# Synthesis and Biological Evaluation of a Lipid A Derivative That Contains an Aminogluconate Moiety

**Balaji Santhanam,[a] Margreet A. Wolfert,[a, b] James N. Moore,[b] and Geert-Jan Boons*[a]**

**Abstract:** A highly convergent strategy for the synthesis of several derivatives of the lipid A of *Rhizobium sin-1* has been developed. The synthetic derivatives are 2-aminogluconate **3** and 2-aminogluconolactone **4**, both of which lack C-3 acylation. These derivatives were obtained by the preparation of disaccharides in which the two amino groups and the C-3′ hydroxy group could be modified individually with acyl or β-hydroxy fatty acyl groups. Detailed NMR spectroscopy and MS analysis of **3** and **4** revealed that, even under neutral conditions, the two compounds equilibrate. The synthetic compounds lack the proinflammatory effects of *Escherichia coli* lipopolysaccharide (LPS), as indicated by an absence of tumor necrosis factor production. Although **3** and **4** were able to antagonize *E. coli* LPS, they were significantly less potent than the synthetic compound **2**, which is acylated at C-3, and *R. sin-1* LPS; these results indicate that the β-hydroxy fatty acyl group at C-3 contributes to the antagonistic properties of *R. sin-1* LPS. Based on a comparison of the biological responses of the synthetic lipid A derivatives with those of the *R. sin-1* LPS and lipid A, the 3-deoxy-D-manno-octulosonic moieties appear to be important for the optimal antagonization of enteric LPS-induced cytokine production.

**Keywords:** carbohydrates · cytokines · glycolipids · inhibitors · lipopolysaccharides

## Introduction

Septicemia is a life-threatening syndrome for which currently no treatment exists other than supportive therapy in an intensive care unit setting.[1,2] The development of Gram-negative sepsis is due to a strong and acute inflammatory response to lipopolysaccharides (LPSs) released from the bacterial outer membrane.[3–5] LPSs initiate the production of multiple host-derived inflammatory mediators, such as tumor necrosis factor (TNF-α), interleukins 1 and 6 (IL-1 and IL-6), arachidonic acid metabolites, and leukotrienes. LPSs induce the production of these mediators after binding either to the cluster differentiation antigen CD14 on mononuclear phagocytes or to soluble CD14 in plasma and then to cells lacking CD14.[6–8] The interaction of LPSs with CD14 is facilitated by a plasma protein called LPS binding protein. As CD14 is a glycosylphosphatidylinositol-anchored protein, it lacks transmembrane and cytoplasmic domains and is therefore unable to directly transmit signals to the interior of the cell. The latter function is performed by Toll-like receptor 4 (TLR4),[9–11] which contains extracellular, transmembrane, and intracellular domains, as well as the accessory protein MD-2.[12,13] While the precise mechanisms involved in the interactions among LPSs, CD14, TLR4, and MD-2 have yet to be discovered,[14,15] 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. The end result is an up-regulation of more than 120 genes, including those for the cytokines, most notably TNF-α, interleukin-1α, and interleukin-1β.[16]

LPSs consist of an O-chain polysaccharide, a core oligosaccharide, and an amphiphilic moiety referred to as lipid A. Lipid A obtained by acid hydrolysis of *E. coli* LPS has lethal toxicity, pyrogenicity, and TNF-α- and other-cytokine-activating properties similar to native LPS; it is thus regarded as toxic principle of the LPS.[17,18] The structure of lipid A is largely conserved among most enteric bacteria; it consists of a β(1–6)-linked glucosamine disaccharide backbone with phosphate monoesters at C-1 and C-4′, β-hydroxy fatty acyl groups at positions 2 and 3, and acyloxyacyl residues at positions 2′ and 3′ (Scheme 1).[19] Small modifications in the acyl-

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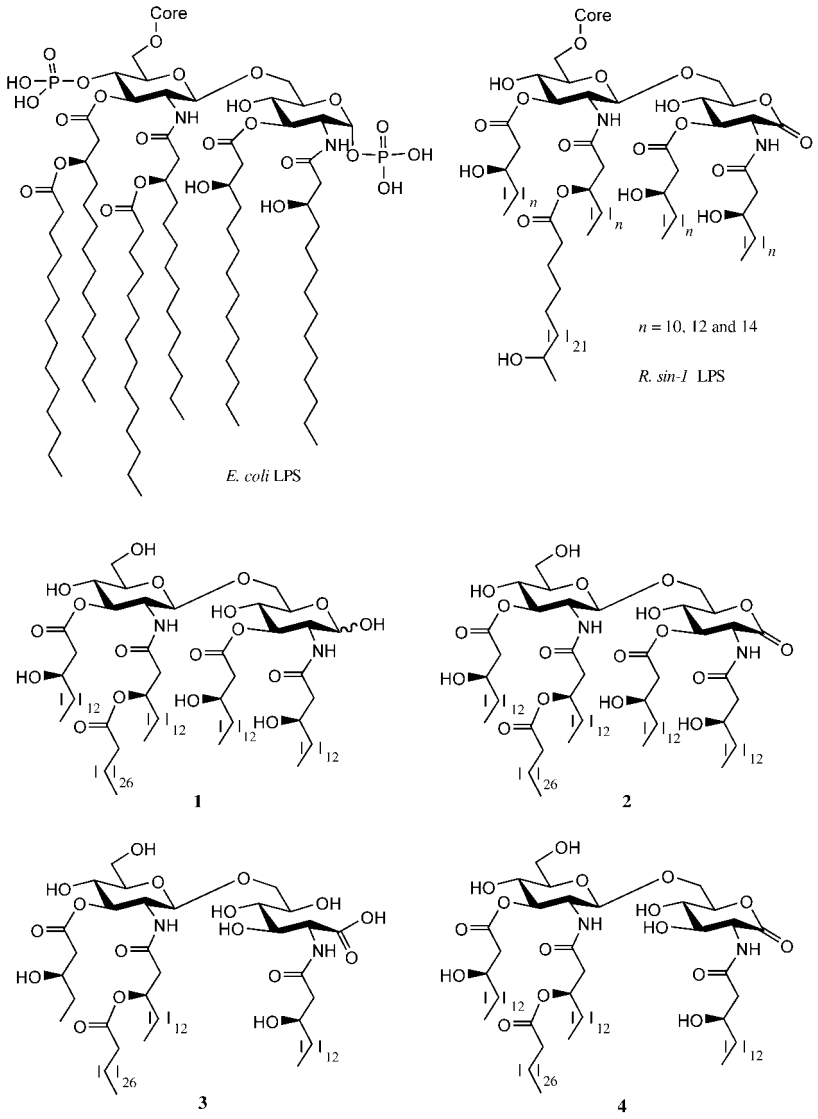



Scheme 1. Structures of *E. coli* and *R. sin-1* LPSs and the lipid A analogues **1**–**4**.

ation 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 lengths, sites of unsaturation, or keto functional groups.[20–23] Other modifications include the addition of a palmitoyl residue, the hydroxylation of a myristoyl substituent, and the addition of aminoarabinosyl and phosphoethanolamine moieties.[24]

An appealing method for the prevention of the deleterious effects of enteric LPSs is to block the interaction of lipid A with its receptors on mononuclear phagocytes.[25,26] Interference at this level may prevent initiation of the cellular reactions that lead to systemic inflammatory responses and septic shock. Efficacious pharmacological receptor antagonists are often derived by modifying a compound possessing agonist activity. It has, however, proven difficult to identify lipid A derivatives that possess these properties. The best-studied derivatives are monosacccharide biosynthetic precursors of lipid A[27–29] and synthetic analogues derived from the lipid A of *Rhodobacter sphaeroides* or *Rhodobacter capsulatus*, two species with very similar lipid A structures.[30–32] Although the *R. sphaeroides*/*R. capsulatus* lipid A has a bis-1,4′-phosphorylated backbone identical to that of *E. coli* lipid A, the fatty acyl pattern is quite different. The *R. sphaeroides*/*R. capsulatus* lipid A consists of two 3-oxomyristic acid residues, two (*R*)-β-hydroxydecanoic acid residues, and one dodecenoic acid residue. The latter fatty acid is the only acyloxyacyl substituent and is located on the 3′-β-hydroxydecanoic acid residue. The *R. sphaeroides*/*R. capsulatus* lipid A lacks toxic effects, fails to induce cytokine synthesis by human monocytes, and is an antagonist of enteric endotoxin.

Recent data from our laboratory indicate that LPS from the nitrogen-fixing symbiont *Rhizobium sin-1* does not stimulate human monocytes.[33] More importantly, *R. sin-1* LPS significantly inhibits *E. coli* LPS dependent synthesis of TNF-α by human monocytes. The lipid A of *R. sin-1* is perhaps the most structurally unusual lipid A reported to date, because its structure (Scheme 1) differs in almost every aspect from those known to contribute to the toxicity of enteric LPSs.[34] In particular, the disaccharide moiety of the rhizobial lipid A is devoid of phosphate groups and the glucosamine phosphate is replaced by a 2-aminogluconolactone. This lipid A contains a very long chain fatty acid, 27-hydroxyoctacosanoic acid, which, in turn, can be esterified by β-hydroxybutyrate.

The lipid A of *R. sin-1* shows considerable microheterogeneity. The fatty acylation pattern is heterogeneous and consists exclusively of β-hydroxy fatty acids. The N-acyl groups can consist of β-hydroxymyristate, β-hydroxypalmitate, or β-hydroxystearate. The O-acyl groups are primarily β-hydroxymyristate, but occasionally can also include β-hydroxypentadecanoate. Furthermore, a significant percentage of *R. sin-1* lipid A moieties lack a fatty acyl residue at the C-3 position. It may also be possible that the 2-aminogluconolactone residue exists as a 2-aminogluconate.

*R. sin-1* LPS cannot be developed as a therapeutic agent for Gram-negative septicemia due to its inherent molecular heterogeneity. Furthermore, the current inability to separate the different species limits the identification of specific

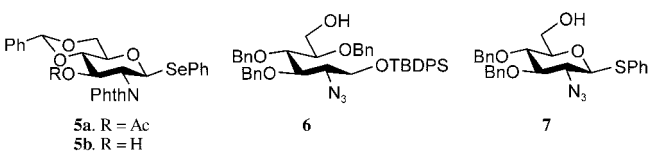
  

structural features that make *R. sin-1* lipid A an antagonist as opposed to an agonist. To address these problems, we are engaged in the development of facile approaches for the synthesis of a wide range of well-defined lipid A derivatives based on the structure of *R. sin-1* LPS. We have already shown[35] that the synthetic compounds **1** and **2** (Scheme 1) lack the proinflammatory effects of *E. coli* LPS, as indicated by an absence of expression of TNF-α mRNA or production of TNF-α protein. Furthermore, the synthetic compound **2** was able to antagonize *E. coli* LPS, whereas compound **1** was devoid of this ability. Based on the known structure of the lipid A of *R. sin-1*, these results suggest that the gluconolactone moiety of *R. sin-1* LPS is important for this property. This finding was significant as **2** is the first example of a synthetic lipid A derivative that lacks the phosphate moieties but can inhibit cytokine production initiated by *E. coli* LPS. In this respect, compounds containing phosphate groups are less attractive candidates for drug development because of their instability.

In this paper, we report efficient approaches for the synthesis of compounds **3** and **4**, which are putative structural elements of *R. sin-1* LPS. As it is unknown whether these derivatives exist as 2-aminogluconolactones or 2-aminogluconates, both forms were prepared. The proinflammatory properties of compounds **3** and **4** and their capability to inhibit *E. coli* LPS dependent synthesis of TNF-α by human monocytes have been determined. These studies highlight the importance of C-3 acylation for biological properties. It was also discovered that the 2-aminogluconolactone and 2-aminogluconate moieties equilibrate under neutral conditions. In order to determine the importance of 3-deoxy-D-manno-octulosonic (KDO) moieties for optimal inhibition of enteric LPS-induced cytokine production, the biological responses of the synthetic lipid A derivatives have been compared with *R. sin-1* LPS and lipid A.

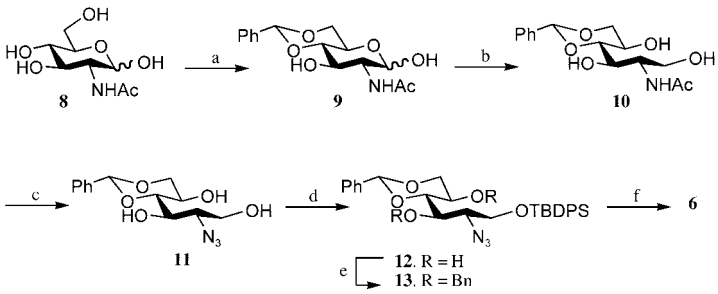
## Results and Discussion

**Synthesis**: Glycosyl donors **5a** and **5b** and glycosyl acceptors **6** and **7** (Scheme 2) were used as appropriately protected building blocks for the synthesis of target compounds **3** and **4**. Coupling of the donor with each of the acceptors will give disaccharides (**14** and **21**, see Schemes 4 and 5) that are appropriately protected for the selective introduction of β-hydroxy fatty acyl and acyloxyacyl residues. In this respect, the phthalimido moiety of the disaccharides can be selectively cleaved with hydrazine hydrate in refluxing ethanol without affecting the azido group. Under these conditions, the acetyl esters will also be removed. However, the resulting amine and hydroxy groups can be selectively acylated by exploiting the fact that primary amines are more nucleophilic than hydroxy groups. The azido function can be reduced to an amine under mild conditions by using propane-1,3-dithiol[36] in a mixture of pyridine, triethylamine, and water and these conditions will not affect any of the other functionalities. Finally, at a late stage of the synthesis, the *tert*-butyldiphenylsilyl (TBDPS) and thiophenyl groups[37,38] at position 1 of the compounds derived from **6** and **7**, respectively, can be selectively removed to install the aminogluconate or lactone moiety.




Scheme 2. Building blocks for the preparation of compounds **3** and **4**. Bn = benzyl, Phth = phthalimido, TBDPS = *tert*-butyldiphenylsilyl.

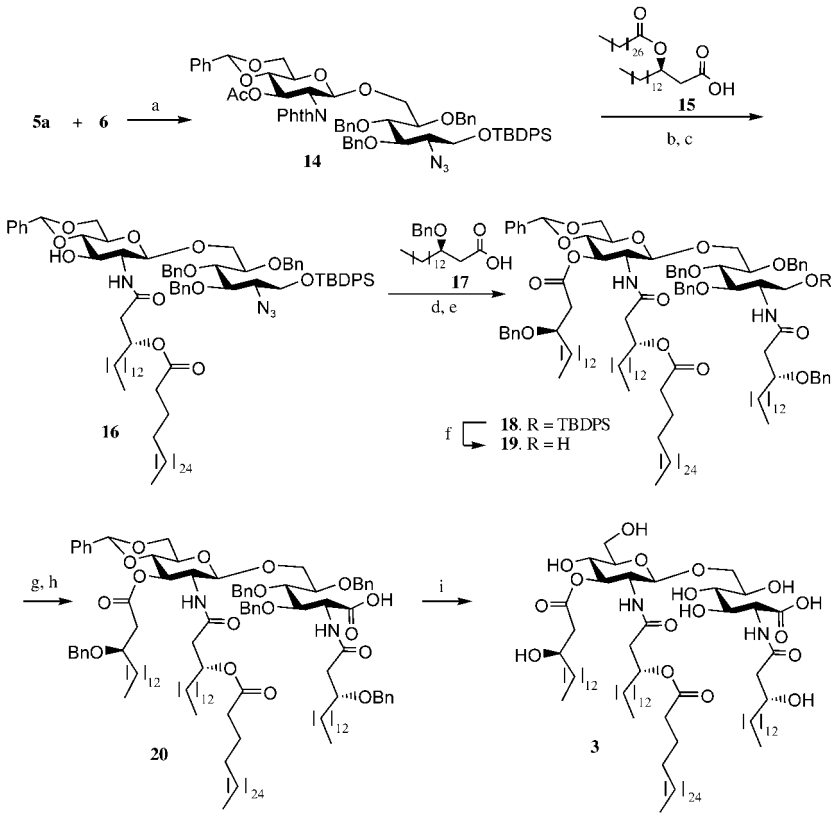
In order to synthesize target compound **3**, an efficient strategy for the preparation of protected 2-azido-2-deoxy-alditol **6** needed to be developed. It was envisaged that this compound could be obtained by reduction of the anomeric center of an appropriate protected *N*-acetylglucosamine derivative followed by conversion of the acetamido moiety into an azido group. Thus, the 4,6-diol of commercially available *N*-acetylglucosamine was protected as a benzylidine acetal by using freshly distilled benzaldehyde and zinc chloride to give compound **9** (Scheme 3).[39] A good yield




Scheme 3. a) PhCHO, $ZnCl_2$; b) $NaBH_4$, MeOH; c) $Ba(OH)_2 \cdot 8H_2O$, MeOH, $H_2O$, 90 °C; then $Tf_2O$, $NaN_3$, $CuSO_4$; d) TBDPSCl, imidazole, DMF; e) BnBr, NaH, DMF; f) 1 M $BH_3$ in THF, 1 M $Bu_2BOTf$ in $CH_2Cl_2$). Tf = trifluoromethanesulfonyl.

was achieved when the reaction mixture was sonicated for two hours. Reduction of the hemiacetal of **9** with $NaBH_4$ in methanol at 0 °C gave alditol **10** in a yield of 95 %.[40] Treatment of **9** with barium hydroxide converted the acetamido group into an amine, which was transformed into an azido moiety by an azido transfer reaction with triflic azide (formed in situ) to give compound **11** in an excellent overall yield of 85 %.[41] Next, the primary alcohol of **11** was selectively protected by treatment with TBDPSCl and imidazole in DMF to give **12**, which was benzylated under standard conditions to afford the fully protected **13**. Finally, regioselective opening of the benzylidine acetal of **13** by treatment with $BH_3$ (1 M in THF) and $Bu_2BOTf$ (1 M in $CH_2Cl_2$) gave the alditol acceptor **6** in an excellent yield of 85 %.[41]

Coupling of the glycosyl donor **5a** with alditol acceptor **6**, by using NIS/TMSOTf[42] as the activator, gave disaccharide **14** in excellent yield (Scheme 4). In this coupling, only the β anomer was formed due to neighboring-group participation of the phthalimido group. Removal of the phthalimido group of **14** by treatment with hydrazine hydrate in refluxing ethanol followed by selective N-acylation with **15** in the



Scheme 4. a) NIS, TMSOTf, 4 Å molecular sieves, $CH_2Cl_2$, −40 °C; b) $H_2NNH_2$, EtOH, Δ; c) DCC, $CH_2Cl_2$; d) $HS(CH_2)_3SH$, pyridine, $Et_3N$, $H_2O$; e) DCC, DMAP, $CH_2Cl_2$; f) 1 M TBAF in THF; g) $(COCl)_2$, DMSO, $Et_3N$; h) $NaClO_2$, 2-methyl-2-butene, $NaH_2PO_4$, THF/*t*BuOH; i) Pd/C, $H_2$, *t*BuOH, THF. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, NIS = *N*-iodosuccinimide, TBAF = tetrabutylammonium fluoride, TMS = trimethylsilyl.

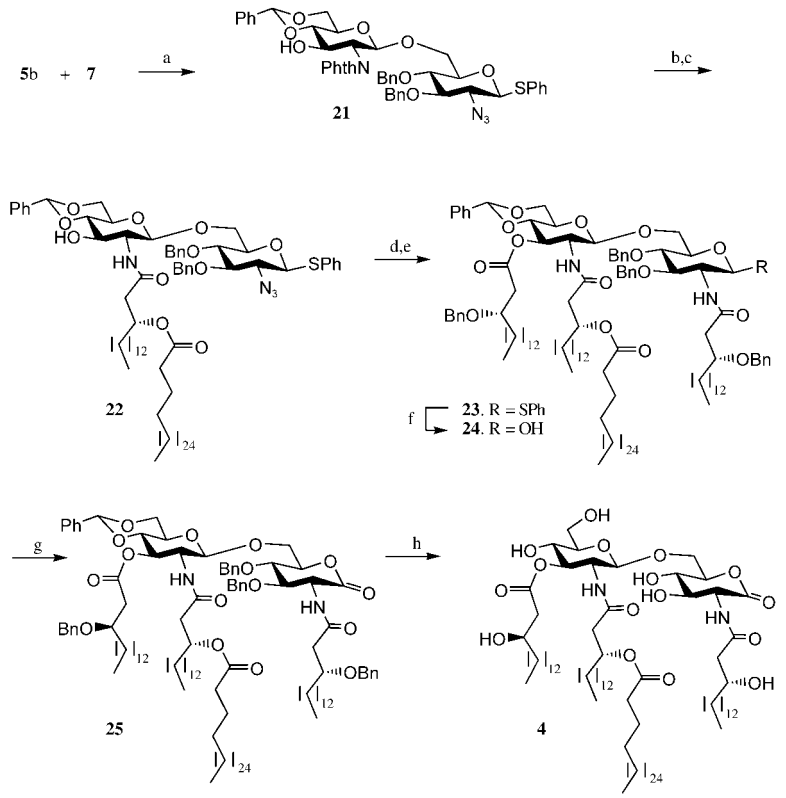
presence of DCC gave **16** in a yield of 71 %. Reduction of the azido moiety of **16** was easily accomplished by reaction with propane-1,3-dithiol[36] in a mixture of pyridine, triethylamine, and water. The C-2 amine and C-3′ hydroxy group of the resulting compound were immediately acylated with **17** by using DCC and DMAP as the activation reagents to afford **18** in an overall yield of 60 %. It is important to note that selective N-acylation could be accomplished by performing the reaction in the absence of DMAP, thereby making it possible to synthesize derivatives with different substituents at the C-2 and C-3′ positions. The TBDPS group of compound **18** was removed by treating with TBAF to afford **19** in good yield (Scheme 4). The oxidation of the primary alcohol of **19** to form carboxylic acid **20** was attempted with a catalytic amount of 2,2,6,6-tetramethyl-1-pipiridinoxyl (TEMPO) and NaBr with $NaOCl_2$ as the cooxidant under biphasic conditions.[43] However, a low yield of this **20** was obtained and the major product was an intermediate aldehyde. Pyridinium dichromate (PDC)/DMF mediated oxidation gave only recovery of the starting material. Fortunately, a two-step procedure was successful; this involved Swern conditions to give an intermediate aldehyde that was immediately used without purification in a second oxidation with $NaClO_2$ and sodium dihydrogenphosphate in a mixture of THF/*tert*-butanol to afford **20** in 75 % overall yield. Finally, the benzyl ethers and benzylidene acetal of **20** were removed by catalytic hydrogenation over Pd/C to give the target compound **3**.

The preparation of target derivative **4** commenced with a chemoselective glycosylation of selenoglycoside **5b** with thioglycoside **7** with NIS/TfOH as the promoter to give disaccharide **21** (Scheme 5).[37,44] Although glycosyl donor **5b** was only partially protected, no products derived from oligomerization were detected. Interestingly, when this donor was used for the glycosylation of **6**, only a low yield of disaccharide was obtained. Apparently, the primary hydroxy group of **7** is significantly more reactive than that of **6**, probably due to its cyclic structure. Disaccharide **21** could be converted into acylated derivative **23** by a similar sequence of reactions to those described for **18**. Hydrolysis of the thiophenyl moiety of **23** with NIS and a catalytic amount of TfOH in wet dichloromethane resulted in mixture of **24** and a 1,2-oxazoline derivative. When the above reaction was carried out without TfOH in wet THF clean hydrolysis gave **24** in high yield. PCC-mediated oxidation of **24** led, after purification by Iatro-bead column chromatography with ethyl acetate/toluene as the eluent, to lactone **25** as a pure compound. Finally, the benzyl ethers and benzylidene acetal of **25** were removed by catalytic hydrogenation over Pd/C to give the target compound **4.**

Surprisingly, the high-resolution $^1$H NMR spectra of the final compounds **3** and **4** were identical and showed the presence of two derivatives, assigned as a mixture of 2-aminogluconate **3** and 2-aminogluconolactone **4**. Thus, even under neutral conditions, the two compounds equilibrate to the open- and closed-ring forms. Key evidence of the presence of the lactone came from the chemical shift of H-5 at $\delta = 4.25$ ppm, whereas the same proton of the aminogluconate appeared at $\delta = 3.35$ ppm. The chemical shift of H-4 at $\delta = 3.54$ ppm for both components demonstrates that a 1,4-lactone has not been formed. Integration of the H-3′ signal of both compounds indicates that they are present in approximately equal quantities. High-resolution MALDI-TOF MS also confirmed the presence of the two compounds.

**Biological evaluation**: Compounds **3** and **4** were tested over a wide concentration range for the ability to activate a human monocytic cell line (Mono Mac 6) and to produce the TNF-α protein. The resulting values were compared with those obtained for *E. coli* and *R. sin-1* LPSs and for the



Scheme 5. a) NIS, TMSOTf, 4 Å molecular sieves, $CH_2Cl_2$, −40 °C; b) $H_2NNH_2$, EtOH, Δ; c) **15**, DCC, $CH_2Cl_2$; d) $HS(CH_2)_3SH$, pyridine, $Et_3N$, $H_2O$; e) **17**, DCC, DMAP, $CH_2Cl_2$; f) NIS, aq. THF; g) PCC, 3 Å molecular sieves, $CH_2Cl_2$; h) Pd/C, $H_2$, THF, *t*BuOH. PCC = pyridinium chlorochromate.

synthetic compound **2** (Figure 1). Incubation with *E. coli* LPS for 6 h yielded a clear dose–response effect of TNF-α production, with maximal supernatant concentrations of TNF-α being caused by 10 ng mL$^{-1}$ of *E. coli* LPS. The results of these experiments yielded an LPS $EC_{50}$ value (that is, the concentration producing 50% activity) of 0.2 ng mL$^{-1}$ and a Hill slope of 2.5. Neither *R. sin-1* LPS nor synthetic compounds **2–4** induced significant production of TNF-α at concentrations up to 10 μg mL$^{-1}$. To exclude the possibility that any effects observed might be due to the presence of THF, the cells were incubated with concentrations of THF up to 0.5%. These concentrations of THF alone did not cause TNF-α production by the cells nor did they alter the response of cells coincubated with *E. coli* LPS at a concentration of 10 ng mL$^{-1}$ (data not shown).

Based on their lack of proinflammatory effects, compounds **3** and **4** were tested over a wide concentration range for their ability to antagonize the responses of monocytic cells incubated with 10 ng mL$^{-1}$ *E. coli* LPS (Figure 2). At the highest concentration tested, compounds **3** and **4** antagonized the effect of *E. coli* LPS by 23%. The synthetic compound **2** and *R. sin-1* LPS were, however, significantly more potent inhibitors with $IC_{50}$ values (that is, the concentration producing 50% inhibition) of 13 μg mL$^{-1}$ (7.3 nmol mL$^{-1}$) and 0.21 μg mL$^{-1}$, respectively, whereas **3/4** gave an estimated $IC_{50}$ value of 330 μg mL$^{-1}$ (216 nmol mL$^{-1}$).

The structure of lipid A of *R. sin-1* shows considerable microheterogeneity.[34] Several major components lack a β-hydroxy fatty acyl group at C-3. The findings of this study indicate that this fatty acyl moiety is important for antagonistic

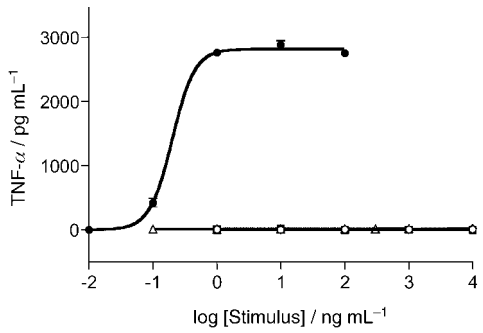



Figure 1. Concentration–response curves of *E. coli* LPS, *R. sin-1* LPS, *R. sin-1* lipid A, and synthetic compounds **2–4**. Mono Mac 6 cells were incubated for 6 h at 37 °C with increasing concentrations of *E. coli* LPS (●), *R. sin-1* LPS (○), *R. sin-1* lipid A (◇), **2** (△), **3** (□) or **4** (■). TNF-α protein in cell supernatants was measured by using an ELISA. (Please note that *R. sin-1* LPS, *R. sin-1* lipid A, and **2–4** show background values and therefore overlap in the figure.) Treatment with the compounds did not affect cell viability, as judged by cellular exclusion of trypan blue.

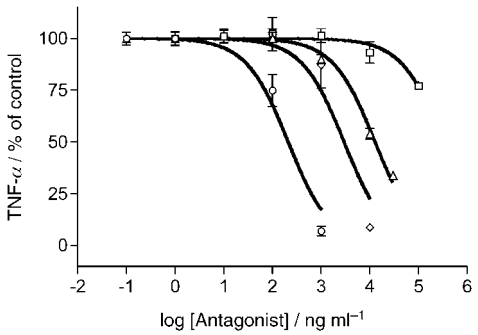



Figure 2. Antagonism of *E. coli* LPS by *R. sin-1* LPS, *R. sin*-1 lipid A, and synthetic compounds **2–4**. TNF-α protein concentrations were measured after preincubation of Mono Mac 6 cells with increasing concentrations of *R. sin-1* LPS (○), *R. sin-1* lipid A (◇), **2** (△), and **3/4** (□) for 1 h at 37 °C followed by incubation for 6 h with 10 ng mL$^{-1}$ *E. coli* LPS. Results are expressed as a percentage of the TNF-α concentration of control cells, which were incubated only with *E. coli* LPS. The effects of **3** and **4** are shown as one curve, since their behavior was exactly the same.

properties. Although lipid A derivative **2** is a significantly more potent antagonist than **3** and **4**, it has a 60-fold reduced activity compared to *R. sin-1* LPS. LPSs consist of an O-chain polysaccharide and a core oligosaccharide linked to a lipid A moiety through a dimeric KDO moiety. Thus, it may be possible that the KDO residues of the core region contribute to the antagonistic potential. To explore this possibility, purified *R. sin-1* LPS was subjected to mild acid hydrolysis to cleave the KDO glycosidic linkages. The obtained lipid A preparations were tested for agonistic and antagonist properties. As can be seen in Figure 1, the *R. sin-1* lipid A does not stimulate monocytic cells to produce TNF-α. However, it could antagonize the effect of *E. coli* LPS with an $IC_{50}$ value (3 $\mu g\,mL^{-1}$) similar to that of compound **2** (Figure 2). Thus, these observations indicate that the oligo-(poly)saccharide moiety of LPS contributes to the antagonistic potential. In this respect, recent studies[45] have shown that meningococcal lipid A expressed by *Meningococci* with defects in KDO biosynthesis or transfer has a 10-fold reduction in bioactivity compared to $KDO_2$-containing meningococcal lipooligosaccharides. Removal of the KDO moieties by mild acidic treatment also dramatically attenuated cellular responses. Thus, it is probable that the cell-surface receptors that recognize LPS bind to the KDO moiety of LPSs as well as to the lipid A.

The results of previous studies indicate that antagonism of the cell-surface receptors that recognize enteric LPSs can prevent the production of cytokines.[25] Hence, such compounds have potential for use as therapeutic interventions for patients with Gram-negative septicemia. Success in this area has been limited and most efforts have been directed towards the synthesis of analogues of lipid A of *R. sphearoides*[31,32] and derivatives of lipid X.[27–29] These compounds, which are either mono- or bisphosphorylated, may possess metabolic instabilities that complicate drug discovery. A unique aspect of our study is that we have identified a lipid A derivative that lacks phosphate groups and that antagonizes the biological effect of enteric LPSs. By comparing the biological responses initiated by the synthetic lipid A derivatives **2**–**4** and the *R. sin-1* LPS and lipid A, it can be concluded that the gluconolactone moiety, the β-hydroxy fatty acyl group at C-3, and the KDO moieties of *R. sin-1* LPS appear to be important for antagonizing enteric LPS-induced cytokine production. The synthesis of other derivatives to unravel other structural features for optimal antagonistic properties is in progress.

## Conclusion

Previous studies from our laboratory have shown that the LPS of *R. sin-1* can inhibit *E. coli* LPS dependent synthesis of TNF-α by human monocytes. Due to the inherent molecular heterogeneity of lipid A, it cannot be developed as a therapeutic agent for Gram-negative sepsis. Organic synthesis provides an attractive approach for obtaining well-defined derivatives of lipid A of *R. sin-1*. Such compounds will enable us to determine the structural features that account for the antagonistic properties. A significant percentage of lipid A derivatives of *R. sin-1* lack a fatty acyl residue at C-3. Furthermore, it was not known whether the lipid A moiety of *R. sin-1* exists as an aminogluconate or 2-aminogluconolactone. In order to address these issues, we have developed highly convergent approaches for the facile synthesis of 2-aminogluconate **3** and 2-aminogluconolactone **4**, both of which lack C-3 acylation. A key aspect of the synthesis of **3** was glycosylation of a properly protected 2-azido-2-deoxy-alditol to give a disaccharide in which the two amino groups and the C-3′ hydroxy group could individually be modified with acyl or β-hydroxy fatty acyl groups. At the end of the synthetic sequence, the C-1 protecting group of the alditol moiety could be selectively removed to give a hydroxy group, which was oxidized to a carboxylic acid by using a two-step procedure. 2-Aminogluconolactone **4** could easily be synthesized by a chemoselective glycosylation of a selenoglycosyl donor with a thioglycosyl acceptor to give a disaccharide. The anomeric center, the two amino groups, and the C-3′ hydroxy group of this compound could individually be modified, thereby providing a flexible route to lipid A analogues. Detailed NMR spectroscopy and MS analysis of **3** and **4** revealed that, even under neutral conditions, the two compounds equilibrate to both forms. The synthetic compounds lack the proinflammatory effects of *E. coli* LPS, as indicated by the absence of the production of TNF-α protein. Although they were able to antagonize *E. coli* LPS, synthetic compound **2**, which is acylated at C-3, and *R. sin-1* LPS were significantly more potent inhibitors; these results indicate that the β-hydroxy fatty acyl group at C-3 contributes to the antagonistic potential of *R. sin-1* LPS. Based on the comparison of the biological responses of the synthetic lipid A derivatives and of the *R. sin-1* LPS and lipid A, it was determined that the KDO moieties are important for antagonizing enteric LPS-induced cytokine production.

## Experimental Section

**General synthetic methods**: Column chromatography was performed on silica gel 60 (EM Science, 70–230 mesh), size-exclusion column chromatography was performed on Sephadex LH-20 (*i*PrOH/$CH_2Cl_2$ or MeOH/$CH_2Cl_2$ (1:1) elution) or G-25 (water elution) columns. Reactions were monitored by TLC on Kiesel gel 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 $<40\,^{\circ}C$. $CH_2Cl_2$, $(ClCH_2)_2$, and MeCN were distilled from $CaH_2$ (twice) 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 was then distilled and stored under argon. Pyridine was dried by refluxing with $CaH_2$ and was then 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 for 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 P-1020 polarimeter. $^1H$ NMR and $^{13}C$ NMR spectra were recorded with Varian Inova 500 and Inova 600 spectrometers equipped with Sun workstations. $^1H$ NMR spectra were recorded in $CDCl_3$ and referenced to residual $CHCl_3$ at 7.24 ppm; $^{13}C$ NMR spectra were referenced to the central peak of $CDCl_3$ at 77.0 ppm. Assignments were made by standard gCOSY and gHSQC techniques. High-resolution mass spectra were obtained on a Bruker Ultraflex MALDI-TOF mass spectrometer. Signals marked with a subscript L symbol belong to the biantennary lipid at C-2′, whereas sig-

nals marked with a subscript L′ symbol belong to the C-28 side chain. Signals marked with a subscript S symbol belong to the monoantennary lipids at C-2, C-3, and C-3′. Signals marked with an asterisk may be interchangeable.

**2-Acetamido-4,6-*O*-benzylidene-2-deoxy-D-glucopyranose (9)**: Freshly distilled benzaldehyde (16.6 mL, 163.3 mmol) was added to *N*-acetylglucosamine **8** (6.65 g, 30.08 mmol) followed by zinc chloride (4.1 g, 30.08 mmol). The mixture was sonicated for 2 h and left stirring overnight. The solid formed was washed with hexanes (3×75 mL) followed by water (3×75 mL). The filtered solid was washed again with methanol (3×50 mL). The white solid thus obtained was dried in vacuo to give **9** as a white powder (5.4 g, 58%). $R_f$=0.34 (15% methanol in $CH_2Cl_2$); $^1$H NMR (300 MHz, $CD_3OD$): $\delta$=7.52–7.26 (m, 5H; aromatic), 5.47 (s, 1H; >CHPh), 4.21 (q, $J$=5.3 Hz, 1H; H-6a), 4.12 (m, 2H; H-2, H-3), 3.87 (m, 1H; H-5), 3.69–3.54 (m, 4H; H-1a, H-1b, H-6b, H-4), 1.79 ppm (s, 3H; NHCOC$H_3$).

**2-Acetamido-4,6-*O*-benzylidene-2-deoxy-D-glucitol (10)**: $NaBH_4$ (5.23 g, 138.3 mmol) was added in four equal parts over a period of 1 h to a suspension of lactol **9** (5.35 g, 17.28 mmol) in methanol (95 mL) at 0 °C. The reaction mixture was allowed to stir for 5 h to give a clear solution. A saturated solution of $NaH_2PO_4$ was added to the reaction mixture until the pH value reached 7. The mixture was kept in the freezer for 8 h. The supernatant was decanted and the resulting semicrystalline mass was washed with MeOH (3×75 mL). The combined methanolic extracts and supernatant were concentrated under reduced pressure. The solid residue was extracted with MeOH (4×20 mL) and the methanolic extracts concentrated to dryness. The resulting white solid was washed with $CH_2Cl_2$ and the residue was taken up in methanol. After filtration and evaporation of the methanol, pure **10** was obtained (5.22 g, 95%). $R_f$=0.24 (15% methanol in $CH_2Cl_2$); $^1$H NMR (300 MHz, $CD_3OD$): $\delta$=7.52–7.26 (m, 5H; aromatic), 5.47 (s, 1H; >C*H*Ph), 4.21 (q, $J$=5.3 Hz, 1H; H-6a), 4.12 (m, 2H; H-2, H-3), 3.87 (m, 1H; H-5), 3.69–3.54 (m, 4H; H-1a, H-1b, H-6b, H-4), 1.79 ppm (s, 3H; NHCOC$H_3$); $^{13}$C NMR (75 MHz, $CD_3OD$): $\delta$=173.1 (NH*C*OCH$_3$), 139.0, 129.4, 128.6, 127.1 (Ph), 102.1 (*C*HPh), 83.7 (C-4), 72.2 (C-6), 67.7 (C-3), 62.3 (C-1), 61.6 (C-5), 55.6 (C-2), 22.6 ppm (NHCO*C*H$_3$).

**2-Azido-4,6-*O*-benzylidene-2-deoxy-D-glucitol (11)**: Barium hydroxide octahydrate (6.32 g, 20 mmol) was added to a solution of **10** (2.5 g, 8.02 mmol) in a mixture of MeOH/$H_2O$ (1:1, 60 mL). The reaction mixture was heated under reflux conditions for 14 h. Sulfuric acid (2.2 mL in 20 mL of $H_2O$) was added dropwise until the pH value reached 5. The precipitated $BaSO_4$ was removed by centrifugation and filtration. The filtrate was concentrated and then dissolved in the minimum amount of MeOH/$H_2O$ (1:1). The resulting solution was eluted through a column packed with Dowex (550 $OH^-$ resin). The eluent was concentrated under reduced pressure to give the free amine compound (1.71 g, 79%). Sodium azide (1.14 g, 17.5 mmol) was dissolved in water (3 mL) and cooled to 0 °C. $Tf_2O$ in $CH_2Cl_2$ (5 mL) was added and the resulting solution was stirred vigorously for 2 h. The organic layer was separated and the aqueous layer was washed with $CH_2Cl_2$ (2×5 mL). The combined $CH_2Cl_2$ extracts were washed with aqueous saturated $NaHCO_3$ solution. The resulting $TfN_3$ solution in $CH_2Cl_2$ and a catalytic amount of $CuSO_4$ were added to a solution of the free amine compound (0.47 g, 1.74 mmol) in the minimum amount of methanol. After stirring the reaction mixture for 2 h, the solvents were evaporated and the residue was coevaporated with toluene (3×50 mL). The residue was purified by silica gel column chromatography (50% gradient of ethyl acetate in hexanes) to afford azide **11** (0.47 g, 90%). $R_f$=0.65 (ethyl acetate); $[\alpha]_D^{23}$=+38.4° ($c$=1.3 in $CH_3OH$); $^1$H NMR (300 MHz, $CD_3OD$): $\delta$=7.55–7.31 (m, 5H; aromatic), 5.53 (s, 1H; >C*H*Ph), 4.23 (q, $J$=5.3 Hz, 1H; H-6a), 3.99 (d, $J$=2.5 Hz, 1H; H-3), 3.93 (q, $J$=4.7 Hz, 1H; H-5), 3.81 (d, $J$=3.0 Hz, 1H; H-1a), 3.74–3.63 (m, 3H; H-1b, H-4, H-2), 3.61 (t, 1H; H-6b) ppm; $^{13}$C NMR (75 MHz, $CD_3OD$): $\delta$=139.4, 129.8, 129.0, 127.4 (Ph), 102.4 (*C*HPh), 82.7 (C-4), 72.4 (C-6), 70.3 (C-3), 67.9 (C-5), 62.8 (C-1), 61.8 ppm (C-2); HRMS: $m/z$: calcd for $C_{13}H_{17}N_3O_5Na$: 318.1066; found: 318.1006.

**2-Azido-1-*O*-(*tert*-butyldiphenylsilyl)-2-deoxy-3,5-di-*O*-benzyl-4,6-*O*-benzylidene-D-glucitol (13)**: *tert*-Butyldiphenylsilylchloride (0.3 mL, 1.15 mmol) and then imidazole (0.157 g, 2.31 mmol) were added to a solution of **11** (0.284 g, 0.963 mmol) in DMF (5 mL). The mixture was stirred at room temperature under an atmosphere of argon. After 12 h, TLC analysis indicated completion of the reaction. The reaction mixture was diluted with ethyl acetate/hexanes (1:1, 80 mL), transferred to a separatory funnel, and washed with ice-cold water (2×25 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (5% gradient of ethyl acetate in hexanes) to afford **12** (0.45 g, 87%). Compound **12** (0.434 g, 0.813 mmol) was dissolved in DMF (6 mL) and sodium hydride (0.078 g, 3.25 mmol) was added. After the mixture was stirred at 0 °C for 30 min, benzyl bromide was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred for 16 h. The reaction mixture was diluted with ethyl acetate/hexanes (1:1; 50 mL), transferred to a separatory funnel, and washed with ice-cold water (2× 15 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (3% gradient of ethyl acetate in hexanes) to afford **13** (0.401 g, 70%). $R_f$=0.68 (20% ethyl acetate in hexanes); $[\alpha]_D^{23}$=+18.3° ($c$=0.49 in $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=7.55–7.31 (m, 5H; aromatic), 5.07 (s, 1H; >C*H*Ph), 4.80–4.38 (m, 4H; C$H_2$Ph), 4.35 (q, 1H; H-6a), 4.07 (dd, 1H; H-1a), 3.99 (dd, 1H; H-4), 3.92–3.82 (m, 3H; H-5, H-3, H-1b), 3.67 (dd, 1H; H-2), 3.52 (t, 1H; H-6b), 1.08 ppm (s, 9H; *t*Bu); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=139.4, 129.8, 129.0, 127.4 (Ph), 102.4 (*C*HPh), 82.7 (C-4), 72.4 (C-6), 70.3 (C-3), 67.9 (C-5), 62.8 (C-1), 61.8 (C-2), 27.0 ppm (C(*C*H$_3$)); HRMS: $m/z$: calcd for $C_{43}H_{49}N_3O_5SiNa$: 736.3183; found: 736.3372.

**2-Azido-1-*O*-(*tert*-butyldiphenylsilyl)-2-deoxy-3,4,5-tri-*O*-benzyl-D-glucitol (6)**: A solution of 1 M $BH_3$ in THF (1.42 mL) was added to compound **13** (96 mg, 0.134 mmol). After the solution was stirred for 10 min at 0 °C, a solution of 1 M $Bu_2BOTf$ in $CH_2Cl_2$ (0.14 mL) was added dropwise. After the mixture was stirred for a further 45 min, TLC analysis showed completion of the reaction. Triethylamine was added (55 μL); this was followed by addition of methanol until the evolution of $H_2$ gas ceased. The solvents were evaporated under reduced pressure and the residue was coevaporated with methanol (3×50 mL). The residue was purified by silica gel column chromatography (5% ethyl acetate gradient in hexanes) to afford acceptor **6** as an oil (83 mg, 85%). $R_f$=0.40 (ethyl acetate/toluene, 1:3); $[\alpha]_D^{25}$=−134.2° ($c$=0.64 in $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=7.64–7.16 (m, 25H, aromatic), 4.76–4.51 (6H; 3× C$H_2$Ph), 3.94 (dd, $J$=5.3, 5.7 Hz, 1H; H-4), 3.84–3.74 (m, 4H; H-6a, H-6b, H-2, H-1a, H-3), 3.6 (q, $J$=4.4 Hz, 1H; H-5), 3.56 (q, $J$=4.9 Hz, 1H; H-1b), 2.11 (t, 1H; OH), 1.06 ppm (s, 9H; *t*Bu); $^{13}$C NMR (75 MHz, $CD_3OD$): $\delta$=127.9–138.3 (Ph), 79.8 (C-5), 79.5 (C-4), 78.5 (C-3), 75.0, 74.9, 71.9 (3×*C*H$_2$Ph), 64.0 (C-1), 63.9 (C-2), 61.0 (C-6), 27.0 ppm (C(*C*H$_3$)); HRMS: $m/z$: calcd for $C_{43}H_{49}N_3O_5SiNa$: 736.3183; found: 736.3372.

**1-*O*-(*tert*-Butyldiphenylsilyl)-(3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-azido-2-deoxy-3,4,5-tri-*O*-benzyl-6-*O*-hydroxy-D-glucitol (14)**: A suspension of glycosyl donor **5a** (0.443 g, 0.767 mmol) and acceptor **6** (0.457 g, 0.639 mmol) in $CH_2Cl_2$ (20 mL) with 4 Å molecular sieves was stirred under an atmosphere of argon for 2 h. The mixture was cooled to −35 °C and this was followed by the addition of NIS (171 mg, 0.767 mmol) and TMSOTf (≈7 μL). The reaction mixture was stirred for 1 h during which the temperature was gradually raised to 0 °C. The reaction was quenched by addition of pyridine (0.15 mL). The reaction mixture was diluted with $CH_2Cl_2$ (35 mL) and the molecular sieves were removed by filtration through a pad of celite and washed with $CH_2Cl_2$ (3×50 mL). The combined filtrates was washed with 10% $Na_2S_2O_3$ (2×20 mL) and water (2×20 mL). The organic phase was dried ($MgSO_4$) and filtered, then the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (5% gradient of ethyl acetate in hexanes) to afford **14** as an oil (0.651 g, 90%). $R_f$=0.57 (40% ethyl acetate in hexanes); $[\alpha]_D^{23}$=−6.1° (c=2.7 in $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=7.73—6.97 (m, 34H; aromatic), 5.92 (dd, $J$=9.6, 9.3 Hz, 1H; H-3′), 5.53 (s, 1H; >CHPh), 5.51 (d, $J$=8.5 Hz, 1H; H-1′), 4.62–4.23 (m, 9H; 3×C$H_2$Ph, H-6a′, H-2′, H-6a), 3.81–3.60 (m, 9H; H-4′, H-5′, H-6b′, H-6b, H-1a, H-2, H-3, H-4, H-5), 3.50 (m, 1H; H-1b), 1.91 (s, 3H; COC$H_3$), 1.04 ppm (s, 9H; *t*Bu); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=170.4 (*C*OCH$_3$), 138.3–123.8 (Ph), 102.4 (*C*HPh), 82.7 (C-4), 72.4 (C-6), 70.3 (C-3), 67.9 (C-5), 62.8 (C-1), 61.8 ppm (C-2); HRMS: $m/z$: calcd for $C_{66}H_{68}N_{412}SiONa$: 1160.3412; found: 1160.7328.

**1-*O*-(*tert*-Butyldiphenylsilyl)-6-*O*-{4′,6′-*O*-benzylidene-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-azido-2-deoxy-**

**3,4,5-tri-*O*-benzyl-D-glucitol (16)**: Hydrazine hydrate (0.34 mL, 6.84 mmol) was added to a solution of **14** (0.389 g, 0.342 mmol) in ethanol (15 mL). After being stirred at 90 °C for 15 h, the reaction mixture was cooled and concentrated under reduced. The residue was coevaporated from toluene (3×25 mL) after which it was purified by silica gel column chromatography (35 % gradient of ethyl acetate in hexanes) to afford an amine (0.274 g, 0.283 mmol). DCC (0.05 g, 0.239 mmol) was added to a solution of **15** (0.163 g, 0.239 mmol) in $CH_2Cl_2$ (5 mL) and stirred for 10 min, followed by the addition of the amine described above (0.193 g, 0.20 mmol) in $CH_2Cl_2$ (10 mL). The reaction mixture was stirred for 16 h at room temperature. The solids were filtered off and the residue was washed with $CH_2Cl_2$ (2×10 mL). The combined filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (10 % gradient of ethyl acetate in hexanes) to afford **16** as a white solid (0.23 g, 71 %). $R_f$=0.57 (30 % ethyl acetate in hexanes); $[\alpha]_D^{23}$=+21.3° ($c$=0.60 in $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=7.64–7.14 (m, 30H; aromatic), 5.98 (d, 1H, $J$=5.8 Hz; NH), 5.53 (s, 1H; >C*H*Ph), 5.05 (m, 1H; H-3$_L$), 4.71 (d, $J$=8.3 Hz, 1H; H-1′), 4.25 (dd, $J$=5.8, 4.9 Hz, 1H; H-6a′), 4.17 (d, $J$=9.3 Hz, 1H; H-6a), 4.08 (t, $J$=9.3 Hz, 1H; H-3′), 3.92 (m, 1H; H-5), 3.79–3.67 (m, 6H; H-6a, H-1a, H-6b′, H-4, H-3, H-2), 3.56–3.50 (m, 3H; H-2′, H-4′, H-1b), 3.45 ppm (m, 1H; H-5); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=174.4 (NH*C*OCH$_3$), 171.5 (*C*OCH$_3$), 138.4–126.6 (Ph), 102.1 (*C*HPh), 101.5 (C-1′), 81.7 (C-4′), 79.8 (C-5), 79.5 (C-4), 78.4 (C-3), 75.1–72.4 (3×*C*H$_2$Ph), 71.5 (C-3′), 71.4 (C-3$_L$), (C-6), 70.4 (C-3), 68.8 (C-6′), 66.6 (C-5′), 63.9 (C-1, C-2), 59.4 ppm (C-2′); HRMS: $m/z$: calcd for $C_{100}H_{148}N_4O_{12}SiNa$: 1649.3401; found: 1649.3158.

**1-*O*-(*tert*-Butyldiphenylsilyl)-6-*O*-{4′,6′-*O*-benzylidene-3′-*O*-[(*R*)-3-benzyloxyhexadecanoyl]-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxyhexadecan]amido-3,4,5-tri-*O*-benzyl-D-glucitol (18)**: A solution of **16** (210 mg, 0.123 mmol) in 1,3-propanedithiol (0.25 mL, 2.46 mmol), pyridine (8.7 mL), and $H_2O$ (1.25 mL) was stirred for 16 h at room temperature. The reaction mixture was concentrated in vacuo and the residue was coevaporated with toluene (2× 5 mL) and ethanol (2×5 mL). The residue was purified by silica gel column chromatography (2 % gradient of methanol in $CH_2Cl_2$) to afford the free amine as a colorless syrup (189 mg, 95 %). DCC (54 mg, 0.261 mmol) and DMAP (20 mg, 0.163 mmol) were added to a stirred solution of **17** (89 mg, 0.244 mmol) in $CH_2Cl_2$ (10 mL). After stirring for 10 min, amine (131 mg, 0.081 mmol) in $CH_2Cl_2$ (1.5 mL) was added. The reaction mixture was stirred for 16 h at room temperature, after which the solids were filtered off and the residue was washed with $CH_2Cl_2$ (2× 10 mL). The combined filtrates were concentrated in vacuo and the residue was purified by silica gel column chromatography (3 % gradient of ethyl acetate in $CH_2Cl_2$) to afford **18** as a white solid (142 mg, 71 %). $R_f$= 0.43 (20 % ethyl acetate in hexanes); $[\alpha]_D^{23}$=+12.5° ($c$=0.8 in $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=7.64–7.14 (m, 35H; aromatic), 6.23 (d, $J$=8.3 Hz, 1H; NH), 5.78 (d, 1H; NH′), 5.39 (s, 1H; >C*H*Ph), 5.38 (t, $J$=9.3 Hz, 1H; H-3′), 4.98 (m, 1H; H-3$_L$), 4.74 (1H; H-1′), 4.73–4.31 (m, 10H; 5×*CH*$_2$Ph), 4.29–4.26 (m, 2H; H-6a′, 2), 4.13 (d, $J$=9.8 Hz, 1H; H-6a), 4.02 (d, $J$=7.8 Hz, 1H; H-3), 3.92–3.85 (m, 3H; H-5, H-6b, H-2′), 3.78 (m, 2H; H-4), 3.67–3.59 (m, 3H; H-6b′, H-4′, H-1b), 3.52 (dd, $J$= 9.8, 9.3 Hz, 1H; H-6a), 3.45 ppm (m, 1H; H-5); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=174.4, 172.8 (NH*C*OCH$_3$), 171.5, 170.2 (*C*OCH$_3$), 138.4–126.6 (Ph), 102.1 (*C*HPh), 101.6 (C-1′), 82.0 (C-4′), 79.7 (C-5), 79.3 (C-4), 78.4 (C-3), 76.2–72.3 (5×*C*H$_2$Ph), 71.8 (C-3′), 71.4 (C-3$_L$), 70.8 (C-6), 70.7 (C-3), 68.9 (C-6′), 66.5 (C-5′), 62.6 (C-1), 55.4 (C-2), 51.5 ppm (C-2′); HRMS: $m/z$: calcd for $C_{146}H_{222}N_2O_{16}SiNa$: 2312.1251; found: 2312.1257.

**6-*O*-{4′,6′-*O*-Benzylidene-3′-*O*-[(*R*)-3-benzyloxyhexadecanoyl]-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxyhexadecan]amido-3,4,5-tri-*O*-benzyl-D-glucitol (19)**: 1 M TBAF in THF (40 μL, 0.039 mmol) was added to a solution of **18** (76 mg, 0.033 mmol) in THF (2 mL). After the reaction mixture was stirred for 30 min, TLC analysis indicated completion of the reaction. The solvent was evaporated in vacuo and the oily residue was subjected to purification by silica gel column chromatography (10 % gradient of ethyl acetate in hexanes) to afford **19** as a fluffy white solid (62 mg, 91 %). $R_f$=0.38 (30 % ethyl acetate in hexanes); $[\alpha]_D^{25}$=+5.0° ($c$=0.7 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$=7.41–7.18 (m, 30H; aromatic), 6.38 (d, $J$=8.8 Hz, 1H; NH), 6.23 (d, $J$=7.32 Hz, 1H; NH′), 5.43 (s, 1H; >C*H*Ph), 5.26 (dd, $J$=9.3, 8.8 Hz, 1H; H-3′), 5.10 (m, 1H; H-3$_L$), 4.52 (m, 1H; H-1′), 4.77 (dd, $J$=9.8, 9.3 Hz, 1H; H-2), 4.83–4.35 (m, 10H; 5×*CH*$_2$Ph), 4.44 (m, 1H; H-5), 4.37–4.32 (m, 2H; H-6a, H-6a′), 4.16 (q, $J$= 7.8 Hz, 1H; H-2′), 4.03–3.99 (m, 2H; H-3′, H-2), 3.91–3.86 (m, 2H; H-5′, H-4′), 3.76 (m, 1H; H-6b), 3.68 (dd, $J$=9.1, 9.8 Hz, 1H; H-4), 3.58–3.55 (m, 2H; H-6b′, H-1a), 3.51–3.47 ppm (m, 2H; H-5, H-1b); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=174.2, 171.8 (NH*C*OCH$_3$), 171.3, 171.2 (*C*OCH$_3$), 139.5–126.4 (Ph), 102.3 (*C*HPh), 101.7 (C-1′), 82.7, 79.1 (C-5), 78.8 (C-4′), 77.9 (C-3), 76.8 (C-4), 75.7–70.8 (5×*C*H$_2$Ph), 71.4 (C-3$_L$), 71.1 (C-3′), 68.8 (C-6), 68.1 (C-6′), 66.8 (C-5′), 62.9, 54.6 (C-2′), 51.9 ppm (C-2); HRMS: $m/z$: calcd for $C_{130}H_{204}N_2O_{16}Na$: 2073.6311; found: 2073.6309.

**6-*O*-{4′,6′-*O*-Benzylidene-3′-*O*-[(*R*)-3-benzyloxyhexadecanoyl]-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxyhexadecan]amido-3,4,5-tri-*O*-benzyl-D-gluconic acid (20)**: Oxalyl chloride (12 μL, 0.136 mmol) was added to $CH_2Cl_2$ (1 mL) cooled to −40 °C under an atmosphere of argon. DMSO (12 μL, 0.168 mmol) was added and the mixture was stirred for 2 min at −40 °C. Compound **19** (36 mg, 17.47 μmol) in $CH_2Cl_2$ (1 mL) was added dropwise through a syringe. $Et_3N$ (50 μL, 0.288 mmol) was added after the reaction mixture had been stirred for 30 min at −40 °C. The reaction was allowed to warm to room temperature and stirred for 1 h. The reaction mixture was then diluted with $CH_2Cl_2$ (15 mL), washed with saturated aqueous $NH_4Cl$ solution, and then concentrated under reduced pressure. The residue was dissolved in THF (0.5 mL), then $NaClO_2$ (5 mg, 50 μmol), $NaH_2PO_4$ (3 mg, 2 μmol), and 2-methyl-2-butene (110 μL of 2 M solution in THF) in *t*BuOH (1 mL) and water (0.2 mL) were added. After stirring vigorously for 3 h, the reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (1 % MeOH gradient in $CH_2Cl_2$) to afford **20** (26 mg, 74 %). $R_f$=0.61 (10 % methanol in $CH_2Cl_2$); $[\alpha]_D^{23}$=−9.34° ($c$=0.6 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 7.41–7.18 (m, 30H, aromatic), 7.14 (d, $J$=8.8 Hz, 1H; NH), 6.36 (d, $J$=7.32 Hz, 1H; NH′), 5.46–5.42 (m, 2H; >C*H*Ph, H-1′), 5.26 (dd, $J$=9.3, 8.8 Hz, 1H; H-3′), 5.10 (m, 1H; H-3$_L$), 4.52 (m, 1H; H-1′), 4.77 (dd, $J$=9.8, 9.3 Hz, 1H; H-2), 4.83–4.35 (m, 10H; 5×*CH*$_2$Ph), 4.44 (m, 1H; H-5), 4.37–4.32 (m, 2H; H-6a, H-6a′), 4.16 (q, $J$=7.8 Hz, 1H; H-2′), 4.03–3.99 (m, 2H; H-3′, H-2), 3.91–3.86 (m, 2H; H-5′, H-4′), 3.76 (m, 1H; H-6b), 3.68 (dd, $J$=9.1, 9.8 Hz, 1H; H-4), 3.58–3.55 (m, 2H; H-6b′, H-1a), 3.51–3.47 ppm (m, 2H; H-5, H-1b); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=174.2, 171.8 (NH*C*OCH$_3$), 171.3, 171.2 (*C*OCH$_3$), 139.5–126.4 (Ph), 102.3 (*C*HPh), 101.7 (C-1′), 82.7, 79.1 (C-5), 78.8 (C-4′), 77.9 (C-3), 76.8 (C-4), 75.7–70.8 (5×*C*H$_2$Ph), 71.4 (C-3$_L$), 71.1 (C-3′), 68.8 (C-6), 68.1 (C-6′), 66.8 (C-5′), 62.9, 54.6 (C-2′), 51.9 ppm (C-2); HRMS: $m/z$: calcd for $C_{130}H_{202}N_2O_{17}Na$: 2087.5119; found: 2087.5149.

**Debenzylation of compound 20**: Pd/C (5 mg) was added to a solution of hydroxy acid **20** (15 mg, 7.26 μmol) dissolved in THF/*t*BuOH (2 mL, 1:1). The mixture was placed under an atmosphere of $H_2$ and stirred for 24 h at room temperature. The catalyst then was filtered off through a pad of celite and subsequently washed with THF (2×3 mL) and $CH_2Cl_2$ (2× 3 mL). The combined filtrates were concentrated under reduced pressure and the residue was purified by size-exclusion column chromatography with Sephadex LH-20 (*i*PrOH/ $CH_2Cl_2$ 1:1) to afford a mixture of **3** and **4** (5 mg, 67 %). $R_f$=0.41 (methanol/$CH_2Cl_2$/$NH_4OH$ 15:80:5); $^1$H NMR (500 MHz, [D$_8$]THF/MeOD 1:1): $\delta$=7.02 (d, 1H, $J$=8.8 Hz, NH), 6.84 (d, $J$=7.32 Hz, 1H; NH′), aminogluconate: 5.05 (t, $J$=10.4, 9.3 Hz, 1H; H-3′), 4.71 (d, $J$=3.6; H-2), 4.54 (d, $J$=8.3 Hz, 1H; H-1′), 4.32 (1H; H-3), 3.82 (1H; H-2′), 3.86 (1H; H-6a′), 3.73–3.70 (1H; H-6a, H-6b′), 3.55–3.53 (2H; H-4′, H-6b), 3.54 (1H; H-4), 3.35–3.32 (1H; H-5, H-5′), aminogluconolactone: 5.01 (t, $J$=10.3, 9.3 Hz, 1H; H-3′), 4.66 (d, $J$=8.3 Hz, 1H; H-1′), 4.25 (1H; H-2, H-5), 3.89 (1H; H-6a), 3.86 (1H; H-2′, H-6a′), 3.69 (1H; H-6b), 3.55–3.54 (1H; H-4, H-4′), 3.32 (1H; H-5); HRMS: aminogluconate: $m/z$: calcd for $C_{88}H_{168}N_2O_{17}Na$: 1548.2811; found: 1548.2816; aminogluconolactone: $m/z$: calcd for $C_{88}H_{166}N_2O_{16}Na$: 1530.2135; found: 1530.2786.

**Phenyl 2-azido-3,4-di-*O*-benzyl-6-*O*-(4′,6′-*O*-benzylidene-2′-deoxy-2′-phthalimido-β-D-glucopyranosyl)-2-deoxy-1-thio-β-D-glucopyranoside (21)**: A mixture of donor **5b** (0.624 g, 1.16 mmol) and acceptor **7** (0.463 g, 0.97 mmol) and activated molecular sieves (4 A, 1.2 g) in $CH_2Cl_2$ (15 mL) was stirred under an atmosphere of argon for 1 h. The mixture was cooled (−35 °C) and NIS (0.26 g, 1.16 mmol) and TfOH (≈10 μL) were added. The reaction mixture was allowed to warm to −10 °C. After the mixture had been stirring for 45 min, TLC analysis indicated completion of the reaction. The reaction was quenched with pyridine (0.2 mL) and

diluted with $CH_2Cl_2$ (50 mL). The molecular sieves were removed by filtration and washed with $CH_2Cl_2$ (3×10 mL). The combined filtrates were washed with 10% $Na_2S_2O_3$ (2×35 mL) and water (2×35 mL). The organic phase was dried ($MgSO_4$) and filtered, then the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (5% gradient of ethyl acetate in toluene) to afford **21** as a white fluffy solid (0.683 g, 80%). $R_f$=0.55 (35% ethyl acetate in hexanes); $[\alpha]_D^{23}$=+36.3° ($c$=2.5 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$=7.78–7.08 (m, 24H; aromatic), 5.59 (s, 1H; >CHPh), 5.41 (d, $J$=8.79 Hz, 1H; H-1′), 4.66 (t, $J$=7.82, 8.3 Hz, 1H; H-3′), 4.39 (dd, $J$=6.35, 1.96 Hz, 1H; H-6a′), 4.34 (dd, $J$=8.3, 10.26 Hz, 1H; H-2′), 4.27 (d, $J$=9.77 Hz, 1H; H-1), 4.78–4.28 (m, 4H; 2×C$H_2$Ph), 4.11 (dd, $J$=1.46, 9.28 Hz, 1H; H-6a), 3.85 (dd, $J$=10.25, 1.96 Hz, 1H; H-6b′), 3.71–3.65 (m, 3H; H-6b, H-4′, H-5′), 3.40–3.36 (m, 2H; H-3, H-5), 3.28 (dd, $J$=9.28, 9.76 Hz, 1H; H-4), 3.20 ppm (t, $J$=9.77 Hz; H-2); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=137.7–123.7 (Ph), 102.2 (*C*HPh), 98.9 (C-1′), 85.1 (C-1), 85.0 (C-5), 82.5 (C-4′), 78.4 (C-3), 77.2 (C-4), 76.0 (*C*$H_2$Ph), 75.0 (*C*$H_2$Ph), 68.9 (C-3′, C-6′), 68.2 (C-6), 66.4 (C-5′), 64.9 (C-2), 56.6 ppm (C-2′); HRMS: $m/z$: calcd for $C_{47}H_{44}N_4O_{10}SNa$: 879.2676; found: 873.2520.

**Phenyl 2-azido-3,4-di-*O*-benzyl-6-*O*-{4′,6′-*O*-benzylidene-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-deoxy-1-thio-β-D-glucopyranoside (22)**: Hydrazine hydrate (0.30 mL, 6.84 mmol) was added to a solution of **21** (0.273 g, 0.304 mmol) in ethanol (15 mL). The reaction mixture was stirred at 90°C for 12 h. After cooling to room temperature, the mixture was concentrated to dryness and the residue was coevaporated from toluene (2×25 mL). The residue was purified by silica gel column chromatography (35% gradient of ethyl acetate in hexanes) to afford the free amine (0.195 g, 88%). DCC (0.10 g, 0.484 mmol) was added to a solution of **15** (290 mg, 0.431 mmol) in $CH_2Cl_2$ (6 mL). After stirring for 10 min, the amine (195 mg, 0.267 mmol) in $CH_2Cl_2$ (4 mL) was added. The reaction mixture was stirred for 16 h, after which the solids were filtered off and the residue was washed with $CH_2Cl_2$ (2× 10 mL). The combined filtrates were concentrated in vacuo and the residue was purified by silica gel column chromatography (10% gradient of ethyl acetate in toluene) to afford **22** as a white solid (0.237 g, 65%). $R_f$=0.74 (25% ethyl acetate in toluene); $[\alpha]_D^{23}$=+10.65° ($c$=0.9 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$=7.52–7.26 (m, 15H; aromatic), 5.75 (d, $J$=6.3 Hz, 1H; NH), 5.55 (d, $J$=5.3 Hz, 1H; H-1), 5.50 (s, 1H; >C*H*Ph), 4.96 (m, 1H; H-3$_L$), 4.76 (dd, $J$=11.2 Hz, 2H; C$H_2$Ph), 4.66 (d, $J$=8.3 Hz, 1H; H-1′), 4.38–4.34 (m, 1H; H-5), 4.28 (dd, $J$=4.9, 10.3 Hz, 1H; H-6′a), 4.11–3.92 (m, 3H; H-3, H-3′, H-6a), 3.82–3.70 (m, 3H; H-2, H-6b, H-6b′), 3.53–3.39 ppm (m, 4H; H-2′, H-4, H-4′, H-5′); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=133.00–127.00 (Ph), 102.63 (>*C*HPh), 101.50 (C-1′), 87.83 (C-1), 82.00 (C-4′), 79.11 (C-4), 75.41 (*C*$H_2$Ph), 74.92 (C-3), 72.03 (C-3$_L$), 71.13 (C-5, C-3′), 69.24 (C-6a), 68.78 (C-6′), 67.08 (C-5′), 64.69 (C-2), 59.28 ppm (C-2′); HRMS: $m/z$: calcd for $C_{83}H_{126}N_4O_{11}SNa$: 1410.0321; found: 1410.0608.

**Phenyl 3,4-di-*O*-benzyl-6-*O*-{4′,6′-*O*-benzylidene-3′-*O*-[(*R*)-3-benzyloxyhexadecanoyl]-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxyhexadecan]amido-2-deoxy-1-thio-α-D-glucopyranoside (23)**: A solution of **22** (230 mg, 0.165 mmol) in 1,3-propanedithiol (0.35 mL, 3.32 mmol), pyridine (12 mL), and $H_2O$ (1.8 mL) was stirred for 16 h at room temperature. The mixture was concentrated in vacuo and the residue was coevaporated with toluene (2×5 mL) and ethanol (2× 5 mL). The residue was purified by silica gel column chromatography (2% gradient of methanol in $CH_2Cl_2$) to afford the free amine as a colorless syrup (193 mg, 86%). DCC (48 mg, 0.232 mmol) and DMAP (6 mg, 0.046 mmol) were added to a stirred solution of **17** (56 mg, 0.155 mmol) in $CH_2Cl_2$ (5 mL). After stirring for 10 min, the amine (53 mg, 0.039 mmol) in $CH_2Cl_2$ (1.5 mL) was added. The reaction mixture was stirred for 16 h at room temperature, after which the solids were filtered off and the residue was washed with $CH_2Cl_2$ (2×5 mL). The combined filtrates were concentrated in vacuo and the residue was purified by silica gel column chromatography (3% gradient of ethyl acetate in toluene) to afford **23** as a white fluffy solid (51 mg, 64%). $R_f$=0.68 (10% ethyl acetate in toluene); $[\alpha]_D^{23}$=−3.8° ($c$=0.52 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$=7.58–7.18 (m, 30H; aromatic), 6.62 (d, $J$=8.31 Hz, 1H; NH), 5.41 (d, 2H; >C*H*Ph, NH′), 5.25 (t, $J$=9.77 Hz, 1H; H-3′), 4.98 (m, 1H; H-3$_L$), 4.85 (d, $J$=8.79 Hz, 1H; H-1), 4.71 (m, 3H; H-1′, C$H_2$Ph), 4.65–4.36 (m, 6H; 3×C$H_2$Ph), 4.32 (dd, $J$=4.8, 5.8 Hz, 1H; H-6a′), 4.02 (d, $J$=11.23 Hz, 1H; H-6a), 3.85–3.68 (m, 5H; H-2′, H-2, H-6b, H-3, H-6a′), 3.63 (t, $J$=9.28 Hz, 1H; H-4′), 3.50 (dd, $J$=8.8, 7.9 Hz, 1H; H-5), 3.41 (m, 1H; H-5′), 3.33 ppm (t, $J$=8.8, 9.3 Hz, 1H; H-4); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=137.7–123.7 (Ph), 101.5 (*C*HPh), 101.4 (C-1′), 85.9 (C-1), 82.9, 79.7 (C-5), 79.1 (C-4′), 78.5 (C-4), 78.1 (C-3), 74.8–70.9 (4×C$H_2$Ph), 71.5 (C-3$_L$), 68.4 (C-6′), 68.2 (C-6), 66.0 (C-5′), 54.8 (C-2), 54.6 ppm (C-2′); HRMS: $m/z$: calcd for $C_{129}H_{200}N_2O_{15}SNa$: 2073.7321; found: 2073.7354.

**3,4-Di-*O*-benzyl-6-*O*-{4′,6′-*O*-benzylidene-3′-*O*-[(*R*)-3-benzyloxyhexadecanoyl]-2′-deoxy-2′-[(*R*)-3-octacosanoyloxyhexadecan]amido-β-D-glucopyranosyl}-2-[(*R*)-3-benzyloxyhexadecan]amido-2-deoxy-D-glucono-1,5-lactone (25)**: *N*-NIS (14 mg, 62 μmol) was added to a stirred solution of **23** (26 mg, 12.4 μmol) in THF/$H_2O$ (3 mL, 10:1) at room temperature. The reaction mixture was vigorously stirred for 16 h until TLC analysis indicated that the reaction was complete. The reaction mixture was diluted with $CH_2Cl_2$ (15 mL) and washed with aqueous $Na_2S_2O_3$ (15%, 5 mL) and water (3×5 mL). The organic phase was dried ($MgSO_4$) and filtered, then the filtrate was concentrated in vacuo. The residue was purified by silica gel chromatography (1% gradient of MeOH in $CH_2Cl_2$) to afford the lactol **24** (17 mg, 8.68 μmol). A suspension of lactol **24** (14.0 mg, 7.1 μmol) and activated molecular sieves (3 Å, 25 mg) in $CH_2Cl_2$ (2 mL) was stirred for 1 h at room temperature under an atmosphere of argon. PCC (8.1 mg, 37 μmol) was then added and the reaction mixture was stirred for another hour until TLC analysis indicated completion of the reaction. The reaction mixture was placed onto a column of Iatro beads and eluted with EtOAc/toluene (1:1) to afford lactone **25** as a colorless film (11.3 mg, 81%). $R_f$=0.66 (25% ethyl acetate in toluene); $[\alpha]_D^{25}$=−13.2° ($c$=0.3 in $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$=7.38–7.18 (m, 25H, aromatic), 7.02 (d, 1H, $J$=8.8 Hz, NH), 6.84 (d, $J$=7.32 Hz, 1H; NH′), 5.70 (dd, $J$=9.3, 8.8 Hz, 1H; H-3′), 5.39 (s, 1H; >C*H*Ph), 5.08 (m, 1H; H-3$_L$), 5.04 (d, $J$=8.3 Hz, 1H; H-1′), 4.77 (dd, $J$=9.8, 9.3 Hz, 1H; H-2), 4.73–4.37 (m, 8H; 4×C$H_2$Ph), 4.44 (m, 1H; H-5), 4.28 (m, 1H; H-6a′), 4.11 (d, $J$=10.7 Hz, 1H; H-6a), 3.95 (t, $J$=7.3 Hz, 1H; H-4), 3.71 (m, 2H; H-3, H-6b′), 3.57–3.52 (m, 3H; H-5′, H-6b, H-4′), 3.46 ppm (q, $J$=8.3, 9.3 Hz, 1H; H-2′); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=137.7–123.7 (Ph), 101.7 (*C*HPh), 101.0 (C-1′), 79.7 (C-5), 79.1 (C-4′, C-3), 78.0 (C-4), 74.1–70.9 (4×C$H_2$Ph), 70.5 (C-3$_L$), 70.3 (C-3′), 69.1 (C-6), 68.5 (C-6′), 66.0 (C-5′), 56.5 (C-2′), 52.9 ppm (C-2); HRMS: $m/z$: calcd for $C_{123}H_{194}N_2O_{16}Na$: 1979.8501; found: 1979.8321.

**Hydrogenation of compound 25**: Pd/C (5 mg) was added to lactone **25** (10 mg, 5.11 μmol) in THF/*t*BuOH (2 mL, 1:1). The reaction mixture was placed under an atmosphere of $H_2$ and stirred for 24 h at room temperature. The catalyst then was filtered off through a pad of celite and subsequently washed with THF (2×3 mL) and $CH_2Cl_2$ (2×3 mL). The filtrate was then concentrated. The residue was purified by size-exclusion column chromatography on a Sephadex LH-20 column (*i*PrOH/ $CH_2Cl_2$ 1:1) to afford a mixture of **3** and **4** (5 mg, 43%). $R_f$=0.41 (methanol/$CH_2Cl_2$/$NH_4OH$ 15:80:5); $^1$H NMR (500 MHz, [$D_8$]THF/MeOD 1:1): $\delta$=7.02 (d, $J$=8.8 Hz, 1H; NH), 6.84 (d, $J$=7.32 Hz, 1H; NH′), aminogluconate: 5.05 (t, $J$=10.4, 9.3 Hz, 1H; H-3′), 4.71 (d, $J$=3.6; H-2), 4.54 (d, $J$=8.3 Hz, 1H; H-1′), 4.32 (1H; H-3), 3.82 (1H; H-2′), 3.86 (1H; H-6a′), 3.73–3.70 (1H; H-6a, H-6b′), 3.55–3.53 (2H; H-4′, H-6b), 3.54 (1H; H-4), 3.35–3.32 (1H; H-5, H-5′), aminogluconolactone: 5.01 (t, $J$=10.3, 9.3 Hz, 1H; H-3′), 4.66 (d, $J$=8.3 Hz, 1H; H-1′), 4.25 (1H; H-2, H-5), 3.89 (1H; H-6a), 3.86 (1H; H-2′, H-6a′), 3.69 (1H; H-6b), 3.55–3.54 (1H; H-4, H-4′), 3.32 ppm (1H; H-5); HRMS: aminogluconate: $m/z$: calcd for $C_{88}H_{168}N_2O_{17}Na$: 1548.2811; found: 1548.2816; aminogluconolactone: $m/z$: calcd for $C_{88}H_{166}N_2O_{16}Na$: 1530.2135; found: 1530.2786.

**Reagents for biological experiments**: *E. coli* 055:B5 LPS was obtained from List Biologicals, phorbol 12-myristate 13-acetate (PMA) was from Sigma, and *R. sin-1* LPS was kindly provided by Dr. R. Carlson (CCRC, Athens, GA). All data presented in this study were generated by using the same batches of *E. coli* 055:B5 LPS and *R. sin-1* LPS. Synthetic compounds were stored lyophilized at −20°C 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 cells, provided by Dr. H. W. L. Ziegler-Heitbrock (Institute for Inhalation Biology, Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with 100 U mL$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin, 1% OPI supplement (containing oxaloacetate, pyruvate, and bovine insulin; Sigma), and 10% fetal calf serum (HyClone). The cells were maintained

in a humid 5% $CO_2$ atmosphere at 37°C. New batches of frozen cell stock were grown up every 2 months and growth morphology evaluated. Before each experiment, Mono Mac 6 cells were incubated with 10 ng mL$^{-1}$ calcitriol (Sigma) for 2 days to differentiate into macrophage-like cells.

**TNF-α ELISA**: Differentiated cells were harvested by centrifugation and gently suspended ($10^6$ cells mL$^{-1}$) in prewarmed (37°C) medium. Cells were then incubated with different combinations of stimuli for 6 h as described below. Cell supernatants were then collected and stored frozen (−80°C) until assayed for TNF-α protein. Concentrations of TNF-α protein in culture supernatants were determined in duplicate by a solid-phase sandwich ELISA. Briefly, 96-well 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 for 2 h at room temperature. Biotinylated mouse anti-human TNF-α antibody (Pharmingen) was then added, thereby 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 by using a microplate reader (Dynatech Laboratories). All TNF-α data are presented as the mean values (± the standard deviation) of duplicate cultures, with each experiment being repeated three times.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