8H, 2 x H-2La, H-$2L_b$, H-2L' x 4, 3a"), 2.19 (dd, 1H, $J_{3a",3b"}$ = 15.0 Hz, $J_{3b",4"}$ = 5.4 Hz, H-3b"), 2.16 (dd, $J_{2La,2Lb}$ = 15.0 Hz, $J_{2Lb,3L}$ = 6.0 Hz, H-2_{Lb}), 1.68-1.58 (m, 15H, 2 x H-4_S, 2 x H-4_L, 2 x $3_{L'}$, C*H*$(CH_3)_2$), 1.46 (s, 3H, *CH*$_3$ of isopropylidiene), 1.39 (s, 3H, *CH*$_3$ of isopropylidiene), 1.22 (bs, 96H, 2 x H-(5_S-11_S), 2 x H-(5_L-13_L), 2 x H-($4_{L'}$-$11_{L'}$)), 0.96-0.87 (m, 30H, 2 x H-12_S, 2 x 14_L, 2 x $12_{L'}$, SiC(CH_3)$_2$CH(CH_3)$_2$), 0.21 (s, 3H, Si(CH_3)), 0.20 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.60 (*C*=O), 173.54 (*C*=O), 171.62 (*C*=O), 171.06 (*C*=O), 169.95 (*C*=O), 169.05 (*C*=O), 168.04 (*C*=O), 139.00-127.43 (m, aromatic), 108.69 (C-2"), 99.60 (C-1'), 98.43 (*C*$(CH_3)_2$ of isopropylidiene), 85.85 (C-1), 77.26 (C-7"), 76.90 (C-4), 75.50-75.41 (3C, C-3, C-3_S x 2), 74.22-74.08 (4C, C-4', 5, *C*H_2Ph x 2), 73.37 (*C*H_2Ph), 72.52 (C-5'), 71.75 (C-3'), 71.27 (*C*H_2Ph), 71.21 (*C*H_2Ph), 70.98 (C-5"), 70.71 (C-3_L x 2), 70.20 (C-8"), 69.88 (C-4"), 68.85 (C-6), 68.62 (C-6"), 68.41 (*C*H_2Ph), 68.13 (*C*H_2Ph), 66.98 (COO*C*H_2Ph), 61.90 (C-6'), 56.74 (C-2'), 56.20 (C-2), 41.56 (C-2_L), 41.47 (C-2_L), 39.60 (C-2_S), 38.96 (C-2_S), -1.56 (Si*C*H_3), -3.31 (Si*C*H_3). HR MS (m/z) calcd for $C_{157}H_{241}N_2O_{29}Si[M+Na]^+$, 2700.6850; found, 2700.6572.

Benzyl (7,8-di-O-benzyl-3-deoxy-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-O-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl-(1→6)-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranose (27): TFA/H_2O (3/2, v/v, 100 μl) was added dropwise to a stirred solution of **26** (30 mg, 11 μmol) in DCM (2 mL). The reaction mixture was stirred at room temperature for 6 h, after which it was diluted with ethyl acetate (10 mL) and then wahsed with saturated aqueous $NaHCO_3$ (2 x 10 mL) and brine (2 x 10 mL). The organic phase was

dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by preparative silica gel TLC (eluent: hexane/ethyl acetate, 3/1, v/v) to afford **27** as pale yellow syrup (25 mg, 89%). R_f = 0.25 (hexane/acetone, 1/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.42-7.19 (m, 32H, aromatic), 6.82 (d, J = 7.8 Hz, aromatic), 6. 64 (d, J = 6.0Hz, aromatic), 5.91 (d, 1H, $J_{2,NH}$ = 9.6 Hz, N*H*), 5.81 (d, 1H, $J_{2',NH'}$ = 7.8 Hz, N*H'*), 5.44 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.38 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1'), 5.32 (dd, 1H, J = 9.6 Hz, J = 10.2 Hz, H-3), 5.19 (d, 1H, J = 12.6 Hz, COOCH_{2a}Ph), 5.14 (d, 1H, J = 12.6 Hz, COOCH_{2b}Ph), 5.11-5.07 (m, 2H, H-1, H-3_L), 5.01-4.88 (m, 3H, H-3_L, CH_2Ph x 2), 4.83 (dd, 1H, J = 12.2 Hz, J = 13.8 Hz, CH_2Ph), 4.74-4.67 (m, 3H, CH_2Ph x 3), 4.62 (dd, J = 9.0 Hz, H-4'), 4.58-4.41 (m, 10H, CH_2Ph x 7), 4.35-4.31 (m, 2H, H-4", CH_2Ph), 4.12-3.94 (m, 5H, H-2, 5, 5", 6", 7"), 3.86-3.72 (m, 7H, H-6a, 6a', 6b', 8a", 8b", 2 x H-3_S), 3.66 (d, 1H, $J_{5',6b'}$ = 7.2 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6b'), 3.46 (bs, H-5'), 3.24 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.6 Hz, H-4), 3.02 (dd, 1H, J = 7.8 Hz, H-2'), 2.68 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sa,3S}$ = 4.8 Hz, H-2_{Sa}), 2.61 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sb,3S}$ = 6.6 Hz, H-2_{Sb}), 2.55 (dd, 1H, $J_{2Sa,2Sb}$ = 15.6 Hz, $J_{2Sa,3S}$ = 7.8 Hz, H-2_{Sa}), 2.41 (dd, 1H, $J_{2Sa,2Sb}$ = 16.2 Hz, $J_{2Sb,3S}$ = 4.8 Hz, H-2_{Sb}), 2.34-2.22 (m, 7H, 2 x H-2La, H-$2L_b$, H-2L' x 4), 2.18 (dd, 1H, $J_{3a",3b"}$ = 12.6 Hz, $J_{3a",4"}$ = 4.6 Hz, H-3a"), 2.11 (dd, $J_{2La,2Lb}$ = 15.6 Hz, $J_{2Lb,3L}$ = 6.0 Hz, H-2_{Lb}), 1.96 (t, 1H, $J_{3a",3b"} = J_{3b",4"}$ = 12.6 Hz, H-3b"), 1.61-1.48 (m, 14H, 2 x H-4_S, 2 x H-4_L, 2 x $3_{L'}$), 1.22 (bs, 96H, 2 x H-(5_S-11_S), 2 x H-(5_L-13_L), 2 x H-($4_{L'}$-$11_{L'}$)), 0.96-0.87 (m, 18H, 2 x H-12_S, 2 x 14_L, 2 x $12_{L'}$). HR MS (m/z) calcd for $C_{146}H_{219}N_2O_{29}Si[M+Na]^+$, 2518.5359; found, 2518.2606.

(3-deoxy-α-D-*manno*-oct-2-ulopyranosyl)onate-(2→6)-3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranosyl-(1→6)--3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoyl]-β-D-glucopyranose 1, 4'-biphosphate (1) To a cooled (-78 ºC) solution of **27** (23 mg, 9.2 μmol) and tetrabenzyl

diphosphate (10 mg, 18.4 μmol) in THF (2 mL) was added dropwise lithium bis(trimethylsilyl)amide in THF (1.0 M, 15 μL, 15 μmol). The reaction mixture was stirred for 1h, and then allowed to warm up to -20 ºC. After stirring the reaction mixture for 1 h at -20 ºC, it was quenched with saturated aqueous $NaHCO_3$ (10 mL), and diluted with ethyl acetate (15 mL). The organic phase was washed with brine (2 x 15 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by Iatrobeads column chromatography (hexane/ethyl acetate, 3/1 – 2/1 – 1/1-3/4, v/v) to give the phosphoryl intermediate as a colorlesssyrup (19 mg, 75%). R_f = 0.50 (hexane/acetone, 1/1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.14 (m, 42H, aromatic), 6.82 (d, J = 8.0 Hz, aromatic), 6.69 (d, J = 7.5 Hz, aromatic), 6.41 (d, 1H, $J_{2',NH'}$ = 8.5 Hz, N*H'*), 6.04 (d, 1H, $J_{2,NH}$ = 8.5 Hz, N*H*), 5.65 (bs, 1H, H-1), 5.35 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3'), 5.24-4.73 (m, 12H, H-1', 3, CH_2Ph x 5), 4.61-4.36 (m, 11H, H-4', CH_2Ph x 5), 4.38 (d, 1H, J = 11.5 Hz, CH_2Ph), 4.27-4.23 (m, 1H, H-4",), 4.19-4.14 (m, 1H, H-2), 4.09-3.96 (m, 4H, H-5, 5", 6", 7"), 3.91-3.74 (m, 8H, H-6a, 6a', 6b, 6b', 8a", 8b", 2 x H-3_S), 3.53-3.44 (m, 3H, H-2', 4, 5'), 2.70 (dd, 1H, $J_{2Sa,2Sb}$ = 16.5 Hz, $J_{2Sa,3S}$ = 7.0 Hz, H-2_{Sa}), 2.65 (dd, 1H, $J_{2Sa,2Sb}$ = 16.5 Hz, $J_{2Sb,3S}$ = 5.0 Hz, H-2_{Sb}), 2.56 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sa,3S}$ = 7.5 Hz, H-2_{Sa}), 2.48 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sb,3S}$ = 5.0 Hz, H-2_{Sb}), 2.31-2.18 (m, 7H, 2 x H-2La, H-$2L_b$, H-2L' x 4), 2.12 (dd, 1H, $J_{3a",3b"}$ = 11.0 Hz, $J_{3a", 4"}$ = 5.0 Hz, H-3a"), 2.09 (dd, $J_{2La,2Lb}$ = 16.5 Hz, $J_{2Lb,3L}$ = 6.0 Hz, H-2_{Lb}), 2.04 (t, 1H, $J_{3a",3b"} = J_{3b", 4"}$ = 12.5 Hz, H-3b"), 1.61-1.49 (m, 14H, 2 x H-4_S, 2 x H-4_L, 2 x $3_{L'}$), 1.26 (bs, 96H, 2 x H-(5_S-11_S), 2 x H-(5_L-13_L), 2 x H-($4_{L'}$-$11_{L'}$)), 0.90-0.88 (m, 18H, 2 x H-12_S, 2 x 14_L, 2 x $12_{L'}$). HR MS (m/z) calcd for $C_{160}H_{232}N_2O_{32}Si[M+Na]^+$, 2778.5961; found, 2778.9185. A mixture of the above obtained intermediate (9 mg, 3.3 μmol) and Pd black (15.0 mg) in anhydrous THF (3 mL) was shaken under an atmosphere of H_2 (65 psi) at room temperature for 30 h, after which it was neutralized with triethylamine (10 μl), and the catalyst was removed by

filteration and the residue washed with THF (2 x 1 mL). The combined filtrates were concentrated *in vacuo* to afford **1** as a colorless film (5.0 mg, 78%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.24 (m, 1H, H-1), 4.91-4.83 (m, 4H, H-3, 3', 2 x H_{3L}), 4.30 (b, 1H, H-1'), 3.86 (m, 1H, H-2), 3.82-3.45 (m, 11H, H-5, 5', 5'', 6a, 6b, 6'a, 6'b, 6'', 7'', 8''a, 8''b), 3.80 (m, 2H, H-4', 4''), 3.76 (m, 1H, H-3S), 3.72 (m, 1H, H-3S), 3.60 (m, 1H, H-2'), 3.29 (m, 1H, H-4), 2.23-2.07 (m, 8H, 2 x H-2S, 2 x H-2L), 2.04-1.98 (m, 4H, 2 x H-2L'), 1.76 (dd, 1H, $J_{3a",3b"}$ = 15.0 Hz, $J_{3a", 4"}$ = 5.4 Hz, H-3a"), 1.61(t, 1H, $J_{3a",3b"}$ = $J_{3b", 4"}$ = 15.0 Hz, H-3b"), 1.41-1.19 (m, 14H, 2 x H-4_S, 2 x H-4_L, 2 x $3_{L'}$),1.12 (bs, 96H, 2 x H-(5_S-11_S), 2 x H-(5_L-13_L), 2 x H-($4_{L'}$-$11_{L'}$)), 0.91-0.88 (m, 18H, 2 x H-12_S, 2 x 14_L, 2 x $12_{L'}$). HR MS (m/z) (negative) calcd for $C_{160}H_{232}N_2O_{32}Si$, 1933.1838; found, 1932.6287[M], 1954.5441[M+Na-H], 1976.4611[M+2Na-2H].

***t*-Butyldimethylsilyl 6-*O*-{3-*O*-allyloxycarbonyl-6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-2-[(*R*)-3-benzyloxy-dodecanoylamino]-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-deoxy-β-D-glucopyranoside** (**29**): A suspension of **28** (180 mg, 0.111 mmol), zinc (72 mg, 1.11 mmol), and acetic acid (25 μL, 0.444 mmol) in DCM (5mml) was stirred at room temperature for 12 h, after which it was diluted with ethyl acetate (20 mL), the solids removed by filtration and the residue was washed with ethyl acetate (2 x 2 mL). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 15 mL) and brine (2 x 15 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/2, v/v) to afford the amine as a pale yellow syrup (160mg, 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. A reaction mixture of (*R*)-3-dodecanoyl-tetradecanoic acid **23** (31 mg, 73 μmol) and DCC (20 mg, 98 μmol) in DCM (2 mL)

was stirred at room temperature for 10 min, and then the above obtained amine (78 mg, 49 μmol) 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 x 1mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by preparative silica gel TLC (eluent: hexane/ethyl acetate, 5/1, v/v) to give **29** as a white solid (82 mg, 84%). R_f = 0.51(hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D$ = -3.0º (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37-7.18 (m, 19H, aromatic), 5.85 (d, 1H, $J_{NH',2'}$ = 7.5 Hz, N*H*'), 5.86-5.79 (m, 1H, OCH_2CH=CH_2), 5.65 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.45 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 10.0 Hz, H-3'), 5.28 (d, 1H, *J* = 16.0Hz, OCH_2CH=CH_2), 5.16 (d, 1H, *J* = 11.0 Hz, OCH_2CH=CH_2), 5.04-4.92 (m, 8H, H-1', 3, 2 x H-3_L, *o*-$C_6H_4(CH_2O)_2P$), 4.63 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.55-4.35 (m, 9H, H-4', 3 x Ph-CH_2, OCH_2CH=CH_2), 3.92 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.78-3.71 (m, 3H, H-2, 6a', H-3_S), 3.67-3.62 (m, 3H, H-5, 6b, 6_b'), 3.50-3.48 (m, 2H, H-4, 5'), 3.39-3.32 (m, 1H, H-2'), 2.48-2.13 (m, 10H, 2 x H-2_L, H-2_S, 2 x H-$2_{L'}$), 1.63-1.42 (m, 10H, 2xH-4_L, H-4_S, 2xH-$3_{L'}$), 1.26 (broad, 82 H, 41 x CH_2), 0.90-0.86 (m, 24H, 5xCH_3, $SiC(CH_3)_3$). 0.11 (s, 3H, $SiCH_3$), 0.09 (s, 3H, $SiCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.66 (C=O), 173.51 (C=O), 170.10 (C=O), 169.19 (C=O), 154.47 (C=O), 138.51-127.48 (aromatic, OCH_2CH=CH_2), 118.78 (OCH_2CH=CH_2), 99.13 (C-1'), 96.11 (C-1), 76.02, 75.41, 74.85, 74.31, 74.17, 73.47, 71.26, 70.99, 70.72, 68.93-68.01 (m), 56.37 (C-2), 56.11 (C-2'), -3.86 ($Si(CH_3)_2$), -5.15 ($Si(CH_3)_2$). HR MS(m/z) for calcd for $C_{115}H_{185}N_2O_{22}PSi[M+Na]^+$, 2028.2824; found, 2028.5043.

***t*-Butyldimethylsilyl 6-*O*-{6-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-2-[(*R*)-3-benzyloxy-dodecanoylamino]-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-deoxy-β-D-glucopyranoside**

(**30**): Tetrakis(triphenylphosphine)palladium (6.9 mg, 6 μmol) was added to a solution of **29** (62 mg, 31 μmol), n-$BuNH_2$ (6.1 μL, 62 μmol), and HCOOH (2.3 μL, 62 μmol) in THF (5 mL). After stirring the reaction mixture at room temperature for 20 min, it was diluted with DCM (10 mL), and washed with water (20 mL), saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 20 mL), successively. The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/3, v/v) to give the alcohol intermediate as a colorless syrup. A solution of (*R*)-3-benzyloxy-dodecanoic acid 7 (14 mg, 47 μmol) and DCC (13 mg, 62 μmol) in DCM (2 mL) was stirrd at room temperature for 10 min, and then the above obtained intermediate and DMAP (1.8 mg, 15 μmol) were added. The reaction mixture was stirred for another 10 h, after which the solids were removed by filtration and washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/1, v/v) to afford **30** as a white solid (49mg, 72%, 2 steps). R_f = 0.45(hexane/ethyl acetate, 2/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.37-7.10 (m, 24H, aromatic), 5.69 (d, 1H, $J_{NH,2}$ = 8.4 Hz, N*H*), 5.63 (d, 1H, $J_{NH',2'}$ = 7.8Hz, N*H'*), 5.59 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.10 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.07 (1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.04-4.85 (m, 6H, 2xH-3_L, *o*-$C_6H_4(CH_2O)_2P$), 4.69 (t, 1H, $J_{1,2}$ = 7.8Hz, H-1), 4.63-4.41 (m, 9H, H-4', 4xPh-CH_2,), 3.97 (d, 1H, $J_{6a,6b}$ = 10.8 Hz, H-6a), 3.88-3.78 (m, 4H, H-2, 5, 2xH-3_S), 3.72-3.67 (m, 3H, H-5', 6b, 6a'), 3.58-3.54 (m, 2H, H-4, 6b'), 3.29-3.25 (m, 1H, H-2'), 2.66-2.01 (m, 12H, 2xH-2_L, 2xH-2_S, 2xH-$2_{L'}$), 1.58-1.54 (m, 12H, 2xH-4_L, 2xH-4_S, 2xH-$3_{L'}$), 1.24 (broad, 96H, 48xCH_2), 0.87-0.84 (m, 27H, 6xCH_3, SiC(CH_3)$_3$), 0.09 (s, 3H, Si(CH_3)), 0.07 (s, 3H, Si(CH_3)). 13C NMR(75 MHz, $CDCl_3$): 173.64 (C=O), 171.63 (C=O), 171.40 (C=O), 169.89 (C=O), 168.15 (C=O), 138.63-127.48 (aromatic), 99.45 (C-1'), 96.16 (C-1), 75.89, 75.57, 75.38, 74.92, 74.38, 74.19, 73.79, 73.50, 71.99, 71.33,

71.28, 70.80, 70.54, 68.93, 68.55, 68.18, 56.26 (C-2'), 56.31 (C-2), -3.81 ($Si(CH_3)_2$), -5.10 ($Si(CH_3)_2$). HR MS (m/z) calcd for $C_{130}H_{209}N_2O_{22}PSi[M+Na]^+$, 2232.4702; found, 2232.7168.

6-*O*-{6-*O*-Benzyl-3-*O*-[(*R*)-3-benzyloxy-dodecanoyl]-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3yl)-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-2-[(*R*)-3-benzyloxy-dodecanoylamino]-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-deoxy-α-D-glucopyranoside (**31**): HF/pyridine (40 μL) was added dropwise to a stirred solution of **30** (31 mg, 14 μmol) in THF (2 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 (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 3/1-4/3, v/v) to give **31** as a white solid (25.8 mg, 88%). R_f = 0.39 (hexane/ethyl acetate, 1/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38-6.81 (m, 24H, aromatic), 5.90 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.83 (d, 1H, $J_{NH',2'}$ = 7.2 Hz, N*H*'), 5.53 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.48 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.34 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.28-5.24 (m, 1H, H-3_L), 5.12-5.10 (m, 2H, H-1, H-3_L), 5.03-4.84 (m, 5H, H-3_L, *o*-$C_6H_4(CH_2O)_2P$), 4.63-4.37 (m, 9H, H-4', 4xPh-CH_2), 4.14-4.11 (m, 1H, H-2), 4.05-4.02 (m, 1H, H-5), 3.88-3.80 (m, 4H, H-6a, 6a', 2xH-3_S), 3.80-3.68 (m, 3H, H-5', 6b', 6b), 3.29 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.6 Hz, H-4), 3.17-3.13 (m, 1H, H-2'), 2.71-2.12 (m, 12H, 2xH-2_L, 2xH-2_S, 2xH-$2_{L'}$), 1.62-1.51(broad, 12H, 2xH-4_L, 2xH-4_S, 2xH-$3_{L'}$), 1.23 (broad, 48H, 96xCH_2), 0.87-0.85 (m, 18H, 6xCH_3). HR MS (m/z) calcd for $C_{124}H_{195}N_2O_{22}PSi[M+Na]^+$, 2118.3837; found, 2118.5320.

6-*O*-{3-*O*-[(*R*)-3-hydroxy-dodecanoyl]-2-deoxy-2-[(*R*)-3-dodecanoyloxy-tetradecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxy-dodecanoylamino]-3-*O*-[(*R*)-3-dodecanoyloxy-dodecanoyl]-2-deoxy-α-D-glucopyranoside 1, 4'-bisphosphate (**2**): To a

cooled (-78 ºC) solution of **31** (14 mg, 6.7 μmol) and tetrabenzyl diphosphate (18 mg, 34 μmol) in anhydrous THF (2 mL) was added dropwise lithium bis(trimethylsilyl)amide in THF (1.0 M, 20 μL, 20 μmol). The reaction mixture was stirred for 1h, and then allowed to warm up to -20 ºC. After stirring the reaction mixture 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 x 15 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by Iatrobeads column chromatography (hexane/ethyl acetate, 5/1 – 3/1 – 1/1, v/v) to give the phosphoryl intermediate as a colorlesssyrup (13 mg, 81%). A reaction mixture of the above obtained intermediate (10 mg, 4.2 μmol) and Pd black (15 mg) in anhydrous THF (5 mL) was shaken under an atmosphere of H_2 (65 psi) at room temperature for 30 h, after which it was neutralized with triethylamine (10 μL), and the catalyst was removed by filtration and the residue was washed with THF (2 x 1mL). The combined filtrates were concentrated *in vacuo* to afford **2** as a colorless film (5.4mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.13 (broad, 1H, H-1), 4.84 (broad, 4H, H-3, 3', 2xH-3_L), 3.86-3.81 (m, 2H, H-2, 4'), 3.74-3.64 (m, H-5, 2xH-3_S), 3.57-3.48 (m, H-2'), 3.16-3.04 (m, 2H, H-4, 5'), 2.33-1.95 (m, 12H, 2xH-2_L, 2xH-2_S, 2xH-$2_{L'}$), 1.27 (broad, 12H, 2xH-4_L, 2xH-4_S, 2xH-3_L), 0.91 (broad, 48H, 96xCH_2), 0.54-0.52 (m, 18H, 6xCH_3). HR MS (m/z) (negative) for $C_{88}H_{166}N_2O_{25}P_2$, 1713.1255; found, 1712.2797[M-H], 1713.2834[M].

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CHAPTER 4

THE INFLUENCE OF THE LONG CHAIN FATTY ACID ON THE ANTAGANISTIC ACTIVITIES OF *RHIZOBIUM SIN*-1 LIPID A [#]

[#]Yanghui Zhang, Margreet A. Wolfert and Geert-Jan Boons*. 2007. *Bioorganic and Medicinal Chemistry.* 15:4800-2812.

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Abstract

The lipid A from nitrogen-fixing bacterial species *R. sin*-1 is structurally unusual due to lack of phosphates and the presence of a 2-aminogluconolactone and a very long chain fatty acid, 27-hydroxyoctacosanoic acid (27OHC28:0), moiety. This structurally unusual lipid A can antagonize TNF-α production by human monocytes induced by *E. coli* LPS. To establish the relevance of the unusual long chain 27-hydroxyoctacosanoic acid for antagonistic properties, a highly convergent strategy for the synthesis of several derivatives of the lipid A of *Rhizobium sin*-1 has been developed. Compound 1 is a natural *R. sin*-1 lipid A having a 27-hydroxyoctacosanoic acid at C-2', compound 2 contains an octacosanoic acid moiety at this position, and compound 3 is modified by a short chain tetradecanoic acid. Cellular activation studies with a human monocytic cell line have shown that the octacosanoic acid is important for optimal antagonistic properties. The hydroxyl of the natural 27-hydroxyoctacosanoic moiety does, however, not account for inhibitory activity. The resulting structure activity relationships are important for the design of compounds for the treatment of septic shock.

Introduction

Septicemia is a serious worldwide health problem associated with mortality rates of 40%-60%. Currently, no effective treatment exists for this life-threatening syndrome other than supportive therapy in an intensive care setting.[1, 2] The development of septicemia is often linked to a systemic inflammatory response to lipopolysaccharide (LPS) in the blood of affected patients.

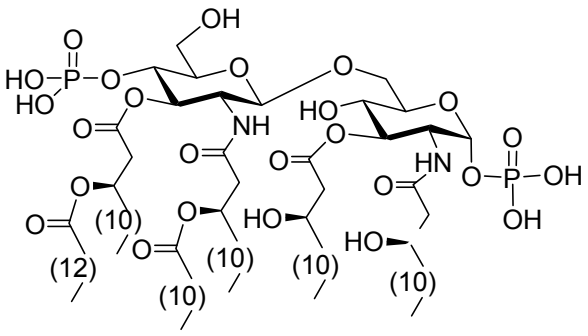
LPS induces cellular responses after binding to the cluster differentiation antigen CD14 on mononuclear phagocytes, or to soluble CD14 in plasma and then to cells lacking CD14.[3-5] As CD14 is a glycosylphosphatidylinositol-anchored protein, it lacks transmembrane and

cytoplasmic domains, and therefore is unable to directly transmit signals to the interior of the cell. The latter function is performed by the Toll-like receptor 4 (TLR4),[6, 7] which contains extracellular transmembrane and intracellular domains, and an accessory protein MD2.[8] While the precise mechanisms involved in the interactions among LPS, CD14, TLR4, and MD2 remain to be discovered,[9, 10] it is clear that cellular activation leads to the induction of cytokine gene expression, primarily through the activation of NF-κB, and the MAP kinases, which results in the biosynthesis of a diverse set of inflammatory mediators such as TNF-α, IL-6 and IL-1β to eradicate the immediate infection. Unfortunately, the presence of a large amount of LPS in the blood can cause the overproduction of the mediators, which can lead to septic inflammatory response syndrome (SIRS) and include life-threatening symptoms such as vascular fluid leakage, tissue damage, hypotension, shock, and organ failure.

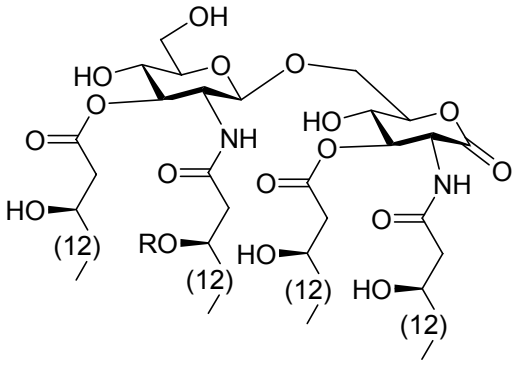
LPS consists of an O-chain polysaccharide, a core oligosaccharide and an amphiphilic moiety referred to as lipid A. The latter moiety has been shown to be the toxic principle of LPS. Lipid A of most enteric bacteria consists of a β(1-6)-linked glucosamine disaccharide backbone with phosphate monoesters at C-1 and C-4' and β-hydroxyl fatty acyl groups and acyloxyacyl residues at C-2, and C-3 and C-2' and C-3', respectively (**Figure 4.1**).[11] Small modifications in the acylation pattern of lipid A are thought to contribute to the virulence of enteric pathogens. For example, fatty acyl components can be present that have shorter chain length, sites of unsaturation or keto functional groups.[12, 13] Other modifications include the addition of a palmitoyl residue, the hydroxylation of a myristoyl substituent and the addition of aminoarabinosyl and phosphoethanolamine moieties.[14]

A number of lipid A derivatives have been shown to lack agonistic properties and instead can inhibit the production of TNF-α induced by enteric LPS.[15-19] Not surprisingly, these compounds

have attracted attention for the treatment of septic shock. For example, the lipid As from *Rhodobacter sphaeroides, R. capsulatus,* and *Helicobacter pylori*, deacylated LPS, and lipid IVa lack toxic properties, but can antagonize cytokine production induced by enteric LPS. Furthermore, chemical synthesis of lipid A analogs patterned after *R. sphaeroides / R. capsulatus* lipid A have been reported. A number of these compounds could prevent the pyrogenic effects of enteric LPS in rabbits,[20] protects against LPS-induced lethality in mice,[19] and blocks TLR4-mediated NF-kB activation by LPS.[21]



E. coli lipid A



1 R = $C(O)(CH_2)_{25}CH(OH)CH_3$
2 R = $C(O)(CH_2)_{26}CH_3$
3 R = $C(O)(CH_2)_{12}CH_3$

Figure 4.1 Structures of *E. coli* lipid A and target *R. sin-1* lipid A derivatives **1**-**3**.

We have identified naturally occurring LPS from *Rhizobium sin*-1, a nitrogen-fixing bacterial species, that does not stimulate TNF-α production by human monocytes,[22-24] and that prevents the induction of TNF-α by *E. coli* LPS. The lipid A of *R. sin*-1 is perhaps the most structurally unusual lipid A reported to date, its structure (**Figure 4.1**; *e.a.* compound **1**) differing in almost every aspect from those known to contribute to the toxicity of enteric lipid A.[25] In particular, the disaccharide moiety of *Rhizobial* lipid A is devoid of phosphate and the glucosamine phosphate is replaced by 2-aminogluconolactone. Furthermore, it contains a very long chain fatty acid, 27-hydroxyoctacosanoic acid (27OHC28:0).

Previously reported phosphate-containing lipid A antagonists are metabolically labile. Hence, it is attractive to study the structure-activity relationship of *R. sin*-1 lipid A to identify specific structural features that render *R. sin*-1 lipid A an antagonist. We have already investigated the importance of the lactone moiety of *R. sin*-1 lipid A for antagonistic properties. Here, we report the chemical synthesis of compounds **1**-**3** (**Figure 4.1**) to establish the relevance of the unusual long chain 27-hydroxyoctacosanoic acid for antagonistic properties. Thus, compound **1** is a natural *R. sin-1* lipid A having a 27-hydroxyoctacosanoic acid at C-2', compound **2** contains an octacosanoic acid moiety at this position, and compound **3** is modified by a short chain tetradecanoic acid.

Results and Discussion

Chemical synthesis In first instance, attention was focused on the preparation of compound **3** which is acylated with a simple tetradecanoic acid at the β-hydroxyl of the C-2 acyloxyacyl residue of the distal sugar moiety. It was envisaged that **3** could be obtained by a regio-, chemo-, and stereoselective glycosylation of glycosyl donor **4**[26] with glycosyl acceptor **5**[27, 28] to give disaccharide **11**, which after a number of selective deprotections can be acylated at the C-2, C-2', C-3 and C-3', oxidized to a lactone and deprotected. Thus, an *N*-iodosuccinimide (NIS) /

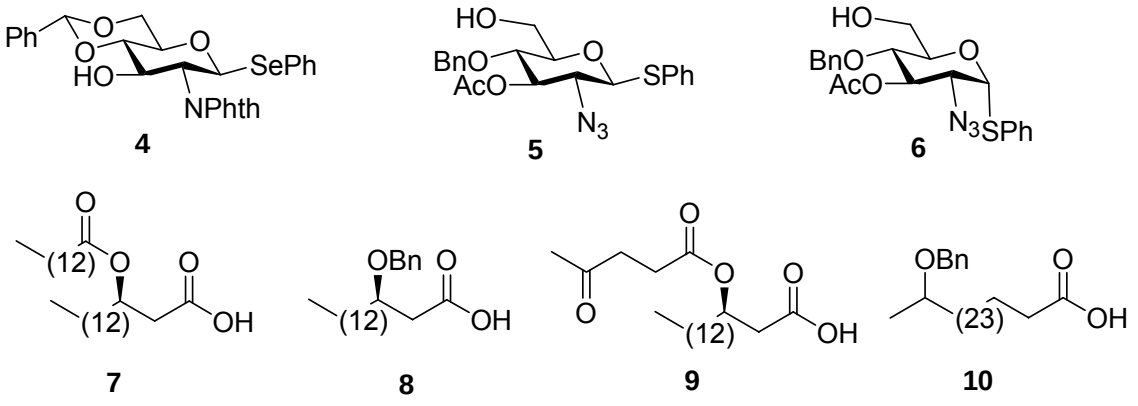
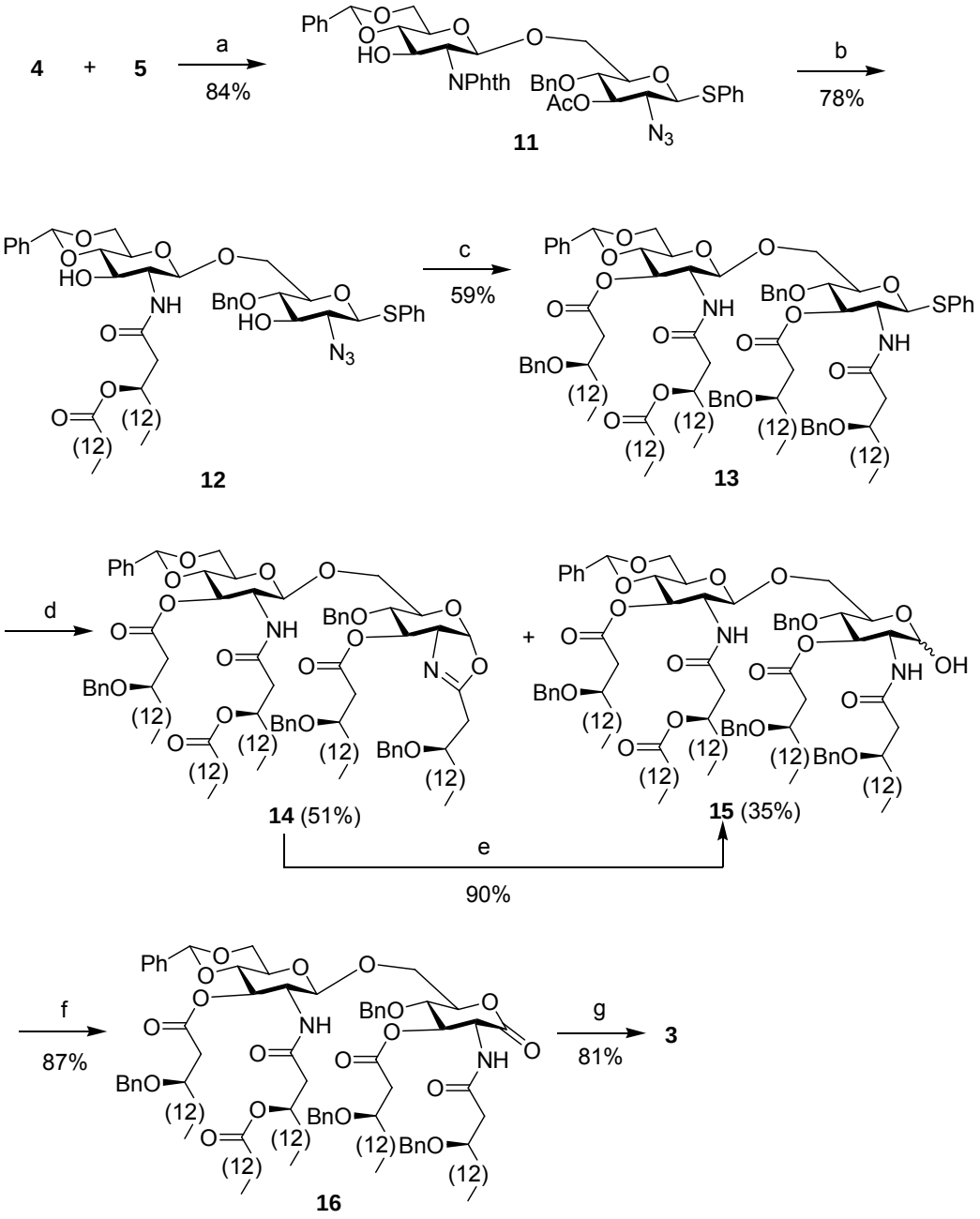


Figure 4.2 Structures of building blocks **4**-**10**.

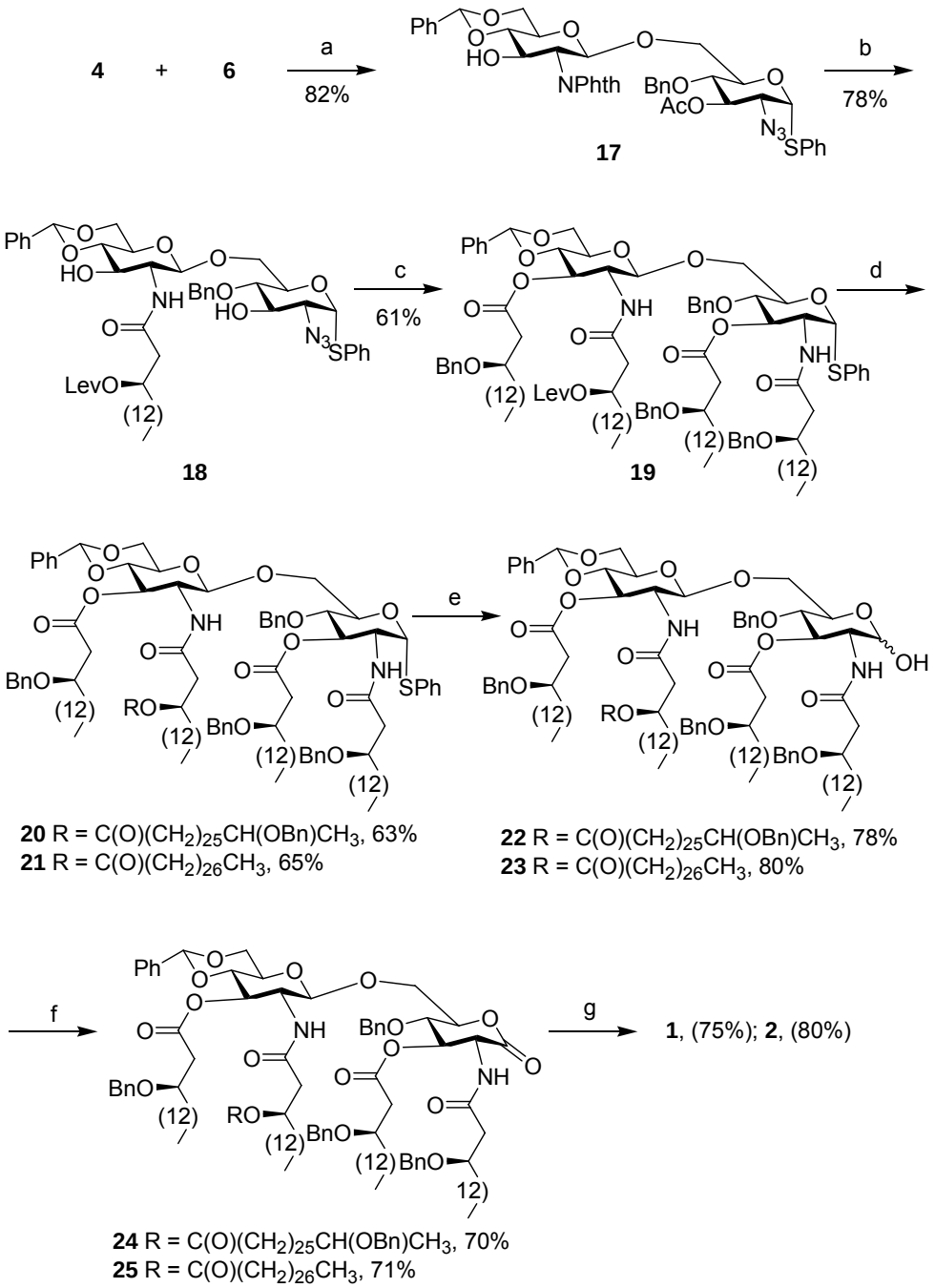
trifluoromethanesulfonic acid (TfOH)[29] mediated coupling of **4** with **5** in dichloromethane (DCM) at -75 ^{o}C gave, after purification by silica gel column chromatography, disaccharide **11** in a yield of 84% (**Figure 4.2** and **Scheme 4.1**). The coupling exploited the higher reactivity of the C-6 hydroxyl of glycosyl acceptor **5** compared to the C-3 hydroxyl of glycosyl donor **4**. Furthermore, the reaction took advantage of the ability to selectively active a selenoglycoside in the presence of a thioglycoside.[30] Interestingly, it was found that NIS/TfOH gave a higher yield of **11** than when AgOTf/K_2CO_3[31] was used as the promoter system.

Having advanced disaccharide **11** in hand, attention was focused on removal of the protecting groups and selective introduction of the fatty acids. Thus, the phthalimido group and acetyl ester of **11** were cleaved by treatment with ethylenediamine in refluxing n-butanol.[32] The resulting C-2' amino was selectively acylated with (*R*)-3-tetradecanoyloxy-hexadecanoic acid **7**[33] using *N*,*N*'-dicyclohexyl carbodiimide (DCC) as the activating reagent to afford **12** in a yield of 78%. Reduction of the azido moiety of **12** was easily accomplished by reaction with propane-1,3-dithiol in a mixture of pyridine, triethylamine, and water, and the amine and hydroxyls of the resulting compound were acylated with (*R*)-3-benzyloxy-hexadecanoic acid (**8**) using DCC and 4-dimethylaminopyridine (DMAP) as activating reagents to afford **13** in an overall yield of 59%. Hydrolysis of the thiophenyl moiety of **13** by treatment with NIS and a catalytic amount of TfOH in wet DCM proved problematic and two products were isolated namely the required lactol **15** and the 1,2-oxazoline derivative **14**. The latter byproduct was formed by neighboring participation of the C-2 amide after activation of the thioglycoside of **13** with NIS followed by elimination of water. Fortunately, the 1,2-oxazoline could be converted into compound **15** by treatment with dibenzylphosphate in wet DCM[34] in an 80% yield. The anomeric hydroxyl of **15** was oxidized using pyridinium chlorochromate (PCC) to give lactone **16** in high yield. Finally,

the benzyl ethers and benzylidene acetal of **16** were removed by catalytic hydrogenation over Pd/C to afford target compound **3**.



Scheme 4.1 Reagents and conditions: (a) NIS, TfOH, MS 3 Å, DCM, -75 ^{o}C; (b) 1: $H_2N(CH_2)_2$, *n*-BuOH, 90 ^{o}C; 2: **7**, DCC, DCM; (c) 1: $HS(CH_2)_3SH$, pyridine, Et_3N, H_2O; 2: **8**, DCC, DMAP, DCM; (d) NIS, TfOH, DCM, H_2O; (e) dibenzylphosphate, H_2O, DCM; (f) PCC, MS 3 Å, DCM; (g) Pd/C, H_2, *t*-BuOH, THF.



Scheme 4.2 Reagents and conditions: (a) NIS, TfOH, MS 3 Å, DCM, -75 ^{o}C; (b) 1: $H_2N(CH_2)_2$, *n*-BuOH, 90 ^{o}C; 2: **9**, DCC, DCM; (c) 1: $HS(CH_2)_3SH$, pyridine, Et_3N, H_2O; 2: **8**, DCC, DMAP, DCM; (d) 1: H_2NNH_2·HOAc, DCM; 2: ROH, DCC, DMAP, DCM; (e) NIS, TfOTMS, DCM, H_2O; (f) PCC, MS 3 Å, DCM; (g) Pd/C, H_2, *t*-BuOH, THF.

To avoid the formation of the unwanted 1,2-oxazoline observed during the preparation of **3**, a different strategy was employed for the preparation of compounds **1** and **2**. In the new approach,

glycosyl acceptor **6**[27, 28] was employed, which contains an α-instead of a β-anomeric thiophenyl moiety (**Scheme 4.2**). It was hoped that the hydrolysis of the α-anomeric thioglycoside would be less problematic because the C-2 amide cannot directly displace the anomeric leaving group. As a result, the hydrolysis will proceed through an oxa-carbenium ion, which can more easily be trapped by water to give a lactol then when the anomeric leaving group can be directly displaced by the C-2 amide leading to an 1,2-oxazoline. Furthermore, the acyloxyacyl moiety at C-2' was introduced by first acylation with (*R*)-3-levulinoyloxy-hexadecanoic acid (**9**)[35] followed by selective removal of the Lev ester and acylation of the resulting alcohol with an appropriate fatty acid. This approach is attractive because it provides an easy route to a variety of structural analogs. Thus, NIS/TMSOTf mediated glycosylation of glycosyl donor **4** with glycosyl acceptor **6** in DCM at -75 ^{o}C provided disaccharide **17** in 82% yield. Treatment of **17** with ethylenediamine in refluxing n-butanol followed by acylation of the resulting C-2' amino with **9** using DCC as the activating reagent afforded **18** in a yield of 78%. The azido moiety of **18** was reduced with propane-1,3-dithiol and the amine and hydroxyls of the resulting compound were acylated with **8** using DCC and DMAP as activating reagents to provide **19** in an overall yield of 61%. Next, the Lev ester of **19** was selectively removed by treatment with hydrazine acetate, and the hydroxyl of the resulting compound was acylated with 27-benzyloxyoctacosanoic- and octacosanoic acid to give compounds **20** and **21**, respectively. Gratifyingly, no 1,2-oxazoline was formed when the anomeric thioglycosides of **20** and **21** were hydrolyzed with NIS/TMSOTf and lactols **22** and **23** were isolated in high yields. Finally, **22** and **23** were oxidized using PCC to give lactones **24** and **25** which were globally deprotected using standard conditions to afford the target compounds **1** and **2**, respectively.

Biological evaluations Compounds **1-3** were tested over a wide concentration range for their ability to activate a human monocytic cell line (Mono Mac 6) to produce TNF-α protein and the resulting values were compared with similar data obtained for *E. coli* LPS, *R. sin*-1 LPS, and *R. sin*-1 lipid A. Thus, incubation of Mono Mac 6 cells with *E. coli* LPS for 5.5 hours yielded a clear dose response effect of TNF-α production with maximal supernatant concentrations of TNF-α being caused by 10 ng/mL (0.093 nM) of *E. coli* LPS. Incubations of concentrations up to 10 μg/mL for *R. sin*-1 LPS (270 nM) and *R. sin*-1 lipid A (6.6 μM) and up to 100 μg/mL (approximately 50 μM) for the synthetic compounds **1-3** did not induce significant production of TNF-α (**Figure 4.3**).

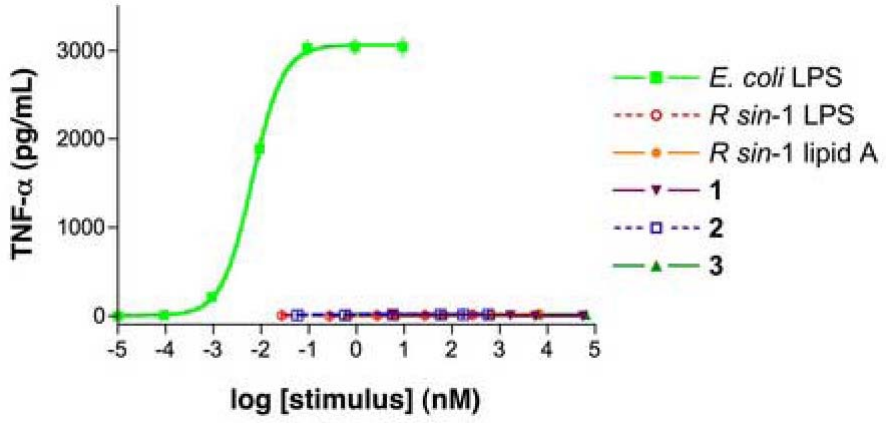


Figure 4.3 Concentration-response curves of *E. coli* LPS, *R. sin*-1 LPS, *R. sin*-1 lipid A, and synthetic compounds **1-3** in human monocytic cells. Mono Mac 6 cells were incubated for 5.5 h at 37 ^{o}C with increasing concentrations of *E. coli* LPS, *R. sin*-1 LPS, *R. sin*-1 lipid A, **1**, **2**, or **3** as indicated. TNF-α protein in cell supernatants was measured using ELISA. (Please note that *R. sin*-1 LPS, *R. sin*-1 lipid A, and **1-3** show background values and therefore overlap in the figure). Treatment with *E. coli* LPS, *R. sin*-1 LPS, *R. sin*-1 lipid A, and **1-3** did not affect cell viability, as judged by cellular exclusion of trypan blue.

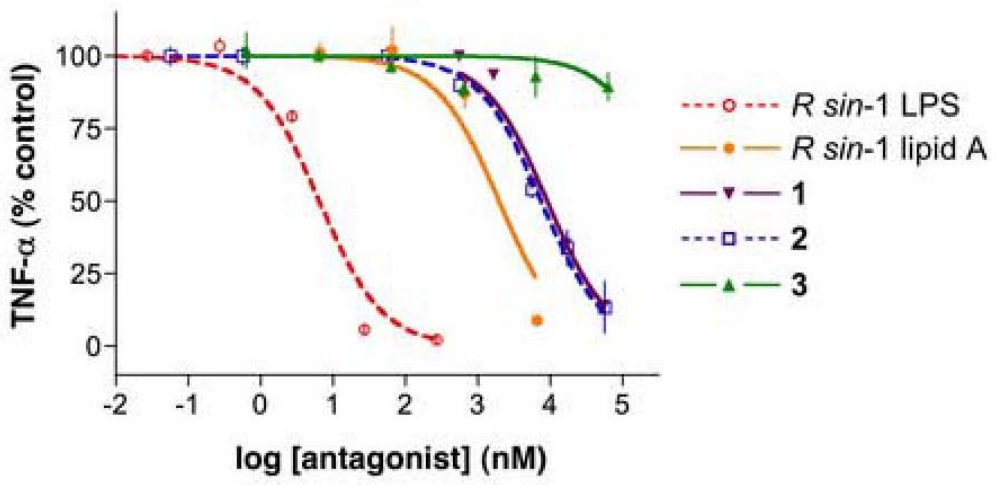


Figure 4.4 Antagonism of *E. coli* LPS by *R. sin*-1 LPS, *R. sin*-1 lipid A, and synthetic compounds **1**-**3** in human monocytic cells. TNF-α concentrations were measured after preincubation of Mono Mac 6 cells with increasing concentrations of *R. sin*-1 LPS, *R. sin*-1 lipid A, **1**, **2**, or **3** as indicated for 1 h at 37 ^{o}C, followed by 5.5 h of incubation with *E. coli* LPS (10 ng/mL). Results are expressed as percentage of cytokine concentration of control cells, which are incubated only with *E. coli* LPS.

Based on its lack of proinflammatory effects, compounds **1**-**3** were tested over a wide concentration range for its ability to antagonize TNF-α production by monocytic cells incubated with *E. coli* LPS (10 ng/mL). At the highest concentration tested, compound **1** antagonized the effect *E. coli* LPS by 86%, and an IC_{50} (concentration producing 50% inhibition) of 9.1 μM (16.4 μg/mL) was established (**Figure 4**.**4**). A similar inhibition experiment with compound **2** gave a similar IC_{50} value of 7.5 μM (13.2 μg/mL). However, compound **3** was only able to marginally inhibit the production of TNF-α. Thus, these data show that the hydroxyl of the 27-hydroxyoctacosanoic acid moiety of *R. sin*-1 lipid A is not important for antagonistic properties. However shortening the octacosanoic acid moiety as in compound **3** resulted in a significant reduction in inhibitory potential. Furthermore, the EC_{50} values for compounds **1** and **2** are similar to that of *R. sin*-1 lipid A, which was obtained by mild acid hydrolysis of the corresponding LPS.

As expected, *R. sin*-1 LPS was a more potent inhibitor and an IC_{50} value of 6.5 nM (239 ng/mL) was determined when incubated with 10 ng/mL of *E. coli* LPS (Figure 4). Probably, the KDO moiety of *R. sin*-1 LPS accounts for the greater biological activity.

Conclusions

Several studies have indicated that compounds that can antagonize cytokine production induced by enteric LPS may have the potential to be developed as therapeutics for the treatment of Gram-negative septicemia.[36] Success in this area has been limited and most efforts have been directed towards the synthesis of analogs of lipid A of *R.sphearoides*[18, 19] and derivatives of lipid X.[15-17] Unique features of *R. sin*-1 lipid A is lack of phosphates, an 2-aminogluconolactone moiety, and a very long chain fatty acid 27-hydroxyoctacosanoic acid. Previously, we reported that the 2-aminogluconolactone is in equilibrium with the corresponding 2-aminogluconate.[37] Furthermore, studies with a compound that was locked in the latter form displayed antagonistic activity. In this report, we have shown that acylation of the C-2' acyloxyacyl moiety with octacosanoic acid is important for optimal antagonistic properties. The hydroxyl of the natural 27-hydroxyoctacosanoic moiety does, however, not account for inhibitory activity.

Chemical Experiments

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 ^{o}C. CH_2Cl_2 was distilled from CaH_2 and tetrahydrofuran (THF) was distilled from sodium directly prior to the application. CH_3OH was dried by refluxing with mageniusm 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 ^{o}C during 8 h in the first instance and then for 2-3 h at 390 ^{o}C directly prior to application. Optical rotations were measured with a Jasco model P-1020 polarimeter. $^{!}$H NMR and ^{13}C NMR spectra were recorded with Varian spectrometers (model Inova500) equipped with Sun workstations. $^{!}$H NMR spectra were recorded in $CDCl_3$ and reference to residual $CHCl_3$ at 7.24 ppm, and ^{13}C NMR spectra were referenced to the center 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 lipid at C-2', whereas signals marked with subscript L' symbol belong to the side chain. Signals marked with a subscript S symbol belong to the monoantennary lipids at C-2, C-3, and C-3'.

(*R*)-3-Tetradecanoyloxy-hexadecanoic Acid (7): Myristoyl chloride (2.22 mL, 8.16 mmol) was added dropwise to a solution of 2-(4-bromophenyl)-2-oxoethyl (*R*)-3-hydroxyhexadecanoate (3.65 g, 7.77 mmol), pyridine (1.26 mL, 15.54 mmol) and DMAP (47 mg, 0.39 mmol) in DCM (70 mL). After stirring the reaction mixture at room temperature for 9 h, it was diluted with DCM (50 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 60 mL) and brine (2 x 60 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo.* The residue was purified by silica gel column chromatography (eluent: toluene) to afford 2-(4-bromophenyl)-2-oxoethyl (*R*)-3-tetradecanoyloxy-hexadecanoate as a colorless syrup (4.95 g, 94%). R_f = 0.68 (DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, 2H, J = 8.7 Hz, aromatic), 7.64 (d, 2H, J = 8.7 Hz, aromatic), 5.29-5.25 (m, 1H, H-3), 5.25 (s, 2H, H-2), 2.78-2.65 (m, 2H, H-2a, 2b), 2.28 (t, 2H, $J_{2',3'}$ = 7.5 Hz, H-2a', H-2b'), 1.63-1.56 (m, 4H, H-3a', H-3b', H-4a, H-4b), 1.23 [broad, 42H, 21 x CH_2], 0.88-0.84 (m, 6H, 2 x CH_3). HR MS (*m*/*z*) calcd for

$C_{38}H_{63}BrO_5[M+Na]^+$, 701.3757; found, 701.3568. Zinc dust (719 mg, 11.1 mmol) was added portionwise to 2-(4-bromophenyl)-2-oxoethyl (*R*)-3-tetradecanoyloxyhexadecanoate (1.50 g, 2.21 mmol) in acetic acid (15 mL). The reaction mixture was stirred at 60 °C for 2 h and then diluted with DCM (20 mL). The solids were filtered off through a pad of Celite, and the residue was washed with DCM (3 x 5 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (eluent: DCM/methanol, 100/1, v/v) to afford **7** as a white solid (1.04 g, 98%). R_f = 0.35 (toluene/ethyl acetate, 4/1, v/v); $[\langle]^{24.9}_D$ = -1.2° (c = 1.0, $CHCl_3$). 1H NMR (300 MHz, $CDCl_3$): δ 5.21-5.14 (m, 1H, H-3), 2.66-2.51 (m, 2H, H-2a, 2b), 2.26 (t, 2H, $J_{2',3'}$ = 7.8 Hz, H-2'), 1.60-1.56 (m, 4H, H-3a', H-3b', H-4a, H-4b), 1.23 (bs, 42H, 21 x CH_2), 0.88-0.83 (m, 6H, 2 x CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.28 (*C*=O), 173.28 (*C*=O), 69.96 (C-3), 38.87 (C-2), 34.34 (C-2'), 33.97-22.69 [C- (4-15), (3'-13')], 14.11 (C-14', 16). HR MS (*m*/*z*) calcd for $C_{30}H_{58}O_4[M+Na]^+$, 505.4233; found, 505.4313.

Phenyl 3-*O*-acetyl-6-*O*-(4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-azido-4-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyanoside (11): A suspension of glycosyl donor **4** (435 mg, 0.81 mmol), acceptor **5** (290 mg, 0.68 mmol) and activated molecular sieves (4 Å, 500 mg) in DCM (15 mL) was stirred under an atmosphere of argon at room temperature for 2 h. The reaction mixture was cooled (-75 ^oC), and NIS (191 mg, 0.85 mmol) and TfOH (5.8 μL, 0.07 mmol) were added. After being warmed up to -35 ^oC in 10 min, the reaction mixture was quenched by addition of pyridine (100 μL) and diluted with DCM (20 mL). The reaction mixture was washed with aqueous $Na_2S_2O_3$ (15%, 2 x 40 mL) and water (2 x 50 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/1, v/v) to afford **6** as an amorphous solid (461 mg, 84 %). R_f = 0.45 (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{24.9}_D$ = -32.7^o

(c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.94-7.76 (m, 19H, aromatic), 5.59 (s, 1H, CH, benzylidene), 5.41 (d, 1H, $J_{1',2'}$ = 8.7 Hz, H-1'), 5.02 (dd, 1H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 4.71-4.63 (m, 1H, H-3'), 4.43-4.33 (m, 3H, H-1, H-2', H-6'a), 4.25 (d, 1H, *J* = 10.8 Hz, C*H*H, Bn), 4.19 (d, 1H, C*H*H, Bn), 4.09 (d, 1H, H-6a), 3.88-3.82 (m, 1H, H-6'b), 3.74 (dd, $J_{5,6a}$ = 11.1 Hz, $J_{6a,6b}$ = 4.2 Hz, H-6b), 3.68-3.64 (m, 2H, H-4', H-5'), 3.46-3.42 (m, 1H, H-5), 3.45 (dd, 1H, $J_{4,5}$ = 9.6 Hz, H-4), 3.18 (t, 1H, H-2), 1.91 (s, 3H, COC*H*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.57 (*C*=O), 168.09 (*C*=O), 137.13-123.52 (aromatic), 102.01 (*C*H, benzylidene), 98.64 (C-1'), 85.46 (C-1), 82.25(C-4'), 75.66(C-3 or 4), 75.59 (C-3 or 4), 74.51 (*C*H$_2$, Bn), 68.68 (C-3', 6'), 67.64 (C-6), 66.20 (C-5'), 62.84 (C-2), 56.37 (C-2), 23.72 (*C*H$_3$). HR MS (m/z) calcd for $C_{42}H_{40}N_4O_{11}S$ $[M+Na]^+$, 831.2312, found, 831.2519.

Phenyl 2-azido-4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-2-deoxy-2[(*R*)-3-tetradecanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-1-thio-β-D-glucopyranoside (12): Compound **11** (250 mg, 0.31 mmol) was dissolved in a mixture of *n*-butanol (25 mL) and ethylenediamine (5 mL, 75 mmol). The reaction mixture was stirred at 90 ^{o}C for 20 h, after which it was concentrated *in vacuo*. The residue was dissolved in DCM (30 mL), and the solids were removed by filtration. The filtrate was concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (eluent: DCM/methanol, 30/1, v/v) to afford phenyl 6-*O*-(2-amino-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-2-azido-4-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside as a white solid (180 mg, 92%). R_f = 0.45 (DCM/methanol, 10/1, v/v). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.59-7.25 (m, 15H, aromatic), 5.54 (s, 1H, CH, benzylidene), 4.78 (d, 1H, *J* = 10.4 Hz, C*H*H, Bn), 4.69 (d, 1H, C*H*H, Bn), 4.50 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 4.33-4.28 (m, 2H, H-1', H-6'a), 4.14 (dd, 1H, $J_{5,6a}$ = 1.8 Hz, $J_{6a,6b}$ = 11.1 Hz, H-6a), 3.80-3.50 (m, 6H, H-3, H-3', H-4', H-5', H-6b, H-6'b), 3.44-3.36 (m, 1H, H-5), 3.34-3.25 (m, 2H, H-3, H-4), 2.78 (t, 1H,

$J_{2,3}$ = 9.6 Hz, H-2), 1.91 (s, 3H, COCH_3, acetyl); HR MS (m/z) calcd for $C_{32}H_{36}N_4O_8S[M+Na]^+$, 659.2151; found, 659.2238. A mixture of DCC (45 mg, 0.218 mmol) and **7** (70 mg, 0.145 mmol) in DCM (2 mL) was stirred at room temperature for 10 min, and then the above mentioned amino derivative (58 mg, 91 μmol) in DCM (1 mL) was added. The reaction mixture was stirred at room temperature for 2 h, after which the solids were removed by filtration, and the residue was washed with DCM (2 x 3 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (eluent: DCM/methanol, 50/1, v/v) to afford **12** as a white solid (85 mg, 85%). R_f = 0.60 (DCM/diethyl ether, 6/1, v/v); $[\alpha]^{24.8}_D$ = -18.3° (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.57-7.31 (m, 15H, aromatic), 5.50 (s, 1H, CH, benzylidene), 5.04 (m, 1H, H-3$_L$), 4.76 (d, 1H, J = 11.5 Hz, C*H*H, Bn), 4.73 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.67(d, 1H, C*H*H, Bn), 4.56 (d, 1H, $J_{1,2}$ = 10.0 Hz, H-1), 4.46 (bs, 1H, O*H*'), 4.34 (dd, 1H, $J_{5,6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 10.0 Hz, H-6'a), 4.16 (d, 1H, H-6a), 4.06 (dd, 1H, $J_{2',3'}$ = 8.5 Hz, $J_{3',4'}$ = 9.5 Hz, H-3'), 3.79 (dd, 1H, $J_{5',6'b}$ = 10.5 Hz, H-6b'), 3.60-3.70 (m, 3H, H-3, H-5, H-6b), 3.58 (dd, 1H, $J_{4',5'}$ = 10.0 Hz, H-4'), 3.52 (m, 1H, H-2'), 3.45 (ddd, 1H, H-5'), 3.27 (dd, 1H, $J_{2,3}$ = 9.5 Hz, H-2), 3.27 (dd, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 2.76(b, 1H, O*H*), 2,25-2.31 (m, 2H, H-2$_L$), 1.53-1.58 (m, 4H, H-4$_L$, H-3$_L$'), 1.25 (m, 42H, 21 x CH_2, lipid), 0.87-0.90 (m, 6H, 2 x CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.31 (*C*=O), 171.72 (*C*=O), 137.65-126.36 (aromatic), 101.96 (*C*H, benzylidene), 101.13 (C-1'), 85.57 (C-1), 81.49 (C-4'), 78.59 (C-5), 77.86 (C-4), 77.21 (C-3), 74.84 (*C*H_2, Bn), 71.72 (C-3'), 71.48 (C-3$_L$), 68.78 (C-6), 68.58 (C-6'), 66.44 (C-2), 59.14 (C-2'). HR MS (m/z) calcd for $C_{62}H_{92}N_4O_{11}S[M+Na]^+$, 1123.6381; found, 1123.9268.

Phenyl 4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-2-[(*R*)-3-tetradecanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-

glucopyranoside (13): 1,3-Propanedithiol (0.18 mL, 1.75 mmol) was added to a stirred solution of **12** (95 mg, 0.086 mmol) and triethylamine (0.2 mL) in a mixture of pyridine and H_2O (7 mL, 6/1, v/v). The reaction mixture was stirred at room temperature for 16 h and then concentrated *in vacuo* to dryness. The residue was purified by silica gel column chromatography (eluent: DCM/methanol, 60/1, v/v) to afford phenyl 2-amino-4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-tetradecanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-deoxy-1-thio-β-D-glucopyranoside as a white solid (85.5 mg, 92.5%). R_f = 0.40 (DCM/diethyl ether, 6/1, v/v). 1H NMR (500 MHz, $CDCl_3$): δ 7.26-7.53 (m, 15H, aromatic), 6.08 (d, 1H, $J_{NH,2}$ = 5.0 Hz, N*H*), 5.57 (s, 1H, CH, benzylidene), 5.06 (m, 1H, H-3_L), 4.82 (d, 1H, *J* = 11.5 Hz, C*H*H, Bn), 4.72 (d, 1H, $J_{1',2'}$ = 9.0 Hz, H-1'), 4.66 (d, 1H, C*H*H, Bn), 4.53 (d, 1H, $J_{1,2}$ = 9.5 Hz, H-1), 4.34 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 4.20 (d, 1H, H-6a), 4.05 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ = 9.0 Hz, H-3'), 3.79 (dd, 1H, $J_{5',6'b}$ = 10.0 Hz, H-6'b), 3.64-3.71 (m, 2H, H-5, H-6b), 3.58 (dd, 1H, $J_{4',5'}$ = 9.0 Hz, H-4'), 3.49-3.52 (m, 2H, H-2', H-3), 3.45 (ddd, 1H, H-5'), 3.26 (dd, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, H-4), 2.63 (dd, 1H, $J_{2,3}$ = 10,0 Hz, H-2), 2.27-2.43 (m, 4H, H-2_L, H-$2_{L'}$), 1.50-1.58 (m, 4H, H-4_L, H-$3_{L'}$), 1.25 (m, 42H, 21 x CH_2, lipid), 0.88-0.90 (m, 6H, 2 x CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 126.60-138.18 (aromatic), 102.18 (*C*H, benzylidene), 101.45 (C-1'), 89.18 (C-1), 81.73 (C-4'), 78.88 (C-5), 78.55 (C-3), 77.45 (C-4), 74.71 (*C*H_2, Bn), 72.04 (C-3'), 71.59 (C-3_L), 69.48 (C-6), 68.83 (C-6'), 66.67 (C-5'), 59.49 (C-2'), 56.50 (C-2). HR MS (m/z) calcd for $C_{62}H_{94}N_2O_{11}S[M+Na]^+$, 1097.6476; found, 1097.6069. A mixture of DCC (57.5 mg, 0.279 mmol) and (*R*)-3-benzyloxy-hexadecanoic acid **8** (85 mg, 0.232 mmol) in DCM (3 mL) was stirred at room temperature for 10 min, after which DMAP (6.2 mg, 0.051 mmol) and the above mentioned amino derivative (50 mg, 46.5 μmol) in DCM (1.5 mL) was added. The reaction mixture was stirred at room temperature for 5 h, and then the solids were removed by filtration

and the residue was washed with DCM (2 x 2 mL). The combined filtrates were concentrated *in vacuo,* and the residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 5/1, v/v) to afford **13** as a white solid (62 mg, 63%). R_f = 0.60 (hexane/ethyl acetate, 3/1, v/v). $[\alpha]^{25.2}_D$ = +9.1° (c = 1.0, $CHCl_3$). ^{1}H NMR (300M Hz, $CDCl_3$): δ 7.14-7.43 (m, 30H, aromatic), 6.46 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N*H*), 5.42 (s, 1H, CH, benzylidene), 5.36 (d, 1H, $J_{NH,2'}$ = 9.0 Hz, N*H'*), 5.24 (dd, 1H, $J_{2',3'}$ = 9.6 Hz, $J_{3',4'}$ = 9.9 Hz, H-3'), 5.10 (dd, 1H, $J_{2,3}$ = 8.7 Hz, $J_{3,4}$ = 10.2 Hz, H-3), 4.96 (m, 1H, H-3_L), 4.67 (d, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.41-4.61 (m, 9H, H-1, 4 x C*H*$_2$, Bn), 4.32 (dd, 1H, $J_{5,6a}$ = 4.8 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6a), 4.04 (m, 1H, H-2), 3.96 (d, 1H, H-6'a), 3.59-3.84 (m, 7H, H-2', H-4', H-6b, H-6'b, 3 x H-3s), 3.36-3.49 (m, 3H, H-4, H-5, H-5'), 2.09-2.69 (m, 10H, H-2_L, H-$2_{L'}$, 3 x H-2_S), 1.57-0.98 (m, 118H, 59 x CH_2, lipid), 0.82 (m, 15H, 5 x CH_3, lipid). ^{13}C NMR (75Hz, $CDCl_3$): δ 126.34-138.73 (aromatic), 101.62 (C-1', *C*H, benzylidene,), 86.95 (C-1), 79.68 (C-5 or 5'), 79.13 (C-4'), 75.67-76.30 (C-3, 3*C-3s), 74.67 (C-4), 71.18-71.63 (C-3',3L, 4 x *C*H_2, Bn), 68.86 (C-6 or 6'), 68.33 (C-6 or 6'), 66.43 (C-5 or 5'), 55.24 (C-2'), 53.10 (C-2); HR MS (m/z) calcd for $C_{131}H_{202}N_2O_{17}S[M+Na]^+$, 2130.4622; found, 2130.6140.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-2-[(*R*)-3-tetradecanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucopyranoside (15): TfOH (0.5 μL, 4.4 μmol) was added to a stirred solution of **13** (31 mg, 14.7 μmol) and NIS (9.9 mg, 44 μmol) in a mixture of DCM and H_2O (4 mL, 100/1, v/v) at 0 °C. The reaction mixture was vigorously stirred for 30 min until TLC analysis indicated that the reaction had gone to completion, and then it was diluted with DCM (10 mL) and washed with aqueous $Na_2S_2O_3$ (15%, 15 mL) and water (2 x 10 mL). The organic phase was dried ($MgSO_4$) and filtered, and the

filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (elulent: hexane/ethyl acetate, 6/1-4/1, v/v) to afford **15** as a white solid (10.5 mg, 35%) and its 1,2-oxazoline derivative **14** (15 mg, 51%). A mixture of the resulting 1,2-oxazoline and dibenzylphosphate (4 mg, 15 μmol) in wet 1,2-dichloroethane (1 mL) was stirred at room temperature for 1 h. The reaction mixture was diluted with DCM (10 mL) and wahsed with saturated aqueous $NaHCO_3$ (2 x 8 mL) and brine (2 x 8 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/1, v/v) to afford **15** as a white solid (13.5 mg, 90%). The overall yield of **15** for the two steps was 80%. R_f= 0.2 (hexane/ethyl acetate, 2.5/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.18-7.40 (m, 25H, aromatic), 6.28 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.95 (d, 1H, $J_{NH',2'}$ = 8.5 Hz, N*H*'), 5.44 (s, 1H, CH, benzylidene), 5.39-5.43 (m, 2H, H-3, H-3'), 5.17 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.09 (m, 1H, H-1), 4.95 (m, 1H, H-3$_L$), 4.40-4.61 (m, 8H, 4 x CH_2, Bn), 4.35 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 10.0 Hz, H-6'a), 4.20 (m, 1H, H-2), 4.07 (m, 1H, H-5), 3.96 (d, 1H, H-6a), 3.74-3.85 (m, 4H, H-6'b, 3 x H-3$_S$), 3.61-3.68 (m, 3H, H-2', H-4', H-6b), 3.52 (ddd, 1H, H-5'), 3.37 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 10.0 Hz, H-4), 2.20-2.66 (m, 10H, H-2$_L$, H-2$_{L'}$, 3 x H-2$_S$), 1.59-1.10 (m, 118H, 59 x CH_2, lipid), 0.84 (m, 15H, 5 x CH_3, lipid). ^{13}C NMR (75Hz, $CDCl_3$): δ 126.0-138.6 (aromatic), 101.5 (*C*H, benzylidene), 100.4 (C-1'), 91.5 (C-1), 79.0 (C-4'), 76.4 (C-4), 75.5-76.4 (C-3x3S), 74.4 (*C*H_2, Bn), 73.5 (C-3 or 3'), 71.6 (C-5), 71.0-71.4 (C-3$_L$, 3 or 3', 3 x *C*H_2, Bn), 68.7 (C-6'), 67.5 (C-6), 66.5 (C-5'), HR MS (m/z) calcd for $C_{125}H_{198}N_2O_{18}[M+Na]^+$, 2038.4537; found, 2038.3850.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-2-[(*R*)-3-tetradecanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-D-glucono-1,5-lactone

(16): A suspension of **15** (8.0 mg, 4.0 μmol) and activated molecular sieves (3 Å, 15 mg) in DCM (2.0 mL) was stirred at room temperature under an atmosphere of argon for 1 h. PCC (4.3 mg, 20 μmol) was then added and the reaction mixture was stirred for 1 h until TLC analysis indicated the reaction had gone to completion. The reaction mixture was purified by Iatro beads column chromatography (eluent: hexane/ethyl acetate, 4/1, v/v) to afford **16** as a white solid (7 mg, 87%). $[\alpha]^{23}_{D}$ = -12.5^{o} (c = 1, $CHCl_3$). R_f = 0.65 (hexane/ethyl acetate, 2.5/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.20-7.38 (m, 25H, aromatic), 6.82 (d, 1H, $J_{NH,2}$ = 8.5 Hz, N*H*), 6.61 (d, 1H, $J_{NH',2'}$ = 7.5Hz, N*H*'), 5.60 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 10.0 Hz, H-3'), 5.39 (s, 1H, CH, benzylidene), 5.34 (dd, 1H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 5.07 (m, 1H, H-3$_L$), 4.95 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.79 (t, 1H, H-2), 4.37-4.60 (m, 9H, H-5, 4 x C*H*$_2$, Bn), 4.28 (dd, 1H, $J_{5',6'a}$ = 3.5 Hz, $J_{6'a,6'b}$ = 9.5 Hz, H-6'a), 4.02-4.06 (m, 2H, H-4, H-6a), 3.80-3.85 (3H, 3 x 3s), 3.75 (t, 1H, $J_{5',6'b}$ = 10.0 Hz, H-6'b), 3.52-3.60 (m, 4H, H-2', H-4', H-5', H-6a), 2.25-2.68 (m, 10H, H-2$_L$, H-2$_{L'}$, 3 x H-2$_S$), 1.60-1.03 (m, 118H, 59 x CH_2, lipid), 0.86 (m, 15H, 5 x CH_3, lipid). HR MS(m/z) calcd for $C_{125}H_{196}N_2O_{18}[M+Na]^+$, 2036.4381; found, 2036.8009.

2-Deoxy-6-*O*-{2-deoxy-3-*O*-[(*R*)-3-hydroxy-hexadecanoyl]-2-[(*R*)-3-tetradecanoyloxy-hexadecanoylamino-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxy-hexadecanoylamino]-3-*O*-[(*R*)-3-hydroxy-hexadecanoyl]-D-glucono-1,5-lactone (3): Pd/C (10 mg) was added to the solution of lactone **16** (7 mg, 3.5 μmol) in a mixture of THF and *t*-BuOH (3 mL, 1/1, v/v). The reaction mixture was shaken under an atmosphere of H_2 (15 psi) at room temperature for 24 h, after which the catalyst was removed by filtration and washed with THF (3 x 0.5 mL). The combined filtrates were concentrated *in vacuo* to afford **3** as a white solid (5 mg, 81%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.34 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 5.10 (m, 1H, H-3$_L$), 5.00 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 10.0 Hz, H-3'), 4.56 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.26 (m, 1H, H-5), 4.21 (d, 1H, H-2), 4.19 (d,

1H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 3.74-3.92 (m, 7H, H-4, H-2', H-6'a, H-6b, 3 x H-3s), 3.63 (m, 1H, H-6'b), 3.47 (dd, 1H, $J_{4',5'}$ = 9.0 Hz, H-4'), 3.27 (m, 1H, H-5'), 2.27-2.18 (m, 10H, H-2_L, $2_{L'}$, 3 x H-2_S), 1.63-0.96 (m, 118H, 59 x CH_2, lipid), 0.84 (m, 15H, 5 x CH_3, lipid). HR MS (m/z) calcd for $C_{90}H_{168}N_2O_{18}[M+Na]^+$, 1588.2190; found, 1588.4548.

Phenyl 3-*O*-acetyl-6-*O*-(4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-azido-4-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyanoside (17): The glycosylation of **4** (87 mg, 0.20 mmol) and **6** (58 mg, 0.136 mmol) was performed similar to the synthesis of **11** using NIS (47 mg, 0.21 mmol) and TfOH (1.7 μL, 0.02 mmol) in DCM (2 mL) to afford **13** as an amorphous solid (90 mg, 82%). R_f = 0.41 (hexane/ethyl acetate, 5/2, v/v). $[\alpha]^{24.9}_D$ = -40.5° (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.70-6.88 (m, 19H, aromatic), 5.56-5.55 (m, 2H, H-1, CH, benzylidene), 5.32-5.23 (m , 2H, H-1', H-3), 4.71-4.63 (m, 1H, H-3'), 4.40-4.27 (m, 3H, H-2', H-5, H-6'a), 4.25 (d, 1H, J = 10.5 Hz, C*H*H, Bn), 4.03-3.96 (m, 2H, H-6a, C*H*H, Bn), 3.85-3.77 (m, 3H, H-2, H-6b, H-6'b), 3.66-3.61 (m, 2H, H-4', H-5'), 3.47 (dd, 1H, $J_{3,4} = J_{4,5}$ = 9.6 Hz, H-4), 1.90 (s, 3H, COCH_3, acetyl). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.30 (*C*=O), 136.89-123.38 (aromatic), 101.79 (*C*H, benzylidene), 98.59 (C-1'), 86.92 (C-1), 81.75 (C-4'), 75.98 (C- 4), 74.26 (CH_2, Bn), 73.01 (C-3), 70.51 (C-5), 68.36 (C-3', C-6'), 67.26 (C-6), 66.17 (C-5'), 61.93 (C-2), 56.28 (C-2), 20.59 (CH_3, acetyl). HR MS (m/z) calcd for $C_{42}H_{40}N_4O_{11}S$ $[M+Na]^+$, 831.2312; found, 831.1761.

Phenyl 2-azido-4-O-benzyl-6-O-{4,6-O-benzylidene-2-deoxy-2-[(*R*)-3-levulinoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-deoxy-1-thio-α-D-glucopyranoside (18): The phthalimido and acetyl group of **17** (210 mg, 24.7 mmol) were removed similar to the deprotection of **11** in a mixture of n-butanol (15 mL) and ethylenediamine (3 mL, 45 mmol) to afford phenyl 6-O-(2-amino-4,6-O-benzylidene-2-deoxy-β-D-glucopyranosyl)-2-azido-4-O-

benzyl-2-deoxy-1-thio-α-D-glucopyranoside as a colorless syrup (142 mg, 91%). R_f = 0.52 (DCM/methanol, 1/9, v/v). DCC (67 mg, 32.4 mmol) was added to a solution of (*R*)-3-levulinoyloxyhexadecanoic acid **9** (100 mg, 27.0 mmol) in DCM (5 mL) and the resulting solution was stirred for 10 min. Next, the above described amino derivative (142 mg, 22.5 mmol) in DCM (2 mL) was added and the reaction mixture was stirred for 12 h at room temperature. The solids were filtered-off and the residue was washed with DCM (2 x 3 mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by silica gel column chromatography (eluent: DCM/methanol, 50/1, v/v) to afford **18** as a white solid (189 mg, 86%). R_f = 0.55 (DCM/diethyl ether, 6/1, v/v). $[\alpha]^{23}_D$ = +4.9^o (c = 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.53-7.18 (m, 15H, aromatic), 6.34 (d, 1H, $J_{NH',2'}$ = 7.0 Hz, N*H*'), 5.61 (d, 1H, $J_{1,2}$ = 5.0 Hz, H-1), 5.56 (s, 1H, CH, benzylidene), 5.07-5.02 (m, 1H, H-3$_L$), 4.91 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.84 (d, 1H, *J* = 11.5 Hz, C*H*H, Bn), 4.74 (d, 1H, C*H*H, Bn), 4.37 (d, 1H, *J* = 10.0 Hz, H-5), 4.32 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 4.23 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3'), 4.13 (d, 1H, $J_{6a,6b}$ = 10.0 Hz, H-6a), 3.98 (dd, 1H, *J* = 10.0 Hz, *J* = 9.0 Hz, H-3), 3.89-3.86 (m, 2H, H-2, H-6'b), 3.80 (t, 1H, $J_{5,6b}$ = 10.0 Hz, H-6b), 3.63 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.5 Hz, H-4), 3.58 (dd, 1H, $J_{4',5'}$ = 9.0 Hz, H-4'), 3.54-3.48 (m, 2H, H-2', H-5'), 2.97-2.24 (m, 6H, H-2$_L$, 2 x CH_2, Lev), 2.17 (s, 3H, CH_3, Lev), 1.59-1.51 (m, 2H, H-4$_L$), 1.24 (broad, 22H, 11 x CH_2, lipid), 0.92-0.90 (m, 3H, CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 137.76-123.74 (aromatic), 102.02 (*C*H, benzylidene), 100.86 (C-1'), 87.64 (C-1), 81.62(C-4'), 78.43 (C-4), 75.26 (*C*H$_2$, Bn), 74.31 (C-3), 72.6 4(C-3$_L$), 71.62 (C-3, 5), 71.45 (C-3'), 69.02 (C-6'), 68.33 (C-6), 66.71 (C-5'), 64.19 (C-2), 59.41 (C-2'). HR MS (*m*/*z*) calcd for $C_{53}H_{72}N_4O_{11}S[M+Na]^+$: calcd, 1012.2143; found, 1012.3649.

Phenyl 4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexaadecanoyl-2-deoxy-2-

[(*R*)-3-levulinoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-α-D-glucopyranoside (19): Triethylamine (1.0 mL) was added to a stirred solution of **18** (450 mg, 0.455 mmol) and propanedithiol (491 mg, 45.5 mmol) in pyridine (15 mL) and H_2O (1.5 mL). The reaction mixture was stirred at room temperature for 14 h, after which it was concentrated *in vacuo* to dryness. The residue was co-evaporated with toluene (2 x 30 mL) and ethanol (2 x 20 mL) and then purified by silica gel column chromatography (eluent: DCM/methanol, 30/1, v/v) to afford phenyl 2-amino-4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-levulinoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-deoxy-1-thio-α-D-glucopyranoside as a colorless syrup (397 mg, 91 %). R_f = 0.45 (DCM/methanol, 15/1, v/v). A mixture of (*R*)-3-benzyloxy-hexadecanoic acid **8** (226 mg, 624 μmol) and DCC (154 mg, 749 μmol) was stirred for 10 min, and then the above described amine (150 mg, 156 μmol) in DCM (2 mL) and DMAP (3.0 mg, 24.7 μmol) were added. After stirring the reaction mixture for 16 h, the solids were filtered off and the residue was washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (eluent: toluene/ethyl acetate, 10/1, v/v) to afford **19** as a white solid (209 mg, 67%). R_f = 0.56 (DCM/methanol, 60/1, v/v). $[\alpha]^{23}_D$ = +11.5° (c = 1.0, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38-7.20 (m, 30H, aromatic), 6.55 (d, 1H, $J_{NH',2'}$ = 9.6 Hz, NH'), 6.45 (d, 1H, $J_{NH,2}$ = 8.4 Hz, NH), 5.71 (d, 1H, $J_{1,2}$ = 4.8 Hz, H-1), 5.33-5.31 (m, 2H, H-3', CH, benzylidene), 5.24 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 4.88 (m, 1H, H-3$_L$), 4.83 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.62-4.41 (m, 9H, H-2, 4 x CH_2, Bn), 4.41-4-27 (m, 2H, H-5, H-6'a), 4.17 (m, 1H, H-2'), 4.01 (d, 1H, $J_{6a,6b}$ = 10.8 Hz, H-6a), 3.92 (d, 1H, H-6b), 3.80-3.76 (m, 4H, H-4, 3 x H-3$_S$), 3.69 (t, 1H, $J_{5',6'b} = J_{6'a,6'b}$ = 10.6 Hz, H-6'b), 3.62 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.6 Hz, H-4'), 3.47 (m, 1H, H-5'), 2.87-2.21 (m, 12H, H-2$_L$, 3 x H-2$_S$, 2 x

CH_2, Lev), 2.07(s, 3H, CH_3, Lev), 1.59-1.51 (m, 8H, H-4_L, 3 x H-4_S), 1.23 (broad, 88H, 44 x CH_2, lipid), 0.88-0.86 (m, 12H, 4 x CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{122}H_{182}N_2O_{18}S[M+Na]^+$, 2018.3006; found, 2018.2588.

Phenyl 4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl-2-deoxy-2-[(*R*)-3-(27-benzyloxy-octacosanoyloxy)-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-α-D-glucopyranoside (20): To a solution of **19** (50.0 mg, 25 μmol) in DCM (2 mL) was added dropwise hydrazine acetate (2.4 mg, 26 μmol) in methanol (0.2 mL). The resulting mixture was stirred at room temperature for 3 h after which TLC indicated completion of the reaction. The reaction mixture was concentrated *in vacuo*, and the residue was purified by preparative silica gel TLC chromatography (eluent: DCM/methanol, 60/1, v/v) to afford the alcohol as a white solid (43 mg, 90%). R_f = 0.48 (DCM/methanol, 60/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.37-7.21 (m, 30H, aromatic), 6.48 (d, 1H, $J_{NH,2}$ = 7.8 Hz, N*H*), 5.68-5.65 (m, 2H, H-1, N*H*'), 5.46 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.41 (s, 1H, CH, benzylidene), 5.25 (t, 1H, $J_{2,3} = J_{3,4}$ = 10.2 Hz, H-3), 4.79 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1'), 4.62-4.41 (m, 9H, H-2, 4 x C*H*$_2$, Bn), 4.32 (m, 1H, H-5), 4.29 (dd, 1H, $J_{5',6'a}$ = 4.8 Hz, $J_{6'a,6'b}$ = 10.2 Hz, H-6'a), 3.98 (d, 1H, $J_{6a,6b}$ = 9.6 Hz, H-6a), 3.84-3.69 (m, 8H, H-H-2', H-4, H-6b, H-6'b**,** 4 x H-3_S), 3.63 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.6 Hz, H-4'), 3.48 (m, 1H, H-5'), 2.62 -2.56 (m, 2H, H-2_S), 2.49 (dd, 1H, *J* = 5.4 Hz, *J* = 15.0 Hz, H-2_{Sa}), 2.43(dd, 1H, *J* = 5.4 Hz, *J* = 15.6 Hz, H-2_{Sb}), 2.32-2.27 (m, 2H, H-2_S), 1.96-1.88 (m, 2H, H-2_S), 1.57-1.55 (m, 8H, 4 x H-4_S), 1.24 (broad, 88H, 44 x CH_2, lipid), 0.87-0.85 (m, 12H, 4 x CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.63 (C=O), 172.10 (C=O), 171.44 (C=O), 171.26 (C=O), 138.45-126.26 (aromatic), 101.49 (*C*H, benzylidene), 100.03 (C-1), 87.53 (C-1), 78.70 (C-4'), 77.20-75.96 (C-4, 3 x C-3_S), 75.96 (C-4), 75.63 (*C*H_2, Bn), 74.56 (C-3), 74.56, 73.46, 71.41, 71.19,

70.95 (C-3', 5, 3 x *C*H_2, Bn), 68.54 (C-6'), 68.19 (C-3_S), 66.94 (C-6), 66.43 (C-5'), 55.40 (C-2'), 52.72 (C-2). A mixture of 27-hydroxyoctacosanoic acid **10** (22 mg, 42 μmol) and DCC (13 mg, 65 μmol) in DCM (1.5 mL) was stirred at room temperature for 10 min, and then the above alcohol (50 mg, 26 μmol) in DCM (1 mL) and DMAP (2.5 mg, 21 μmol) were added. The reaction mixture was stirred at room temperature for 15 h, the solids were filtered off and the residue was washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by preparative silica gel TLC chromatography (eluent: DCM/methanol, 60/1, v/v) to afford **20** as a white fluffy solid (35.7 mg, 63%). R_f = 0.65 (DCM/methanol, 60/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.40-7.18 (m, 30H, aromatic), 6.48 (d, 1H, $J_{NH,2}$ = 8.5 Hz, N*H*), 5.72 (d, 1H, $J_{1,2}$ = 4.5 Hz, H-1), 5.61 (d, 1H, $J_{NH',2'}$ = 8.5 Hz, N*H*'), 5.39 (s, 1H, CH, benzylidene), 5.39 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3'), 5.27 (dd, 1H, *J* = 9.5 Hz, *J* = 10.0Hz, H-3), 4.97 (m, 1H, H-3_L), 4.89 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.62-4.42 (m, 11H, H-2, 5 x CH_2, Bn), 4.38 (m, 1H, H-5), 4.31 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 11.0 Hz, H-6'a), 3.98 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.89-3.83 (m, 2H, H-6b, H-3_S), 3.80-3.79 (m, 2H, 2 x H-3_S), 3.73-3.66 (m, 3H, H-2', H-4, H-6'b), 3.63 (t, 1H, $J_{4',5'}$ = 9.5 Hz, H-4'), 3.52-3.47 (m, 2H, H-5', H-$27_{L'}$), 2.65 -2.57 (m, 2H, H-2_S), 2.51 (dd, 1H, *J* = 5.5 Hz, *J* = 15.0 Hz, H-2_{Sa}), 2.45 (dd, 1H, *J* = 5.5 Hz, *J* = 15.5 Hz, H-2_{Sb}), 2.33-2.19 (m, 4H, 2 x H-2_S, H-2_{La}, H-$2_{La'}$), 2.09-1.98 (m, 2H, H-2_{Lb}, H-$2_{Lb'}$), 1.59-1.40 (m, 12H, H-4_L, H-$3_{L'}$, H-$26_{L'}$, 3 x H-4_S), 1.25 (broad, 176H, 88 x CH_2, lipid), 1.18 (d, 3H, $J_{27L',28L'}$ = 6.0 Hz, H-$28_{L'}$), 0.89-0.85 (m, 12H, 4 x CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.98 (*C*=O), 172.45 (*C*=O), 171.40 (*C*=O), 171.26 (*C*=O), 169.96 (*C*=O), 139.65-126.31 (aromatic), 101.96 (*C*H, benzylidene), 101.01 (C-1'), 87.5 8 (C-1), 79.05 (C-4'), 77.26 (C-3_S), 76.68 (C-3_S), 75.96 (C-3_S), 75.26 (C-$27_{L'}$), 74.51 (*C*H_2, Bn), 73.88 (C-3), 71.86 (C-5), 70.96 (C-3'), 70.68-70.42 (C-4, C-3_L, 3 x *C*H_2, Bn), 68.85 (C-6'), 67.78 (C-6), 66.56 (C-5'),

55.86 (C-2'), 53.12 (C-2). HR MS (*m*/*z*) calcd for $C_{152}H_{236}N_2O_{18}S[M+Na]^+$, 2432.7232; found, 2432.9846.

Phenyl 4-*O*-benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-2-[(*R*)-3-octacosanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-α-D-glucopyranoside (21): The above alcohol intermediate **(**40 mg**,** 20 μmol) was acylated similar to the synthesis of **20** with octacosanoic acid (12 mg, 30 μmol), using DCC (9.3 mg, 45 μmol) and DMAP (2 mg, 16.5 μmol) as activating agents, to afford **21** as a white fluffy solid (30 mg, 65%). R_f = 0.65 (DCM/methanol, 60/1, v/v). $[\alpha]^{26}_D$ = +14.8° (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41-7.11 (m, 30H, aromatic), 6.46 (d, 1H, $J_{NH,2}$ = 8.4 Hz, N*H*), 5.70 (d, 1H, $J_{1,2}$ = 5.3 Hz, H-1), 5.59 (d, 1H, $J_{NH',2'}$ = 8.1 Hz, N*H*'), 5.37 (s, 1H, CH, benzylidene), 5.37 (dd, 1H, $J_{3',4'}$ = 9.3 Hz, H-3'), 5.24 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.0 Hz, H-3), 4.95 (m, 1H, H-3$_L$), 4.80 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.37-4.61 (m, 9H, H-2, 4 x C*H*$_2$, Bn), 4.33 (m, 1H, H-5), 4.29 (dd, 1H, $J_{5',6'a}$ = 5.4 Hz, $J_{6'a,6'b}$ = 11.1 Hz, H-6'a), 3.64-4.00 (m, 8H, H-2', H-4, H-6a, H-6b, H-6'b, 3 x H-3$_S$), 3.60 (dd, 1H, $J_{4',5'}$ = 9.3 Hz, H-4'), 3.48 (m, 1H, H-5'), 1.90-2.64 (m, 10H, H-2$_L$, H-2$_{L'}$, 3 x H-2$_S$), 1.63-1.05 ((m, 146H, 73 x CH_2, lipid), 0.85 (m, 15H, 5 x CH_3, lipid). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.91 (*C*=O), 172.35 (*C*=O), 171.45 (*C*=O), 171.28 (*C*=O), 169.91 (*C*=O), 135.59-125.00 (aromatic), 101.23 (*C*H, benzylidene), 100.92 (C-1'), 87.03 (C-1), 78.08 (C-4'), 75.93 (C-4, 2 x C-3$_S$), 75.21 (C-3$_S$), 74.52 (*C*H$_2$, Bn), 73.00 (C-3), 71.2 (C-5), 70.91 (C-3'), 70.42-70.64(3 x *C*H$_2$, Bn), 70.33 (C-3$_L$), 68.26 (C-6'), 67.41 (C-6), 66.03 (C-5'), 55.52 (C-2'), 52.66 (C-2). HR MS (*m*/*z*) calcd for $C_{145}H_{230}N_2O_{17}S[M+Na]^+$, 2326.6847; found, 2326.7550.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-2-[(*R*)-3-(27-benzyloxy-octacosanoyloxy)-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-

benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-D-glucopyranose (22): NIS (10.0 mg, 44.4 μmol) and TMSOTf (0.3 μL, 1.5 μmol) were added to a stirred solution of **20** (25 mg, 10.4 μmol) in DCM/H_2O (3 mL, 100/1, v/v) at 0 ^oC. The reaction mixture was vigorously stirred at room temperature for 20 min until TLC analysis indicated that reaction had gone to completion. The reaction mixture was diluted with DCM (5 mL), and then washed with aqueous $Na_2S_2O_3$ (10%, 5 mL) and water (2 x 5 mL). The organic phase was dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (eluent: toluene/ethyl acetate, 20/1, v/v) to afford lactol **22** as a white solid (18 mg, 78%). R_f = 0.45 (DCM/methanol, 50/1, v/v). ^1H NMR (600 MHz, $CDCl_3$): δ 7.39-7.16 (m, 25H, aromatic), 6.28 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.92 (d, 1H, $J_{NH',2'}$ = 7.8 Hz, N*H*'), 5.43 (s, 1H, CH, benzylidene), 5.42-5.39 (m, 2H, H-3, H-3'), 5.20 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.09 (m, 1H, H-1), 4.96 (m, 1H, H-3_L), 4.61-4.40 (m, 8H, 4 x CH_2, Bn), 4.35 (dd, 1H, $J_{5',6'a}$ = 5.4 Hz, $J_{6'a,6'b}$ = 10.8 Hz, H-6'a), 4.20 (m, 1H, H-2), 4.06 (m, 1H, H-5), 3.95 (d, 1H, $J_{6a,6b}$ = 12.0Hz, H-6a), 3.84-3.74 (m, 4H, H-6'b, 3 x H-3_S), 3.69-3.58 (m, 3H, H-2', H-4', H-6b), 3.54-3.48 (m, 2H, H-5', H-$27_{L'}$), 3.37 (t, 1H, $J_{4,3}$ = $J_{4,5}$ = 9.6 Hz, H-4), 2.65 -2.56 (m, 2H, H-2_S), 2.50 (dd, 1H, *J* = 5.4 Hz, *J* = 15.0 Hz, H-2_{Sa}), 2.41 (dd, 1H, *J* = 4.8 Hz, *J* = 15.6 Hz, H-2_{Sb}), 2.35-2.20 (m, 4H, 2 x H-2_S, H-2_{La}, H-$2_{La'}$), 2.12-2.02 (m, 2H, H-2_{Lb}, $2_{Lb'}$), 1.59-1.40 (m, 12H, H-4_L, H-$3_{L'}$,$26_{L'}$, 3 x H-4_S), 1.25 (broad, 176H, 88 x CH_2, lipid), 1.18 (d, 3H, $J_{27L',28L'}$ = 6.0 Hz, H-$28_{L'}$), 0.89-0.87 (m, 12H, 4 x CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{146}H_{232}N_2O_{19}[M+Na]^+$, 2340.7147; found, 2340.7925.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl-2-deoxy-2-[(*R*)-3-octacosanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-D-glucopyranose (23):

Compound **21** (30 mg, 13 μmol) was hydrolyzed similar to the synthesis of **22** with NIS (12 mg, 52 μmol) and TMSOTf (1.0 μL, 2.7 μmol) in DCM/H_2O (4 mL, 100/1) to afford **23** as a white solid (23.4 mg, 80%). R_f = 0.45 (DCM/methanol, 50/1, v/v). 1H NMR (300 MHz, $CDCl_3$): δ 7.38-7.12 (m, 26H, aromatic), 6.26 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N*H*), 5.90 (d, 1H, $J_{NH',2'}$ = 8.1 Hz, N*H*'), 5.41 (s, 1H, CH, benzylidene), 5.40-5.36 (m, 2H, H-3, H-3'), 5.18 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.10 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1), 4.95 (m, 1H, H-3_L), 4.59-4.37 (m, 9H, H-2, 4 x CH_2, Bn), 4.36 (m, 1H, H-6'a), 4.17 (m, 1H, H-2), 4.03 (m, 1H, H-5), 3.92 (m, 1H, H-6a), 3.83-3.47 (m, 8H, H-2', H-4', H-5', H-6b, H-6'b, 3 x H-3_S), 3.35 (t, 1H, $J_{4,5}$ = 9.6 Hz, H-4), 1.64-1.99 (m, 10H, H-2_L, H-$2_{L'}$, 3 x H-2_S), 1.63-1.05 (m, 146H, 73 x CH_2, lipid), 0.88-0.84 (m, 15H, 5 x CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{139}H_{226}N_2O_{18}[M+Na]^+$, 2234.6728; found, 2234.6657.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl-2-deoxy-2-[(*R*)-3-(27-benzyloxy-octacosanoyloxy)-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-D-glucono-1,5-lactone (24): A suspension of **22** (10 mg, 4.3 μmol) and activated molecular sieves (3 Å, 25 mg) in DCM (2 mL) was stirred at room temperature under an atmosphere of argon for 1 h. PCC (9.3 mg, 43 μmol) was then added and the reaction mixture was stirred for another 1.5 h until TLC analysis indicated completion of the reaction. The reaction mixture was purified by Iatro beads column chromatography (eluent: hexane/ethyl acetate, 5/1-3/1, v/v) to afford lactone **24** as a colorless film (7.0 mg, 70%). R_f = 0.35 (hexane/ethyl acetate, 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.39-7.18 (m, 25H, aromatic), 5.60 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.34 (s, 1H, CH, benzylidene), 5.32 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.06 (m, 1H, H-3_L), 4.94 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1'), 4.78 (t, 1H, $J_{1,2} = J_{2,3}$ = 10.2 Hz, H-2), 4.59-4.36 (m, 8H, 4 x CH_2, Bn), 4.40 (m, 1H, H-5), 4.26 (dd, 1H, $J_{5',6'a}$ = 4.8 Hz, $J_{6'a,6'b}$ = 9.6 Hz, H-6'a), 4.04-4.01 (m, 2H, H-4, H-6a),

3.83 (m, 1H, H-3_S), 3.80-3.76 (m, 2H, 2 x H-3_S), 3.69 (t, 1H, $J_{5',6'b}$ = 10.2 Hz, H-6'b), 3.56-3.47 (m, 5H, H-2', H-4', H-5', H-6b, H-$27_{L'}$), 2.64 (dd, 1H, J = 6.0 Hz, J = 14.4 Hz, H-2_{Sa}), 2.52 (dd, 1H, J = 7.2 Hz, 15.6 Hz, H-2_{Sb}), 2.46 (dd, 1H, J = 6.0 Hz, J = 18.0 Hz, H-2_{Sa}), 2.39-2.23 (m, 7H, H-2_S x 3, H-2_L, $2_{L'}$), 1.59-1.40 (m, 12H, H-4_L, H-$3_{L'}$,$26_{L'}$, 3 x H-4_S), 1.24 (broad, 176H, 88 x CH_2, lipid), 1.17 (d, 3H, $J_{27L',28L'}$ = 6.0 Hz, H-$28_{L'}$), 0.88-0.86 (m, 12H, 4 x CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{146}H_{230}N_2O_{19}[M+Na]^+$, 2338.6990; found, 2338.8489.

4-*O*-Benzyl-6-*O*-{4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl-2-deoxy-2-[(*R*)-3-octacosanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-tetradecanoyl]-2-deoxy-D-glucono-1,5-lactone (25): Compound **23** (12 mg, 5.4 μmol) was oxidized similar to the synthesis of **24** with PCC (9.4 mg, 43.4 μmol) to afford lactone **25** as a colorless film (8.5 mg, 71%). R_f = 0.35 (hexane/ethyl acetate, 3/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.17 (m, 25H, aromatic), 5.60 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 9.5 Hz, H-3'), 5.39 (s, 1H, CH, benzylidene), 5.31 (dd, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.04 (m, 1H, H-3_L), 4.93 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.78 (t, 1H, $J_{2,3}$ = 10.5 Hz, H-2), 4.59-4.36 (m, 9H, H-5, 4 x CH_2, Bn), 4.27 (dd, 1H, $J_{5',6'a}$ = 5.5 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 4.04-4.01 (m, 2H, H-4, H-6a), 3.87-3.75 (m, 3H, 3 x H-3_S), 3.69 (t, 1H, $J_{5,6'b}$ = 9.5 Hz, H-6'b), 3.57-3.50 (m, 4H, H-2', H-4', H-5', H-6b), 2.30-1.93 (m, 10H, H-2_L, H-$2_{L'}$, 3 x H-2_S), 1.00-1.75 (m, 146H, 73 x CH_2, lipid), 0.82 (m, 15H, 5 x CH_3, lipid). HR MS (*m*/*z*) calcd for $C_{139}H_{224}N_2O_{18}[M+Na]^+$, 2232.6572; found, 2232.6703.

2-Deoxy-6-*O*-{2-deoxy-3-*O*-[(*R*)-3-hydroxy-tetradecanoyl]-2-[(*R*)-3-(27-hydroxy-octacosanoyloxy)-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxy-hexadecanoylamino]-3-*O*-[(*R*)-3-hydroxy-hexadecanoyl]-D-glucono-1,5-lactone (1): Compound **24** (7.0 mg, 3.0 μmol) was dissolved in THF/*t*-BuOH (2 mL, 1/3, v/v) and Pd/C (10

mg) was added. The reaction mixture was shaken under an atmosphere of H_2 (15 psi) at room temperature for 36 h, and the catalyst was filtered off and the residue was washed with THF (2 x 1 mL). The combined filtrates were concentrated to afford **1** as a colorless film (4.2 mg, 75%). ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ = 5.40 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.12 (bs, 1H, H-3_L), 4.98 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 4.62 (bs, 1H, H-1'), 4.30-3.95 (m, H-2, H-4, H-5, H-6'a, H-6'b), 3.81-3.33 (m, H-2', H-4', H-5', H-6a, H-6b, H-$27_{L'}$), 2.44-2.24 (m, 10H, H-2_L, H-$2_{L'}$, 3 x H-2_S,). HR MS (*m*/z) calcd for $C_{104}H_{196}N_2O_{19}[M + Na]^+$, 1800.4330; found, 1800.6962.

2-Deoxy-6-*O*-{2-deoxy-3-*O*-[(*R*)-3-hydroxy-tetradecanoyl]-2-[(*R*)-3-octacosanoyloxy-hexadecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxy-hexadecanoylamino]-3-*O*-[(*R*)-3-hydroxy-hexadecanoyl]-D-glucono-1,5-lactone (2): Compound **25** (7.5 mg, 3.4 μmol) was deprotected similar to the synthesis of **1** by hydrogenation (H_2, 15 psi) over Pd/C (10 mg) in THF/*t*-BuOH (2 mL, 1/3, v/v) to afforded **2** as a colorless film (4.8 mg, 80%). ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ 5.39 (t, 1H, $J_{3,4} = J_{3,4}$ = 9.6 Hz, H-3), 5.09 (bs, 1H, H-3_L), 4.97 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 4.55 (bs, 1H, H-1'), 4.32 (m, 1H, H-5), 4.17 (m, 1H, H-2), 4.13 (m, 1H, H-6'a), 3.95 (m, 1H, H-4), 3.85-3.81 (m, 2H, H-2', H-6'b), 3.76- 3.70 (m, 2H, H-6a, H-6b), 3.54 (t, 1H, $J_{4',5'}$ = 9.3 Hz, H-4'), 3.33 (m, 1H, H-5'), 2.55-2.13 (m, 10H, H-2_L, H-$2_{L'}$, 3 x H-2_S), HR MS (*m*/z) calcd for $C_{104}H_{196}N_2O_{18}[M+Na]^+$, 1784.4381; found, 1784.4586.

Biological Experiments

Reagents: *E. coli* 055:B5 LPS was obtained from List Biologicals, and *R. sin-1* LPS and lipid A were kindly provided by Dr. R. Carlson (CCRC, Athens, GA). All data presented in this study were generated using the same batches of *E. coli* 055:B5 LPS and *R. sin-1* LPS. Synthetic compounds **1**-**3** were stored lyophilized at -80 ^oC and reconstituted in dry THF on the day of the

experiment; final concentrations of THF in the biological experiments never exceeded 0.5% to avoid toxic effects.

Cell maintenance: Mono Mac 6 (MM6) cells, provided by Dr. H.W.L. Ziegler-Heitbrock (Institute for Inhalationbiology, Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with penicillin (100 u/mL) / streptomycin (100 µg/mL; Mediatech, OPI supplement (1%; Sigma; containing oxaloacetate, pyruvate and bovine insulin) and fetal calf serum (FCS; 10%; HyClone). New batches of frozen cell stock were grown up every 2 months and growth morphology evaluated. Before each experiment, MM6 cells were incubated with calcitriol (10 ng/mL; Sigma) for 2 days to differentiate into macrophage like cells. The cells were maintained in a humid 5% CO_2 atmosphere at 37 ^{o}C.

Cytokine induction and TNF-α ELISA: On the day of the exposure assay differentiated MM6 cells were harvested by centrifugation and gently suspended (10^6 cells/mL) in prewarmed (37 ^{o}C) medium. MM6 cells were then incubated with different combinations of stimuli for 5.5 hours. Culture supernatants were then collected and stored frozen (-80 ^{o}C) until assayed for TNF-α production. Concentrations of human TNF-α protein in culture supernatants were determined by a solid phase sandwich ELISA. Plates (96-well MaxiSorp plates; Nalge Nunc International) were coated with purified mouse anti-human TNF-α antibody (Pharmingen). TNF-α in standards and samples was allowed to bind to the immobilized antibody. Biotinylated mouse anti-human TNF-α antibody (Pharmingen) was then added, producing an antibody-antigen-antibody "sandwich". After addition of avidin-horseradish peroxidase conjugate (Pharmingen) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories), a green color was produced in direct proportion to the amount of TNF-α present in the sample. The reaction was stopped by adding peroxidase stop solution (Kirkegaard & Perry Laboratories) and the absorbance was measured at 405 nm using a

microplate reader (BMG Labtech). TNF-α values are presented as the means ± SD of triplicate measurements, with each experiment being repeated three times.

Data analysis: Concentration-response and inhibition data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). Concentration-response 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. Inhibition data were fit with the following logistic equation: $Y = Bottom + (Top – Bottom) / (1 + 10^{(X - Log\ IC50)})$, where Y is the TNF-α response, X is the logarithm of the concentration of the inhibitor, and IC_{50} is the concentration of the inhibitor that reduces the TNF-α response by half.

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CHAPTER 5

DETERMINATION OF THE ACTIVATION PATHWAYS OT *PORPHYROMONAS GINGIVALIS* LIPOPOPLYSACCHARIDE USING SYNTHETIC LIPID A DERIVATIVES[#]

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Abstract

There is some indication that *P. gingivalis* LPS initiates innate immune responses through TLR2 and/or TLR4. The heterogeneity of LPS and lipid A preparations limits the identification of specific structural features that are responsible for this unusual activation pathway. To address this problem, two lipid As of *P. gingivalis* LPS have been synthesized by a highly convergent approach, which can provide easy access to a wide range of lipid As to study their structure-activity relationship. Furthermore, an efficient method has been developed for the synthesis of optically pure 3-hydroxy fatty acids with a terminal isopropyl group, which are the important constituents and synthetic intermediates of a wide rang of biologically interesting natural compounds including *P. gingivalis* lipid As.

Introduction

Mammalian Toll-like receptors (TLR) comprise a large family containing extracellular leucine-rich repeats and a cytoplasmic Toll/IL-1R homology domain, and are implicated in the recognition of pathogen-associated microbial products.[1,2] So far, 10 members (TLR1-10) have been reported,[3-7] among which TLR4 has been shown to be a critical receptor and signal transducer for lipopolysaccharide (LPS),[8] the major constituent of the outer surface of the gram-negative bacterial cell wall. TLR2 is known to be essential for the signaling of some bacterial components, such as peptidoglycan,[9,10] bacterial lipoprotein,[11-14] zymosan,[15] and bacterial fimbriase and their peptides.[16]

Porphyromonas gingivalis is a periopathogen strongly associated with the development of adult-type periodontitis.[17] The innate host response to *P. gingivalis* LPS is unusual in that it activates murine macrophages through TLR2 and/or TLR4,[18] with different studies reporting that it can be an antagonist[19] or agonist[20] for TLR4. *P. gingivalis* LPS contains an unusual amount of

lipid A heterogeneity. At least four structures have now been characterized which differ in the number of phosphate groups and fatty acids.[17] The presence of multiple lipid A structures has complicated the interpretation of innate responses elicited by *P. gingivalis* LPS preparations, thus hindering a more complete understanding of the contribution of *P. gingivalis* LPS to the periodontitis pathogen. Two lipid As **3** and **4** (**Figure 5.1**) from *P. gingivalis* have been chemically synthesized, and biological assays showed that both of these lipid As activate the cell through TLR4 rather than TLR2.[8,21] However, highly purified native *P. gingivalis* LPS or lipid A preparations consistently demonstrate TLR2 activity.[8,21,22,23] It has been proposed that trace amounts of tightly associated endotoxin proteins[24] or other as-yet-undefined *P. gingivalis* components[8] are responsible for TLR2 activity in native *P. gingivalis* LPS preparations.[18] A recent study showed that an LPS preparation enriched for lipid A species at m/z 1435 and 1450, whose structures have been elucidated as being tetra-acylated monophosporylated compounds **1** and **2** (**Figure 5.1**), can activate TLR2 and TLR4.[18] This result could account for the apparent discrepancy among studies employing chemically synthesized lipid A species and native preparations. However, just as the paper said, the presence of minor amounts of other lipid A species in the employed LPS preparations can not be excluded, and it was difficult to quantify

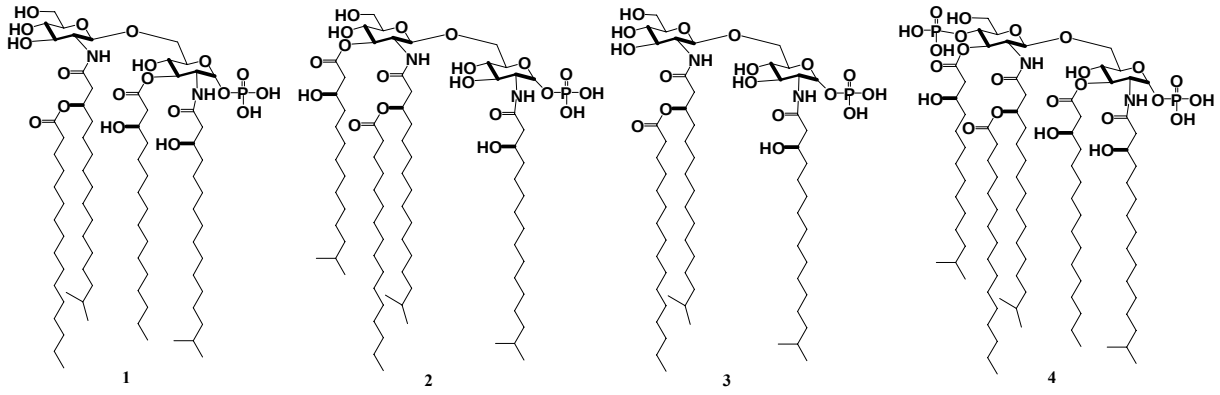


Figure 5.1 Chemical structures of *Porphyromanas gingivalis* Lipid As **1-4**

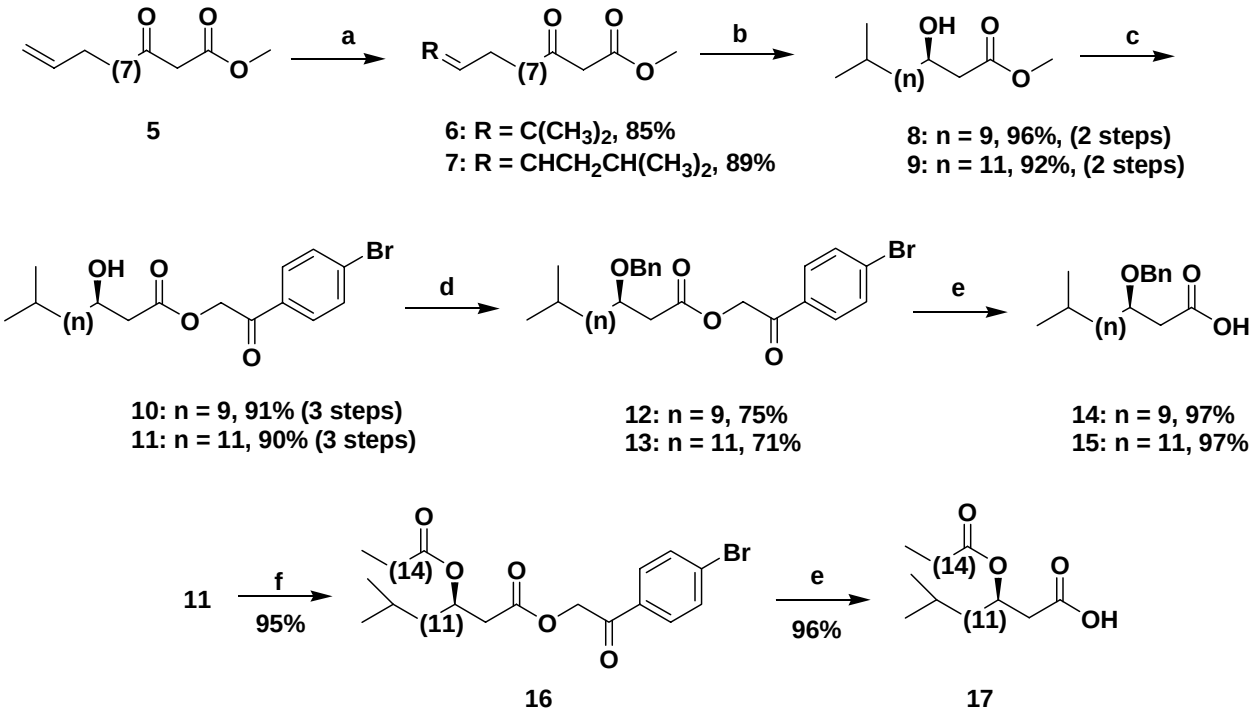
the amount of each lipid A species by MALDI-TOF analysis, which limits the identification of specific structural features responsible for the TLR2 and TLR4 activation pathway of *P. gingivalis* LPS. Fortunately, chemical synthesis can afford pure compounds of well-defined structure. Hence, we have chemically synthesized the two lipid As (compound **1**, **2**) from *P. gingivalis* LPS. To synthesize these two compounds we developed a highly convergent approach, which can provide easy access to a wide range of lipid As to study their structure-activity relationship. Biological assay of these two compounds would afford convincing evidence to clarify which lipid As of *P. gingivalis* LPS are responsible for TLR2 activation.

Result and discussion

Optically pure 3-hydroxy fatty acids with a terminal isopropyl group are important constituents or synthetic intermediates of a wide range of biologically interesting natural compounds, including Flavolipin,[25] N-4909 (a stimulator of apolipoprotein E secretion),[26] liposidomycin-B,[27] and some lipid As.[18] While several approaches have been developed for the synthesis of such compounds by both chemical and enzymatic methods,[28-32] surprisingly these methods involve too many synthetic steps, some of which are conducted in harsh and difficult to handle conditions and as such give low overall yields and are time-consuming.

We aimed to develop a convenient approach to synthesize this type of fatty acid. Thus, known compound **5** (**scheme 5.1**), which could be easily prepared by two synthetic steps, was reacted with olefins in the presence of the Grubbs catalyst 2nd generation to afford **6** and **7** respectively. In the synthesis of intermediate **6**, to avoid using special instruments we used 2-methyl-2-butene rather than 2-methyl-propene,[33] which is a gas at room temperature, as the reacting alkene. The ketone of the resulting cross metathesis of products **6** and **7** was reduced enantioselectively by catalytic hydrogenation in the presence of (*R*)-$RuCl_2$(BINAP) to give

optically pure **8** and **9** with *R*-configuration.[34] Although it is almost putative that the reduction of β-keto esters catalyzed by *R/S* $RuCl_2$(BINAP) gives 3-hydroxy esters with high enantiomeric excess,[35] the optical purity of



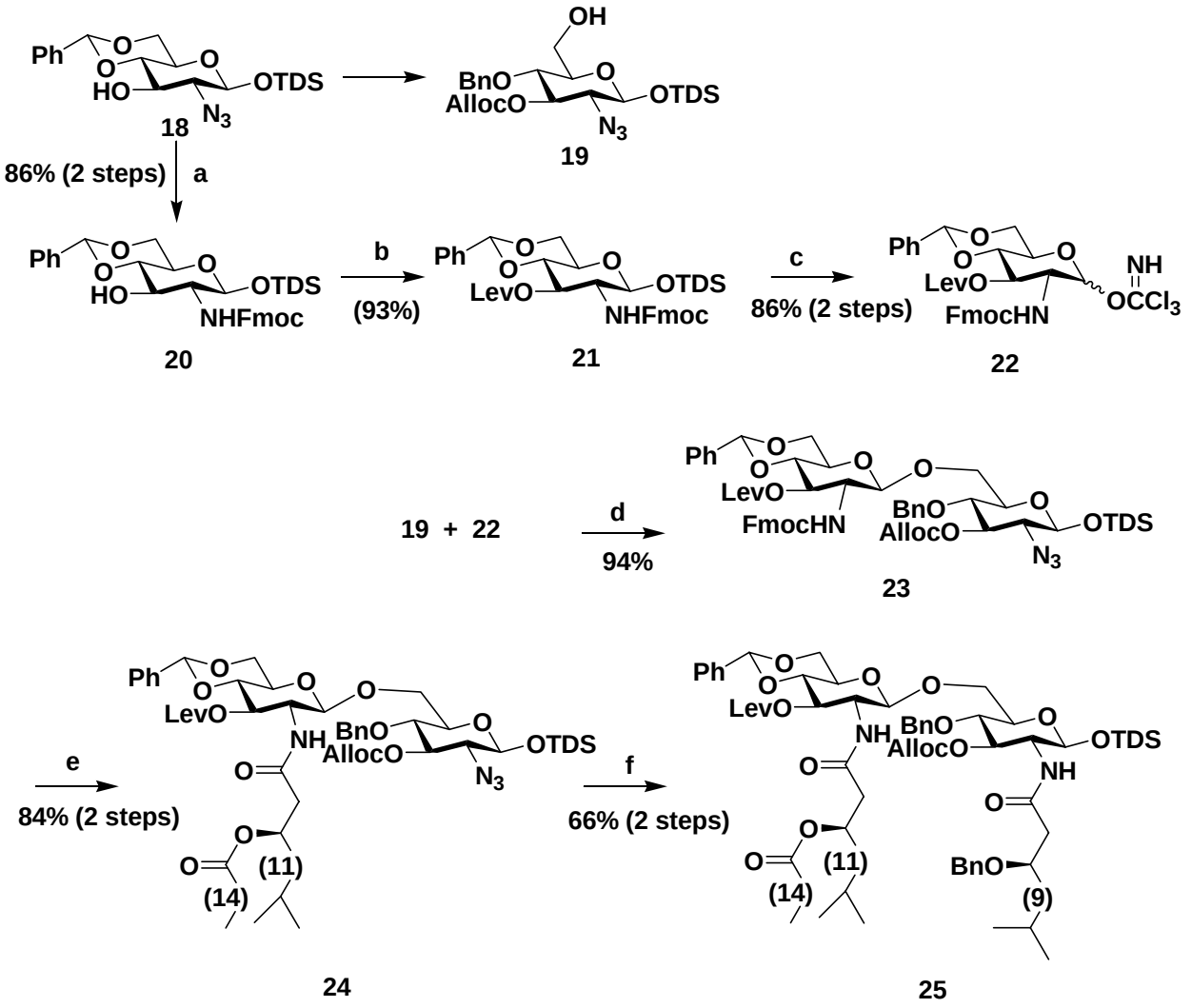
Scheme 5.1 Reagents and conditions: (a) R=CH_2, Grubbs 2nd generation cat. (b) 1: $RuCl_2$[(*R*)-BINAP], H_2 (65 psi), 2 M HCl, CH_3OH, 40 ºC; 2: H_2 (1 atm), Pd/C, CH_3OH; (c) 1: LiOH, THF/H_2O; 2: DCHA, CH_3CN, reflux; 3: Et_3N, EtOAc; (d) benzaldehyde, $(TMS)_2O$, TMSOTf, THF; then Et_3SiH; (e) Zn/HOAc, 60 ºC; (f) hexadecanoyl chloride, pyridine, DMAP, DCM.

8 was determineded by NMR spectroscopic analysis.[36] Thus, NMR experiments of compound **8** and its *R*/*S*-racemic mixture were conducted in the presence of optically active chemical shift reagent $Eu(hfmc)_3$ in $CDCl_3$. It was found that the difference in the chemical shift for the methyl proton of methoxyl of *R*/*S*-racemic mixture was 46.8 Hz, and no (*S*)-enantiomer was observed in the NMR spectrum. The *R*/*S*-racemic mixture was prepared by reduction of **6** with $NaBCNH_3$. It

should be mentioned the *S*-isomers can be easily prepared using (*S*)-$RuCl_2(BINAP)_2$ as the catalyst. Next, compounds **8** and **9** were hydrolysed under standard conditions, and the resulting acids were neutralized with dicyclohexaneamine to give solid salts, which were recrystallized from CH_3CN to improve their optical purity. The carboxylate of the intermediates was protected with 2-(4-bromophenyl)-2-oxoethyl to give key intermediates **10** and **11.**[34] This protecting group can be removed by treatment with zinc in acetic acid without affecting an ether or ester group, so the 3-hydroxy of **10** and **11** can be protected as a benzyl ether or modified with an acyl group, both of which are important intermediates for the synthesis of natural forms of this type of lipid. Thus, the hydroxyl of **10** and **11** was protected as a benzyl ether by reaction with benzaldehyde with the aid of TMSOTf in a mixture of $(TMS)_2O$ and THF, followed by reduction by Et_3SiH.[37] The resulting compounds **12** and **13** were deprotected by treatment with zinc in acetic acid to give lipids **9** and **11**, respectively. Alternatively, compound **11** could be modified with hexadecanoyl chloride in the presence of pyridine and DMAP to yield **16**, which was deprotected to produce lipid **17**. As it has been shown, all the reactions involved in this method could be conducted under mild conditions and handled easily, and were high-yielding. Furthermore, it is to be expected that other fatty acids with various terminal substituents can be synthesized employing this method by using different olefins, which makes it a convergent synthesis.

Target compound **1** and **2** have a similar structure. Both of them have only a phosphate group at the anomeric position and the same fatty acids at the 2- and 2'- amino groups. Their structures differ only in the acylation pattern of the 3- and 3'- hydroxyls. While compound **1** has a fatty acid only at the 3-hydroxyl, the 3'-hydroxyl of compound **2** is acylated with a different fatty acid. To synthesize these two compounds with a similar structure, we have developed a convergent approach (**scheme 5.2**). This approach employed the advanced disaccharide intermediate **23**,

which is protected with a set of orthogonal protecting groups: the levulinicate (Lev), the 9-fluorenylmethoxycarbamate (Fmoc), the allyloxycarbonate (Alloc), the azido, and the anomeric dimethylthexylsilyl (TDS). Disaccharide **23** can be selectively modified with any lipid at C-2, C-3, C-2', and C-3', so this strategy provides easy access to a wide range of lipid As, which can be



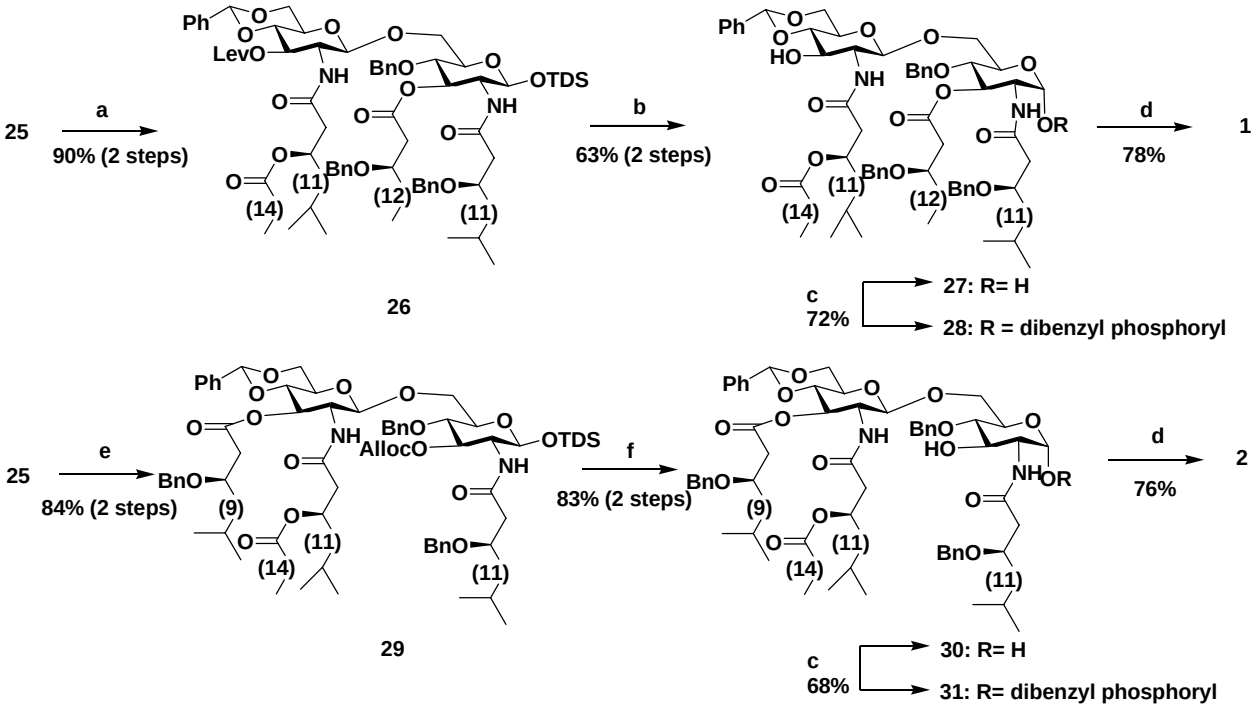
Scheme 5.2 Reagents and conditions: (a) 1: Zn/HOAc, DCM; 2: FmocCl, DIPEA, DCM; (b) Levoulic acid, DCC, DMAP, DCM; (c) 1: Bu_4NF/HOAc, THF; 2: $CNCCl_3$, NaH, THF; (d) TfOH, DCM, -50 ºC; (e) 1: DBU, DCM; 2: (*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoic acid **17**, DCC, DCM; (f) 1: Zn/HOAc, DCM; 2: (*R*)-3-benzyloxy-15-methyl-hexadecanoic acid **15**, DCC, DCM.

used for SAR studies. Furthermore, it has been found that 4'-phosphate of lipid As tends to migrate to the 3'-hydroxyl,[38] and therefore it was necessary to introduce the phosphate after modification of the 3'-hydroxyl with a fatty acid using standard conditions. The 4',6'-diol of **23** is protected as a benzylidene acetal, and therefore the phosphate can be introduced at a late stage by regioselectively opening of the benzylidiene acetal to give the free 4'-hydroxyl.

Another attractive feature of the approach is that glycosyl donor **22** and acceptor **19** can be synthesized from the common intermediate **18**, which can be easily prepared from glucosamine. Thus, glycosyl acceptor **19** was synthesized from **18** according to the procedure developed in the synthesis of lipid A derivatives containging Kdo. The azido moiety of **18** could be easily converted to Fmoc carbomate by treatment with zinc and acetic acid followed by reaction with FmocCl in the presence of DIPEA. The hydroxyl of resulting compound **20** was protected with Lev to afford **21**. Next, removal of the anomeric TDS of **21** was easily accomplished by treatment with Bu_4NF in the presence of acetic acid. The resulting hydroxyl was allowed to react with trichloroacetonitrile with the aid of NaH to afford trichloroacetimidate **22**.[39] A trifluoromethanesulfonic acid (TfOH)-mediated glycosylation of **19** with **22** proceeded in a stereoselective manner to give disaccharide **23** in an excellent yield of 92%.

Having the advanced disaccharide **23** and lipids **14**, **15** and **17** at hand, attention focused on the selective acylation of relevant hydroxyls and amines. Thus, removal of the Fmoc protecting group of **23** using 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) in DCM followed by acylation of the resulting amino group with lipid **17** using dicyclohexylcarbodiimide (DCC) as the activation reagent gave compound **24** (**scheme 5.3**). Next, the azido moiety of **24** was reduced by treatment with zinc and acetic acid in DCM, and the amine of the resulting compound reacted with lipid **11** in the presence of DCC to afford **25** as the common intermediate for the synthesis of target

molecules **1** and **2**. Thus, for the synthesis of **1**, the Alloc protecting group of **25** was removed by reaction with $Pd(PPh_3)_4$ in the presence of HCOOH and n-$BuNH_2$,[40] and the resulting hydroxyl acylated with (*R*)-3-benzyloxy-hexadecanoic acid using DCC and DMAP as the activation reagent to give **26**. Next, removal of the Lev group of **26** was easily accomplished by treatment with hydrazine acetate. The anomeric TDS of the resulting compound was removed with Bu_4NF



Scheme 5.3 Reagents and conditions: (a) 1: $Pd(PPh_3)_4$, HCO_2H, n-$BuNH_2$, THF; 2: (*R*)-3-benzyloxy-hexadecanoic acid, DCC, DMAP, DCM; (b) 1: H_2NNH_2·HOAc, DCM/CH_3OH; 2: Bu_4NF/HOAc, THF; (c) tetrabenzyl diphosphate, $LiN(TMS)_2$, THF, -78 ºC; (d) H_2 (50 psi), Pd black, THF; (e) 1: H_2NNH_2·HOAc, DCM/CH_3OH; 2: (*R*)-3-benzyloxy-13-methyl-tetradecanoic acid **14**, DCC, DMAP, DCM; (f) 1: $Pd(PPh_3)_4$, HCO_2H, n-$BuNH_2$, THF; 2: Bu_4NF/HOAc, THF.

in the presence of acetic acid to give the desired product **27** in a yield of 72% and a small amount of side product from elimination of the 3-acyloxyl group was also isolated. Finally, the anomeric

alcohol of **27** was phosphorylated using tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide in THF at -78 ºC to give **28** as only the α-anomer. Global deprotection of **28** by catalytic hydrogenolysis over Pd-black gave requisite lipid A **1**.[41] The synthesis of **2** could be accomplished in a manner similar to that of **1**. Thus, removal of the Lev protecting group of the common intermediate **25** followed by acylation with lipid **14** under standard conditions produced **29**. Next, the Alloc and anomeric protecting groups were removed to give **30**. No elimination was observed in this reaction, which was to be expected because a hydroxyl group is a poor leaving group compared to an acyloxyl group. In a manner similar to the synthesis of **1**, compound **30** was phosphorylated and deprotected to give target lipid A **2**.

Conclusion

An efficient method has been developed for the synthesis of optically pure 3-hydroxy fatty acids with a terminal isopropyl group by olefin cross metathesis between a β-keto ester with a terminal double bond and different olefins. Two lipid As of *P. gingivalis* LPS have been synthesized by a highly convergent approach, which should provide easy access to a wide range of lipid As to study their structure-activity relationship. Biological assay of these two compounds should clarify which lipid As of *P. gingivalis* LPS are responsible for TLR2 activation.

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 F254 (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 ºC. CH_2Cl_2 was distilled from NaH and stored over molecular sieves (3 Å). Tetrahydrofuran (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 ºC during 8 h in the first instance and then for 2-3 h at 390 ºC 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.

Methyl 13-methyl-3-oxo-12-teradecenoate (**6**): Grubbs 2nd catalyst was added to a stirring solution of compound **5** (1.0 g, 4.17 mmol) in 4-methyl-pentene (20 mL) under an atmosphere of nitrogen. After stirring the reaction mixture at room temperature for 24 h, it was concentrated *in vacuo* to 0.5 mL, and subjected to purification by silica gel column chromatography (eluent: hexane/ethyl acetate, 30/1, v/v) to afford **6** as a colorless oil (949 mg, 85%). R_f = 0.50 (hexane/ethyl acetate, 10/1, v/v); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 5.08 (t, 1H, J = 6.9 Hz, H-12), 3.71 (s, 3H, OCH_3), 3.42 (s, 2H, H-2), 2.50 (t, 2H, $J_{4,5}$ = 7.5 Hz, H-4), 1.91 (m, 2H, H-11), 1.66 (s, 3H, H-14), 1.57-1.54 (m, 5H, H-5, H-14), 1.25 [bs, 10H, H-(6-10)]. HR MS (m/z) calcd for $C_{16}H_{28}O_3$ $[M + Na]^+$, 291.1931; found, 291.1965.

Methyl 15-methyl-3-oxo-12-hexadecenoate (7): Grubbs 2nd catalyst was added to a stirring

solution of compound **5** (125 mg, 0.52 mmol) in 4-methyl-pentene (2 mL) under an atmosphere of nitrogen. After stirring the reaction mixture at room temperature for 16 h, it was concentrated *in vacuo* to 0.5 mL, which was subjected by silica gel column chromatography (eluent: hexane/ethyl acetate, 30/1, v/v) to afford **7** as a colorless oil (137 mg, 89%). R_f = 0.50 (hexane/ethyl acetate, 10/1, v/v); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 5.32-5.29 (m, 2H, H-12, H-13), 3.66 (s, 3H, OC*H*$_3$), 3.38 (s, 2H, H-2), 2.46 (t, 2H, $J_{4,5}$ = 7.2 Hz, H-4), 1.92 (m, 2H, H-14), 1.79 (m, 2H, H-11), 1.55-1.47 (m, 4H, H-5, H-15), 1.22 [bs, 10H, H-(6-10)], 0.80 (d, 6H, $J_{15,16}$ = 6.9 Hz, H-16). HR MS (m/z) calcd for $C_{18}H_{32}O_3$ $[M + Na]^+$, 319.2238; found, 319.2607.

Methyl (*R*)-3-hydroxy-13-methyl-tetradecanoate (8): A solution of **6** (800 mg, 2.99 mmol) in methanol (15 mL) was degassed with nitrogen for 10 min, after which 2 M HCl (0.2 mL) and $RuCl_2$[(*R*)-BINAP] (20 mg) were added under a nitrogen atmosphere. The reaction mixture was shaken under an atmosphere of H_2 (65 psi) at 45 ºC for 12 h, after which it was quenched with Et_3N (100 μL). The solids were filtered off, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 6/1, v/v) to afford the alcohol intermediate as a colorless oil. The intermediate was shaken with Pd/C (10 mg) in methanol (15 mL) under an atmosphere of H_2 (1 atm) for 12 h, after which the catalyst was filtered off, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography ((hexane/ethyl acetate, 6/1, v/v) to **8** as a colorless oil (780 mg, 96%, two steps). R_f = 0.45 (hexane/ethyl acetate, 4/1, v/v); $[\alpha]^{25}_D$ = -7.2º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 3.98 (m, 1H, H-3), 3.69 (s, 3H, OC*H*$_3$), 2.50 (dd, 1H, $J_{2a,2b}$ = 13.5 Hz, $J_{2a,3}$ = 3.6 Hz, H-2a), 2.38 (dd, 1H, $J_{2a,2b}$ = 13.5 Hz, $J_{2b,3}$ = 8.7 Hz, H-2b), 1.53-1.36 (m, 3H, H-4, H-13), 1.23-1.09 [m, 16H, H-(5-12)], 0.84 (d, $J_{13,14}$ = 6.9 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.51 (*C*=O), 68.03 (C-3), 51.71 (*C*H_3O), 41.08 (C-2), 39.03 (C-12), 36.52 (C-4), 22.64 (C-

14). HR MS (m/z) calcd for $C_{16}H_{32}O_3$ $[M + Na]^+$, 295.2244; found, 295.2194.

Methyl (*R*)-3-hydroxy-15-methyl-hexadecanoate (9): In a manner similar to the synthesis of compound **8**, compound **7** (100 mg, 0.338 mmol) was reduced by a two step procedure to afford **9** as a colorless oil (93 mg, 92%, two steps). R_f = 0.50 (hexane/ethyl acetate, 4/1, v/v); $[\alpha]^{25}_D$ = -6.0º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 3.95 (m, 1H, H-3), 3.66 (s, 3H, OC*H*$_3$), 2.47 (dd, 1H, $J_{2a,2b}$ = 16.2 Hz, $J_{2a,3}$ = 3.3 Hz, H-2a), 2.36 (dd, 1H, $J_{2a,2b}$ = 16.2 Hz, $J_{2b,3}$ = 9.0 Hz, H-2b), 1.53-1.32 (m, 3H, H-4, H-15), 1.21-1.03 [m, 20H, H-(5-14)], 0.81 (d, $J_{15,16}$ = 6.3 Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.47 (*C*=O), 67.97 (C-3), 51.67 (*C*H_3O), 41.08 (C-2), 39.02 (C-12), 36.51 (C-4), 22.62 (C-14). HR MS (m/z) calcd for $C_{18}H_{36}O_3$ $[M + Na]^+$, 323.2557; found, 323.1925.

2-(4-Bromophenyl)-2-oxoethyl-(*R*)-3-hydroxy-13-methyl-tetradecanoate (10): LiOH·H_2O (101 mg, 4.4 mmol) in H_2O (10 mL) was added to a stirring solution of **8** (600 mg, 2.2 mmol) in THF (150 mL). After stirring the reaction mixture at room temperature for 10 h, the THF was removed *in vacuo*. The aqueous residure was neutralized with 1N HCl (4.4 mL), and extracted with ethyl acetate (20 mL). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo* to afford an acid intermediate. Next, this intermediate was refluxed with dicyclohexaneamine (0.52 mL, 2.64 mmol) in CH_3CN (80 mL) for 2 h. After the reaction mixture cooled down to room temperature, the precipitated solid was collected by filtration to give a salt as a white solid. This product was dissolved in EtOAc (25 mL), and then Et_3N (0.37 mL, 2.64 mmol) and 2,4'-dibromoacetophenone (672 mg, 2.42 mmol) were added. After stirring the reaction mixture at room temperature for 12 h, it was diluted with DCM (50 mL) and washed with brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (DCM) to afford **10** as a white solid (910 mg, 91%, three

steps). R_f = 0.35 (DCM); $[\alpha]^{25}_D$ = -0.8º (*c* = 1.0, $CHCl_3$)^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.7 Hz, aromatic), 7.63 (d, 2H, *J* = 8.7 Hz, aromatic), 5.41 (d, 1H, *J* = 16.5 Hz, CH'$_{2a}$), 5.29 (d, 1H, *J* = 16.5 Hz, CH'$_{2b}$), 4.10 (m, 1H, H-3), 2.67 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2a,3}$ = 2.4 Hz, H-2a), 2.54 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2b,3}$ = 9.0 Hz, H-2b), 1.60-1.45 (m, 3H, H-4, H-13), 1.24-1.12 [m, 16H, H-(5-12)], 0.84 (d, 6H, $J_{13,14}$ = 6.6 Hz, H-14). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.63 (*C*=O), 171.95 (*C*=O), 132.54-129.29 (m, aromatic), 68.45 (C-3), 65.78 (C-2'), 41.99 (C-2), 39.04 (C-12), 36.56 (C-4), 22.65 (C-14). HR MS (m/z) calcd for $C_{23}H_{35}BrO_4$ $[M + Na]^+$, 477.1611; found, 477.1241.

2-(4-Bromophenyl)-2-oxoethyl (*R*)-3-hydroxy-15-methyl-hexadecanoate (11): In a manner similar to the synthesis of 10, compound 9 (960 mg, 3.2 mmol) was hydrolyzed with LiOH (115 mg, 4.8 mmol), recrytallized by refluxing with DCHA (0.76 mL, 3.84 mmol), and protected by reacting with 2,4'-dibromoacetophenone (979 mg, 3.52 mmol) to afford 11 as a white solid (1.39 g, 90%). R_f = 0.35 (DCM); $[\alpha]^{25}_D$ = -1.2º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, 2H, *J* = 8.4 Hz, aromatic), 7.60 (d, 2H, *J* = 8.4 Hz, aromatic), 5.39 (d, 1H, *J* = 16.5 Hz, CH'$_{2a}$), 5.29 (d, 1H, *J* = 16.5 Hz, CH'$_{2b}$), 4.08 (m, 1H, H-3), 2.65 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2a,3}$ = 3.0 Hz, H-2a), 2.54 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2b,3}$ = 9.0 Hz, H-2b), 1.58-1.46 (m, 3H, H-4, H-15), 1.24-1.13 [m, 20H, H-(5-14)], 0.83 (d, 6H, $J_{15,16}$ = 6.6 Hz, H-14). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.60 (*C*=O), 171.86 (*C*=O), 132.49-129.24 (m, aromatic), 68.38 (C-3), 65.74 (C-2'), 41.96 (C-2), 39.00 (C-14), 36.54 (C-4), 22.61 (C-16). HR MS (m/z) calcd for $C_{25}H_{39}BrO_4$ $[M + Na]^+$, 505.1924; found, 505.1160.

2-(4-Bromophenyl)-2-oxoethyl (*R*)-3-benzyloxy-13-methyl-tetradecanoate (12): To a cooled (0 ºC) solution of **10** (405 mg, 0.89 mmol), benzaldehyde (0.27 mL, 2.67 mmol) amd TMS_2O (1.13 mL, 5.34 mmol) in dry THF (20 mL) was added dropwise TMSOTf (77 μL, 0.445 mmol).

After stirring the reaction mixture for 15 min, Et_3SiH (0.50 mL, 3.12 mmol) was added dropwise. The stirring continued at room temperature for another 4 h, after which the reaction mixture was neutralized with Et_3N (60 μL), diluted with ethyl acetate (40 mL), and washed with brine (2 x 25 mL). The organic phase was dried ($MgSO_4$), concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 25/1, v/v) to afford **12** as a white solid (363 mg, 75%). R_f = 0.55 (hexane/ethyl acetate, 6/1, v/v); $[\alpha]^{25}_D$ = -6.2º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77-7.23 (m, 10H, aromatic), 5.27 (d, 1H, *J* = 16.5 Hz, CH'$_{2a}$), 5.21 (d, 1H, *J* = 16.5 Hz, CH'$_{2b}$), 4.58 (d, 1H, *J* =11.4 Hz, CH_{2a} of benzyl), 4.53 (d, 1H, *J* =11.4 Hz, CH_{2b} of benzyl), 3.93 (m, 1H, H-3), 2.77 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2a,3}$ = 7.2 Hz, H-2a), 2.64 (dd, 1H, $J_{2a,2b}$ = 15.0 Hz, $J_{2b,3}$ = 5.4 Hz, H-2b), 1.66-1.24 (m, 3H, H-4, H-13), 1.24-1.11 [m, 16H, H-(5-12)], 0.84 (d, 6H, $J_{13,14}$ = 6.9 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.36 (*C*=O), 171.19 (*C*=O), 138.56-127.56 (m, aromatic), 75.85 (C-3), 71.54 (CH_2 of benzyl), 65.76 (C-2'), 39.54 (C-2), 39.00 (C-12), 34.36 (C-4), 22.62 (C-14). HR MS (m/z) calcd for $C_{30}H_{41}BrO_4$ $[M + Na]^+$, 567.2080; found, 567.2116.

2-(4-Bromophenyl)-2-oxoethyl (*R*)-3-benzyloxy-15-methyl-hexadecanoate (13): In a manner similar to the synthesis of **12**, the hydroxyl of compound **11** (627 mg, 1.30 mmol) was benzylated to afford **13** as a white solid (528 mg, 71%). R_f = 0.60 (hexane/ethyl acetate, 6/1, v/v); $[\alpha]^{24.4}_D$ = -6.7º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76-7.24 (m, 10H, aromatic), 5.27 (d, 1H, *J* = 16.5 Hz, CH'$_{2a}$), 5.21 (d, 1H, *J* = 16.5 Hz, CH'$_{2b}$), 4.58 (d, 1H, *J* =11.4 Hz, CH_{2a} of benzyl), 4.52 (d, 1H, *J* =11.4 Hz, CH_{2b} of benzyl), 3.92 (m, 1H, H-3), 2.77 (dd, 1H, $J_{2a,2b}$ = 15.3 Hz, $J_{2a,3}$ = 7.2 Hz, H-2a), 2.64 (dd, 1H, $J_{2a,2b}$ = 15.3 Hz, $J_{2b,3}$ = 5.4 Hz, H-2b), 1.66-1.36 (m, 3H, H-4, H-13), 1.24-1.12 [m, 20H, H-(5-14)], 0.84 (d, 6H, $J_{15,16}$ = 6.9 Hz, H-16). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.22 (*C*=O), 171.15 (*C*=O), 138.58-127.53 (m, aromatic), 75.95 (C-3), 71.56

(CH_2 of benzyl), 65.79 (C-2'), 39.51 (C-2), 39.06 (C-14), 34.39 (C-4), 22.65 (C-16). HR MS (m/z) calcd for $C_{32}H_{45}BrO_4$ $[M + Na]^+$, 595.2393; found, 595.2437.

(*R*)-3-Benzyloxy-13-methyl-tetradecanoic acid (14): Zinc dust (382 mg, 5.87 mmol) was added portionwise to a solution of **12** (320 mg, 0.587 mmol) in acetic acid (15 mL). The reaction mixture was stirred at 60 °C for 2 h and then diluted with DCM (20 mL). The solids were filtered off through a pad of Celite, and the residue was washed with DCM (3 x 5 mL). The combined filtrates were concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (DCM/methanol, 100/1, v/v) to afford **7** as a white solid (198 mg, 97%). R_f = 0.40 (toluene/ethyl acetate, 3/1, v/v); $[\alpha]^{25}_D$ = -2.3º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35-7.25 (m, 5H, aromatic), 4.55 (s, 2H, CH_2 of benzyl), 3.86 (m, 1H, H-3), 2.62 (dd, 1H, $J_{2a,2b}$ = 15.6 Hz, $J_{2a,3}$ = 6.9 Hz, H-2a), 2.53 (dd, 1H, $J_{2a,2b}$ = 15.6 Hz, $J_{2b,3}$ = 5.1 Hz, H-2b), 1.66-1.45 (m, 3H, H-4, H-13), 1.38-1.10 [m, 16H, H-(5-12)], 0.85 (d, 6H, $J_{15,16}$ = 6.9 Hz, H-14); δ 176.76 (*C*=O), 138.04-127.73 (aromatic), 75.70 (C-3), 71.53 (*C*H_2 of benzyl), 39.42 (C-2), 39.03 (C-12), 34.09 (C-4), 22.66 (C-14); HR MS (m/z) calcd for $C_{22}H_{36}O_3$ $[M + Na]^+$, 371.2557; found, 371.1906.

(*R*)-3-Benzyloxy-15-methyl-hexadecanoic acid (15): In a manner similar to the synthesis of 14, compound 13 (350 mg, 0.611 mmol) was treated with zinc (397 mg, 6.11 mmol) to afford **15** as a white solid (207 mg, 97%). R_f = 0.45 (toluene/ethyl acetate, 3/1, v/v); $[\alpha]^{25}_D$ = -2.5º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34-7.26 (m, 5H, aromatic), 4.57 (s, 2H, CH_2 of benzyl), 3.87 (m, 1H, H-3), 2.64 (dd, 1H, $J_{2a,2b}$ = 15.6 Hz, $J_{2a,3}$ = 6.9 Hz, H-2a), 2.55 (dd, 1H, $J_{2a,2b}$ = 15.6 Hz, $J_{2b,3}$ = 5.1 Hz, H-2b), 1.68-1.38 (m, 3H, H-4, H-15), 1.26-1.14 [m, 20H, H-(5-14)], 0.86 (d, 6H, $J_{15,16}$ = 6.9 Hz, H-16); δ 176.95 (*C*=O), 138.08-127.71 (aromatic), 75.71 (C-3), 71.53 (*C*H_2 of benzyl), 39.47 (C-2), 39.05 (C-12), 34.12 (C-4), 22.65 (C-14). HR MS (m/z) calcd for

$C_{24}H_{40}O_3$ $[M + Na]^+$, 399.2870; found, 399.2552.

2-(4-Bromophenyl)-2-oxoethyl-(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoate (16): Palmitoyl chloride (0.41 mL, 1.34 mmol) was added dropwise to a solution of **11**(540 mg, 1.12 mmol), pyridine (0.22 mL, 2.68 mmol) and DMAP (13 mg, 0.11 mmol) in DCM (10 mL). After stirring the reaction mixture at room temperature for 10 h, it was diluted with DCM (20 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo.* The residue was purified by silica gel column chromatography (toluene) to afford **16** as a white solid (767 mg, 95%). R_f = 0.70 (DCM); $[\alpha]^{25}_D$ = -0.1º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (d, 2H, *J* = 8.4 Hz, aromatic), 7.59 (d, 2H, *J* = 8.4 Hz, aromatic), 5.29-5.24 (m, 3H, H-3, OC*H*$_2$COPhBr), 2.70 (m, 2H, H-2), 2.28 (t, 2H, *J* = 7.5 Hz, H-2$_{L'}$), 1.64-1.42 (m, 3H, H-4, H-15), 1.23-1.11 [m, 20H, H-(5-14)], 0.83 (d, 6H, $J_{15,16}$ = 6.3 Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.78 (*C*=O), 173.20 (*C*=O), 169.80 (*C*=O), 132.83-129.06 (m, aromatic), 70.08 (C-3), 65.84 (O*C*H$_2$COPhBr). HR MS (m/z) calcd for $C_{41}H_{69}BrO_5$ $[M + Na]^+$, 743.4221; found, 745.4365.

(*R*)-3-Hexadecanoyloxy-15-methyl-hexadecanoic acid (17): In a manner similar to the synthesis of **14**, compound **16** (500 mg, 0.666 mmol) was treated with zinc (430 mg, 6.66 mmol) to afford **17** as a white solid (335 mg, 96%). R_f = 0.35 (toluene/ethyl acetate, 4/1, v/v); $[\alpha]^{25}_D$ = -0.6º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.19 (m, 1H, H-3), 2.58 (m, 2H, H-2), 2.25(t, 2H, *J* = 7.5 Hz, H-2$_{L'}$), 1.60-1.43 (m, 3H, H-4, H-15), 1.23-1.14 [m, 20H, H-(5-14)], 0.84 (d, 6H, $J_{15,16}$ = 6.9 Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.24 (*C*=O), 173.27 (*C*=O), 69.95 (C-3). HR MS (m/z) calcd for $C_{33}H_{64}O_4$ $[M + Na]^+$, 547.4697; found, 547.5009.

Dimethylthexylsilyl 4,6-*O*-benzylidene-2-deoxy-2-(9-fluorenylmethoxycarbonylamino-β-D-glucopyranoside (**20**): A suspension of compund 1**8** (1.02 g, 2.34 mmol) and zinc (1.52 g, 23.4

mmol) in a mixture of acetic acid (250 µl) and DCM (12 ml) was stirred at room temperature for 4 h, after which it was diluted with ethyl acetate (40 mL). The solids were removed by filtration, and the residue was washed with ethyl acetate (2 x 4 ml). The combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 30 mL) and brine. The organic phase was dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* to afford the crude amine as a pale yellow oil. The resulting amine was dissolved in DCM (12 mL), and then FmocCl (664 mg, 2.57 mmol) and DIPEA (447 µl, 2.57 mmol) were added. The reaction mixture was stirred at room temperature for 3 h, after which it was diluted with DCM (20 mL) and washed with brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 3/1, v/v) to yield **20** as a colorless solid (1.38 g, 90%, two steps). R_f = 0.55 (hexane/ethyl acetate, 3/2, v/v); $[\alpha]^{25}_D$ = -13.9º (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 7.86-7.23 (m, 13H, aromatic), 6.64 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.61 (s, 1H, C*H* of benzylidene), 4.92 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.32-4.19 (m, 4H, H-6a, OCH_2C*H* of Fmoc), 3.88 (m, 1H, H-3), 3.78 (t, 1H, $J_{5,6b}$ = $J_{6a,6b}$ = 9.9 Hz, H-6b), 3.56 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 3.54 (m, 1H, H-2), 3.44 (m, 1H, H-5), 1.61 (m, 1H, C*H*(CH_3) of TDS), 0.86-0.84 (m, 12H, SiC(CH_3)$_2$CH(CH_3)$_2$), 0.15 (s, 3H, Si(CH_3)), 0.14 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.96 (*C*=O), 144.99-120.57 (m, aromatic), 101.93 (*C*H of benzylidene), 97.80 (C-1), 82.77 (C-4), 71.75 (C-3), 69.09 (C-6), 67.11 (C-5), 66.86 (O*C*H_2 of Fmoc), 61.28 (C-2), 47.84 (OCH$_2$*C*H of Fmoc), 34.59 (*C*H of TDS), -1.83 (Si*C*H_3), -3.23 (Si*C*H_3). HR MS (m/z) calcd for $C_{36}H_{45}NO_7Si[M+Na]^+$, 654.2857; found, 654.2962.

Dimethylthexylsilyl 4,6-*O*-benzylidene-2-deoxy-2-(9-fluorenylmethoxycarbonylamino-3-*O*-levulinoyl-β-D-glucopyranoside (21): A solution of levulinic acid (234 mg, 2.02 mmol) and DCC (499 mg, 2.42 mmol) in DCM (8 mL) was stirred at room temperature for 10 min, after

which compound **20** (1.16 g, 1.84 mmol) and DMAP (12 mg, 0.1 mmol) were added, and stirring was continued for another 10 h. The insoluble materials were removed by filtration, and the residue was washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by silica gel column chromatography (DCM/CH_3OH, 60/1, v/v) to give **21** as a white solid (1.16g, 86%). R_f = 0.55 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{25}_D$ = -14.6º (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3COCD_3): δ 7.86-7.29 (m, 13H, aromatic), 6.62 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N*H*), 5.63 (s, 1H, C*H* of benzylidene), 5.31 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 5.09 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.32-4.19 (m, 4H, H-6a, OCH_2C*H* of Fmoc), 3.83 (t, 1H, $J_{5,6b} = J_{6a,6b}$ = 9.9 Hz, H-6b), 3.78 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.3 Hz, H-4), 3.68 (m, 1H, H-2), 3.54 (m, 1H, H-5), 2.64 (t, 2H, *J* = 6.9 Hz, CH_2 of Lev), 2.49 (t, 2H, *J* = 6.9 Hz, CH_2 of Lev), 2.01 (s, 3H, CH_3 of Lev), 1.62 (m, 1H, C*H*(CH_3) of TDS), 0.86-0.84 (m, 12H, SiC(CH_3)$_2$CH(CH_3)$_2$), 0.17 (s, 6H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): 172.49 (*C*=O), 156.73 (*C*=O), 145.07-120.64 (m, aromatic), 101.67 (*C*H of benzylidene), 97.55 (C-1), 79.80 (C-4), 72.64 (C-3), 69.03 (C-6), 67.11 (C-5, OCH_2 of Fmoc), 59.33 (C-2), 47.83 (OCH_2*C*H of Fmoc), 38.13 (*C*H_2 of Lev), 34.66 (*C*H of TDS), -1.85 (Si*C*H_3), -3.25 (Si*C*H_3). HR MS (m/z) calcd for $C_{41}H_{51}NO_9Si[M+Na]^+$, 752.3225; found 752.2672.

4,6-*O*-Benzylidene-2-deoxy-2-(9-fluorenylmethoxycarbonylamino-3-*O*-levulinoyl-D-glucopyranosyl trichloroacetimidate (22): A mixture of Bu_4NF (1 M in THF, 5 mL) and acetic acid (500 μl) was added dropwise to a stirred solution of **21** (800 mg, 1.10 mmol) in THF (15 mL). After stirring at room temperature for 24 h, the reaction mixture was diluted with DCM (20 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 30 mL) and brine (2 x 30 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: DCM/CH_3OH, 30/1, v/v) to afford the lactol as a pale

yellow solid (606 mg, 94%). R_f = 0.60 (hexane/ethyl acetate, 3/5, v/v). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.88-7.31 (m, 13H, aromatic), 6.62 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N*H*), 5.64 (s, 1H, C*H* of benzylidene), 5.39 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 5.09 (bs, 1H, H-1), 4.43-4.17 (m, 4H, H-6a, OCH_2C*H* of Fmoc), 4.13-3.97 (m, 2H, H-2, H-5), 3.81 (t, 1H, $J_{5,6b} = J_{6a,6b}$ = 9.9 Hz, H-6b), 3.80 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.6 Hz, H-4), 2.65 (t, 2H, J = 6.6 Hz, CH_2 of Lev), 2.50 (t, 2H, J = 6.6 Hz, CH_2 of Lev), 2.00 (s, 3H, CH_3 of Lev). HR MS (m/z) calcd for $C_{33}H_{33}NO_9$[M+Na]$^+$, 610.2048; found, 610.2293. The resulting lactol (606 mg, 1.03 mmol) was dissolved in a mixture of trichloroacetonitrile (2.0 mL) and DCM (10 mL), and then Cs_2CO_3 (163 mg, 0.50 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, after which it was diluted with DCM (20 mL), and then washed with saturated aqueous $NaHCO_3$ (2 x 30 mL) and brine (2 x 30 mL). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: hexane/ethyl acetate, 4/3, v/v) to yield **22** as a pale yellow solid (700 mg, 93%). R_f = 0.45 (hexane/ethyl acetate, 3/2, v/v).

Dimethylthexylsilyl 6-*O*-[4,6-*O*-benzylidene-2-deoxy-2-(9-fluorenylmethoxycarbonylamino)-3-*O*-levulinoyl-β-D-glucopyranosyl]-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**23**): A suspension of trichloroacetimidate **22** (600 mg, 0.82 mmol), acceptor **19** (407 mg, 78 mmol) and molecular sieves (4 Å, 500 mg) in DCM (10 mL) was stirred at room temperature for 1 h. The mixture was cooled (-50 ºC) and then trifluoromethanesulfonic acid (TfOH) (10 μL, 0.078 mmol) was added. After stirring the reaction mixture for 15 min, it was allowed to warm up to -20 ºC in 1 h, after which it was quenched with solid $NaHCO_3$. The solids were removed by filtration, and the filtrate was washed with saturated aqueous $NaHCO_3$ (2 x 50 mL) and brine (2 x 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, 3/1, v/v) to give **23** as a colorless solid (840 mg, 94%). R_f = 0.40 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{26}_D$ = -15.5° (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, CD_3COCD_3): δ 7.84-7.22 (m, 18H, aromatic), 6.79 (d, 1H, $J_{NH',2'}$ = 9.3 Hz, N*H*'), 5.87 (m, 1H, OCH_2CH=CH_2), 5.65 (s, 1H, C*H* of benzylidene), 5.37 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.9 Hz, H-3',), 5.30 (d, 1H, *J* = 18.3 Hz, OCH_2CH=CH_2), 5.17 (d, 1H, *J* = 10.5 Hz, OCH_2CH=CH_2), 4.94 (d, 1H, $J_{1,2}$ = 8.7 Hz, H-1), 4.86-4.80 (m, 2H, H-1, 3), 4.71 (d, 1H, *J* = 10.8 Hz, CH_2 of benzyl), 4.59-4.55 (m, 3H, OCH_2CH=CH_2, CH_2 of benzyl), 4.32-4.29 (m, 2H, H-6'a, CO_2CH_2 of Fmoc), 4.17-4.08 (m, 3H, H-6a, CO_2CH_2CH of Fmoc), 3.92-3.67 (m, 6H, H-2', H-4, H-4', H-5, H-6b, H-6'b), 3.56 (m, 1H, H-5'), 3.41 (dd, 1H, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 10.2 Hz, H-2), 2.61 (t, 2H, *J* = 6.6 Hz, CH_2 of Lev), 2.47 (t, 2H, *J* = 6.6 Hz, CH_2 of Lev), 1.95 (s, 3H, CH_3 of Lev), 1.70 (m, 1H, C*H* of thexyl), 0.91 (bs, 12H, CH_3 of thexyl), 0.26 (s, 3H, Si(CH_3)), 0.25 (s, 3H, Si(CH_3)). ^{13}C NMR (75 MHz, CD_3OCD_3): δ 172.47 (*C*=O), 156.64 (*C*=O), 154.94 (*C*=O), 144.94-120.54 (aromatic), 132.67 (OCH_2CH=CH_2), 118.63 (OCH_2CH=CH_2), 102.42 (C-1'), 101.58 (*C*H of benzylidene), 97.12 (C-1), 79.49 (C-4'), 79.24 (C-3), 76.68 (C-4), 75.09 (*C*H_2 of benzyl), 74.50 (C-5), 72.64 (C-3'), 68.96-68.92 (m, C-6', OCH_2CH=CH_2), 68.57 (C-6), 67.28 (C-2), 67.08 (*C*H_2 of Fmoc), 66.92 (C-5'), 57.26 (C-2'), 47.64 (*C*H of Fmoc), 38.04 (*C*H_2 of Lev), 34.49 (*C*H of thexyl), 29.33 (*C*H_3 of Lev), 28.44 (*C*H_2 of Lev), 25.26 (Si*C* of thexyl), 20.23, 20.19, 18.69, 18.63 (*C*H_3 of thexyl), -1.77 (Si(*C*H_3)$_2$), -3.22 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{58}H_{70}N_4O_{15}Si$ $[M + Na]^+$, 1113.4499; found, 1113.6394.

Dimethylthexylsilyl 6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-3-*O*-levulinoyl-β-D-glucopyranosyl}-3-*O*-allyloxycarbonyl-2-azido-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**24**): 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (60 μl) was added dropwise to a solution of **23** (620 mg, 0.569 mmol) in DCM (8 mL). The reaction

mixture was stirred at room temperature for 4 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 (454 mg, 92%). R_f = 0.30 (hexane/ethyl acetate, 1/1, v/v); HR MS (m/z) calcd for $C_{43}H_{60}N_4O_{13}Si$ $[M + Na]^+$, 891.3818; found, 891.2115. 1,3-Dicyclohexylcarbodiimide (DCC) (188 mg, 0.913 mmol) was added to a stirred solution of (*R*)-3-hexadecanoyl-15-methylhexadecanoic acid **17** (345 mg, 0.659 mmol) in DCM (5 mL). After stirring the reaction mixture for 10 min, the amine (440 mg, 0.507 mmol) in DCM (2 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 x 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 **24** as a white solid (634 mg, 91%). R_f = 0.65 (hexane/ethyl acetate, 2/1, v/v). $[\alpha]^{25}_D$ = -15.2° (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.23-7.03 (m, 10H, aromatic), 5.81 (d, 1H, $J_{NH',2'}$ = 8.4 Hz, N*H*'), 5.69 (m, 1H, OCH_2CH=CH_2), 5.27 (s, 1H, C*H* of benzylidene), 5.19 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.14 (d, 1H, J = 17.1 Hz, OCH_2CH=CH_2), 5.04 (d, 1H, J = 10.2 Hz, OCH_2CH=CH_2), 4.83 (m, 1H, H-3_L), 4.72 (d, 1H, $J_{1',2}$ = 8.4 Hz, H-1'), 4.54 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 4.42-4.37 (m, 5H, H-1, CH_2 of benzyl, OCH_2CH=CH_2), 4.08 (dd, 1H, $J_{5',6'a}$ = 4.8 Hz, $J_{6'a,6'b}$ = 10.2 Hz, H-6'a), 3.74 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.63-3.36 (m, 5H, H-2', H-4, H-4', H-6b, H-6'b), 3.33-3.26 (m, 2H, H-5, H-5'), 3.11 (dd, 1H, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 9.9 Hz, H-2), 2.59-2.30 (m, 4H, CH_2 of Lev), 2.17 (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}), 1.91 (s, 3H, CH_3 of Lev), 1.51-1.28(m, 5H, H-4_L, H-$3_{L'}$, C*H* of thexyl), 1.04 (broad, 44H, CH_2 of lipid), 0.70-0.64 (m, 21H, CH_3 of thexyl, CH_3 of lipid), 0.00 (s, 6H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.45 (*C*=O), 173.75 (*C*=O), 172.17 (*C*=O), 170.05 (*C*=O), 154.32 (*C*=O), 137.51-126.19 (aromatic),

131.26($OCH_2CH{=}CH_2$), 119.21 ($OCH_2CH{=}CH_2$), 101.42 (*C*H of benzylidene), 100.91 (C-1'), 96.92 (C-1), 78.84 (C-4'), 78.58 (C-3), 76.02 (C-4), 74.63 (CH_2 of benzyl), 74.35 (C-5), 71.42 (C-3'), 70.75 (C-3L), 68.90 ($OCH_2CH{=}CH_2$), 68.63 (C-6'), 68.06 (C-6), 66.46 (C-2), 66.19 (C-5'), 55.80 (C-2'), -1.86 ($Si(CH_3)_2$), -3.56 ($Si(CH_3)_2$). HR MS (m/z) calcd for $C_{76}H_{122}N_4O_{16}Si$ [M + Na]$^+$, 1397.8517; found, 1397.7814.

Dimethylthexylsilyl 6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-3-*O*-levulinoyl-β-D-glucopyranosyl}-3-*O*-allyloxycarbonyl-2-4-*O*-benzyl-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-β-D-glucopyranoside (25): A suspension of **24** (256 mg, 0.186 mmol), zinc (121 mg, 1.86 mmol), and acetic acid (100 μL) in DCM (5 mL) was stirred at room temperature for 2 h, after which it was diluted with ethyl acetate (30 ml). The solids were removed by filtration and washed with ethyl acetate (2 x 4 ml), and the combined filtrates were washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (2 x 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 (DCM/methanol, 50/1, v/v) to afford the amine as a pale yellow syrup (188 mg, 75%). R_f = 0.30 (hexane/ethyl acetate, 1/1, v/v); HR MS (m/z) calcd for $C_{76}H_{124}N_2O_{16}Si$ [M + Na]$^+$, 1371.8612; found, 1371.9028. DCC (51 mg, 0.246 mmol) was added to a stirred solution of (*R*)-3-benzyloxy-15-methyl-hexadecanoic acid **15** (69 mg, 0.185 mmol) in DCM (3 mL). After stirring the reaction mixture for 10 min, the amine (166 mg, 0.123 mmol) in DCM (1 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 x 1 mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 6/1, v/v) to give **25** as a white solid (184 mg, 88%). R_f = 0.55 (hexane/ethyl acetate, 4/1, v/v).

$[\alpha]^{25}_{D}$ = -9.5° (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36-7.18 (m, 15H, aromatic), 6.28 (d, 1H, $J_{NH,2}$ = 8.7 Hz, N*H*), 5.90 (d, 1H, $J_{NH',2'}$ = 8.4 Hz, N*H*'), 5.77 (m, 1H, OCH_2CH=CH_2), 5.40 (s, 1H, C*H* of benzylidene), 5.34 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.23 (d, 1H, *J* = 17.1 Hz, OCH_2CH=CH_2), 5.13 (d, 1H, *J* = 9.9 Hz, OCH_2CH=CH_2), 4.99-4.86 (m, 1H, H-1', H-3, H-3_L), 4.56-4.37 (m, 7H, H-1, 2 x CH_2 of benzyl, OCH_2CH=CH_2), 4.25 (dd, 1H, $J_{5',6'a}$ = 5.1 Hz, $J_{6'a,6'b}$ = 10.8 Hz, H-6'a), 3.88 (d, 1H, $J_{6a,6b}$ = 11.4 Hz, H-6a), 3.75-3.51 (m, 7H, H-2, H-2', H-4, H-4', H-6b, H-6'b, H-3_S), 3.46-3.38 (m, 2H, H-5, H-5'), 2.73-2.41 (m, 4H, CH_2 of Lev), 2.40-2.18 (m, 6H, H-2_S, H-2_L, H-$2_{L'}$), 2.05 (s, 3H, CH_3 of Lev), 1.53-1.39 (m, 7H, H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.17-1.07 (m, 64H, CH_2 of lipid), 0.79-0.73 (m, 27H, CH_3 of thexyl, CH_3 of lipid), 0.06 (s, 3H, Si(CH_3)$_2$), 0.00 (s, 3H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.47 (*C*=O), 173.75 (*C*=O), 172.12 (*C*=O), 170.82 (*C*=O), 170.03 (*C*=O), 154.85 (*C*=O), 138.22-126.22 (aromatic), 131.40 (OCH_2*C*H=CH_2), 118.94 (OCH_2CH=*C*H_2), 101.41 (*C*H of benzylidene), 100.92 (C-1'), 95.85 (C-1), 78.90 (C-4'), 78.75 (C-3), 76.37 (C-4), 76.05 (C-3_S), 74.46 (*C*H_2 of benzyl), 74.27 (C-5), 71.42 (C-3'), 70.80 (C-3_L, *C*H_2 of benzyl), 68.63-68.54 (C-6, C-6', O*C*H_2CH=CH_2), 66.20 (C-5'), 56.04 (C-2, C-2'), -1.52 (Si(*C*H_3)$_2$), -3.28 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{100}H_{162}N_2O_{18}Si$ $[M + Na]^+$, 1730.1484; found, 1730.1412.

Dimethylthexylsilyl 6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-3-*O*-levulinoyl-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-*O*-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-β-D-glucopyranoside (26):Tetrakis(triphenylphosphine)palladium (11 mg, 0.01 mmol) was added to a solution of **25** (80 mg, 0.047 mmol), n-$BuNH_2$ (9.4 μL, 0.094 mmol), and HCOOH (3.5 μL, 0.094 mmol) in THF (2 mL). After stirring the reaction mixture at room temperature for 30 min, it was diluted with DCM (15 mL), and washed with water (10 mL), saturated aqueous $NaHCO_3$

(2 x 10 mL) and brine (2 x 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 chromatography (hexane/ethyl acetate, 3/2, v/v) to give the alcohol intermediate (72 mg, 95%). R_f = 0.55 (hexane/ethyl acetate, 3/2, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.43-7.24 (m, 15H, aromatic), 6.37 (d, 1H, $J_{NH,2}$ = 6.0 Hz, N*H*), 5.90 (d, 1H, $J_{NH',2'}$ = 8.5 Hz, N*H*'), 5.46 (s, 1H, C*H* of benzylidene), 5.37 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3'), 5.03 (m, H-3_L), 4.90 (d, 1H, *J* = 11.0 Hz, CH_2 of benzyl), 4.87 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.63 (d, 1H, *J* = 11.0 Hz, CH_2 of benzyl), 4.58 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.55 (d, 1H, *J* = 12.0 Hz, CH_2 of benzyl), 4.49 (d, 1H, *J* = 12.0 Hz, CH_2 of benzyl), 4.28 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz, $J_{6'a,6'b}$ = 11.0 Hz, H-6'a), 3.98 (d, 1H, $J_{6a,6b}$ = 10.0 Hz, H-6a), 3.80-3.67 (m, 5H, H-2', H-3, H-6b, H-6'b, H-3_S), 3.63 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.5 Hz, H-4'), 3.50-3.36 (m, 4H, H-2, H-4, H-5, H-5'), 2.78-2.48 (m, 4H, CH_2 of Lev), 2.43-2.23 (m, 6H, H-2_S, H-2_L, H-$2_{L'}$), 2.11 (s, 3H, CH_3 of Lev), 1.67-1.45 (m, 7H, H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.23-1.12 (m, 64H, CH_2 of lipid), 0.87-0.80 (m, 27H, CH_3 of thexyl, CH_3 of lipid), 0.14 (s, 3H, Si(CH_3)$_2$), 0.09 (s, 3H, Si(CH_3)$_2$). HR MS (m/z) calcd for $C_{96}H_{158}N_2O_{16}Si$ $[M + Na]^+$, 1646.1273; found, 1646.1384. A solution of (*R*)-3-benzyloxy-hexadecanoic acid (15 mg, 0.042 mmol) and DCC (11.5 mg, 0.056 mmol) in DCM (2 mL) was stirred for 10 min at room temperature, after which the alcohol intermediate (45 mg, 0.028 mmol) and DMAP (1 mg, 8 μmol) were added. The reaction mixture was stirred for 10 h at room temperature, after which the solids were removed by filtration and washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by preparative silica gel TLC (hexane/ethyl acetate, 5/2, v/v) afforded **26** as a white solid (52 mg, 95%). R_f = 0.45 (hexane/ethyl acetate, 5/2, v/v); $[\alpha]^{26}_D$ = -8.8^o (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37-7.14 (m, 20H, aromatic), 6.12 (d, 1H, $J_{NH,2}$ = 9.3 Hz, N*H*), 5.88 (d, 1H, $J_{NH',2'}$ = 8.1 Hz,

N*H*'), 5.39 (s, 1H, C*H* of benzylidene), 5.34 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.9$ Hz, H-3'), 5.34 (t, 1H, $J_{2,3} = J_{3,4} = 9.9$ Hz, H-3), 5.00 (m, 1H, H-3_L), 4.85 (d, 1H, $J_{1',2'} = 8.1$ Hz, H-1'), 4.52-4.35 (m, 7H, H-1, 3 x CH_2 of benzyl), 4.25 (dd, 1H, $J_{5',6'a} = 4.5$ Hz, $J_{6'a,6'b} = 10.5$ Hz, H-6'a), 3.87 (d, 1H, $J_{6a,6b} = 10.5$ Hz, H-6a), 3.81-3.46 (m, 8H, H-2, H-2', H-4, H-4', H-6b, H-6'b, 2 x H-3_S), 3.46-3.36 (m, 2H, H-5, H-5'), 2.76-2.61 (m, 2H, CH_2 of Lev), 2.52-2.44 (m, 3H, CH_2 of Lev, H-2_S), 2.35-2.15 (m, 7H, H-2_S x 3, H-2_L, H-$2_{L'}$), 2.06 (s, 3H, CH_3 of Lev), 1.54-1.37 (m, 9H, 2 x H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.23-1.06 (m, 86H, CH_2 of lipid), 0.83-0.73 (m, 30H, CH_3 of thexyl, CH_3 of lipid), 0.07 (s, 3H, Si(CH_3)$_2$), 0.00 (s, 3H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.43 (*C*=O), 173.70 (*C*=O), 172.12 (*C*=O), 171.38 (*C*=O), 170.73 (*C*=O), 169.96 (*C*=O), 138.59-126.19 (aromatic), 101.36 (*C*H of benzylidene), 100.88 (C-1), 96.08 (C-1'), 78.86 (C-4'), 75.91 (C-4), 75.76 (C-3_S), 75.43 (C-3_S), 74.58 (C-3), 74.40 (C-5), 74.08 (*C*H_2 of benzyl), 71.43 (C-3'), 71.33 (*C*H_2 of benzyl), 70.77 (C-3_L), 70.54 (CH_2 of benzyl), 68.15 (C-6, C-6'), 66. 15 (C-5'), 55.91 (C-2'), 55.80 (C-2), -1.50 (Si(*C*H_3)$_2$), -3.24 (Si(*C*H_3)$_2$).CHR MS (m/z) calcd for $C_{119}H_{194}N_2O_{18}Si$ $[M + Na]^+$, 1990.3988; found, 1990.3204.

6-*O*-{4,6-*O*-Benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-β-D-glucopyranosyl}-4-*O*-benzyl-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-*O*-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-α-D-glucopyranose (27): A reaction mixture of **26** (25 mg, 0.013 mmol) and hydrazine acetate (1.3 mg, 0.014 mmol) in a mixture of DCM (2 mL) and methanol (0.2 mL) was stirred at room temperature 6 h, after which it was concentrated *in vacuo*. The residue was purified by preparative silica gel TLC (hexane/ethyl acetate, 5/2, v/v) afforded the alcohol intermediate as a pale yellow syrup (23 mg, 96%). R_f = 0.40 (hexane/ethyl acetate, 5/2, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30-7.12 (m, 20H, aromatic), 6.15 (d, 1H, $J_{NH,2} = 9.3$ Hz, N*H*), 5.87 (d, 1H, $J_{NH',2'} = 5.7$ Hz, N*H*'), 5.47 (s, 1H, C*H*

of benzylidene), 5.08 (t, 1H, $J_{2,3} = J_{3,4} = 9.9$ Hz, H-3), 5.05 (m, 1H, H-3_L), 4.73 (d, 1H, $J_{1',2'} = 8.1$ Hz, H-1'), 4.53-4.36 (m, 7H, H-1, 3 x CH_2 of benzyl), 4.23 (dd, 1H, $J_{5',6'a} = 5.2$ Hz, $J_{6'a,6'b} = 10.2$ Hz, H-6'a), 4.16 (t, 1H, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, H-3'), 3.92 (d, 1H, $J_{6a,6b} = 10.2$ Hz, H-6a), 3.83-3.76 (m, 2H, H-2, H-3_S), 3.70-3.58 (m, 3H, H-5', H-6b, H-3_S), 3.52-3.73 (m, 4H, H-4, H-4', H-5, H-6'b), 3.26 (m, 1H, H-2'), 2.50 (dd, 1H, $J_{2Sa,2Sb} = 15.9$ Hz, $J_{2Sa,3S} = 6.9$ Hz, H-2_{Sa}), 2.38-2.18 (m, 7H, H-2_S x 3, H-2_L, H-$2_{L'}$), 1.54-1.38 (m, 9H, 2 x H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.26-1.09 (m, 86H, CH_2 of lipid), 0.81-0.73 (m, 30H, CH_3 of thexyl, CH_3 of lipid), 0.06 (s, 3H, Si(CH_3)$_2$), 0.00 (s, 3H, Si(CH_3)$_2$). MS (m/z) calcd for $C_{114}H_{188}N_2O_{16}Si$ $[M + Na]^+$, 1892.3620; found, 1892.4476.

Acetic acid (100 μL) was added to a solution of Bu_4NF (1 N in THF, 1mL), and then the alcohol (35 mg, 0.019 mmol) was added. The reaction mixture was stirred at room temperature for 10 h, after which it was diluted with ethyl aetate (10 mL), and washed with saturated aqueous $NaHCO_3$ (2 x 10 mL) and brine (2 x 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 (DCM/acetone, 6/1, v/v) afforded **27** as a pale yellow syrup (21 mg, 65%). $R_f = 0.40$ (DCM/acetone, 6/1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51-7.19 (m, 20H, aromatic), 6.31 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 6.19 (d, 1H, $J_{NH',2'} = 5.5$ Hz, N*H*'), 5.55 (s, 1H, C*H* of benzylidene), 5.43 (t, 1H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3), 5.15-5.09 (m, 2H, H-1, H-1'), 5.01 (m, 1H, H-3_L), 4.63-4.45 (m, 6H, 3 x CH_2 of benzyl), 4.36 (m, 1H, H-6'a), 4.22 (m, 1H, H-2), 4.14 (m, 1H, H-3'), 4.02 (d, 1H, $J_{6a,6b} = 11.5$ Hz, H-6a), 3.85-3.76 (m, 3H, H-6'b, 2 x H-3_S), 3.67-3.47 (m, 3H, H-4', H-5', H-6b), 3.41 (m, 1H, H-4), 3.30 (m, 1H, H-2'), 2.61-2.24 (m, 8H, 2 x H-2_S, H-2_L, H-$2_{L'}$), 1.66-1.49 (m, 8H, 2 x H-4_S, H-$3_{L'}$, H-4_L), 1.26-1.17 (m, 86H, CH_2 of lipid), 0.91-0.87 (m, 18H, CH_3 of lipid). MS (m/z) calcd for $C_{106}H_{170}N_2O_{16}$ $[M + Na]^+$ 1750.2443; found, 1750.2439.

6-*O*-{2-Deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-β-D-

glucopyranosyl}-3-*O*-[(*R*)-3-hydroxy-hexadecanoyl]-2-deoxy-2-*O*-[(*R*)-3-hydroxy-15-methyl-hexadecanoylamino]-α-D-glucopyranose 1-phosphate (1): To a cooled (-78 ºC) solution of **27** (10 mg, 0.0058 mmol) and tetrabenzyl diphosphate (12 mg, 0.022 mmol) in THF (1.5 mL) was added dropwise lithium bis(trimethylsilyl)amide in THF (1.0 M, 15 μL, 0.015 mmol). The reaction mixture was stirred for 1h, and then allowed to warm up to -20 ºC. After the reaction mixture was stirred at -20 ºC for 1 h, it was quenched with saturated aqueous $NaHCO_3$ (10 mL), and extracted with ethyl acetate (10 mL). The organic phase was washed with brine (2 x 10 mL), dried (Na_2SO_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 **28** as a pale yellow oil (8.3 mg, 72%). R_f = 0.55 (hexane/ethyl acetate, 3/2, v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44-7.18 (m, 30H, aromatic), 6.30 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 6.19 (d, 1H, $J_{NH',2'}$ = 5.5 Hz, N*H*'), 5.55 (s, 1H, C*H* of benzylidene), 5.31 (t, 1H, $J_{2,3} = J_{3,4}$ = 10.0 Hz, H-3), 5.23 (m, 1H, H-3_L), 4.61 (d, 1H, *J* = 11.0 Hz, CH_2 of benzyl), 4.52-4.45 (m, 4H, 2 x CH_2 of benzyl), 4.39 (d, 1H, *J* = 12.0 Hz, CH_2 of benzyl), 4.34-4.26 (m, 2H, H-2, H-6'a), 4.14 (m, 1H, H-5), 3.95-3.91 (m, 1H, H-3', H-6a), 3.84-3.74 (m, 3H, H-6b, H-6'b, H-3_S), 3.70 (m, 1H, H-3_S), 3.61 (m, 1H, H-2'), 3.55 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.5 Hz, H-4'), 3.46 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.5 Hz, H-4), 3.36 (m, 1H, H-5'), 2.57 (dd, 1H, $J_{2Sa,2Sb}$ = 16.0 Hz, $J_{2Sa,3S}$ = 8.0 Hz, H-2_{Sa}), 2.51-2.40 (m, 3H, H-2_S, H-2_L), 2.26-2.18 (m, 4H, H-2_S, H-$2_{L'}$), 1.63-1.50 (m, 8H, 2 x H-4_S, H-$3_{L'}$, H-4_L), 1.32-1.17 (m, 86H, CH_2 of lipid), 0.90-0.87 (m, 18H, CH_3 of lipid). MS (m/z) calcd for $C_{120}H_{183}N_2O_{19}P$ $[M + Na]^+$, 2010.3045; found, 2010.2429. A mixture of **28** (10.5 mg, 0.0053 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 26 h, after which it was neutralized with triethylamine (10 μl), and the catalyst removed by filteration and the residue washed with THF (2 x 1 mL). The combined filtrates were concentrated *in vacuo* to

afford **1** as a colorless film (6.0 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 1/1, v/v): δ 5.28 (broad, 1H, H-1), 4.96-4.82 (m, 3H, H-1', H-3, H-3_L). HR MS (m/z) (negative) calcd for $C_{78}H_{149}N_2O_{19}P$, 1449.0492; found, 1449.7284.

Thexylsilyl 6-*O*-{4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-3-*O*-[(*R*)-3-benzyloxy-13-methyl-tetradecanoylamino]-β-D-glucopyranosyl}-3-*O*-allyloxycarbonyl-2-4-*O*-benzyl-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-β-D-glucopyranoside (29): A reaction mixture of **25** (80 mg, 0.047 mmol) and hydrazine acetate (4.7 mg, 0.052 mmol) in a mixture of DCM (3 mL) and methanol (0.3 mL) was stirred at room temperature 6 h, after which it was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 3/1, v/v) afforded the alcohol intermediate as a pale yellow syrup (69 mg, 92%). R_f = 0.40 (hexane/ethyl acetate, 5/2, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46-7.17 (m, 15H, aromatic), 6.35 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.99 (d, 1H, $J_{NH',2'}$ = 5.7 Hz, N*H*'), 5.77 (m, 1H, OCH_2CH=CH_2), 5.46 (s, 1H, C*H* of benzylidene), 5.23 (d, 1H, *J* = 17.1 Hz, OCH_2CH=CH_2), 5.14 (d, 1H, *J* = 10.2 Hz, OCH_2CH=CH_2), 5.02 (m, 1H, H-3_L), 4.94 (dd, 1H, J = 8.7 Hz, J = 10.5 Hz, H-3), 4.75 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1'), 4.58-4.37 (m, 7H, H-1, 2 x CH_2 of benzyl, OCH_2CH=CH_2), 4.23 (dd, 1H, $J_{5',6'a}$ = 4.5 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 4.13(m, 1H, H-3), 3.88 (d, 1H, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.76-3.31 (m, 8H, H-2, H-4, H-4', H-5, H-5', H-6b, H-6'b, H-3_S), 3.27 (m, 1H, H-2'), 2.33-2.17 (m, 6H, H-2_S, H-2_L, H-$2_{L'}$), 1.55-1.37 (m, 7H, H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.17-1.07 (m, 64H, CH_2 of lipid), 0.82-0.73 (m, 27H, CH_3 of thexyl, CH_3 of lipid), 0.06 (s, 3H, Si(CH_3)$_2$), 0.00 (s, 3H, Si(CH_3)$_2$). HR MS (m/z) calcd for $C_{95}H_{156}N_2O_{16}Si$ $[M + Na]^+$, 1632.1116; found, 1631.8767. A solution of (*R*)-3-benzyloxy-13-methyl-tetradecanoic acid **14** (21 mg, 0.061 mmol) and DCC (17 mg, 0.081 mmol) in DCM (2 mL) was stirred for 10 min at room temperature,

after which the alcohol intermediate (65 mg, 0.040 mmol) and DMAP (1 mg, 8 μmol) were added. The reaction mixture was stirred at room temperature for 12 h, after which the solids were removed by filtration and washed with DCM (2 x 1 mL). The combined filtrates were concentrated *in vacuo* and the residue was purified by preparative silica gel TLC (hexane/ethyl acetate, 4/1, v/v) afforded **29** as a white solid (71 mg, 91%). R_f = 0.50 (hexane/ethyl acetate, 3/1, v/v); $[\alpha]^{24}_D$ = -11.1° (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.37-7.21 (m, 20H, aromatic), 6.35 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.84 (m, 1H, OCH_2CH=CH_2), 5.79 (d, 1H, $J_{NH',2'}$ = 9.0 Hz, N*H*'), 5.41 (s, 1H, C*H* of benzylidene), 5.41 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.6 Hz, H-3'), 5.29 (d, 1H, *J* = 17.4 Hz, OCH_2CH=CH_2), 5.19 (d, 1H, *J* = 10.2 Hz, OCH_2CH=CH_2), 5.00-4.96 (m, 2H, H-3, H-3_L), 4.87 (d, 1H, $J_{1'2'}$ = 7.8 Hz, H-1'), 4.61-4.37 (m, 9H, H-1, 3 x CH_2 of benzyl, OCH_2CH=CH_2), 4.29 (dd, 1H, $J_{5',6'a}$ = 5.4 Hz, $J_{6'a,6'b}$ = 10.8 Hz, H-6'a), 3.94 (d, 1H, $J_{6a,6b}$ = 10.2 Hz, H-6a), 3.81-3.78 (m, 3H, H-2, H-6b, H-3_S), 3.74-3.67 (m, 3H, H-2', H-6'b, H-3_S), 3.64-3.58 (m, 2H, H-4, H-4'), 3.50-3.45 (m, 2H, H-5, H-5'), 2.64-2.12 (m, 8H, H-2_S, H-2_L, H-$2_{L'}$), 1.59-1.46 (m, 9H, H-4_S, H-$3_{L'}$, H-4_L, C*H* of thexyl), 1.23-1.13 (m, 83H, CH_2 of lipid), 0.86-0.79 (m, 33H, CH_3 of thexyl, CH_3 of lipid), 0.13 (s, 3H, Si(CH_3)$_2$), 0.06 (s, 3H, Si(CH_3)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.80 (*C*=O), 171.06 (*C*=O), 170.82 (*C*=O), 169.65 (*C*=O), 154.86 (*C*=O), 138.41-126.14 (aromatic), 131.40 (OCH_2*C*H=CH_2), 118.94 (OCH_2CH=*C*H_2), 101.41 (*C*H of benzylidene), 100.93 (C-1'), 95.86 (C-1), 78.93 (C-4'), 78.75 (C-3), 76.31 (C-4), 76.05 (C-3_S), 75.65 (C-3_S), 74.45 (*C*H_2 of benzyl), 74.21 (C-5), 71.24 (C-3'), 71.16 (*C*H_2 of benzyl), 70.80 (*C*H_2 of benzyl), 70.74 (C-3_L), 68.63 (C-6, O*C*H_2CH=CH_2), 68.28 (C-6'), 66.27 (C-5'), 56.03 (C-2), 55.73 (C-2'), -1.52 (Si(*C*H_3)$_2$), -3.27 (Si(*C*H_3)$_2$). HR MS (m/z) calcd for $C_{117}H_{190}N_2O_{18}Si$ $[M + Na]^+$, 1962.3675; found, 1962.3035.

6-*O*-{4,6-*O*-Benzylidene-2-deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoyl

amino]-3-*O*-[(*R*)-3-benzyloxy-13-methyl-tetradecanoylamino]-β-D-glucopyranosyl}-3-*O*-allyloxycarbonyl-2-4-*O*-benzyl-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-β-D-glucopyranose (30): Tetrakis(triphenylphosphine)palladium (6.3 mg, 0.0054 mmol) was added to a solution of **29** (35 mg, 0.018 mmol), n-$BuNH_2$ (3.6 μL, 0.036 mmol), and HCOOH (1.4 μL, 0.036 mmol) in THF (2 mL). After stirring the reaction mixture at room temperature for 1 h, it was diluted with DCM (10 mL), and washed with water (10 mL), saturated aqueous $NaHCO_3$ (2 x 10 mL) and brine (2 x 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 chromatography (hexane/ethyl acetate, 5/2, v/v) to give the alcohol intermediate as a pale yellow syrup (31 mg, 94%). R_f = 0.50 (hexane/ethyl acetate, 3/2, v/v); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29-7.13 (m, 20H, aromatic), 6.29 (d, 1H, $J_{NH,2}$ = 5.7 Hz, N*H*), 5.69 (d, 1H, $J_{NH',2'}$ = 8.7 Hz, N*H*'), 5.31 (s, 1H, C*H* of benzylidene), 5.28 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 8.7 Hz, H-3'), 4.89 (m, 1H, H-3_L), 4.80 (d, 1H, *J* = 11.7 Hz, CH_2 of benzyl), 4.70 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 4.53-4.26 (m, 6H, H-1, CH_2 of benzyl), 4.18 (dd, 1H, $J_{5',6'a}$ = 4.5 Hz, $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 3.89 (d, 1H, $J_{6a,6b}$ = 10.8 Hz, H-6a), 3.78-3.58 (m, 5H, H-2', H-6b, H-6'b, 2 x H-3_S), 3.53 (t, 1H, *J* =8.4 Hz, *J* = 9.6 Hz, H-4), 3.44-3.25 (m, 4H, H-2, H-4, H-5, H-5'), 2.54 (dd, 1H, $J_{2Sa,2Sb}$ = 15.0 Hz, $J_{2Sa,3S}$ = 6.0 Hz, H-2_{Sa}), 2.33-2.17 (m, 6H, H-2_S, H-2_L, H-$2_{L'}$), 2.6 (dd, 1H, $J_{2La,2Lb}$ = 15.0 Hz, $J_{2La,3L}$ = 5.7 Hz, H-2_{La}), 1.49-1.36 (m, 9H, H-4_S, H-$4_{L'}$, H-4_L, C*H* of thexyl), 1.14-1.06 (m, 83H, CH_2 of lipid), 0.76-0.71 (m, 33H, CH_3 of thexyl, CH_3 of lipid), 0.05 (s, 3H, Si(CH_3)$_2$), 0.00 (s, 3H, Si(CH_3)$_2$). HR MS (m/z) calcd for $C_{113}H_{186}N_2O_{16}Si$ $[M + Na]^+$, 1878.3464; found, 1878.3721. Acetic acid (100 μL) was added to a solution of Bu_4NF (1 N in THF, 1mL), and then the alcohol (26 mg, 0.014 mmol) was added. The reaction mixture was stirred at room temperature for 20 h, after which it was diluted with ethyl aetate (10 mL), and washed with saturated aqueous $NaHCO_3$ (2 x

10 mL) and brine (2 x 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 (hexane/ethyl acetate, 1/1, v/v) afforded **29** as a pale yellow syrup (21 mg, 88%). R_f = 0.40 (hexane/ethyl acetate, 1/1, v/v);^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40-7.25 (m, 20H, aromatic), 6.67 (d, 1H, $J_{NH,2}$ = 7.8 Hz, N*H*), 5.91 (d, 1H, $J_{NH',2'}$ = 8.1 Hz, N*H*'), 5.45-5.39 (m, 2H, H-3', C*H* of benzylidene), 5.24 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.08 (d, 1H, $J_{1,2}$ = 2.7 Hz, H-1), 4.96 (m, 1H, H-3_L), 4.92 (d, 1H, *J* = 11.7 Hz, CH_2 of benzyl), 4.64-4.32 (m, 6H, H-6'a, CH_2 of benzyl), 4.05-3.51 (m, 10H, H-2', H-3, H-4', H-5, H-5', H-6a, H-6b, H-6'b, 2 x H-3_S), 3.53 (dd, 1H, *J* =8.4 Hz, *J* = 9.6 Hz, H-4), 3.44-3.25 (m, 4H, H-2, H-4, H-5, H-5'), 2.63 (dd, 1H, $J_{2Sa,2Sb}$ = 16.4 Hz, $J_{2Sa,3S}$ = 6.0 Hz, H-2_{Sa}), 2.53-2.17 (m, 7H, H-2_S, H-2_L, H-$2_{L'}$), 1.64-1.47 (m, 8H, H-4_S, H-$4_{L'}$, H-4_L), 1.25-1.15 (m, 83H, CH_2 of lipid), 0.87-0.85 (m, 21H, CH_3 of lipid). HR MS (m/z) calcd for $C_{105}H_{168}N_2O_{16}Si$ $[M + Na]^+$, 1736.2286; found, 1736.3901.

6-*O*-{2-Deoxy-2-[(*R*)-3-hexadecanoyloxy-15-methyl-hexadecanoylamino]-3-*O*-[(*R*)-3-hydroxy-13-methyl-tetradecanoylamino]-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxy-15-methyl-hexadecanoylamino]-2-deoxy-α-D-glucopyranose 1-phosphate(2): Compound **30** (15 mg, 0.0088 mmol) was phosphorylated in a manner similar to the synthesis of **1** to afford **31** as a pale yellow syrup (11.8 mg, 68%). R_f = 0.60 (hexane/ethyl acetate, 3/2, v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39-7.26 (m, 30H, aromatic), 6.65 (d, 1H, $J_{NH',2'}$ = 8.0 Hz, N*H*'), 6.50 (d, 1H, $J_{NH,2}$ = 8.5 Hz, N*H*), 5.65 (bs, 1H, H-1), 5.42 (m, 1H, C*H* of benzylidene), 5.35 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 10.0 Hz, H-3'), 5.10-4.99 (m, 5H, H-3_L, CH_2 of benzyl), 4.81 (d, 1H, *J* = 10.5 Hz, CH_2 of benzyl), 4.61 (d, 1H, *J* = 10.5 Hz, CH_2 of benzyl), 4.52-4.42 (m, 4H, CH_2 of benzyl), 4.32 (m, 1H, H-6'a), 4.13 (m, 1H, H-2), 3.95-3.74 (m, 6H, H-2', H-6a, H-6b, H-6'b, 2 x H-3_S), 3.66-3.60 (m, 2H, H-3, H-4'), 3.43 (m, 1H, H-5'), 3.36 (m, 1H, H-5), 2.69 (dd, 1H, $J_{2Sa,2Sb}$ = 14.5 Hz, $J_{2Sa,3S}$ = 6.0 Hz, H-

2_{Sa}), 2.52-2.26 (m, 7H, H-2_S, H-2_L, H-$2_{L'}$), 1.59-1.50 (m, 8H, H-4_S, H-$4_{L'}$, H-4_L), 1.27-1.17 (m, 83H, CH_2 of lipid), 0.89-0.87 (m, 21H, CH_3 of lipid). HR MS (m/z) calcd for $C_{119}H_{181}N_2O_{19}P$ [M + Na]$^+$, 1996.2888; found, 1996.0125. Compound **31** (9.6 mg, 0.0049 mmol) was deprotected in a manner similar to the synthesis of **1** to provide **2** as a colorless film (5.3 mg, 76%). ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD, 1/1, v/v): δ 5.18 (broad, 1H, H-1), 4.80-4.64 (m, 2H, H-3', H-3_L), 4.56 (broad, 1H, H-1'). HR MS (m/z) calcd for $C_{77}H_{147}N_2O_{19}P$[M + Na]$^+$, 1458.0233; found [M + Na -2H]$^+$, 1456.2222.

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CHAPTER 6

CONCLUSION

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. The structures of lipid As vary considerably in acylation patterns and phosphorylation among bacterial species, which likely accounts for the highly variable *in-vivo* and *in-vitro* host responses to LPS.

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. An important concern of such therapies is that over-activation of innate immunity may lead to the clinical symptoms of septic shock. Thus, an important issue for the development of lipid A as immuno-modulators requires a detailed knowledge of structure-activity relationship to harness beneficial effects without causing toxicity. We have developed a convergent approach that allows for the convenient synthesis of a panel lipid As employing a common disaccharide building block functionalized with a versatile set of protecting groups. The strategy was employed for the preparation of lipid As 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 and shortening of lipids resulted in higher potencies. Furthermore, LPS gave much higher potencies than the synthetic lipid As. Differences in potencies were also observed for the production of cytokines. For example, large differences were observed between the EC_{50} values

of secreted TNF-α and IL-1β. 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 As gave lower efficacies for the production of RANTES and IL-6 compared to LPS. Collectively, our results 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 As 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 in the underlying mechanism of immune modulation.

All of the above synthetic lipid A derivatives have lower efficacies than *E. coli* 055:B5 LPS for all the tested cytokines. Although lipid A is endotoxically active principle of LPS, the nature and number of attached sacchardie residues and substituents have considerable impact on modulating this activity. Interestingly, for almost every LPS known to date, the first sugar bound to lipid A is 3-deoxy-D-manno-octulosonic acid (Kdo). To exploit the impact of Kdo on modulating immunological activities of lipid A, we synthesized lipid A derivative containing Kdo from *Neisseria meningitidis* with a novel approach, which allows for the convenient synthesis of a panel of analogues differing in fatty acid acylation patterns and degree of phosphorylation. The compound was tested for cytokine production along with the synthetic *N. meningitidis* lipid A and its parent LOS. Examination of the biological results showed that the lipid A derivative containing Kdo was much more active than the lipid A derivative without Kdo

and just slightly less active than its parent LPS, indicating that one Kdo moiety is sufficient for the restoration of biological activity.

Several studies have indicated that compounds that can antagonize cytokine production induced by enteric LPS may have the potential to be developed as therapeutics for the treatment of Gram-negative septicemia. Recent data from our laboratory indicate that LPS from *Rhizobium sin*-1 does not stimulate human monocyte and significantly inhibit *E. coli* LPS-dependent synthesis of TNF-α, making its lipid A an attractive lead compound for drug development. The lipid A of *R. sin*-1 is perhaps the most structurally unusual lipid A reported to date. Unique features of *R. sin*-1 lipid A is lack of phosphates and a very long chain fatty acid 27-hydroxyoctacosanoic acid. To establish the relevance of the 27-hydroxyoctacosanoic acid for antagonistic properties, we synthesized several derivatives of *R. sin*-1 lipid A differing in C-2' acylation. Cellular activation studies with a human monocytic cell line have shown that the octacosanoic acid is important for optimal antagonistic properties. The hydroxyl of the natural 27-hydroxyoctacosanoic moiety does, however, not account for inhibitory activity.

There is some indication that *Porphyromonas gingivalis* LPS initiates innate immune responses through TLR2 and/or TLR4. The heterogeneity of LPS and lipid A preparations limits the identification of specific structural features that are responsible for this unusual activation pathway. To address this problem, we have synthesized two lipid As of *P. gingivalis* LPS by a highly convergent approach, which provides easy access to a wide range of lipid A analogues. Furthermore, an efficient method has been developed for the synthesis of optically pure 3-hydroxy fatty acids with a terminal isopropyl group by olefin cross metathesis between a β-keto ester with a terminal double bond and different olefins.