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Progress in the Synthesis and Biological Evaluation of Lipid A and Its Derivatives

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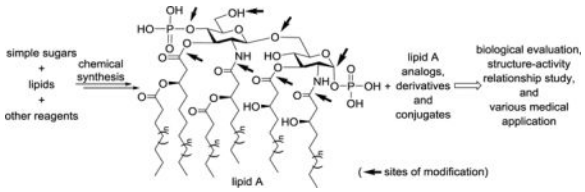
Abstract

Lipid A is one of the core structures of bacterial lipopolysaccharides (LPSs), and it is mainly responsible for the strong immunostimulatory activities of LPS through interactions with the toll-like receptors and other molecules in the human immune system. To obtain structurally homogeneous and well-defined lipid As and their derivatives in quantities meaningful for various biological studies and applications, their chemical synthesis has become a focal point. This review has provided a survey of significant progresses made in the synthesis of lipid A and its derivatives that carry diverse saturated and unsaturated lipids, have the phosphate group at its reducing end replaced with a more stable phosphate or carboxyl group, or lack the reducing end phosphate or both phosphate groups, as well as progresses in the synthesis of LPS analogs and other lipid A conjugates. These synthetic molecules have facilitated the elucidation of the structure-activity relationships of lipid A useful for the design and development of lipid A-based therapeutics, such as those utilized to treat sepsis, and other medical applications, such as the use of monophosphoryl lipid A as a carrier molecule for the study of fully synthetic self-adjuvanting conjugate vaccines. These topics are also briefly covered in the current review.

Abstract

This review covers the chemical synthesis of natural lipid As, their unnatural analogs and derivatives, and lipid A conjugates, as well as biological evaluations, structure-activity relationship studies and medical applications of the synthetic molecules.



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Keywords

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1. Introduction

Lipopolysaccharide (LPS) is one of the most abundant constituents of the outer membrane and one of the major virulent factors for Gram-negative bacteria.[1–3] Structurally, LPS is composed of three components, that is, a repetitive polysaccharide *O*-antigen, a core polysaccharide including an inner core and an outer core, and a glycolipid portion referred to as lipid A (Figure 1). Lipid A is the hydrophobic domain of LPS that anchors LPS to the bacterial cell membrane and is also the main LPS epitope that is recognized by the human innate immune system.[4–6] Consequently, lipid A is the most immunologically active component of LPS.

Immunologically, lipid A functions mainly through interacting with toll-like receptor 4 (TLR-4) and the myeloid differentiation factor 2 (MD-2) complex in association with toll or interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β (TRIF) and MyD88, to activate downstream signaling that stimulates cytokine and chemokine releases and upregulates immune cell expression.[6–9] Thus, on lipid A activation of TLR-4/MD2, there are two consecutive intracellular signaling cascades that mediate gene expression and proinflammatory cytokine secretion. One is dependent upon MyD88, which results in the up-regulation of cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein 1 (MCP-1), etc. The other is TRIF-dependent, which leads to the generation of interferon-β (IFN-β). Subsequently, IFN-β stimulates the signal transducer and activator of transcription 1(STAT-1) pathway to provoke the production of interferon-inducible protein-10 (IP-10) and nitric oxide.[10,11] However, lipid A is highly pro-inflammatory and highly septic to cause fatal complications, such as endotoxic shock, and thus, represents a severe clinical problem.[12–15]

Lipid As from different Gram-negative bacterial strains share a highly conserved construct, i.e., a β-D-glucosamine-(1→6)-α-D-glucosamine (GlcN) disaccharide with 1,4′-di-*O*-phosphorylation and 2,2′-*N*-/3,3′-*O*-acylation. As exemplified in Figure 2, the structures of lipid As isolated from different bacteria, and even from one bacterial strain, can vary in the number, length, saturation, and distribution of fatty acid chains and in the modification of the terminal phosphate moiety.[16–21] This diversity can have a significant impact on the bioactivity of lipid A in that it alters recognition by the TLR4/MD2 complex.[22–25] For example, the lipid A from *Escherichia coli*, which carries six lipid chains made of 12–14 carbons in length, exhibits the full spectrum of immunostimulant activities. Changing the number and length of the pendent fatty acid chains could decrease the magnitude of stimulated signals in human cells.[26–28]

However, extensive and detailed investigations of the structure-activity relationships (SARs) and the functional mechanisms of lipid As and LPSs at the molecular level are restricted by the difficulty to obtain structurally homogeneous lipid As and LPSs in quantities and in varied forms from biological sources. Moreover, lipid A and LPS of natural origins may be

contaminated with other components from the bacterial cell wall, which can further complicate the investigations. Therefore, chemical synthesis as an important tool to resolve the accessibility problem of lipid A and related derivatives has become an attractive topic.

In the literature, there are a number of excellent reviews about the structures, biosynthesis, and bioactivities of lipid As and LPSs derived from natural sources.[29–33] However, no review has been mainly focused on the chemical synthesis of lipid A and derivatives. The present article reviews the recent progress made in the synthesis of lipid A and its derivatives, as well as LPS analogs such as 3-deoxy-D-manno-octulosonic acid (Kdo)-lipid A conjugates. Their biological evaluations including SAR studies and their medical applications are also briefly discussed.

2. Chemical synthesis of lipid A and its derivatives

In 1984, Shiba *et al.* reported the first chemical synthesis of a lipid A derivative, i.e., lipid IV_A – a biosynthetic precursor of lipid A found in a temperature sensitive *E. coli* mutant.[34] Thereafter, from 1984 to early 1990's, several lipid As and their analogs have been chemically achieved.[35–39] Although the efficiency of these earlier syntheses was not remarkably high, these studies have unequivocally verified that lipid A is the primary endotoxin of LPS and established some general principles for lipid A synthesis, such as: (1) using the benzyl group for permanent hydroxyl group protection; (2) installing the phosphate groups at a late stage, e.g., just before the final deprotection; and (3) using *N*-Troc-protected glycosyl donors for the construction of the β-linked disaccharide backbone. In recent years, many more efficient syntheses have been developed, which facilitated various biological studies and SAR analysis of lipid A.[40–45]

Due to the presence of different acyl chains at various positions of the disaccharide backbone of lipid A, two general strategies have been adopted for lipid A and related derivative synthesis.[46] One is to construct a versatile disaccharide and then install individual lipid chains at the specific positions. In this case, orthogonal protection of various disaccharide positions was a prerequisite, especially when lipid chains in the synthetic target had an asymmetrical distribution.[41–44,47–49] The other strategy is to synthesize the lipidated monosaccharides first and then stitch them together by a glycosylation reaction, which can be challenging sometimes due to the increased steric hindrance of the glycosyl donors and acceptors.[37,40,45,50–54] Both strategies will be discussed in great detail with individual examples presented below.

2.1 Synthesis of natural lipid As bearing saturated lipids and both phosphate groups

In 2007, Boons group reported the total synthesis of two natural lipid As derived from *E. coli* (**1**) and *Salmonella typhimurium* (**10**), as well as their derivatives **11**–**13** having short lipid chains or only one phosphate group (Figure 3).[43] Innate immune response studies on *E. coli* LPS and the synthetic lipid As and derivatives **1**, **10**–**13** revealed the impact of various structural modifications on the potency of lipid A to induce the production of cytokines, such as TNF-α, IFN-β, IL-6, IP-10, IL-1β, RANTES, pro- IL-1β, and NF-κB. It was found that the EC_{50} values of these compounds to activate immune cells could differ by as much as 100 folds and no bias between MyD88- and TRIF-dependent immune responses were

noticed. For example, the potencies and the efficacies of these compounds to induce TNF-α and IFN-β secretion differed slightly; in contrast, they had great differences in the potency to elicit TNF-α and IL-1β production. In addition, *E. coli* LPS (EC_{50}: 0.004–1.74 nM) showed much higher activity for all the eight cytokines tested than the synthetic lipid A's **1** (EC_{50}: 21–674 nM) and **11** (EC_{50}: 3.6–43 nM), suggesting the biological role of the Kdo residues in *E. coli* LPS. Moreover, *S. typhimurium* lipid A **10** with seven acyl chains exhibited much lower potency than *E. coli* lipid A **1** with six acyl chains, whereas shortening of the lipid chains (see **11** and **12**) resulted in higher potencies. For *E. coli* lipid As **1** and **11**, the difference in their EC_{50} values was relatively small, whereas the difference of *S. typhimurium* lipid As **10** and **12** was approximately 3 orders of magnitude. These results indicated the biological importance of the number and length of the fatty acid chains.

The syntheses of **1** and **10**–**13** employed orthogonally protected disaccharide **16** as the key and common intermediate, which was obtained upon regio- and stereoselective glycosylation reaction of glycosyl acceptor **15** with *N*-Fmoc-protected glycosyl donor **14** in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) (Scheme 1). This reaction gave β-1,6-linked disaccharide **16** selectively as a result of the neighboring Fmoc group participation and the higher reactivity of the primary C-6 hydroxyl group as compared to that of the secondary C-3 hydroxyl group in **15**. It is worth noting that the 4′-*O*-phosphate group was introduced at an early stage of the synthesis, that is, in glycosyl donor **14**. The phosphate group was protected as a benzyl type of xylidene diester, which was stable to subsequent reactions but readily removable in the final deprotection step. In contrast, phosphoric acid dibenzyl ester, another commonly used strategy for phosphate protection, is less stable and can be sometimes partially deprotected.[29] Orthogonally protected disaccharide **16** was then utilized to prepare both **1** and **10**, as well as their derivatives.

In the synthesis of **10** (Scheme 1), **16** was first treated with 1,8-diazabicycloundec-7-ene (DBU) to remove the 2′-*N*-Fmoc group, which was followed by acylation of the resultant free amino group with (*R*)-3-dodecanoyl-tetradecanoic acid in the presence of 1,3-dicyclohexylcarbodiimide (DCC). Thereafter, the 3-*O*-position was acylated using (*R*)-3-benzyloxy-tetradecanoic acid with DCC and 4-dimethylaminopyridine (DMAP) as condensation reagents to provide **17**. The allyloxycarbonyl (Alloc) group in **17** was removed with tetrakis(triphenylphosphine)palladium [$Pd(PPh_3)_4$], which was followed by acylation with (*R*)-3-(*para*-methoxybenzyloxy)-tetradecanoic acid. Cleavage of the *p*-methoxybenzyl (PMB) ether in **18** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and then acylation of the exposed β-hydroxyl group on the lipid with myristoyl chloride gave **19**. The azido group in **19** was reduced with Zn and HOAc, and the resultant amino group was finally acylated with (*R*)-3-hexadecanoyl-tetradecanoic acid in the presence of DCC to produce **20**. A phosphate group was introduced to the anomeric position in two steps, including HF-pyridine mediated removal of the *tert*-butyldimethylsilyl (TBS) group in **20** and anomeric phosphorylation employing tetrabenzyl diphosphate in the presence of lithium bis(trimethyl)silylamide [$LiN(TMS)_2$]. Eventually, the resultant **21** was globally deprotected through Pd-black catalyzed hydrogenolysis to obtain the synthetic target **10**.

Compounds **11**–**13** were synthesized by a similar strategy with minor modifications, i.e., using orthogonal Alloc and azido groups to protect the 3′-*O*- and 2-*N*-positions (Scheme 2). Specifically, after stepwise acylation of **16** to get **22**, the azido group was reduced with Zn/HOAc, under which condition Alloc was unaffected. Acylation of the free amine with (*R*)-3-hexadecanoyl-tetradecanoic acid in the presence of DCC afforded **23**. Thereafter, the Alloc group was removed with $Pd(PPh_3)_4$ to generate **24**, which was converted into the target molecule **12** through a series of transformations including acylation, phosphorylation, and hydrogenolysis as mentioned above.

The above syntheses used functionalized and orthogonally protected disaccharide **16** carrying Alloc, Fmoc, TBS, and azido protecting groups as the key intermediate, which gave easy access to regioselective modifications of various positions in the disaccharide. The strategy should be useful for parallel synthesis of different lipid As and related analogs. In addition, the revealed SAR based on these structurally defined lipid A derivatives should be very helpful for further analysis and understanding of the immune modulation mechanisms of lipid A and LPS.

The natural lipid As derived from *Rubrivivax gelatinosus* (**26**) and *Chlamydia trachomatis* (**27** and **28**), as depicted in Figure 4, were synthesized by a strategy different from that for **1** and **10**–**13**.[45,54] In this case, lipidated monosaccharides were prepared and used as building blocks for glycosylation reactions to result in the disaccharide backbone of **26**–**28**.

As represented by the synthesis of **26** (Scheme 3), in the presence of TMSOTf, regioselective coupling of the *N*-Troc protected imidate donor **29** and the diol acceptor **30** afforded the desired disaccharide **31** in a 72% yield. It is worth noting that the hydroxyl groups in the 3-*O*-linked lipid chains of **29** and **30** were protected with *p*-trifluoromethylbenzyl group. An unsubstituted benzyl group at this position was prone to oxidation in air and gradual conversion into the corresponding benzoyl group, which was also observed by Kusumoto *et al.*[53] Cleavage of the 2′-*N*-Troc group in **31** with Zn/HOAc was followed by acylation of the amino group with (*R*)-3-dodecanoyl-decanoic acid utilizing 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (WSCD•HCl) as condensation reagents to give the fully lipidated intermediate **32**. Next, removal of the anomeric allyl (All) group in **32** with Ir-complex and phosphorylation of the freed anomeric hydroxyl group with tetrabenzyl pyrophosphate under the influence of $LiN(TMS)_2$ gave **33**. Finally, all of the benzyl type of protecting groups in **33** were removed by Pd-catalyzed hydrogenolysis to afford *R. gelatinosus* lipid A **26**.[45]

The above syntheses employed lipidated monosaccharides as building blocks to accomplish the disaccharide backbone. This strategy should provide an efficient approach to access lipid As and lipid A analogs bearing asymmetrically distributed lipid chains, and the products should be useful for precise and detailed analysis of the immunological activities and SARs of lipid A.

2.2 Synthesis of lipid As bearing unsaturated lipids

Qureshi *et al.* discovered a unique type of lipid A in *Rhodobacter sphaeroides*, which carried unsaturated lipid chains.[55,56] It was a mixture composed of three inseparable components,

and the major products were suggested to be **34a** and **34b**, named RsDPLA (Figure 5). Interestingly, this type of lipid A was a potent LPS antagonist, which could suppress chemokine and cytokine release induced by LPS, demonstrating the significant impact of the unsaturated lipid chain in lipid A on its biological activity.

For the chemical synthesis of lipid As carrying unsaturated lipids, the common strategy using the benzyl group for permanent hydroxyl group protection is unsuitable, as unsaturated lipids are incompatible with Pd-catalyzed hydrogenolysis employed to remove the benzyl protecting groups. Christ *et al.* established a synthetic strategy for these lipid As based on allylic protecting groups (Scheme 4).[37] In both the glycosyl donor **35** and acceptor **36**, Alloc and All groups were used to protect the hydroxyl and phosphate groups, respectively. Glycosylation of **36** with **35** under the promotion of AgOTf produced the desired β-disaccharide **37** and its anomer (β:α 2:1). Reduction of the azido group in **37** with tin(II) tris(benzenethiolate)-triethylamine complex and acylation of the resulting amino group with unsaturated lipids generated **38a** and **38b**. Thereafter, the anomeric TBS group was selectively removed with HF, which was followed by installation of the α-linked diallyl phosphate group to yield **39a** and **39b**. Finally, all allylic protecting groups were removed smoothly with $Pd(Ph_3P)_4$ to afford *cis*- and *trans*-lipid A **34a** and **34b**, respectively. Unfortunately, neither **34a** nor **34b** was a perfect match to the natural lipid A. Nevertheless, both **34a** and **34b** exhibited strong *in vitro* antagonistic activity to suppress TNF-α generation induced by LPS in human monocytes (average $IC_{50} \approx 1$ nM) and were devoid of LPS agonistic properties even at a 100 μM concentration. Moreover, the above synthetic method may be generally applicable to lipid As and related derivatives that contain unsaturated lipid chains.

Although **34a** and **34b** exhibited strong LPS antagonistic activity, the acyl groups at their *O*-3- or *O*-3′- or both positions can be easily hydrolyzed to result in agonistic byproducts. To deal with this issue, Christ *et al.* designed and prepared an artificial analog of *R. sphaeroides* lipid A, E5531 (**40**, Figure 6), which had the *O*-3- and *O*-3′-acyl groups replaced with hydrolytically stable ether linkages.[57] In addition, the C-6′-hydroxyl group was blocked as methyl ether to avoid potential contaminants generated from side reactions at this position. The synthetic E5531 was shown to possess the desired chemical properties including purity, stability, and solubility. Biological study of E5531 using human monocytes revealed that it strongly antagonized the release of a variety of cytokines induced by a number of Gram-negative bacterial LPSs. More importantly, E5531 was completely devoid of LPS agonistic activity, even at the concentration 10,000-fold higher than that required for the antagonistic activity. In addition, *in vivo* evaluation demonstrated that E5531 could protect BCG-primed mice from LPS-induced lethality and viable *E. coli* infection-caused death. Structurally stable E5531 has thus exhibited great therapeutic potential for the treatment of Gram-negative sepsis.[57–59]

E5564 (**41**, Figure 6), the second generation of synthetic analogs of RsDPLA, which carried an unsaturated lipid as well, was proved to be a highly active antagonist for endotoxin-mediated immune cell activation. Compared to E5531, structural variations of E5564 were mainly focused on 3,3′-*O*- and 2′-*N*-lipid chains as marked in Figure 6, and these alterations further improved the antagonist activity.[60] Currently, E5564 is utilized in clinic in

the trade name of *Eritoran* for the treatment of severe sepsis.[61,62] Due to the strong anti-sepsis activity and broad clinical prospects of E5564, many of its glucose-containing derivatives were synthesized, also based on the allylic protection strategy.[63–65]

2.3 Synthesis of unnatural lipid A derivatives bearing two phosphate groups

It was reported that the lipid chains of lipid A could affect its binding with polymyxin B and polymyxin B nonapeptide.[66–68] To study in detail the interactions between lipid A and polymyxin B or polymyxin B nonapeptide and obtain more accurate information about the SARs that would be difficult to obtain by using heterogeneous biological lipid As, Savage group[40] designed and synthesized lipid A derivatives **42**–**44** bearing incrementally shortened lipid chains (Figure 7). The shortening of lipid chains could also help improve the solubility in water.

Lipid A analogs **42**–**44** were obtained by a convergent strategy with lipidated monosaccharides as building blocks, as shown in the synthesis of **43** (Scheme 5). First, lipidated glycosyl acceptor **48** and partially lipidated glycosyl donor **51** were prepared from **45** and **49** through a series of transformations including regioselective protecting group manipulation, lipidation, phosphorylation, and so on. Glycosylation of **48** with **51** gave disaccharide **52**, of which the 2′-*N*-Troc group was replaced with another lipid chain to afford **53**. Finally, the TBS group in **53** was removed for the introduction of anomeric phosphate group, which was followed by global deprotection to give the target molecule **43**. This strategy can be useful for other similar lipid A analogs.

In the structure of natural lipid A, the reducing end phosphate group is directly linked to the anomeric hydroxyl group to form glycosyl phosphate, which is rather unstable. To overcome this problem and improve the stability of lipid A, Kusama group designed and synthesized three *E. coli* lipid A analogs **54**–**56** (Figure 8) as phosphonooxyethyl glycosides to replace the labile α-glycosyl phosphate.[69] It was anticipated that these lipid A analogs might have improved bioactivities, such as antitumor activity,[70–73] owing to their enhanced chemical stability.

In the synthesis of **54**–**56** (Scheme 6), the phosphonooxyethyl group was introduced at an early stage. Immediately after the conversion of **57** into **58** upon protection of the 4,6-*O*-positions with an isopropylidene group, the free primary hydroxyl group at the reducing end of **58** was selectively phosphorylated. Therefore, the phosphate group was introduced at the stage of monosaccharide. Acylation of the remaining free hydroxyl group in **58** and removal of the isopropylidene group afforded diol **59**. Glycosylation of **59** with **60** under the promotion of mercuric cyanide provided the desired β-1,6-disaccharide **61**, which was transformed into the synthetic target **54** following deprotection of the 2′-*N*- and 6′-*O*-Troc groups, regioselective acylation of the resulting amine, and two-step global deprotection.

According to the literature,[70–73] *E. coli* lipid A and its derivatives had antitumor activities, thus the cytotoxicity of natural lipid A **1** and its synthetic analogs **54**–**56** to methylcholanthrene-induced murine fibrosarcoma (Meth A fibrosarcoma) was studied *in vivo*.[69] Compounds were administered to the mice at a dose of 100 μg/mL through the tail veins three times (7, 12, and 17 days after tumor inoculation). The tumor weight ratio of

tested group/control group (T/C) was measured on the 21th day, and it was revealed that phosphonooxyethyl α-glycosides **54** and **55** had potent antitumor activities (T/C: 14% for **54**; 12% for **55**), which were similar to natural *E. coli* lipid A **1** (T/C 10%) However, the β-glycoside **56** (T/C 41%) was significantly less active. It was thus suggested that the glycosyl phosphate moiety may not be critical for the antitumor activity of lipid A and can be substituted with a more stable α-linked phosphonooxyethyl moiety, but the reducing end anomeric α-conformation was important. Moreover, replacing the (*R*)-3-hydroxytetradecanoyl groups with tetradecanoyl groups did not exhibit a major impact on its antitumor activity. Unfortunately, these phosphonooxyethyl analogs of lipid A were still quite toxic.

To further improve the antitumor activity of the above compounds and reduce their side effects, Kusama and coworkers synthesized **64** and **65** (Figure 9), which were derivatives of the less toxic biosynthetic precursor of *E. coli* lipid A **63**, by the same synthetic strategy for **54**.[74] The antitumor activities of compounds **1** and **63**–**65** were evaluated by the same protocol described for **54**–**56**. It was shown that **64** (T/C 32%) and **65** (T/C 24%) had comparable antitumor activity as that of the parent lipid A analog **63** (T/C 41%), but were slightly less active than the natural *E. coli* lipid A **1** (T/C 10%). However, like the precursor **63**, compounds **64** and **65** were weakly toxic. Particularly, **64** had no detectable toxicity at a dose (50 μg/kg) that showed significant antitumor activity. It was thus concluded that replacing the glycosidic phosphate with an α-linked phosphonooxyethyl moiety could decrease the toxicity of lipid A while maintaining its antitumor activity. Moreover, it was found that the presence or absence of a hydroxyl group on the fatty acid chains had very little influence on the antitumor activity.

Above investigations helped establish the first generation of potent antitumor lipid A analogs with enhanced stability and decreased toxicity. They should contain four linear fatty acid chains on the disaccharide backbone and an α-linked phosphonooxyethyloxyl group, instead of a phosphate, at the reducing end anomeric position. This structural scaffold should be useful for the design and development of novel lipid A-based antitumor agents.

Tritium-labeled lipid A derivatives, such as compounds **66** and **67** which had the tritium label on the phosphonooxyethyl group (Scheme 7),[75] were also prepared. These molecules with high chemical purity and specific radioactivity were utilized to analyze the interactions between lipid A and their receptors.[76] Several lipid A binding proteins were clearly detected on macrophages of both LPS-responder C3H/HeN and LPS-hyporesponder C3H/HeJ mice. For example, the p80 (a 80 kDa protein), p66, p65, p60, and p60′ proteins were found as specific lipid A-binding proteins, and no difference in the pattern of **66**-binding proteins was observed between C3H/HeN and C3H/HeJ macrophages. However, these proteins had different structural requirements for the binding with lipid A and LPS. The p80, p66, and p65 proteins preferentially bind to lipid A, while the p80 protein, but not p66 or p65, was also able to bind to LPS. The p60 protein preferentially bind to LPS. In these syntheses, tritium was regiospecifically introduced via reducing aldehyde **69** with ^{3}H-borohydride. Phosphorylation of **70** by the phosphoramidite method, followed by global deprotection through Pd-catalyzed hydrogenolysis, afforded the synthetic target **66**. Compound **67** was prepared by the same method.

2.4 Synthesis of lipid A analogs bearing carboxylic acid at the reducing end

Another direction to discover biologically functional lipid A analogs with improved stabilities is replacing the labile glycosyl phosphate with an anomeric carboxylic acid moiety, which should help improve stability but can still form a negative charge. In the study of GLA-60 (**72**, Figure 10),[77] a lipid A-related monosaccharide analog with LPS-agonistic activity, it was found that the carboxylic acid analog **73** had LPS-antagonistic property toward human monoblastic U937 cell.[78] Therefore, Mochizuki *et al.*[79,80] designed and synthesized a series of artificial carboxylic acid derivatives of lipid A, **74a-c** and **75a-c** (Figure 10), as potential LPS antagonists.

Biological evaluations of **74a-c**, which carried six fatty acid chains with the acyl substitution pattern similar to that of *E. coli* lipid A **1**, revealed that they induced the production of TNF-α in human monoblastic U937 cell, indicating LPS-agonistic activity. Clearly, replacing the phosphate group in *E. coli* lipid A **1** with a carboxyl group did not significantly affect its biological activity. This provided the feasibility to design more stable lipid A analogs, in which the unstably anomeric phosphate group is substituted. In contrast, **75a-c** containing four fatty acid chains inhibited TNF-α production induced by LPS in a dose-dependent manner (IC_{50}: 7, 10, and 9 nM respectively), indicating their LPS-antagonistic activity. Thus, the number of lipid chains in a lipid A analog is of great importance for its LPS-agonistic or antagonistic activity. Additionally, there is no significant difference among **75a-c** in their LPS-antagonistic activity. In the meantime, **74a-c** exhibited the similar level of LPS-agonistic activity. Consequently, the substituents on the 6′-*C*-position of these molecules appeared to be not very important for their biological activities.

In the synthesis of **74a-c** and **75a-c**, all of the glycosyl donors and donor precursors **76**–**78** were prepared from **79**[39] (Scheme 8). For example, **76** was readily obtained from **79** through a series of transformations including regioselective primary hydroxyl group protection, 4-*O*-phosphorylation, and Ir-complex mediated removal of the anomeric All group. On the other hand, **79** was converted into **80** after regioselective 6-*O*-silylation, 4-*O*-phosphorylation, and 6-*O*-desilylation. Compound **80** was then methylated or fluorinated to produce **81** and **82**, respectively. Similarly, Ir-complex mediated removal of the anomeric All group in **81** and **82** afforded **77** and **78**.

In the meantime, glycosyl acceptor **83** was synthesized from intermediate **84** upon protection of the 4,6-*O*-positions, 3-*O*-esterification, and removal of the 4,6-*O*-protection, whereas hemiacetals **76–78** were converted into glycosyl donors **86**–**88** on DBU and CCl_3CN treatments (Scheme 9). Regioselective glycosylation of the less hindered and more reactive 6-*O*-position of diol **83** with imidates **86**–**88** in the presence of TMSOTf at −50 °C gave disaccharides **89a**–**c** (Scheme 9). The 2′-*N*-Troc protection in **89a**–**c** was removed with Zn and HOAc, and the resultant free amino group was then *N*-acylated with EDCI•HCl and (*R*)-3-(dodecanoyloxy)tetradecaonic acid or acetic acid to afford fully protected intermediates **90a**–**c** and **91a**–**c** that were globally deprotected via catalytic hydrogenolysis in two steps to yield the synthetic targets **74a**–**c** and **75a**–**c**, respectively.

To obtain lipid A analogs with additionally increased structural stabilities, Shiozaki designed and synthesized another series of carboxylic acid derivatives **92a**–**c**, **93a**–**c**, and **94a**–**c** (Figure 11), which contained unnatural ether linkages at the 3,3′-*O*-positions.[81] These compounds were also evaluated for their bioactivities to inhibit or promote LPS-induced TNF-α production in human monoblastic U937 cells. Compounds **92a**–**c** and **94a**–**c**, which contained four lipid chains, inhibited TNF-α production with IC_{50} values of 0.86, 0.61, 1.2, 11, 6.4, and 10 nM, respectively, indicating their strong LPS antagonist activity. On the other hand, **93b** carrying six lipid chains was almost inactive, while **93a** and **93c** were moderate LPS agonists to promote TNF-α production. Therefore, the total number of lipid chains in the structure of lipid A analogs was again proved to be of great importance for their LPS-antagonistic or agonistic activity, whereas the C-6′ substitution pattern had only a small impact on their bioactivity. These observations were in accordance with previous findings.[79,80] Additionally, the 3-tetradecanoyloxytetradecyl or 3-dodecyloxytetradecyl group on the 3′-*O*-position was not critical for the inhibitory activity to human monoblastic U937 cell.

As outlined in Scheme 10, the synthesis of **94c** commenced with **95**, which had the amino group protected by a trifluoroacetyl group. First, **95** was converted into **98** via a series of conventional transformations including 3-*O*-alkylation, replacing the *N*-trifluoroacetyl group with a Troc group, regioselective 6-*O*-silylation, 4-*O*-phosphorylation, and selective 6-*O*-desilylation under acidic conditions. The 6-OH group in **98** was then substituted with a fluorine atom upon treatment with diethylaminosulfur trifluoride (DAST), which was followed by anomeric deallylation to generate hemiacetal **99**. Activation of **99** was achieved by reacting with DBU and CCl_3CN, and the resultant trichloroacetimidate was used to glycosylate **100**[82] with TMSOTf as the promoter. The reaction was regioselective for the more reactive primary hydroxyl group to afford disaccharide **101**, which was readily converted into the synthetic target **94c** after swapping the 2′-*N*-Troc group with an acetyl group and then global deprotection by a two-step hydrogenolysis procedure. The same synthetic method was also employed to achieve **92** and **93**.

Based on the findings that the carboxymethyl α-glycoside analog **103**[83] of GLA-60 and lipid A analog **92b** bearing an anomeric carboxylic group and 3,3′-*O*-ether linkages[81] exhibited potent LPS-antagonistic activities (IC_{50}: 5.0 and 0.61 nM, respectively), Shiozaki group designed and synthesized **104a-c** (Figure 12) that contained the structural components of both **103** and **92b**.[51] Biological evaluations showed that **104a**–**c** were LPS-antagonists, inhibiting TNF-α production by human monoblastic U937 cell with IC_{50} values of 6.5, 6.1, and 12.4 nM, respectively. On the other hand, previously studies revealed that the IC_{50} values of corresponding carboxylic acid analogs **94a**–**c** were 11.0, 6.4, and 10.0 nM, respectively.[81] Therefore, substituting the anomeric carboxylic acid group with a carboxymethyl group did not have a significant impact on their activity to inhibit TNF-α production. However, the LPS-antagonistic activities of **94a**–**c** and **104a**–**c** were much lower than that of **92b**, indicating that the ester linkage at the 3′-*O*-position played an important role in the antagonistic property.

The synthesis of **104a-c** (Scheme 11) started with the preparation of diphenylmethyl protected carboxymethyl glycoside **107** as a glycosyl acceptor. First, **105** was prepared and converted into **107** on dihydroxylation of its double bond with osmium tetroxide (OsO_4) and

4-methylmorpholine *N*-oxide (NMO), oxidative cleavage of the resultant vicinal diol with lead acetate, oxidation of the aldehyde with $NaClO_2$, esterification of the carboxylic acid with diphenyldiazomethane (Ph_2CN_2), and then removal of the acetonide group with 80% aq. HOAc. Glycosylation of **107** with glycosyl imidate donors **108a**-c in the presence of TMSOTf gave **109a-c**, respectively. Finally, swapping the 2′-*N*-Troc group with an acetyl group was followed by global deprotection in two steps of Pd- and Pt-catalyzed hydrogenolysis generated the synthetic targets **104a-c**.

Fukase group synthetized and investigated a series of monosaccharide analogs **111a**,**b**–**116a**,**b** (Figure 13) of lipid A, which contained aspartic acid or phosphoserine residue as replacement of the non-reducing end phosphorylated glucosamine residue.[84] These compounds were employed to elucidate the structural and conformational requirements of lipid A derivatives for their endotoxic and antagonistic activities. Biological evaluations revealed that most of the analogs did not induce IL-6 production, but **115a** did exhibit a concentration-dependent induction at concentrations over 100 ng/mL. Compound **115b** also showed some agonistic activity, but it was about 100-fold weaker than that of **115a**. In addition, the phosphoserine-containing derivatives exhibited stronger antagonistic activity than corresponding aspartic acid-containing analogs with the same acylation pattern. As for the aspartic acid analogs, the L-form showed a stronger inhibition than the corresponding D-form, whereas D- and L-phosphoserine derivatives did not exhibit obvious differences. Furthermore, **112a**,**b** exhibited the antagonistic activity, whereas **115a,b** showed the immunostimulating activity. Thus, the endotoxic and antagonistic activities were switched by changing the phosphoryl group and the carboxylic group.

2.5 Synthesis of monophosphoryl lipid A and related derivatives

Although lipid A possesses strong immunostimulatory activities, its clinical applications have been limited as it can cause fatal septic shock.[13] Fortunately, it was discovered that the toxicity of lipid A could be significantly reduced after its anomeric phosphate group was removed to generate 4′-*O*-monophosphorylated lipid A, usually known as monophosphoryl lipid A (MPLA), whereas its beneficial immunostimulatory activities were retained.[2] For example, the MPLA derivative of *Salmonnella minnesota* lipid A was at least 99.92% less toxic than the parent compound.[85,86] In fact, a MPLA preparation (MPL™) obtained by selective hydrolysis of the anomeric phosphate group of *S. minnesota* R595 lipid A has been approved for clinical use as a vaccine adjuvant.[87] However, MPLAs derived from bacterial sources are heterogeneous and thus lack consistency in structure, property, and biological performance. To overcome this problem and to find new MPLA adjuvants with improved immunostimulatory properties, various MPLA derivatives with defined structures have been designed, synthesized, and immunologically evaluated.[50,52,87–92]

As mentioned above, *S. minnesota* R595 lipid A **117** (Figure 14) is a potent immunostimulant and endotoxin. Fortunately, its endotoxic activity could be alleviated after selective removal of its 1-phosphono and 3-*O*-(*R*)-3-hydroxytetradecanoyl groups through acidic hydrolysis.[86,93] The resultant MPLA was shown to enhance both antibody responses and T cell-mediated immunity, and is an effective adjuvant for prophylactic and therapeutic vaccines.[73] However, because of the inherent heterogeneity of cognate LPS and the

incomplete and nonspecific reactions of hydrolysis, the resultant MPLA is highly heterogeneous, containing differently acylated products in addition to the desired hexaacyl components such as **118** (Figure 14).

To explore the SARs of MPLA and discover novel MPLA analogs with favorable properties, Jiang *et al.* prepared MPLAs **119**–**121** bearing varied 3-*O*-substitution and tested their adjuvant activity.[50] As shown in Scheme 12, the synthesis of **119**–**121** employed *N*-Troc protected glycosyl donor **122** and lipidated glycosyl acceptor **123** as building blocks for constructing the lipid A disaccharide backbone due to the asymmetric distribution of lipid chains in the synthetic targets. Additionally, the anomeric position of the acceptor was blocked with a benzyl ether group, which could be easily deprotected at the final stage without affecting other functionalities. Glycosylation of **123** with imidate **122** in the presence of BF_3•OEt_2 generated disaccharide **124** in an 88% yield. Removal of the 3-*O*-All group in **124**, acylation of the resulting hydroxyl group, and subsequent cleavage of the 2′-*N*-Troc group were followed by *N*-acetylation to afford **125**. Treatment of **125** with $NaBH_3CN$ and HCl•Et_2O exposed regioselectively the 4′-*O*-hydroxyl group, which was then phosphorylated by the phosphoramidite method to generate **126**. Finally, **126** was deprotected by Pd/C catalyzed hydrogenolysis to produce the target MPLA **121**.

The immune adjuvant activities of the synthetic MPLAs **119**–**121** and the natural *S. minnesota* R595 MPLA were assessed using a synthetic BLP25 liposomal vaccine system. All three synthetic MPLAs **119**–**121** exhibited strong adjuvant activities, similar to that of natural *S. minnesota* R595 MPLA, including promoting antigen specific T cell-mediated immune responses and interferon-γ (IFN-γ) production. Compound **121** bearing a 3-*O*-(*R*)-3-hydroxytetradecanoyl group was proved to be the most active, whereas **120** containing a propyl group had only slightly lower activity. Thus, the substituent at the 3-*O*-position of the reducing-end sugar unit had only a small impact on the adjuvant activity of these MPLA analogs. However, it is worth noting that the preliminary lethal toxicity investigation showed that **121** containing seven lipid chains was not much more toxic than MPLA derived from natural R595 lipid A, of which the main constituent is 3-*O*-deacylated MPLA **118** carrying six lipid chains. This result was in contrast to the previous finding that cleavage of the 3-*O*-acyl group could further reduce the toxicity of MPLA.[93] Consequently, the synthetic lipid As with well-defined structures were helpful for gaining insights into precise SARs.

In another study, **127a-f** (Scheme 13), analogs of **118** with the side chain lipids replaced with different fatty acids containing 4 to 14 carbon atoms, were designed and synthesized for systematic analysis of the biological functions of fatty acids.[52] In their synthesis, Koenigs-Knorr method was used for glycosylation of **129a-f** with **128a-f** as glycosyl donors and AgOTf as promoter to afford disaccharides **130a-f** in 70–80% yields. Then, the Troc group was removed with Zn and HOAc, which was followed by regioselective 2′-*N*-acylation with **131a-f** and subsequent deprotection in two steps to yield the synthetic targets **127a-f**.

Biological assays of **127a-f** revealed that the fatty acid chain length was a critical determinant of iNOS gene expression in activated mouse macrophages and proinflammatory cytokine (TNF-α and IL-1β) induction in human peripheral monocytes. For example, the

ED_{50} of **127c-f** to induce iNOS were 0.3, 0.05, 1, and 5 ng/mL, whereas the ED_{50} of **127a**,**b** was more than 10 mg/mL, and the potencies of **127a-f** to induce proinflammatory cytokines reached the maximum when $n = 8$ (**127d**). Thus, in contrast to the relatively small biological significance of the 3-*O*-substituent in MPLAs **119**–**121** carrying three large side chain lipids, the length of these side chains in 3-*O*-deacylated *S. minnesota* R595 MPLA **118** and related analogs **127a-f** was of great importance for their immunostimulatory activities.[52]

Corsaro *et al* developed a semisynthetic method to prepare novel lipid A derivatives **133**–**140** (Figure 15).[94] Based on the lipid A **2** purified from the fed-batch fermentation of *E. coli* K4, a series of selective chemical reactions including functionalization of the C-6′ hydroxyl group, modification of the lipid pattern, and methylation of the phosphate group gave novel lipid A derivatives **133–140**. This facile methodology represented an alternative to lengthy total synthesis or extraction from bacterial sources. Preliminary *in vitro* immunological test showed that some of them, especially **136**, were the promising, novel immunoadjuvant candidates. For example, **136** (20 μg/mL) exhibited a stronger potency in inducing TNF-α than that of the clinical *S. minnesota* R595 MPL with the TNF-α concentration were about 700 pg/mL and 40 pg/mL, respectively.

Since MPLA has strong immunostimulatory and adjuvant activities but is almost nontoxic, it can be a useful molecular carrier for the development of fully synthetic vaccines that may also have self-adjuvanting properties. In this context, Guo and co-workers designed and synthesized a series of MPLA derivatives carrying functionalities to facilitate their further elaboration, such as coupling with antigens or other biomolecules, and applied them to biological studies. For example, they prepared a unique monophosphoryl derivative **146** of *Neisseria meningitidis* lipid A, which carried an amino group at the reducing end.[95] This amino group could be selectively derivatized for coupling **146** or its deprotected form with other molecules. In the synthesis of **146** (Scheme 14), monosaccharide **142** with a 2-azidoethyl group attached to its reducing-end was used as a glycosyl acceptor, and the azido group could be selectively reduced later on to form the desirable free amino group. Glycosylation of **142** with **141** in the presence of NIS and AgOTf afforded the β-linked disaccharide backbone **143**. Thereafter, the phthalyl and acetyl groups in **143** were simultaneously removed with hydrazine to pave the way for regioselective acylation of the amino groups and then acylation of the remaining 3-OH and 3′-OH groups to obtain **144**. Regioselective opening of the benzylidene ring in **144** using $NaBH_3CN$ and $HCl{\bullet}Et_2O$ was followed by phosphorylation of the resulting free hydroxyl group to give fully protected MPLA derivative **145**. Selective reduction of its azido group was achieved with Zn and HOAc to yield **146**, which was eventually coupled with tumor-associated carbohydrate antigens (TACAs) for the development of fully synthetic carbohydrate-based cancer vaccines.

The above synthetic strategy was also used to prepare other *N. meningitidis* MPLA derivatives **147**–**150** (Figure 16) carrying symmetric 2-*N*/3-*O*- and 2′-*N*/3′-*O*-lipids of varied chain length and structure. These MPLA derivatives were employed to systematically study the SARs of MPLA, such as how the lipid chain length and structure may affect the immunostimulatory and adjuvant activity of MPLA, to discover optimized vaccine carriers and adjuvants.[96]

For the synthesis of 2-*N*/3-*O*- and 2′-*N*/3′-*O*-asymmetrically acylated MPLA derivatives, such as that of *E. coli* lipid A **156**, partially and fully lipidated monosaccharides **151** and **152** were used as building blocks (Scheme 15).[46] The 2-*N*-position in **151** was protected with a Troc group to minimize steric hindrance of the donor and to assistant β-glycosylation through neighboring group participation as well. Moreover, the Troc group could be readily removed later on to facilitate the introduction of the desired lipid moiety. In the presence of NIS and AgOTf, glycosylation of **152** with **151** afforded the desired β-anomer **153** in a 75% yield. Subsequently, the benzylidene group in **153** was regioselectively opened with Et_3SiH and BF_3•Et_2O to expose the 4′-OH group, which was phosphorylated upon reaction with dibenzyl phosphoramidite in the presence of 1*H*-tetrozole and then in situ oxidation with *t*-BuO_2H to generate **154**. The azide group in **154** was reduced with 1,3-dithiopropane, which was followed by the introduction of an alkyne group via reaction with 4-pentynoic acid under the influence of EDC. Finally, treatment of **155** with Zn/AcOH to remove the 2′-*N*-Troc group and reaction of the resulting amine with (*R*)-3-dodecanoyloxy-tetradecanoic acid in the presence of HATU and DIPEA afforded the target MPLA analog **156**, which was eventually coupled with TACA via a click reaction for biological studies.

In contrast, the lipid A unit of the *Porphyromonas gingivalis* LPS possessed antagonist activity of human TLR4.[97,98] The absence of ester-linked phosphate at the 4′-*O* position and the presence of unusual branched fatty acids such as *R*-(3)-hydroxy-13-methyltetradecanic acid and *R*-(3)-hydroxy-15-methyltetradecanic acid make *P. gingivalis* lipid As unique. To explore their accurate SAR and explore MPLA derivatives with promising antagonistic activity, Boons and Ogawa groups synthesized *P. gingivalis* lipid As **157**–**158** and their tetra-acylated analogs **159**–**160** (Figure 17).[99,100] Biological evaluation showed that both **159** and **160** were able to antagonize TNF-α production induced by *E. coli* LPS in human monocytic cells and that **159** was a significantly more potent antagonist than **160** (IC_{50} for **159** and **160** were 160 nM and 3.2 μM, respectively). Thus, the acylation pattern of **159** was of great importance for optimal activity.

2.6 Synthesis of non-phosphorylated lipid As and related derivatives

Vandenplas *et al.* isolated a LPS from the symbiotic and nitrogen-fixing bacterial species *Rhizobium sin*-1, which could significantly inhibit the TNF-α release by human monocytes.[101,102] The *R. sin*-1 LPS contained structurally unusual lipid As **161** and **162** (Figure 18), which are different from other lipid As in that they are devoid of both phosphate moieties and the reducing end glucosamine is replaced with a 2-aminogluconate residue. Interestingly, under mildly acidic conditions, 2-aminogluconate in **161** and **162** can easily convert into 2-aminogluconolactone (→ **163** and **164**, Figure 18) with elimination of the fatty acid chain at the 3-*O*-position. In addition, the *R. sin*-1 lipid As contain a long lipid chain, 27-hydroxyoctacosanoic acid, which in turn can be esterified by β-hydroxybutyrate.

To explore the intriguing structure and bioactivity of *R. sin*-1 lipid A, Boons and co-workers carried out extensive studies to establish synthetic strategies for these molecules and their analogs and investigate the SAR.[41,42,44,49,103] For example, they employed an advanced intermediate **169** derived from selective glycosylation of **168** with **167** to obtain *R. sin*-1 lipid A derivatives **165** and **166** (Scheme 16).[41] Compound **169** was useful to access various

lipid A analogs, as its anomeric center, 2- and 2′-amino groups, and 3- and 3′-hydroxyl groups were orthogonally protected and could be regioselectively functionalized. Thus, treatment of **169** with ethylenediamine in refluxing *n*-butanol to deprotect the phthalimido and acetyl ester groups and regioselective 2′-*N*-acylation with (*R*)-3-octacosanoyloxy-hexadecanoic acid using DCC generated **170**. Reduction of the azido group in **170** with 1,3-dithiopropane and then synchronous acylation of the free amino group and two hydroxyl groups of the resultant intermediate with (*R*)-3-benzyloxy-hexadecanoic acid, DCC, and DMAP afforded fully protected **171**. Finally, NIS/TfOH-mediated hydrolysis of the thioglycoside in **171** and removal of the benzyl ethers and benzylidene acetal via Pd/C-catalyzed hydrogenolysis gave the target lipid A analog **165**. En route to **166**, PCC-mediated oxidation of the anomeric position after NIS/TfOH-mediated anomeric hydrolysis of **171** produced lactone **172**, whereas oxidation reactions under the Moffatt (DMSO/Ac_2O), Swern (DMSO/oxallyl chloride) or NMO/TPAP conditions led mainly to 1,2-oxazoline and elimination byproducts. Pd/C-catalyzed hydrogenolysis of **172** finally generated **166**.

Compounds **165** and **166** were structurally similar, but their reducing end anomeric carbon atoms had different oxidation states. Biological studies showed that, in contrast to *E. coli* LPS, both **165** and **166** lacked proinflammatory activities as indicated by their absence of abilities to induce TNF-α secretion or TNF-α mRNA expression. In addition, **166** was able to antagonize enteric *E. coli* LPS, but **165** was not, indicating that the gluconolactone moiety in *R. sin*-1 lipid A was important for its antagonistic property.

Based on the above SAR findings, Boons and co-workers synthesized two other putative *R. sin*-1 lipid A analogs **173** and **174** (Figure 19), which were devoid of 3-*O*-acylation, to further examine the biological effect of the 3-*O*-acyl group.[42] It was shown that **173** and **174** not only lacked the proinflammatory activity but also antagonized *E. coli* LPS. However, its antagonistic activity was significantly less potent than that of **166**.[41] Thus, the β-hydroxyl fatty acyl group at the 3-*O*-position was important for the antagonistic activity of *R. sin*-1 lipid A. Moreover, *R. sin-1* LPS, which consists of two Kdo units, an *O*-polysaccharide and a core oligosaccharide, exhibited 60 times stronger activity than that of synthetic *R. sin*-1 lipid A analog **166**, indicating that the Kdo units contributed to the antagonistic effects of *R. sin-1* LPS as well.

The synthesis of **173** (Scheme 17) was commenced with the reduction of the hemiacetal group in **175** with $NaBH_4$, which was followed by $Ba(OH)_2$-mediated hydrolysis of the acetamido group and swap of the amine for azide to give **176**. Subsequently, the primary hydroxyl group in **176** was regioselectively silylated, and the remaining hydroxyl groups were benzylated to afford **177**. Since the synthetic target **173** did not contain the 3-*O*-acyl group, this position was protected with a benzyl group. Regioselective ring-opening of the benzylidene group in **177** using BH_3•THF and Bu_2BOTf converted it to glycosyl acceptor **178**. Glycosylation of **178** with **179** in the presence of NIS and TMSOTf gave β-linked disaccharide **180** stereoselectively as a result of the neighboring phthalimido group participation. Compound **180** was then consecutively deprotected and acylated. The phthalimido and 3′-*O*-acetyl groups were removed upon treatment with hydrazine hydrate in *n*-BuOH, which was followed by selective 2′-*N*-acylation. Thereafter, the azido group was

reduced, and the resulting amine and the free 3′-OH group were concomitantly acylated in the presence of DCC and DMAP to yield **181**. Compound **181** was transformed into carboxylic acid **182** through TBAF treatment to remove the TBDPS group and then a two-step oxidation procedure including Swern oxidation and *in situ* oxidation of the resulting aldehyde with $NaClO_2/NaH_2PO_4$. Finally, global deprotection of **182** via Pd/C-catalyzed hydrogenolysis gave the target lipid A **173**.

Compound **174** was synthesized by a similar strategy (Scheme 18). After selenoglycoside **184** was selectively activated with NIS/TMSOTf for the glycosylation of **183**, the resultant **185** was consecutively deprotected and acylated at the 2′-*N*- and 2-*N*-/3′-*O*-positions, as discussed above, to afford **186**. It is worth noting that NIS/TfOH-mediated hydrolysis of thioglycoside **186** afforded the synthetic target together with a 1,2-oxazoline byproduct, while the NIS/aq. THF system in the absence of TfOH gave a high yield of the target compound. In addition, NMR and MS analyses revealed that **173** and **174** were in equilibrium even under neutral conditions.

The relationship between the unusual long lipid chain, i.e., the 27-hydroxyoctacosanoic group, at the 2′-*N*-position of *R. sin*-1 lipid A and its antagonistic property was also studied by comparing the natural product **189** and its derivatives **166** and **188** bearing different 2′-*N*-lipid chains (Figure 20).[44] All of these compounds, as well as the natural *R. sin-1* lipid A and LPS, were evaluated for their ability to antagonize TNF-α secretion by monocytic cells in the presence of *E. coli* LPS (10 ng/mL). Compounds **189** and **166** exhibited the similar and strong inhibition on TNF-α production with IC_{50} values of 9.1 and 7.5 μM, respectively. However, **188** only showed very weak inhibitory activity. Thus, while the hydroxyl group on the 27-hydroxyoctacosanoic residue did not account for its antagonistic properties, the length of the lipid chain played an important role in the activity. In addition, the *R. sin-1* LPS was found to possess the strongest antagonized potency (IC_{50} = 6.5 nM), indicating that the KDO moiety of *R. sin-1* LPS was also important for the biological activity, which was in accordance with the previous report.[42]

Synthesis of **188** was commenced with the glycosylation of the β-isomer of **168** with **184** (Scheme 19). At a low temperature, NIS and TfOH could selectively activate selenoglycoside **184** in the presence of thioglycoside acceptor, and the reaction gave a higher yield (84%) of disaccharide **190** when compared to that with $AgOTf/K_2CO_3$ as the promoters.[104] Treatment of **190** with ethylenediamine to remove the acetyl and phthalimido groups was followed by regioselective acylation of the 2′-*N*-position with (*R*)-3-tetradecanoyloxy-hexadecanoic acid under the influence of DCC. Thereafter, the azido moiety was reduced to free amine, which was acylated together with the 3-OH and 3′-OH groups to afford **191**. NIS/TfOH-promoted hydrolysis of the thiophenyl moiety in **191** generated **193**, and the reaction also formed a byproduct, that is, 1,2-oxazoline **192**, which could be converted into **193** after treatment with dibenzylphosphate. Finally, the anomeric hydroxyl group in **193** was oxidized with PCC, which was followed by global deprotection via Pd/C-catalyzed hydrogenolysis to yield the synthetic target **188**.

In the synthesis of **166** and **189** (Scheme 20), α-thioglycoside **194**, instead of its β-isomer **190**, was used as a key intermediate. By the similar deprotection and acylation procedures as

shown in Scheme 19, **194** was readily converted into **195**. One of the side chain hydroxyl groups of its lipids was selectively deprotected and acylated to give **196**, which was subjected to NIS/TfOH-promoted hydrolysis of the thiophenyl moiety, oxidation of the resultant hemiacetal with PCC, and global deprotection via Pd/C-catalyzed hydrogenolysis to get the synthetic target **189**. It was worth noting that hydrolysis of α-thioglycoside did not give the 1,2-oxazoline byproduct, possibly because the C-2 amide could not directly displace the anomeric leaving group, resulting in effective trapping of the generated oxa-carbenium ion with water to give the desired lactol.

In summary, the above synthetic strategies provided easy access to different *R. sin-1* lipid A derivatives with well-defined structures, which are useful for understanding their SARs and action mechanisms at the molecular level. Importantly, the antagonistic properties of *R. sin-1* lipid A derivatives and *R. sin-1* LPS make them leading compounds for the design and development of therapeutic antagonists for endotoxin-related diseases.[37,57]

2.7 Synthesis of miscellaneous lipid A analogs

To obtain structurally simplified lipid A mimics, peptides were used to replace the disaccharide moiety of lipid A. For example, mono- and bis-*O*-phosphorylated N^{δ}-L-homoserinyl-D-ornithinol pseudodipeptides **197** and **198** with *N*-acylation were designed and synthesized to mimic *E. coli* lipid A OM-174[105] (Scheme 21).[106] Compounds **197** and **198** were demonstrated to have partial immunostimulatory or immunomodulating activities of OM-174. Importantly, they exhibited very low endotoxicity and pyrogenicity.

3. Synthesis of LPS and Kdo-lipid A derivatives

Lipid A is the artificial product produced by acidic hydrolysis of LPS, thus it is not exposed freely on the living bacterial cell surface. As depicted in Figure 1, the 6′-*O*-position of lipid A is covalently linked to a Kdo residue, which is in turn linked to the outer core oligosaccharide and *O*-antigen. Kdo is important for the LPS molecule as it not only serves as the linkage between lipid A and *O*-antigen but also plays a critical role in the bioactivity of LPS.[41,42,107,108] As mentioned above,[43] *E. coli* LPS that contains two Kdo units exhibited more potent cytokine-inducing activity than *E. coli* lipid A and its analogs. Furthermore, *Meningococcal* lipid A expressed by a bacterial strain that is defect of Kdo biosynthesis possessed considerably reduced activity compared to Kdo bearing *Meningococcal* lipooligosaccharide.[109] However, these findings are still difficult to be more vigorously verified due to the difficulty to purify LPSs from bacteria, which results in heterogeneous and even contaminated products that may affect the observed immunostimulatory activity. Therefore, totally chemical synthesis of homogeneous and structurally well-defined LPS or Kdo-containing lipid A derivatives would be of great value for elucidating their precise SARs and for other biological studies.

3.1 Total synthesis of Re LPS

Re LPS **199** (Figure 21) was originally isolated from an *E. coli* mutant.[110] Consisting of lipid A and two Kdo residues, it is known as the simplest LPS identified so far. In 2001,

Kusumoto *et al.* reported the first total synthesis of Re LPS **199** in 23 steps and a 0.9% overall yield.[53]

As shown in Scheme 22, regioselective glycosylation of **201** with imidate **200** in the presence of polymer-supported Nafion-TMS[111] afforded disaccharide **202** in a 73% yield. Acylation of the remaining free hydroxyl group in **202** was followed by successive cleavage of the 2′-*N*-Troc group, 2′-*N*-acylation, and removal of the benzylidene group with TFA in DCM-H_2O to give tetraacylated **203**. It is worth noting that the side chain hydroxyl groups on the lipids at the 2,3-*N,O*-positions in **203** were protected with the *p*-trifluoromethylbenzyl group, which was found to be resistant to oxidation but readily removable by hydrogenolysis.[112]

Model reactions showed that TBS-protected Kdo fluoride **205** and triethylsilyl (TES)-protected glycosyl acceptors are the best coupling partners to provide Kdo glycosides in high yields and stereoselectivity. Consequently, **203** was converted to 6′-*O*-TES ether **204**, which was glycosylated with TBS-protected Kdo donor **205** under the promotion of BF_3•Et_2O to generate an 89% yield of the desired α-2,6-linked disaccharide **206** with high regio- and stereoselectivity. Phosphorylation of the 4′-hydroxyl group in **206** was followed by desilylation and regioselective triethylsilylation to give trisaccharide **207**, which was ready for introducing the second Kdo unit. Glycosylation of **207** was achieved with the less bulky isopropylidene-protected Kdo fluoride donor α-**208** to afford tetrasaccharide **209** in a 75% yield. It is worth noting that glycosylation of **207** with **205** resulted in a low yield (26%) of the desired product, probably because of the steric hindrance. Removal of the isopropylidene group in **209** with TFA/DCM-H_2O was followed by Ir-complex mediated cleavage of the anomeric All group to afford the hemiacetal intermediate **210**. Finally, regioselective phosphorylation of the anomeric hydroxyl group, catalytic hydrogenolysis to remove all of the benzyl-type of protecting groups, and purification of the product using liquid-liquid partition chromatography on a Sephadex LH-20 column produced the target molecule **199**. Consecutive cytokine-inducing tests showed that natural Re LPS was slightly more potent than synthetic **199**. It was speculated that natural Re LPS contained some potent contaminants, most probably molecules bearing longer saccharide residues.

It was the first chemical synthesis of a LPS molecule. This work established the strategy for α-glycosylation using Kdo fluorides bearing bulky protecting groups, such as isopropylidene and TBS groups, at the 4,5-*O*-positions, which prevented the undesired β-side attack. Nevertheless, stepwise installation of Kdo units was not especially efficient, which restricted parallel preparation of various LPS analogs and further investigation of their SAR.

3.2 Synthesis of *H. pylori* Kdo-lipid A conjugates

Chemical synthesis of Kdo-lipid A conjugates bearing one Kdo unit, especially those related to *Helicobacter pylori* LPS, was also accomplished.[107,113–117] Compared to enterobacterial LPS, *H. pylori* LPS has significantly lower endotoxicity.[118,119] In addition, the lipid A component of *H. pylori* LPS is greatly different from the enterobacterial lipid A in that: (1) it

has fewer and longer fatty acid chains; (2) it is devoid of the 4′-*O*-phosphate group; and (3) its 1-*O*-phosphate group is occasionally modified with an ethanolamine group.[120,121]

In 2007, Fukase *et al.* described the synthesis of *H. pylori* lipid A **211** and its Kdo conjugate **212** (Figure 22).[107] As shown in Scheme 23, acylated **214** was glycosylated with *N*-Troc-protected imidate **213** at −78 °C, under the promotion of TMSOTf, to give disaccharide **215** in an 80% yield. The 2′-*N*-Troc group in **215** was removed upon treatment with Zn-Cu/HOAc, which was followed by acylation of the resulting amine group to generate **216**. Thereafter, the 6′-*O*-Alloc group was swapped for a TES group after sequential removal of the Alloc group with $Pd(PPh_3)_4$ and then reaction with TESCl in the presence of imidazole to produce **217**.

Consecutively, **217** was glycosylated with **205**[53] using BF_3•Et_2O as the promoter to afford **219** in an 85% yield. Compound **205** was obtained from hemiketal **218** in a high yield on fluorination with *N,N*-diethyl-α,α-difluoro-(*m*-methylbenzyl)amine (DFMBA) at a low temperature,[122] under which condition the formation of glycal byproduct was suppressed. For the glycosylation reaction between **217** and **205**, the MS 5Å was proved to be more effective than MS 4Å, probably because the calcium ions in MS 5Å could trap fluoride anions generated from the reaction, thus promoting the glycosylation. Subsequently, the TBS and All groups in **219** were selectively removed with HF and Ir-complex, respectively, which was followed by phosphorylation of the anomeric hydroxyl group with tetrabenzyl pyrophosphate and, finally, debenzylation via catalytic hydrogenolysis to produce the target Kdo-lipid A conjugate **212**.

The immunostimulatory activity of **211** and **212** was tested by measuring their influence on IL-6 secretion from human whole blood cells (HWBC).[123] It was demonstrated that neither **211** nor **212** induced IL-6 secretion; instead, both competitively inhibited IL-6 secretion induced by *E. coli* LPS, verifying that **211** and **212** were TLR-4 antagonists. Moreover, **212** showed a 10-fold increase in inhibitory activity than **211**, demonstrating the biological significance of the Kdo unit. In combination with the previous finding that the ethanolamine-containing *H. pylori* lipid A **220** (Figure 23) showed weak immunostimulatory activity,[112,124] it was concluded that the number of anionic charges were of importance for the bioactivity of lipid A.

Fukase *et al.* also synthesized *H. pylori* lipid A-Kdo **221** (Figure 23) bearing an aminoethanol phosphate through a divergent strategy.[114] As shown in Scheme 24, the 4′,6′-*O*-benzylidene group in **222** was regioselectively opened with Me_2NH•BH_3 and BF_3•Et_2O to obtain **223**. Previous study showed that the acid-labile TBS or isopropylidene group in the Kdo donor could be occasionally cleaved when a large excess of Lewis acid was employed to activate Kdo fluoride donors, which decreased the glycosylation reaction stereoselectivity.[53] Based on this finding, they probed a new synthetic method using Kdo *N*-phenyltrifluoroacetimidate **224** as a donor for the coupling reaction with disaccharide **223**.[113,115] Although in α-promoting solvent cyclopentyl methyl ether (CPME) the reaction under the promotion of TBSOTf afforded the desired trisaccharide **225** in a good yield (70%) and stereoselectivity, a large excess of donor **224** (5 equiv.) had to be utilized because the reaction produced a significant amount of glycal byproduct **226**.

The low efficiency of the above glycosylation reaction might be ascribed to the difficulties in temperature control since imidate **224** was highly reactive and the reaction produced substantial heat. To address the issue, a microfluidic glycosylation protocol was developed,[125] in which the CPME solution of donor **224** (1.5 equiv.) and acceptor **223** (1 equiv.) was mixed with the TBSOTf (1 equiv.) solution in a microreactor at a flow rate of 0.5 mL/min (Scheme 25). Then, the desired trisaccharide **225** was formed in a 72% yield, with the glycal byproduct suppressed. In addition, the protecting groups were intact when relatively high concentration of Lewis acid was used. This represented some significant improvement over the previous glycosylation methods.[53] After the trisaccharide backbone **225** was obtained, its 2′-*N*-Troc group was removed with Zn-Cu/HOAc, followed by acylation of the resultant amino group with acyloxycarboxylic acid in the presence of 2-methyl-6-nitrobenzoic anhydride (MNBA) and *N,N*-diisopropylethylamine (DIPEA). Thereafter, the 2-*N*-position was deprotected with $Pd(PPh_3)_4$ and acylated with benzyloxycarboxylic acid in the presence of HATU and Et_3N to produce **227**. Its isopropylidene group was then swapped with a benzylidene group, as the isopropylidene group was relatively unstable under the condition of 1-*O*-phosphorylation. Ir-complex mediated removal of 1-*O*-All group in **228** and phosphorylation of the exposed anomeric hydroxyl group using phosphoramidate were followed by catalytic hydrogenolysis to afford the target molecule **221**.

The immunomodulatory activities of **211**, **212**, **220** and **221** were evaluated via the analysis of their impacts on cytokine production, using *E. coli* strain O111:B4 LPS as a positive control. The results showed that **220** exhibited weak stimulating effects on IL-1β, IL-6, and IL-8 release. On the other hand, **211**, **212**, and **221** possessed inhibitory activities toward cytokine induction by *E. coli* LPS. Thus, elongation of **220** with a Kdo unit reversed its bioactivity, complementing previous findings that the Kdo residue enhanced the activities of other lipid A derivatives.[108] More detailed SARs of these compounds were summarized in a review.[116]

In summary, to address the challenge of efficient and stereoselective glycosylation with Kdo in the synthesis of LPS or Kdo-lipid A conjugates, glycosylation strategies using Kdo fluoride and imidate donors were exploited in combination with microfluidic reaction conditions. Studies on the synthetic compounds contributed to understanding the SARs of LPS or Kdo-lipid A conjugates at molecular levels. Nevertheless, Kdo installation to lipid A remains a great synthetic challenge, especially in the synthesis of complex LPS analogs containing more Kdo units and *O*-antigens. As a result, more efficient glycosylation and synthetic strategies for LPS are in high demand.

In recent years, great advancements have been made towards understanding the biosynthetic processes of LPSs,[30,126] such as the characterization of various glycosyltransferases, polymerases, and other enzymes involved in and controlling *O*-antigen and LPS assembly.[127–129] These studies have significantly enhanced the potential of using enzymatic procedures to achieve some key steps of LPS synthesis *in vitro*. It is therefore anticipated that chemoenzymatic synthesis of structurally defined LPSs and their analogs will be an attractive and promising research topic.

4. Application of MPLA to vaccine development

TACAs are useful targets for the development of therapeutic cancer vaccines, but TACAs are typically poorly immunogenic and T cell independent antigens. To make them more immunogenic and T cell dependent antigens, they have to be covalently linked to immunologically active carrier molecules to form conjugate vaccine. In recent years, fully synthetic TACA-based conjugate cancer vaccines have become a hot topic, because they possess a number of advantages, such as defined structures, reproducible physical, chemical and biological properties, and promising immunologic activity.[130–133] For the development of fully synthetic cancer vaccine, MPLA can be a particularly useful carrier molecule. First, MPLA is an extremely strong immunostimulator. Second, owing to its bacterial origin, MPLA can function as a “danger signal” to the immune system to enhance the immune responses to TACAs covalently linked to it.[134] Third, as mentioned, the endotoxicity of lipid A could be dramatically reduced after removal of its anomeric phosphate group, whereas its beneficial immunostimulatory activity was unaffected.[2] In reality, MPLA has been approved for clinical use as a vaccine adjuvant and immunostimulator.[87]

To probe the potential of MPLA as a carrier molecule for the development of conjugate cancer vaccines, Guo *et al.* designed and synthesized a monophosphoryl derivative **146** (Scheme 14) of *N. meningitidis* lipid A, which had lipid chains symmetrically distributed on the two sugar units. In **146**, the reducing end phosphate of lipid A was replaced with an aminoalkyl group, to which TACAs could be linked.[95] For example, as depicted in Scheme 26, after acylation of the amino group in **146** with succinic anhydride and then esterification, **146** was converted into active ester **229** to enable the coupling of MPLA with a modified form of GM3, NPhAcGM3 **230**. Finally, the resultant conjugate **231** was deprotected to afford conjugate vaccine **232**.

Immunological studies of the MPLA–NPhAcGM3 conjugate **232** in mice revealed that it alone, namely without the use of an external adjuvant, elicited robust IgG antibody responses. The results have demonstrated that MPLA could act not only as a carrier molecule to significantly improve the immunogenicity of TACAs but also as a built-in adjuvant to form self-adjuvanting vaccines.[135,136] Therefore, MPLA was identified as a highly promising carrier for the design and development of fully synthetic glycoconjugate cancer vaccines.

Guo and coworkers have also developed a convergent synthetic strategy, as shown in Scheme 15, for a monophosphorylated *E. coli* lipid A derivative **156**, which carried lipids asymmetrically on the two sugar units.[46] Compound **156** was smoothly coupled with an NPhAcGM3 derivative **233** that carried an azido group through click reaction in the presence of CuI/DIPEA to create the desired MPLA-GM3NPhAc conjugate **234** (Scheme 27).

To optimize MPLA as a vaccine carrier, Guo and coworkers prepared several *N. meningitidis* MPLA derivatives **147**–**150** (Figure 16), which contained different lipids, and studied their SAR in the form of NPhAcsTn conjugates **240**–**243**.[96] Following the above mentioned synthetic strategy for **229**, **147**–**150** were converted into active esters **235**–**238**,

which were coupled with NPhAcsTn **239** (Scheme 28). The resultant conjugates were deprotected via Pd/C-catalyzed hydrogenolysis to afford the desired MPLA-NPhAcsTn conjugates **240**–**243**.

Immunological studies of **240**–**243** revealed that all of them provoked robust antigen-specific immune responses in mice without the use of an external adjuvant. Moreover, they stimulated high titers of IgG3 and IgG1 antibodies, indicating T cell dependent immune responses.[137,138] Despite that **240**–**243** elicited similar patterns of immune responses, they did show significant difference in the immunological activity. For example, **241** provoked much higher and consistent titers of both total and IgG3 antibodies than **240**, **242**, and **243**. Structurally, **241** contained the monophosphoryl form of natural *N. meningitidis* (H44/76 strain) lipid A.[139] In **240**, the two free hydroxyl groups on the lipid chains were removed; **242** and **243** were different from **241** in the lipid chain length and number. Thus, the lipid chain length and number and the free hydroxyl groups on the lipid chains in MPLA exhibited a significant impact on its immunological property.

After an optimized MPLA as vaccine carrier was established, it was employed to couple with globo H, a TACA expressed by several tumors, to formulate MPLA and globo H-based anticancer vaccines.[140] As shown in Scheme 29, coupling of the active ester **236** of MPLA with a globo H derivative **244**[141] followed by Pd/C-catalyzed hydrogenolysis generated the target MPLA-globo H conjugate **245**. It was evaluated in mouse and compared with the keyhole limpet hemocyanin (KLH) conjugate of globo H **246**, the positive control, which is at Phase III clinical trial for the treatment of colon cancer. It was discovered that MPLA-globo H conjugate **245** elicited much faster and stronger immune responses than that of the KLH conjugate **246**, in terms of both total and IgG antibodies. It was demonstrated that both **245** and **246** induced the similar patterns of immune responses. These results suggested the great promise of conjugate **245** as a therapeutic cancer vaccine.

Recently, MPLA was also employed as a carrier molecule in the development of fully synthetic antibacterial glycoconjugate vaccines.[142] For this purpose, a series of α-2,9-linked oligosialic acids, which were short analogs of α-2,9-polysialic acid – a unique and important capsular polysaccharide antigen of serotype C *N. meningitidis*, were synthesized[143] and coupled with MPLA by the same strategy described above. Immunological studies of the resultant conjugates **247**–**251** (Figure 24) in mouse provided detailed SARs about the antigens and the carrier molecule. It was revealed that all of the conjugates alone elicited robust T cell-dependent immunities, suggesting their self-adjuvanting properties. It was also shown that their antisera had strong and specific binding to α-2,9-polysialic acid and meningococcal cells, indicating the ability of the elicited antibodies to selectively target the bacteria. It was eventually demonstrated that the antisera could mediate strong bactericidal activities. It was thus concluded that the MPLA conjugates of α-2,9-oligosialic acids, especially tri- and tetrasialic acids, are promising self-adjuvanting anti-group C meningitis vaccines worthy further investigation.

Introduction of unique functional groups, such as an azido or alkyne group, to the reducing end of MPLA can provide the flexibility for its regioselective coupling with other molecules, such as carbohydrates, peptides, and proteins to quickly generate complex multicomponent

conjugates in a convergent manner. This should be very helpful for the broad applications of MPLA. For example, current results have proved that MPLA can be a useful carrier molecule in the development of fully synthetic self-adjuvanting anticancer and antibacterial glycoconjugate vaccines.

5. Conclusion

Since the first chemical synthesis of a lipid A derivative reported by Shiba *et al.* in 1984, great progresses have been made in the synthesis and biological studies of lipid A and related molecules. As a result, a series of convergent and efficient strategies are currently available for the preparation of both natural lipid As and their artificial analogs, such as various lipid A mimics and derivatives that contain different lipids, truncated carbohydrate chains and no or only one phosphate group or have one or both of the two phosphate groups replaced with more stable functionalities (Table 1). Chemical syntheses of simple LPS analogs were also accomplished. These synthetic studies have facilitated access to structurally defined lipid As and their rationally designed derivatives or analogs, thus enabling thorough and systematic examination of the structures, functions, and SARs of lipid As at molecular levels. This has led to not only in-depth understanding of the functional mechanisms of lipid A and LPS but also the discovery of new, lipid A-based therapeutics. For example, several lipid A analogs have found clinical applications as endotoxin antagonists for the treatment of severe sepsis, and recently MPLA has been approved for human use as a vaccine adjuvant. MPLA has also been extensively inspected as a promising carrier molecule for the development of fully synthetic self-adjuvanting glycoconjugate vaccines.

It has been demonstrated that the Kdo residues and the *O*-antigen in LPS can have a significant influence on the activities of lipid A. To gain more insights into the mechanisms of these structural motifs to affect the functions of lipid A, it is necessary to have access to structurally defined LPSs and LPS derivatives. However, this remains a significant challenge despite the great achievements discussed above. The sheer complex structure of LPSs makes their chemical assembly particularly difficult. In addition, the efficiency of some glycosylation reactions, such as that of Kdo, involved in LPS synthesis is still far from satisfactory. Therefore, an important future direction in this research area would be development of new efficient strategies and glycosylation methods for the assembly of LPSs. In this regard, chemoenzymatic synthesis that combines enzyme-catalyzed glycosylation with chemical synthesis should be particularly attractive, as enzymatic glycosylation reactions can be especially efficient and selective. In addition, various oligosaccharide transferases, polymerases, and transglycosidases are also powerful tools that can be employed for rapid and highly convergent assembly of complex polysaccharides.

Owing to the extensive biological activities of lipid A, its derivatives can have broad medical applications. As mentioned above, some therapies have already been developed based on lipid A and utilized in clinic. Therefore, another anticipated important direction in this research area would be to develop new lipid A-based therapeutics.[144] However, although in general a series of efficient synthetic strategies have been established for lipid As, their chemical synthesis is still challenging because of their complex structure. This is at least the

partial reason for why the MPLA adjuvant currently used in clinic is obtained through controlled hydrolysis of LPSs derived from biological sources, which can cause a number of problems. Consequently, it is preferable that the new lipid A-based therapeutics should have simplified structures, increased stabilities, enhanced beneficial activities, and improved toxicological profiles. In this regard, the SAR analysis results of synthetic lipid As and related structures should be very useful, as they can be employed to guide the design of novel molecular entities with desirable properties.

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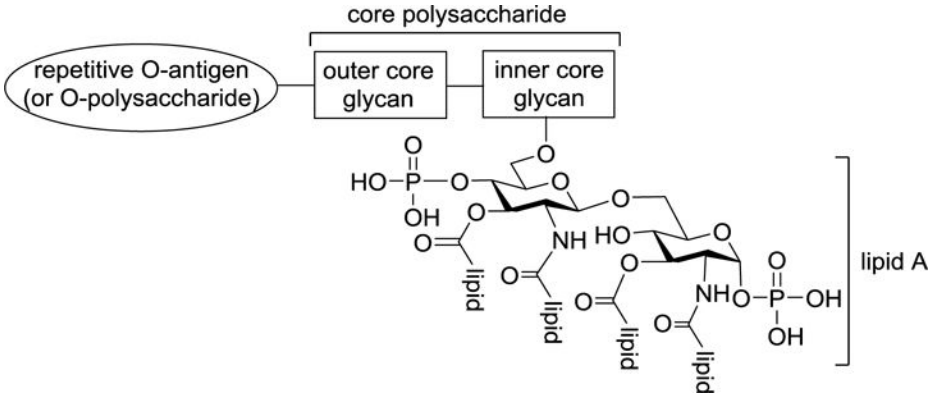


Figure 1.
Schematic representation of the general structure of LPSs of Gram-negative bacteria.

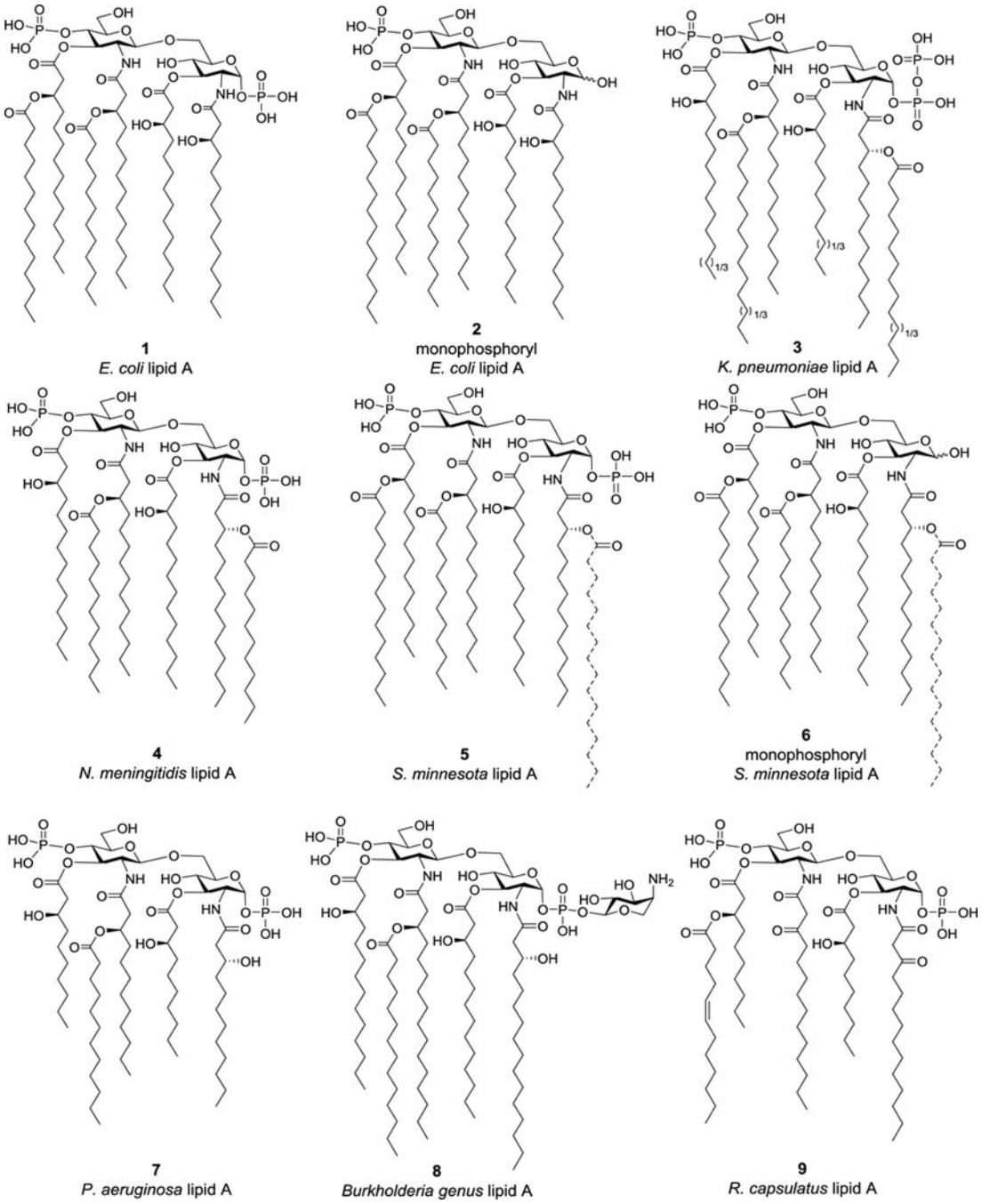


Figure 2.

The structure of selected lipid As derived from different Gram-negative bacteria. Dashed lines represent partial structures that result from incomplete biosynthesis, and the numbers in parentheses indicate the potential variation of chain lengths.

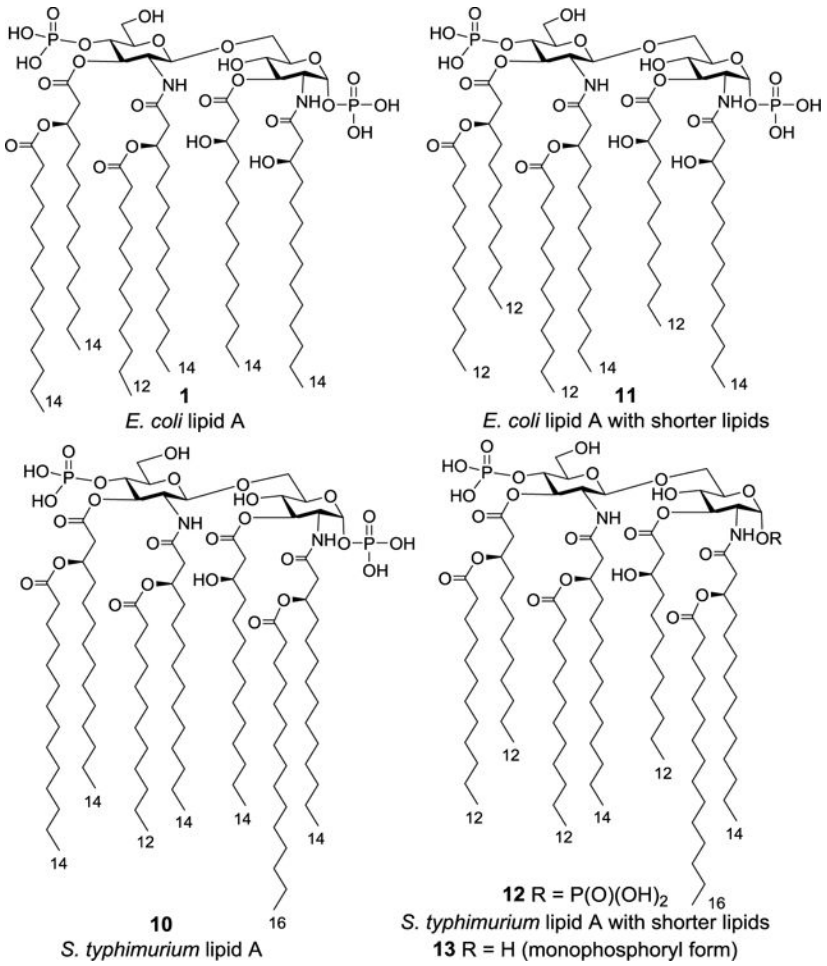


Figure 3.
Structures of *E. coli* and *S. typhimurium* lipid As **1** and **10** and their derivatives **11**–**13**.

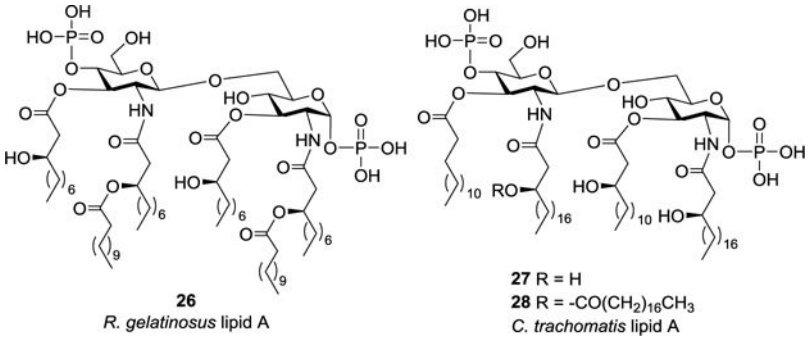


Figure 4.
Structures of *R. gelatinosus* and *C. trachomatis* lipid As **26–28**.

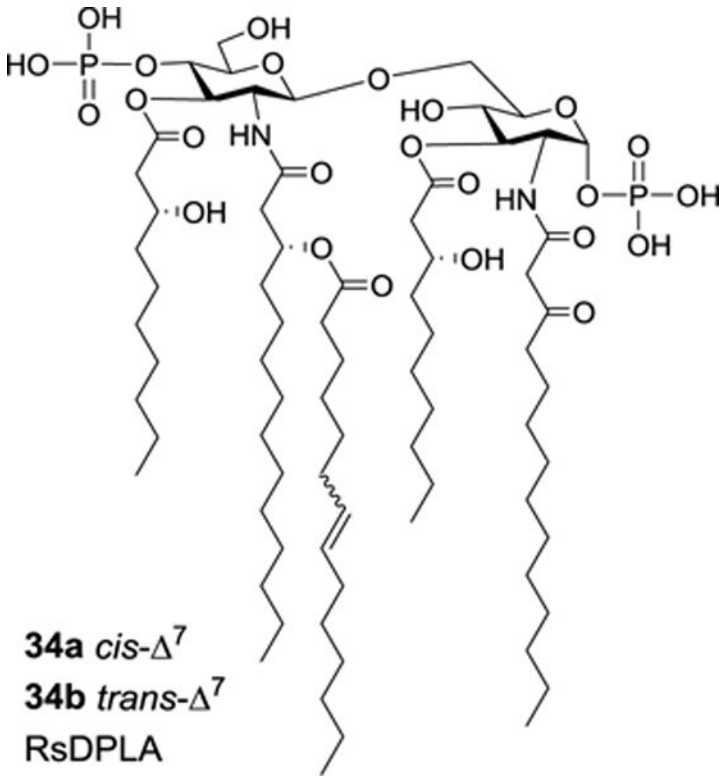


Figure 5.
Structures of the proposed *R. sphaeroides* lipid As **34a** and **34b** bearing an unsaturated lipid.

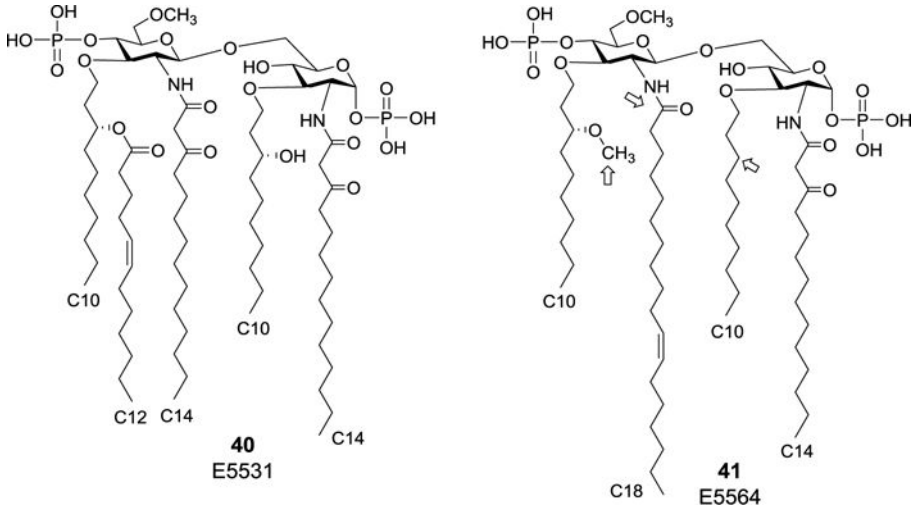


Figure 6.
Structures of *R. sphaeroides* lipid A analogs E5531 and E5564 as potent LPS antagonists.

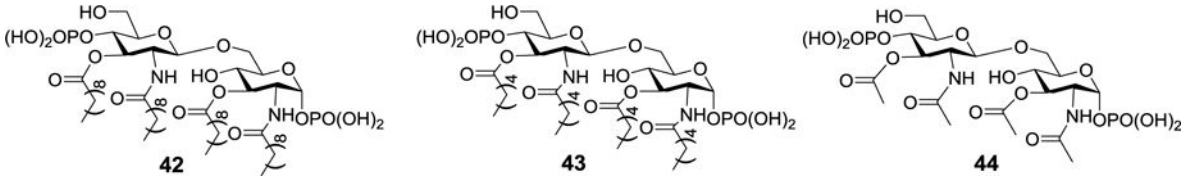


Figure 7.
Structures of artificial diphosphorylated lipid A derivatives **42**–**44**.

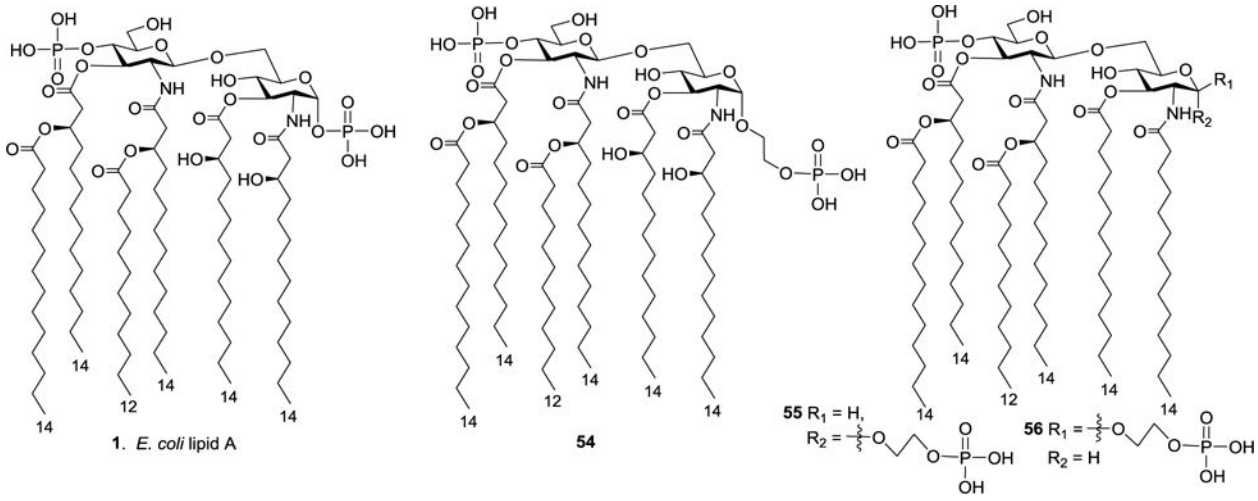


Figure 8.
Structures of *E. coli* lipid A analogs **54**–**56**.

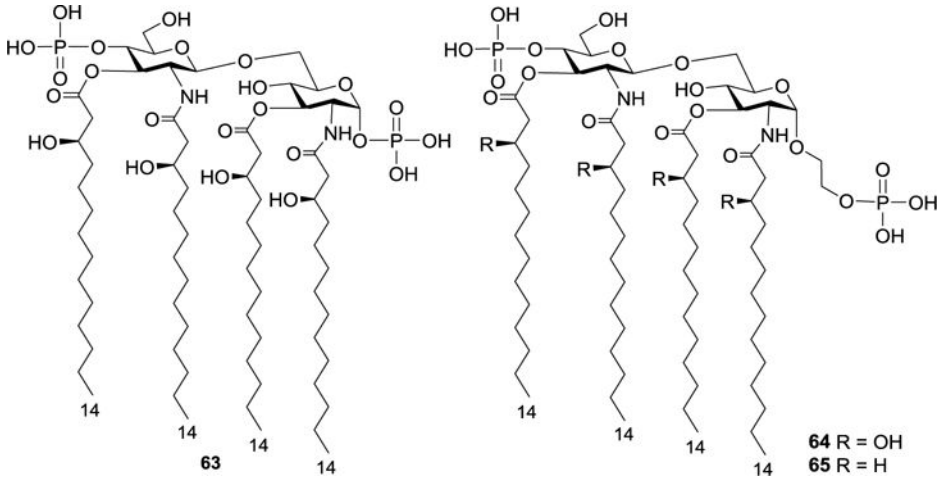


Figure 9.

Structures of the biosynthetic precursor **63** of *E. coli* lipid A **1** and its artificial analogs **64** and **65**.

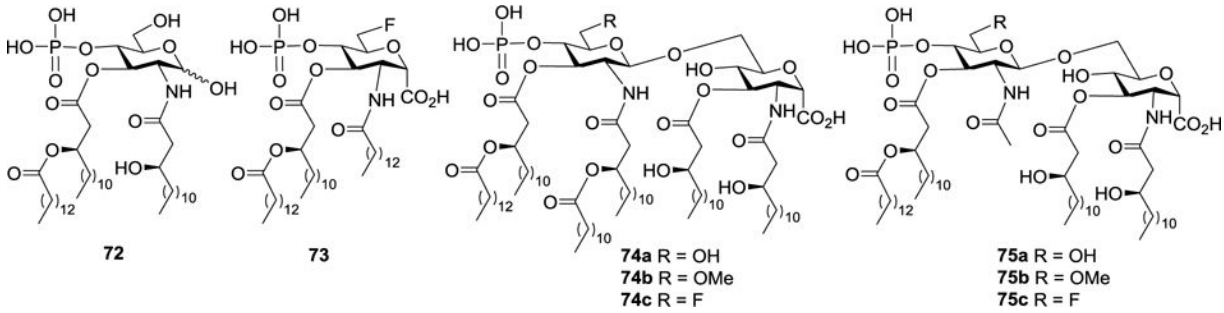


Figure 10.

Structures of GLA-60 **72**, its monosaccharide pyranosyl carboxylic acid derivative **73**, and pyranosyl carboxylic acid derivatives of lipid A **74a-c** and **75a-c**.

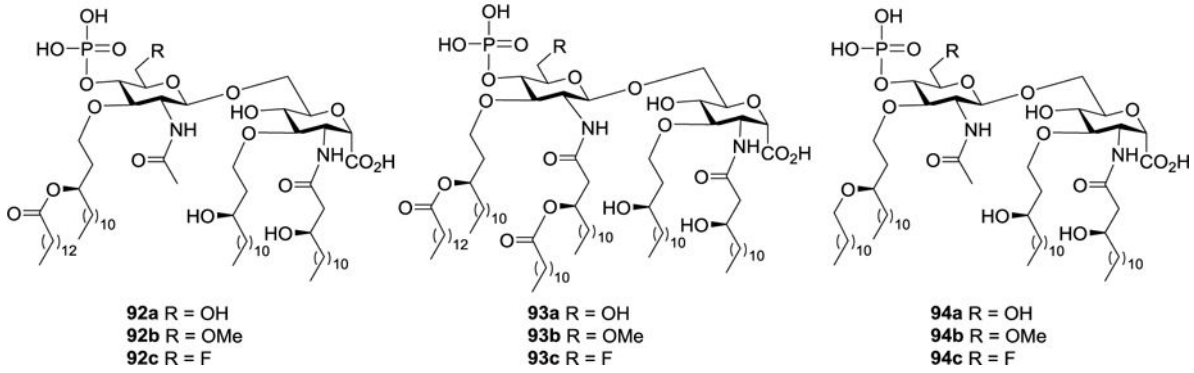


Figure 11.

Structures of pyranosyl carboxylic acid analogs of lipid A **92a–c**, **93a–c**, and **94a–c** bearing unnatural ether chains.

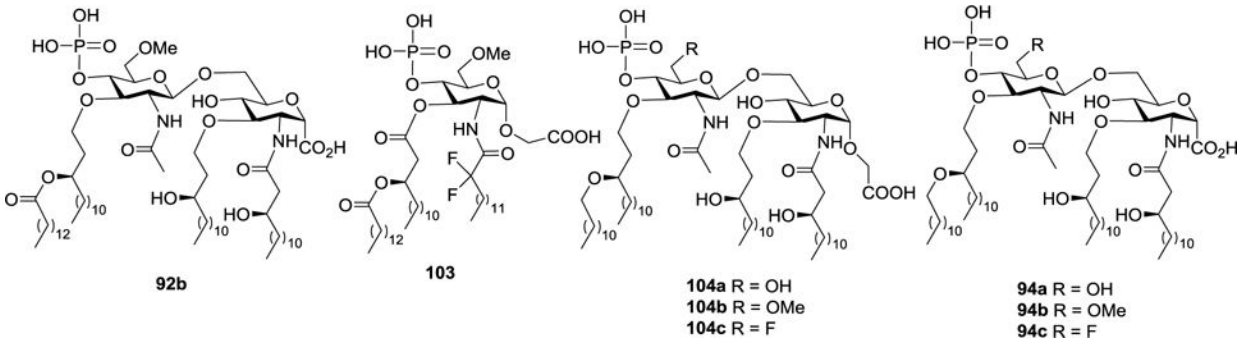


Figure 12.
Structures of GLA-60 analog **103** and lipid A analogs **92b**, **104a–c** and **94a-c**.

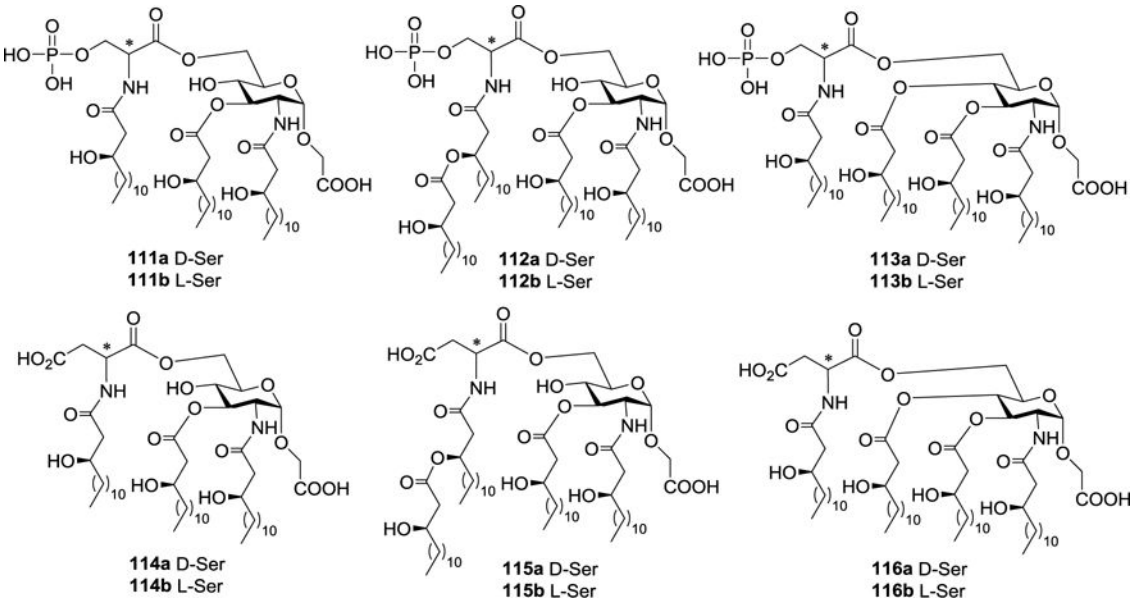


Figure 13.

Structures of monosaccharide analogs of lipid A **111a**,**b**–**116a**,**b** containing acidic *N*-acyl-amino acids as replacement of the non-reducing end phosphorylated glucosamine residue.

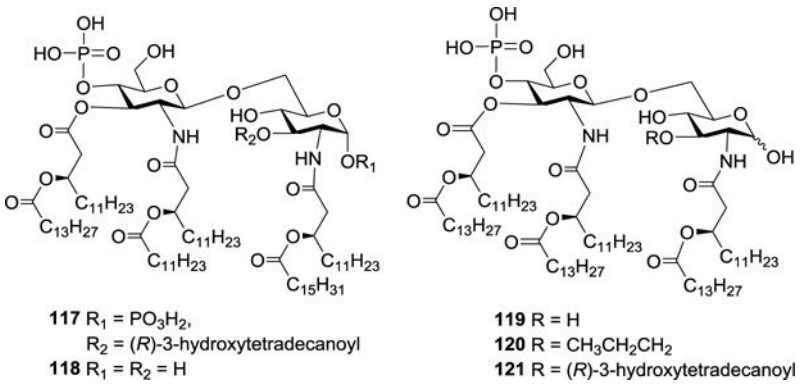


Figure 14.
Structures of MPLAs **117–121**.

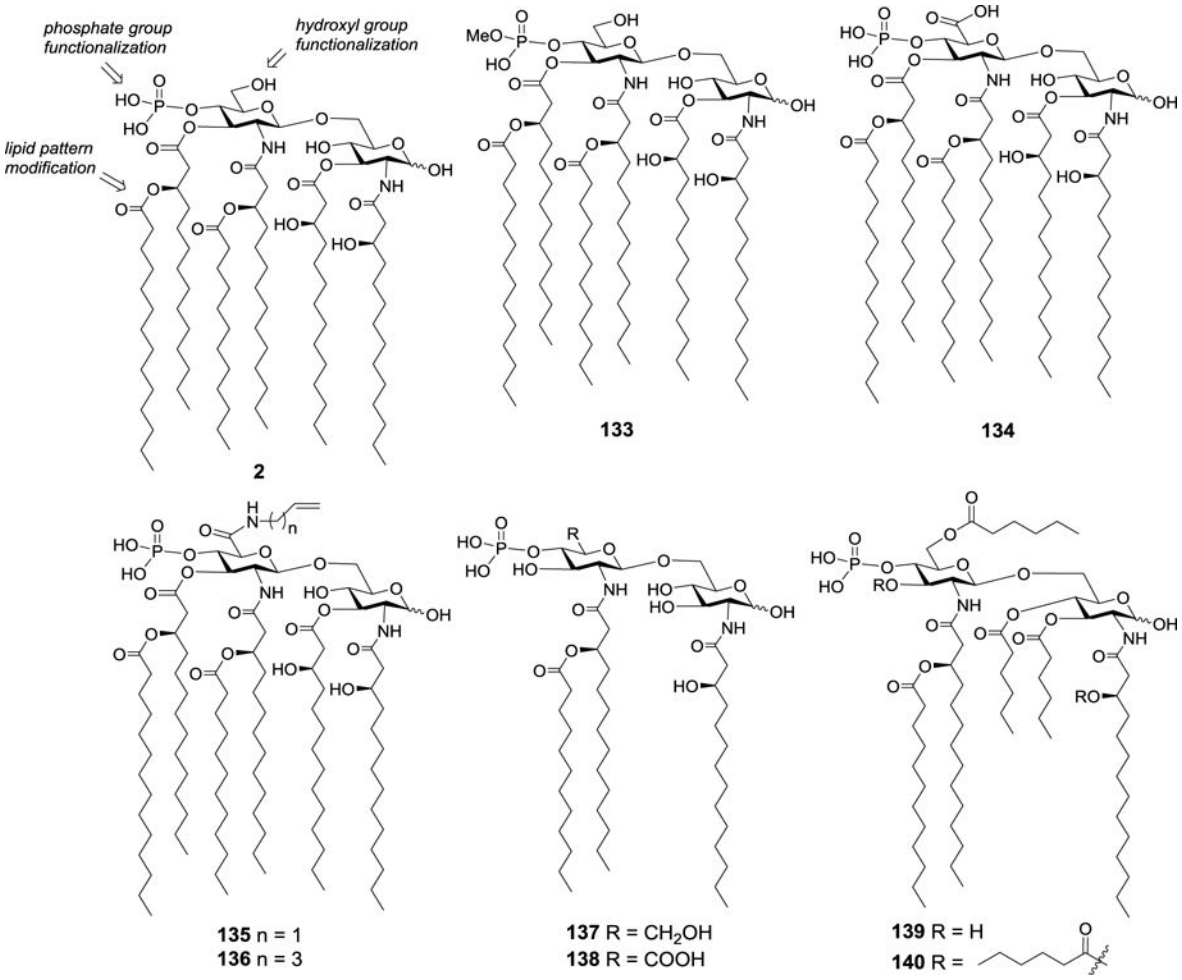


Figure 15.
Structures of *E. coli* MPLA **2** and its semisynthetic derivatives **133**–**140**.

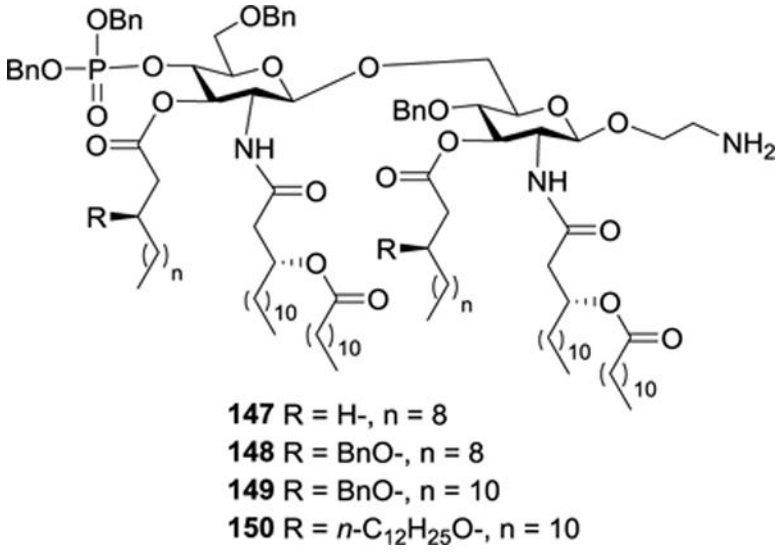


Figure 16.
Structures of *N. meningitidis* MPLA derivatives **147–150**.

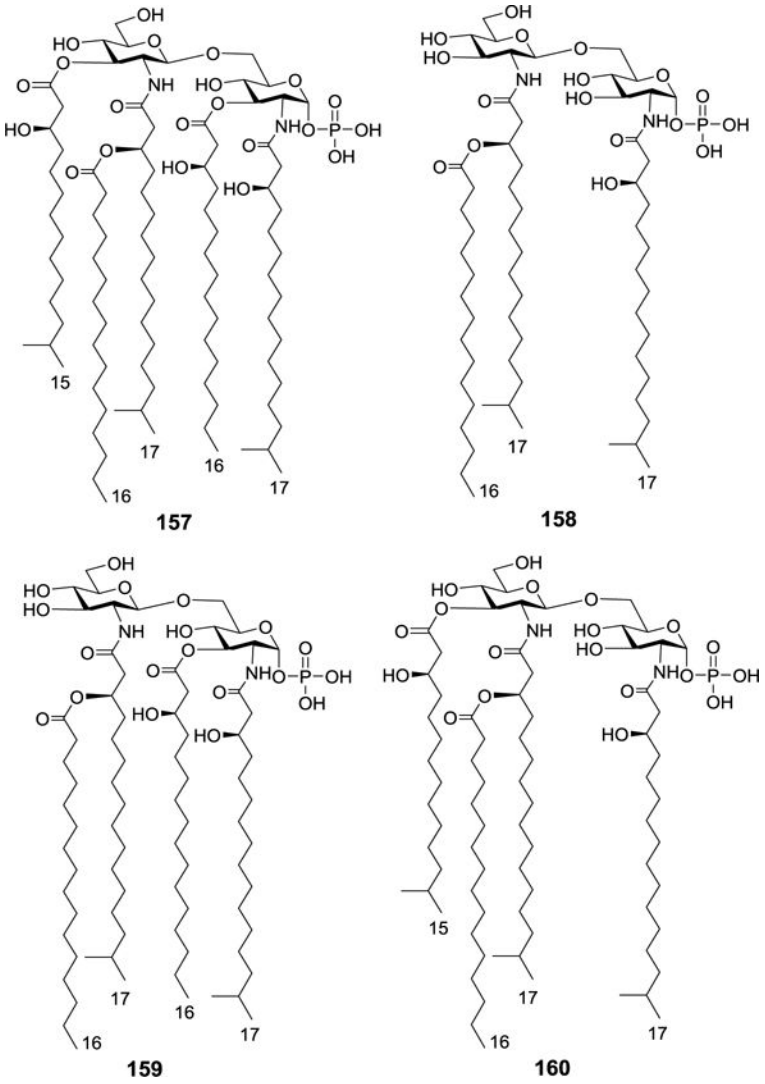


Figure 17.

Structures of P. gingivalis MPLA **157**, **158** and tetra-acylated derivatives **159**, **160**.

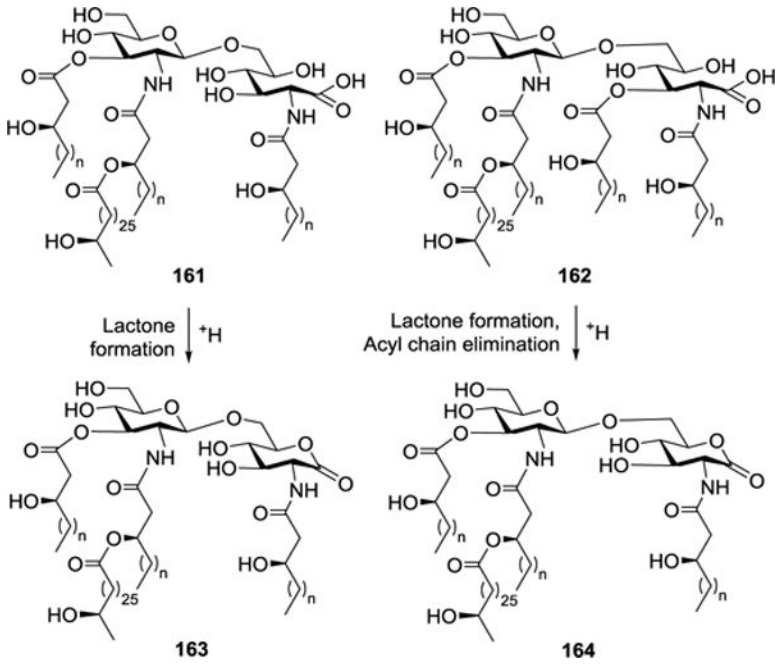


Figure 18.
Structures of *R. sin*-1 lipid As **161**–**164**.

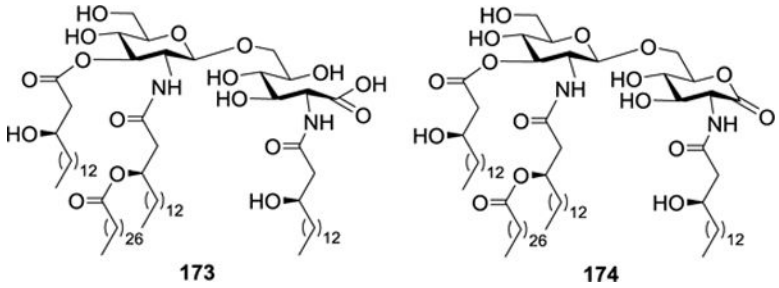
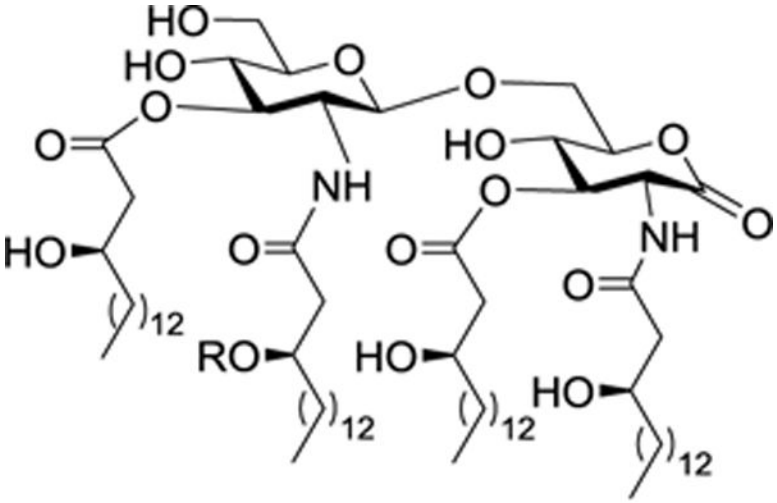


Figure 19.
Structures of *R. sin*-1 lipid A analogs **173** and **174**.



166 R = $C(O)(CH_2)_{26}CH_3$
188 R = $C(O)(CH_2)_{12}CH_3$
189 R = $C(O)(CH_2)_{25}CH(OH)CH_3$

Figure 20.
Structures of *R. sin*-1 lipid A **189** and its derivatives **166** and **188**.

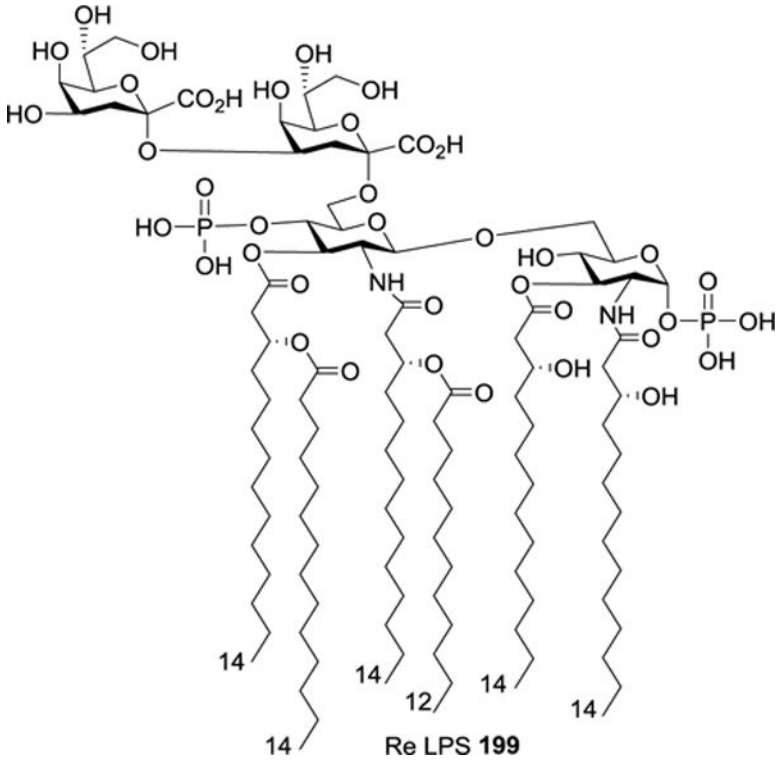


Figure 21.
Structure of Re LPS **199**.

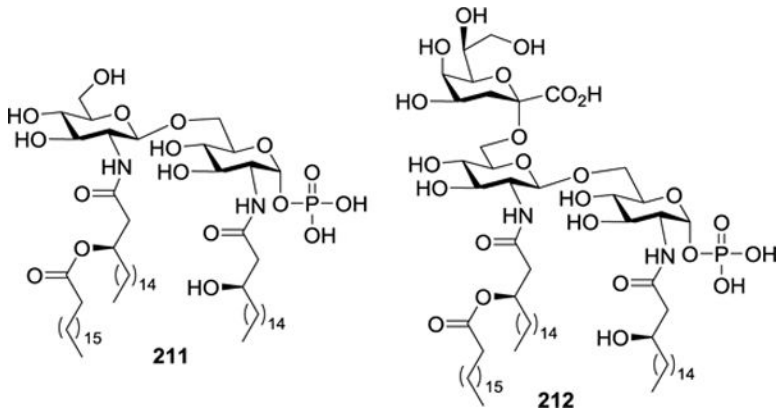


Figure 22.
Structures of *H. pylori* lipid A **211** and its Kdo conjugate **212**.

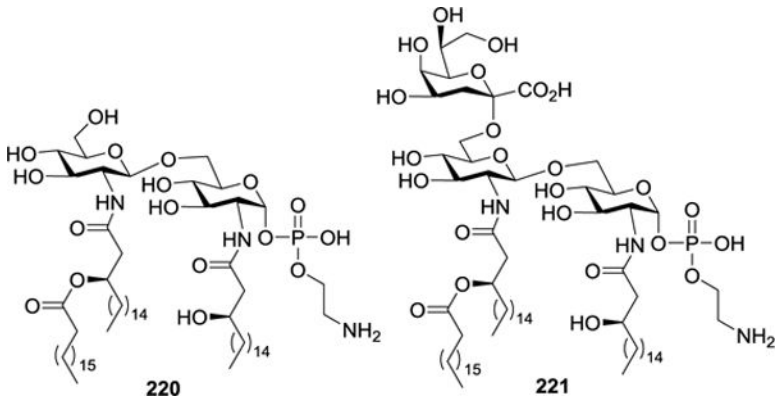


Figure 23.
Structures of the ethanolamine-containing *H. pylori* lipid A **220** and its Kdo conjugate **221**.

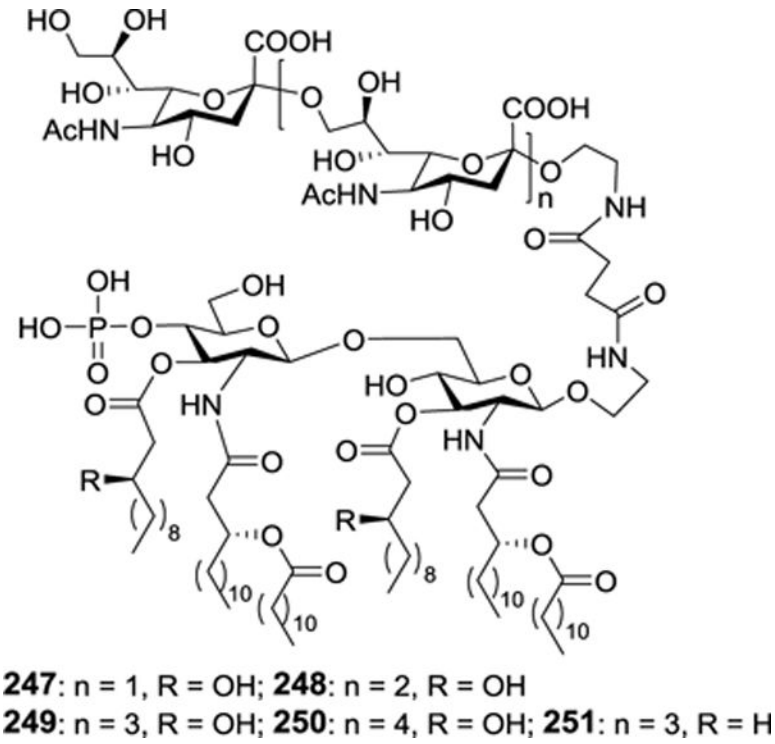
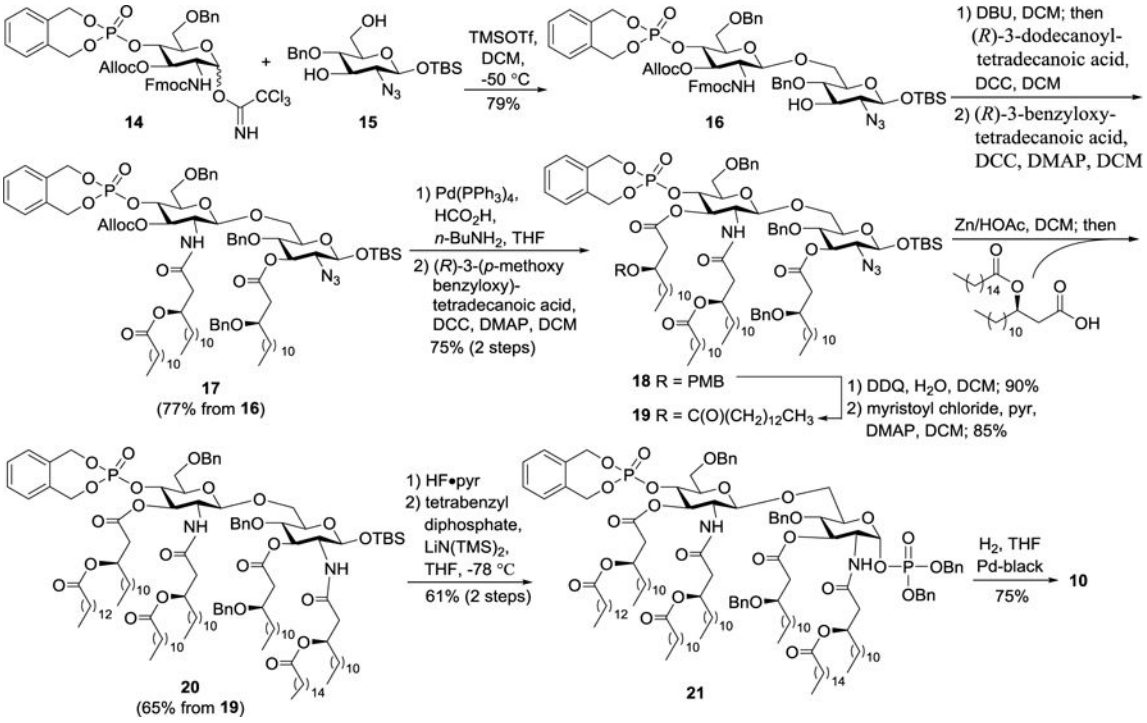


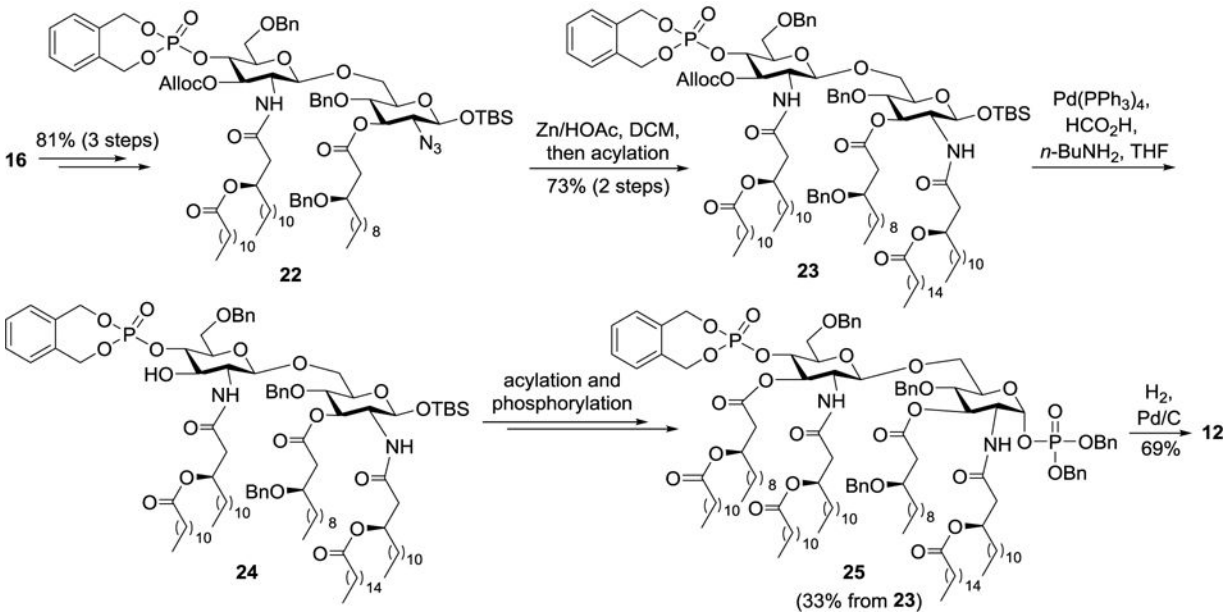
Figure 24.

Structures of MPLA-oligosialic acid conjugates **247**–**251** explored as fully synthetic anti-group C meningitis vaccines.

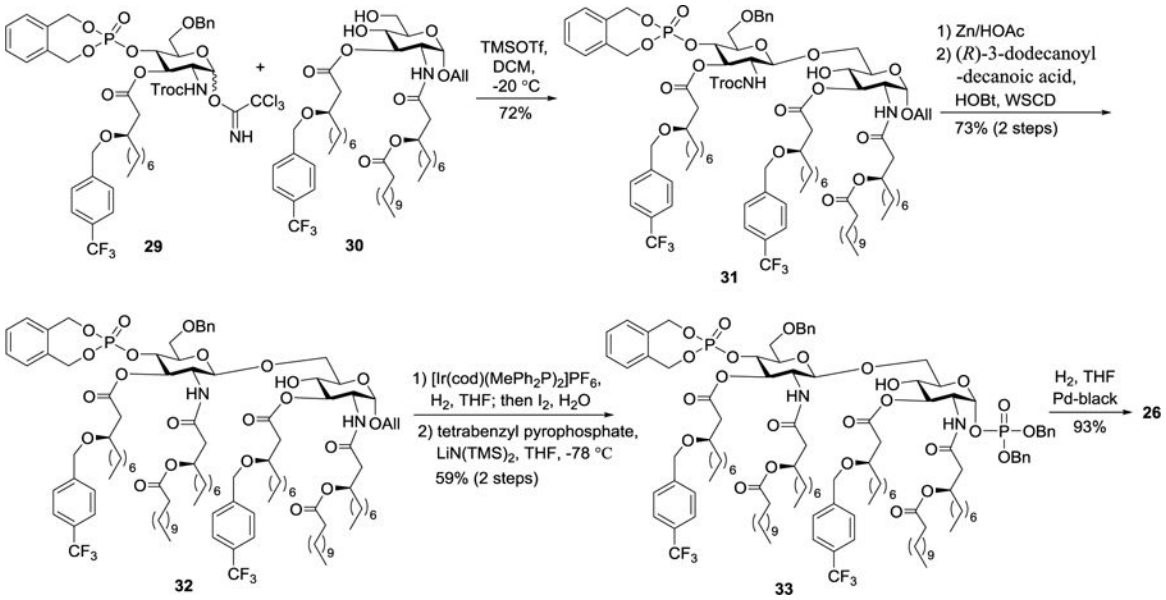


Scheme 1.

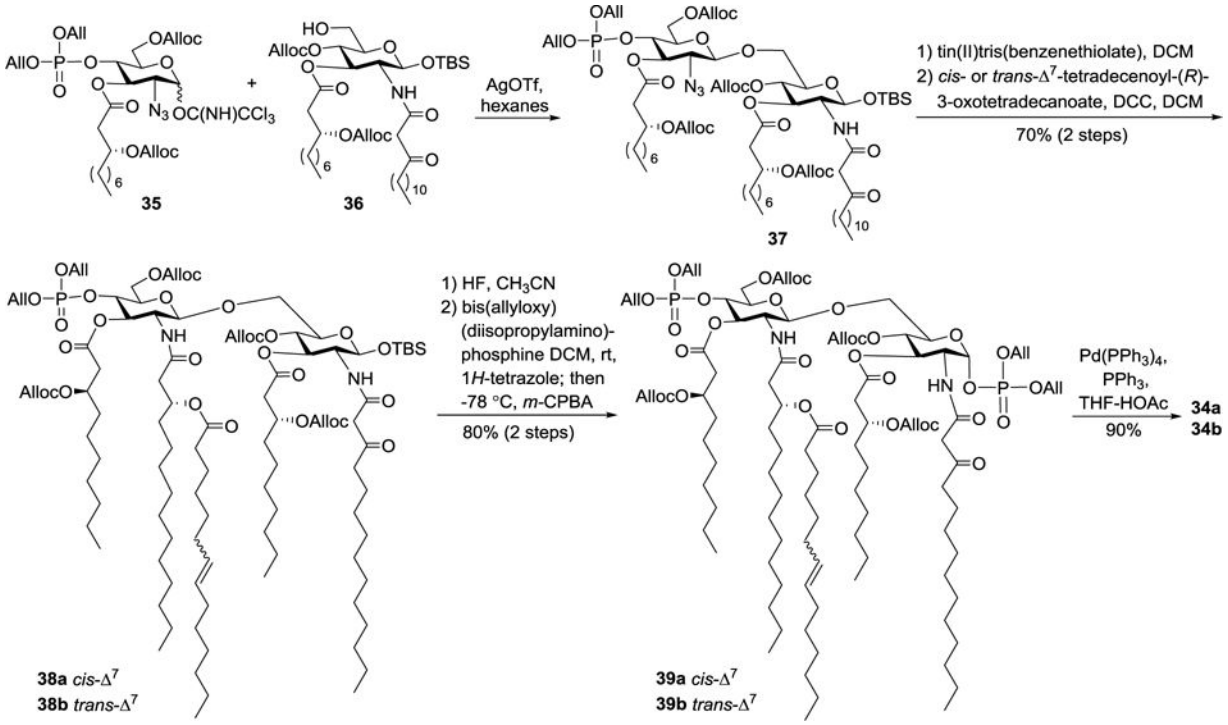
Synthesis of *S. typhimurium* lipid A **10**.



Scheme 2.
Synthesis of lipid A **12**.

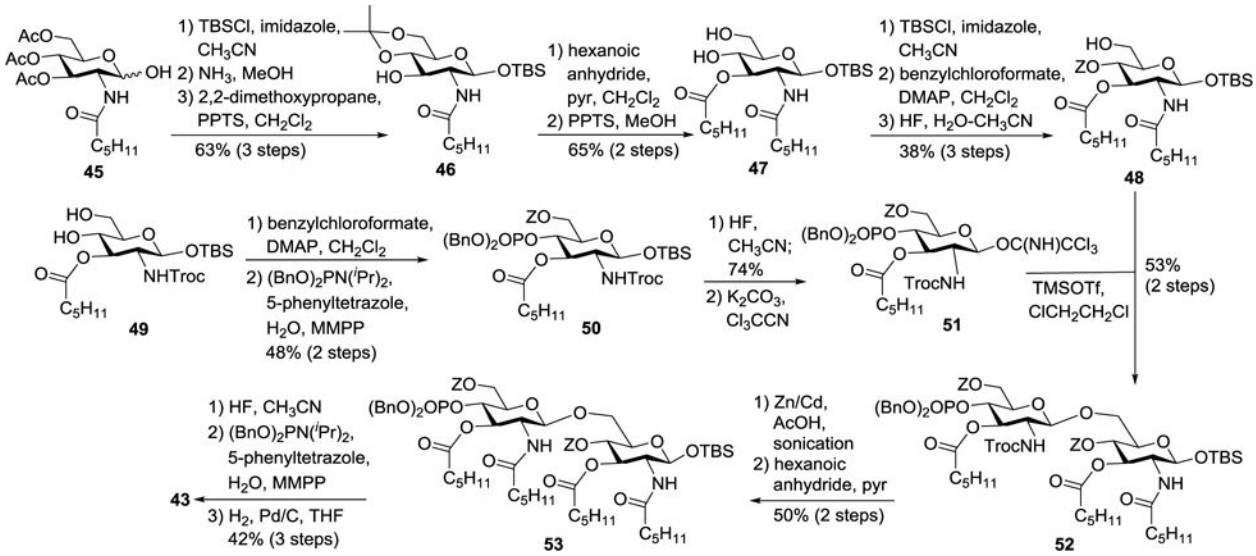


Scheme 3.
Synthesis of lipid A **26**.



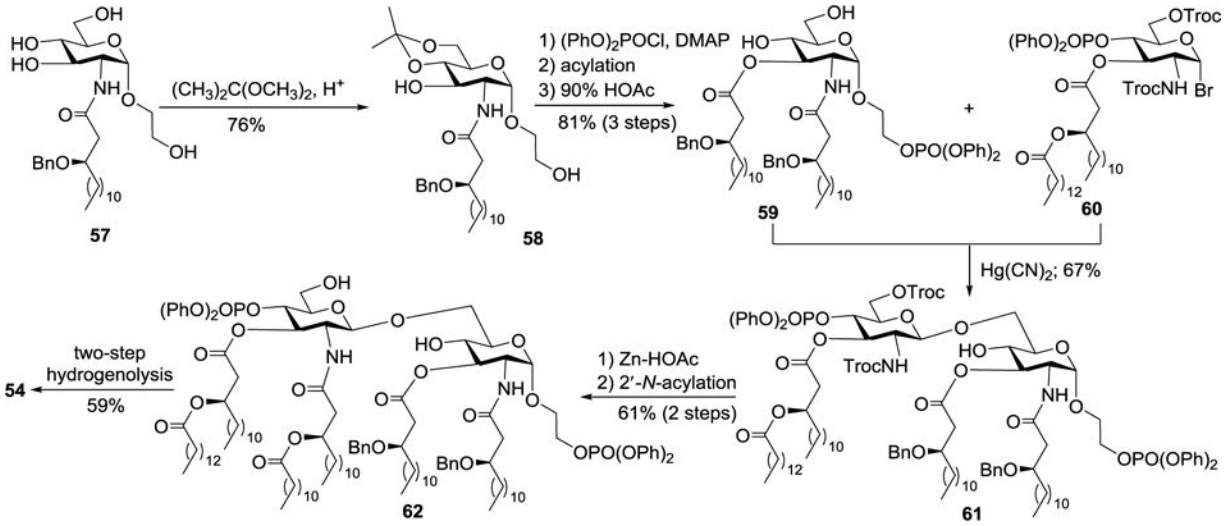
Scheme 4.

Synthesis of **34a** and **34b**.



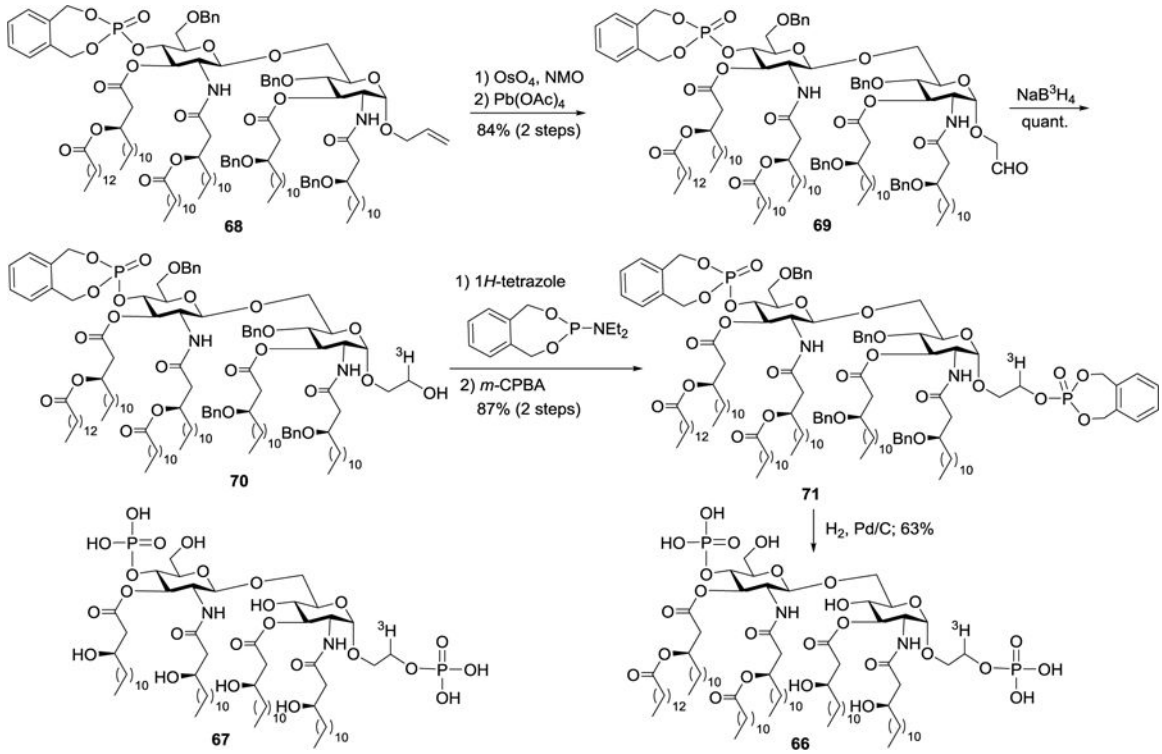
Scheme 5.

Synthesis of lipid A derivative **43**.



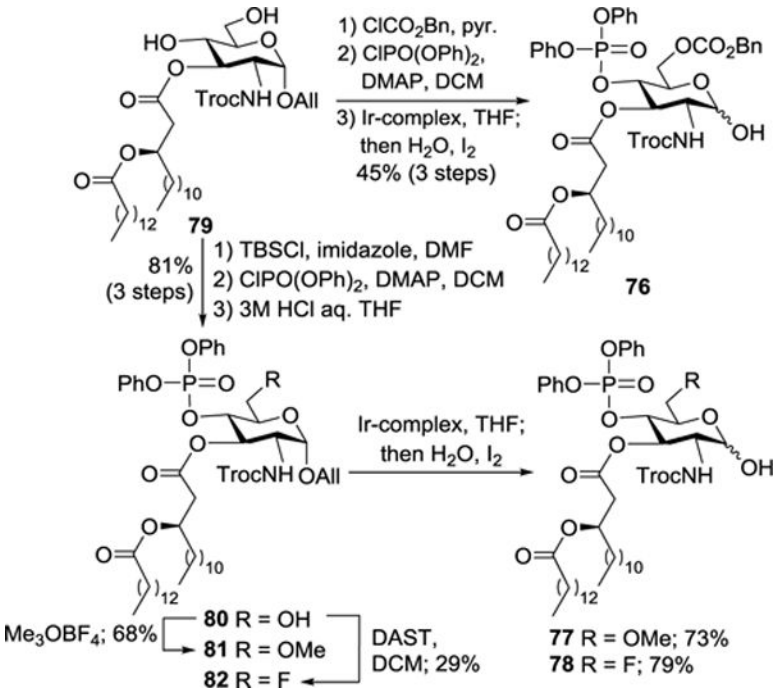
Scheme 6.

Synthesis of lipid A derivative **54**.



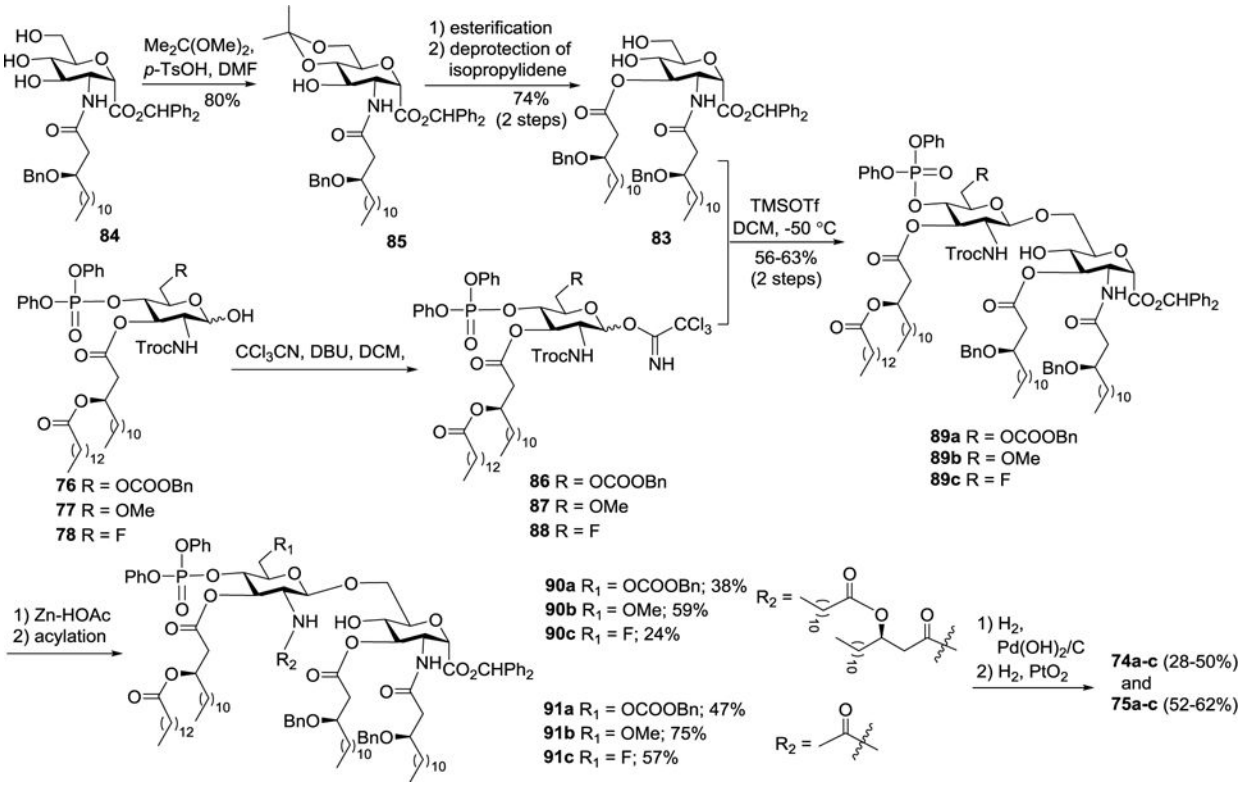
Scheme 7.

Structures of tritium-labeled lipid A analogs **66** and **67**, as well as the synthesis of **66**.



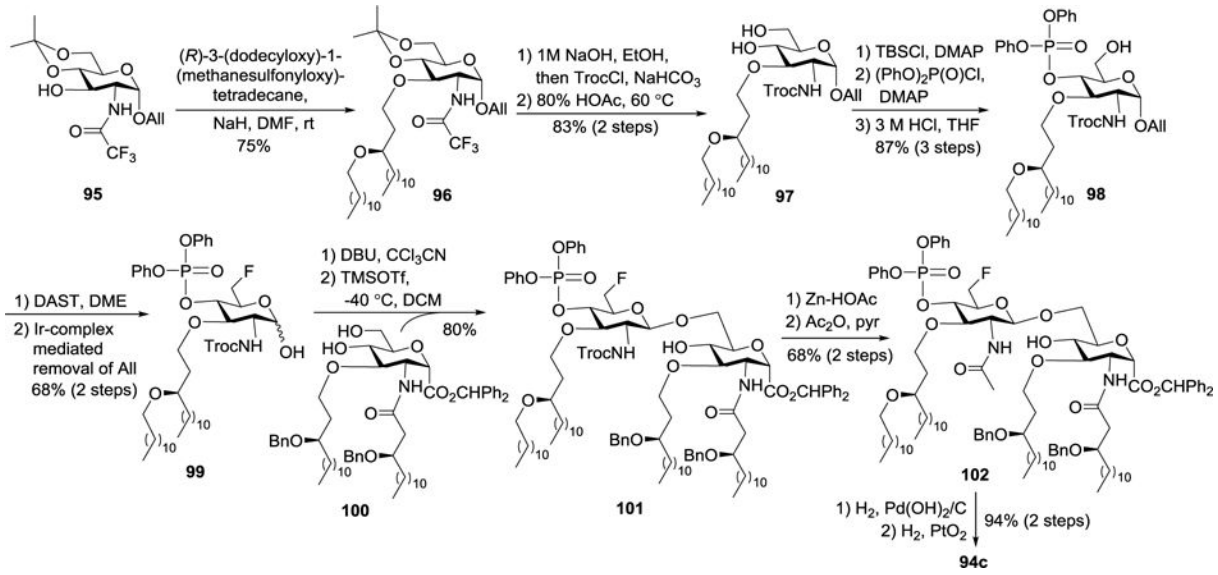
Scheme 8.

Synthesis of glycosyl donor precursors **76**–**78**.



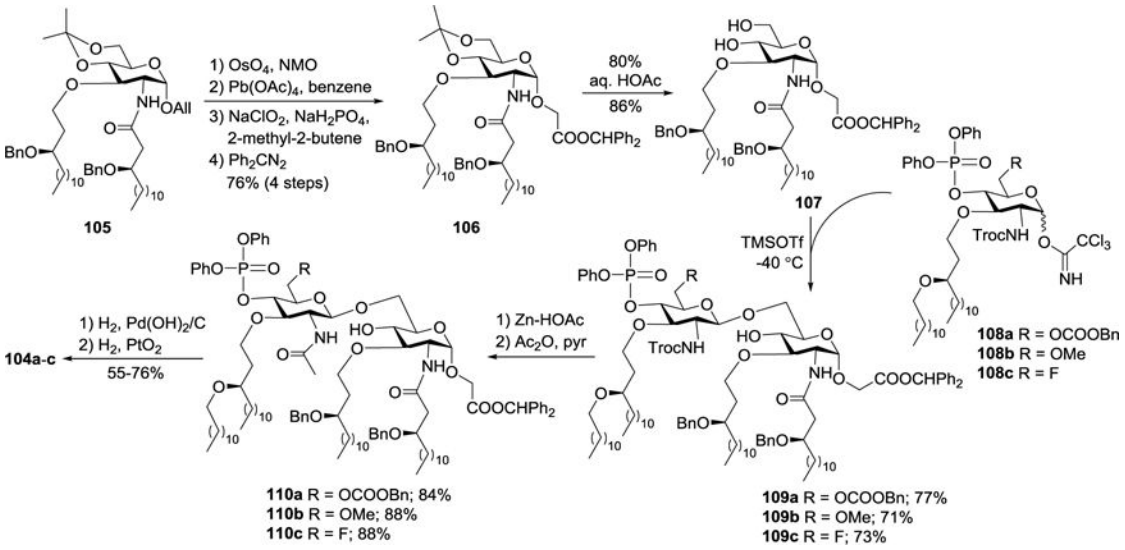
Scheme 9.

Synthesis of carboxylic acid analogs of lipid A **74a-c** and **75a-c**.



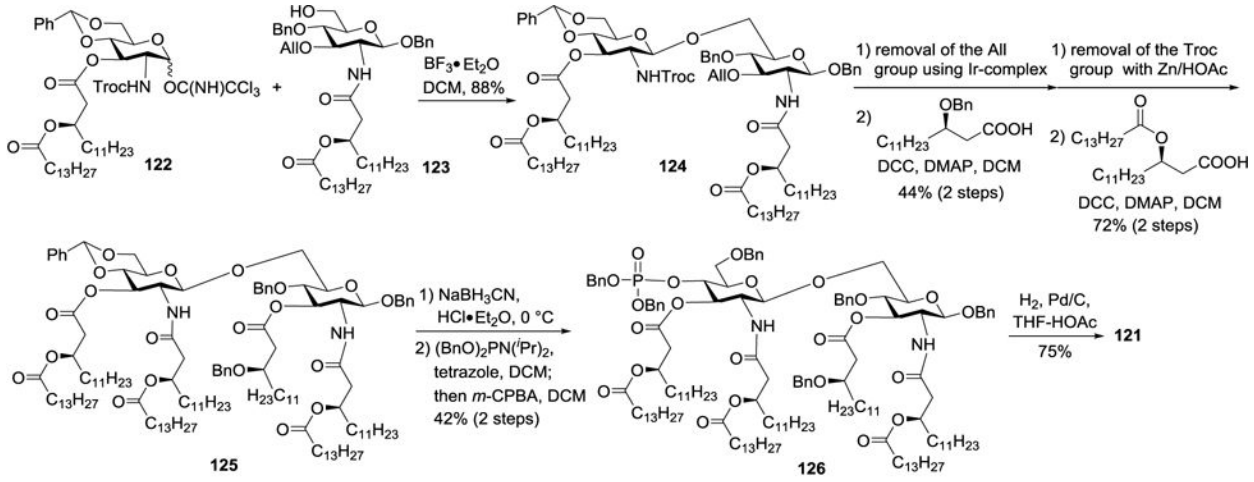
Scheme 10.

Synthesis of glycosyl carboxylic acid derivative of lipid A **94c** bearing ether linked lipids.

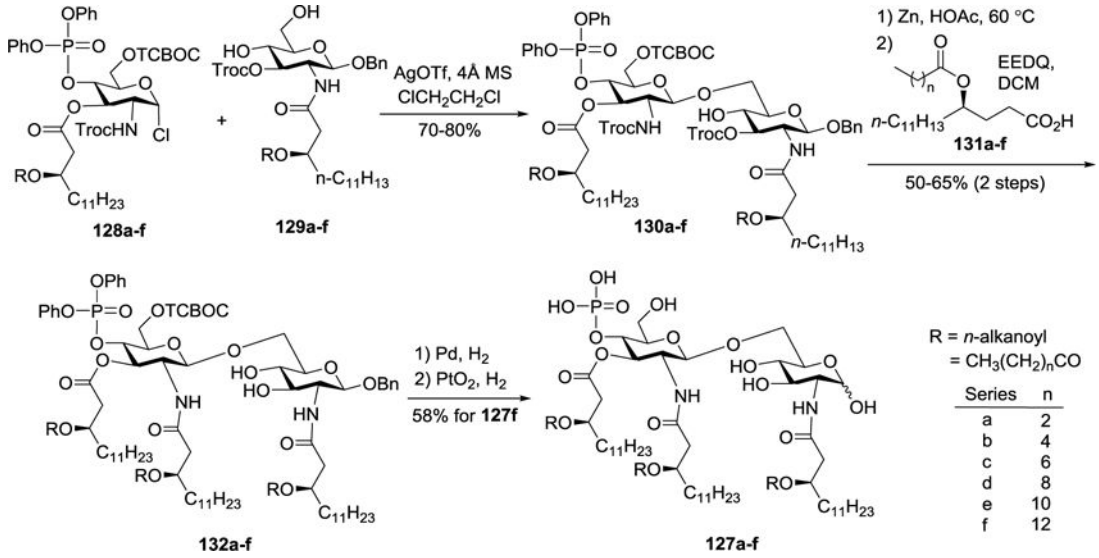


Scheme 11.

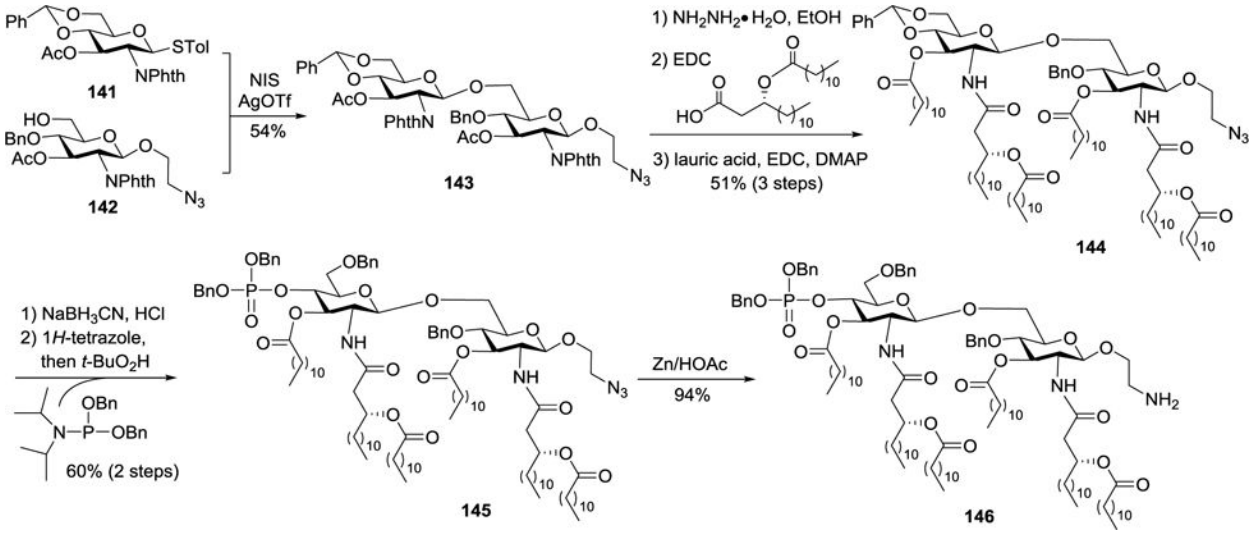
Synthesis of carboxymethyl glycoside derivatives of lipid A **104a–c**.



Scheme 12.
Synthesis of MPLA **121**.

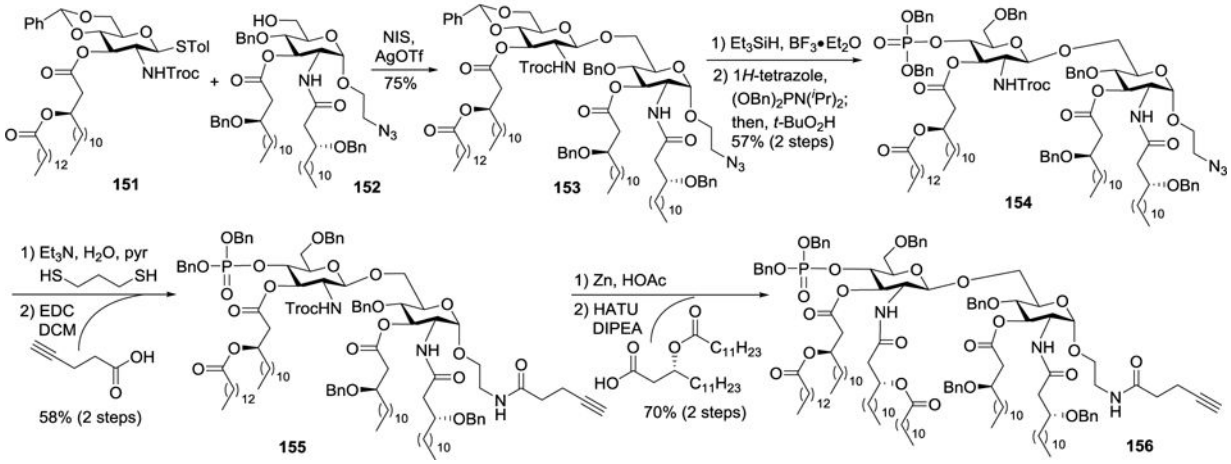


Scheme 13.
Synthesis of MPLAs **127a-f**.



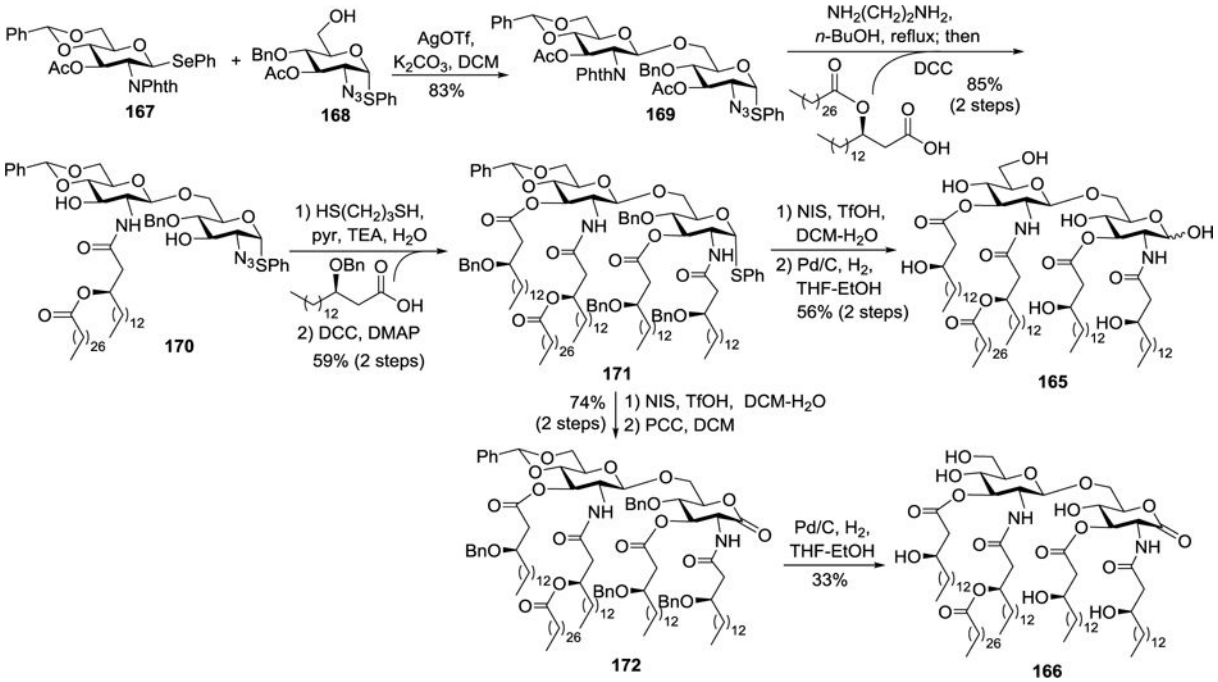
Scheme 14.

Synthesis of *N. meningitidis* MPLA derivative **146**.



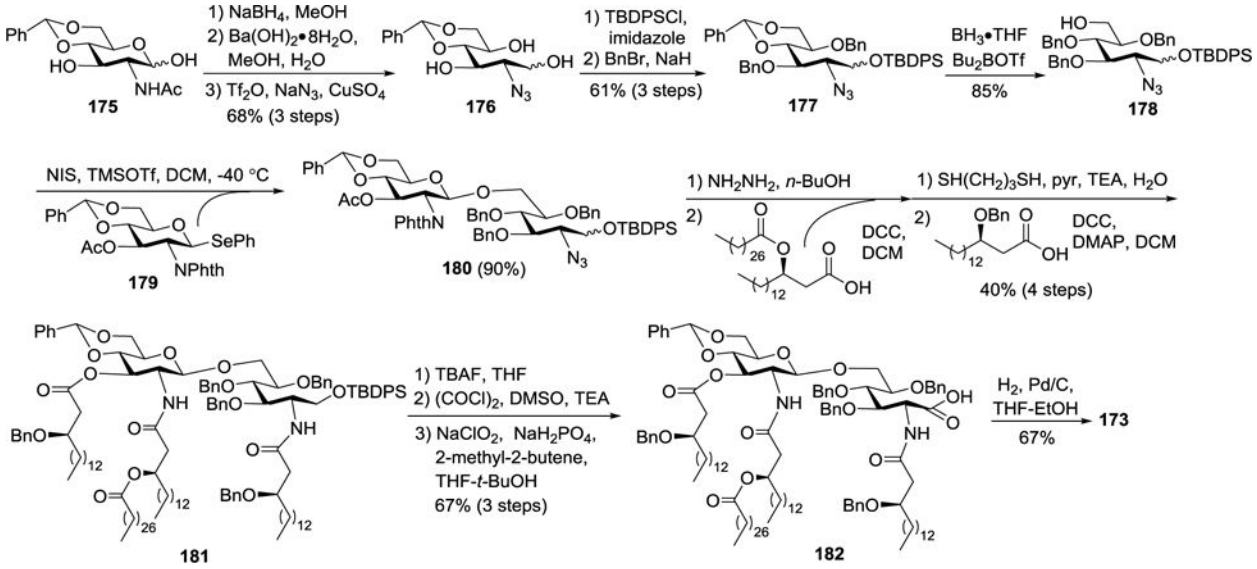
Scheme 15.

Synthesis of E. coli MPLA derivative **156**.



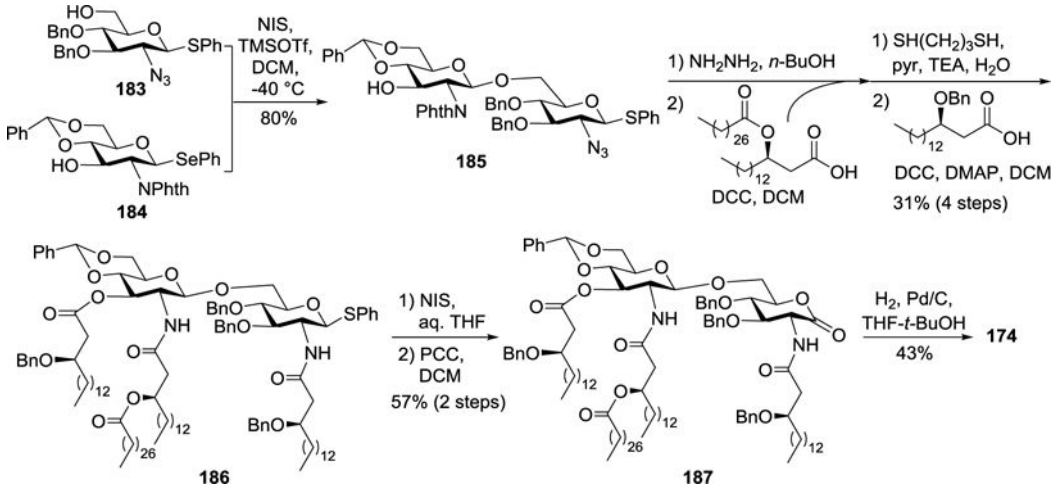
Scheme 16.

Synthesis of *R. sin*-1 lipid A derivatives **165** and **166**.



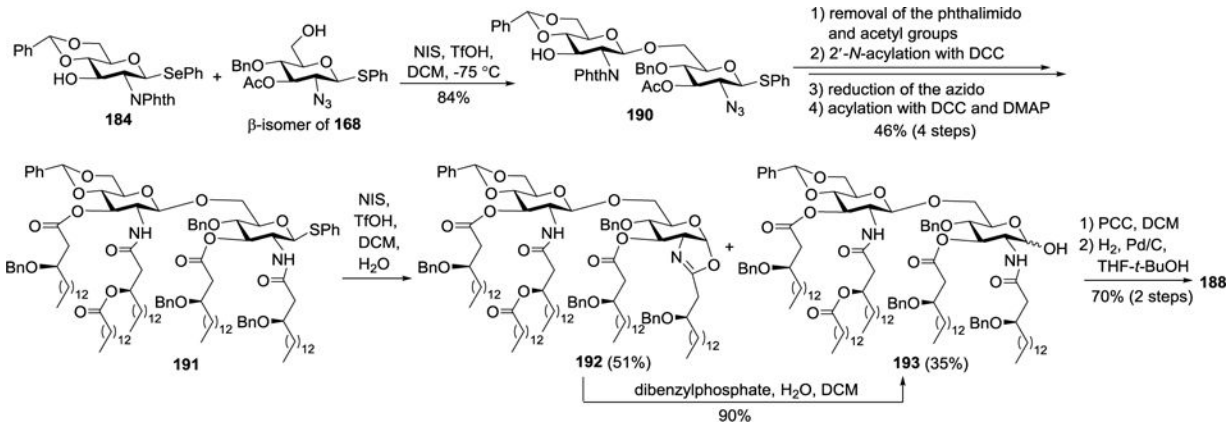
Scheme 17.

Synthesis of putative *R. sin*-1 lipid A **173**.



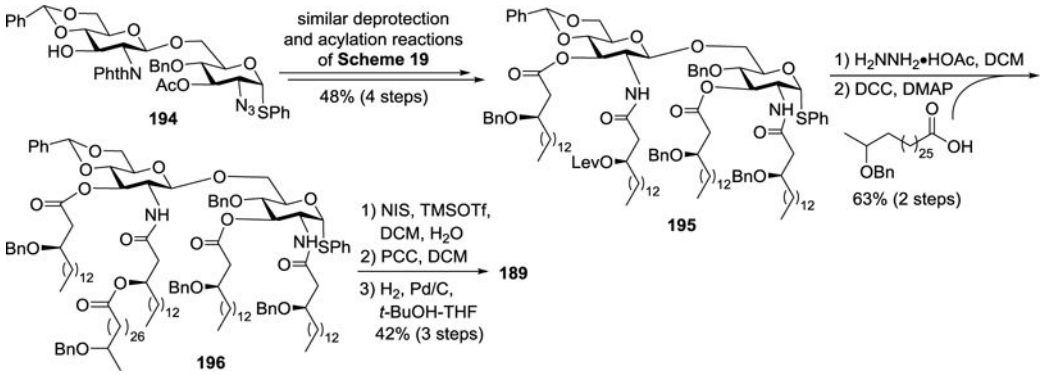
Scheme 18.

Synthesis of putative *R. sin*-1 lipid A **174**.

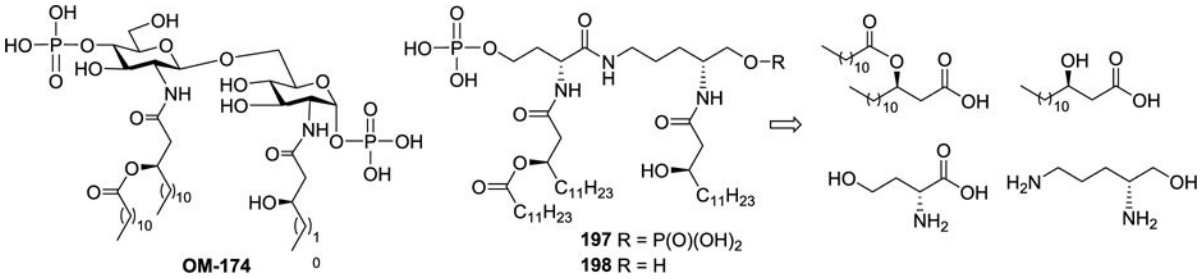


Scheme 19.

Synthesis of *R. sin*-1 lipid A derivative **188**.

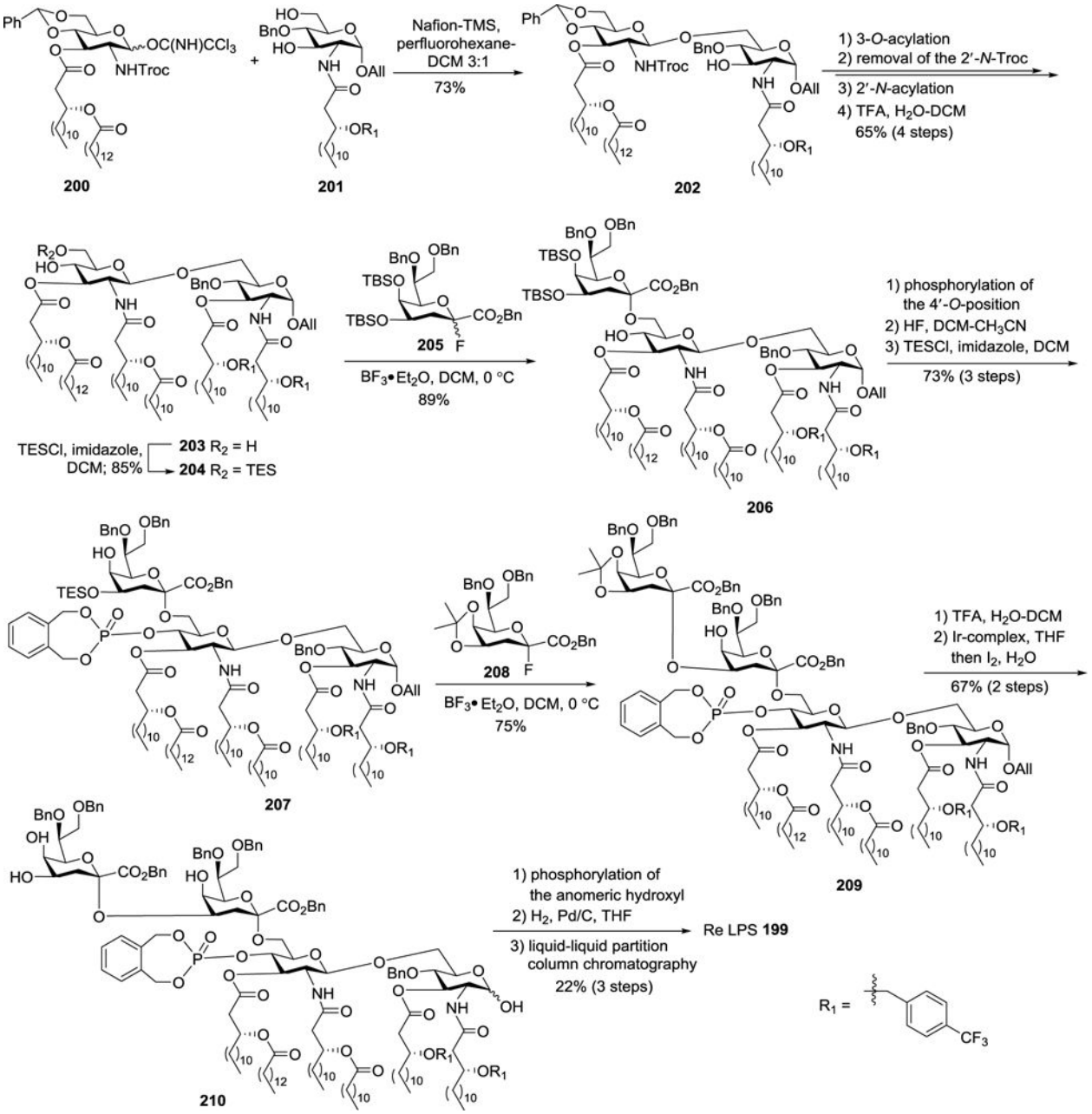


Scheme 20.
Synthesis of *R. sin*-1 lipid A **189**.

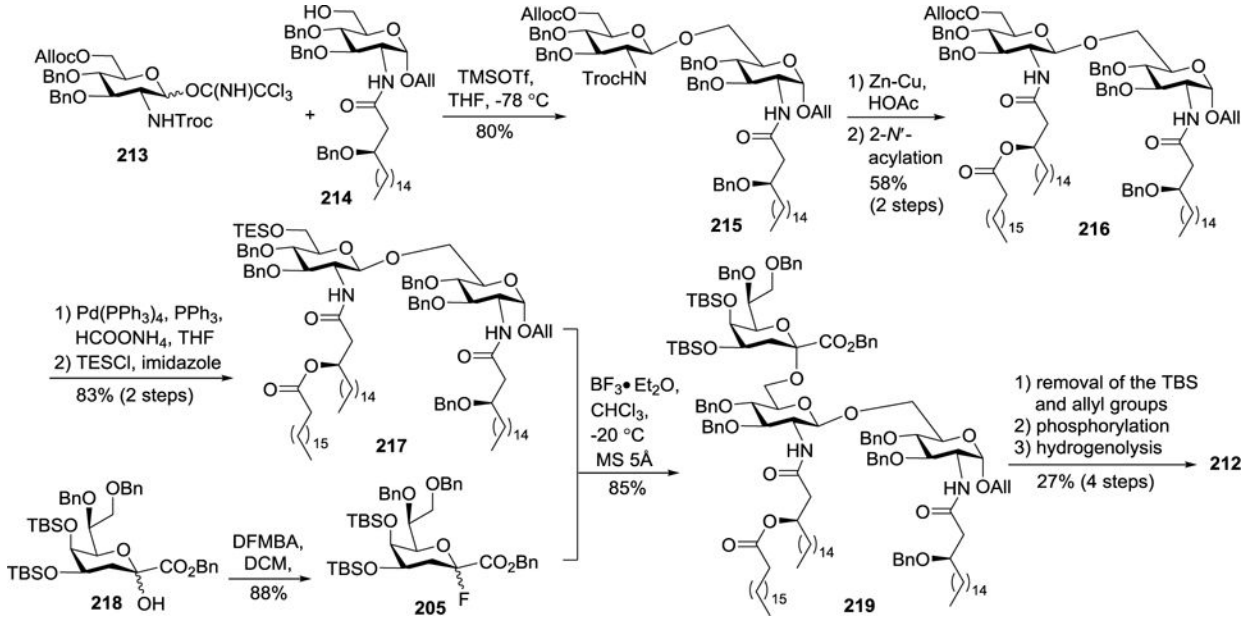


Scheme 21.

Structure of *E. coli* lipid A **OM-174** and retrosynthesis of its analogs **197** and **198**.

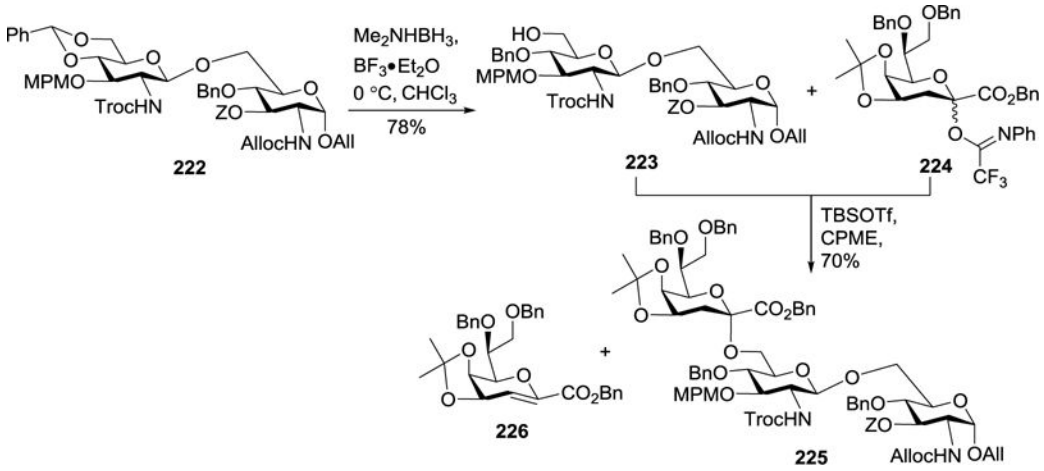


Scheme 22.
Total synthesis of Re LPS **199**.



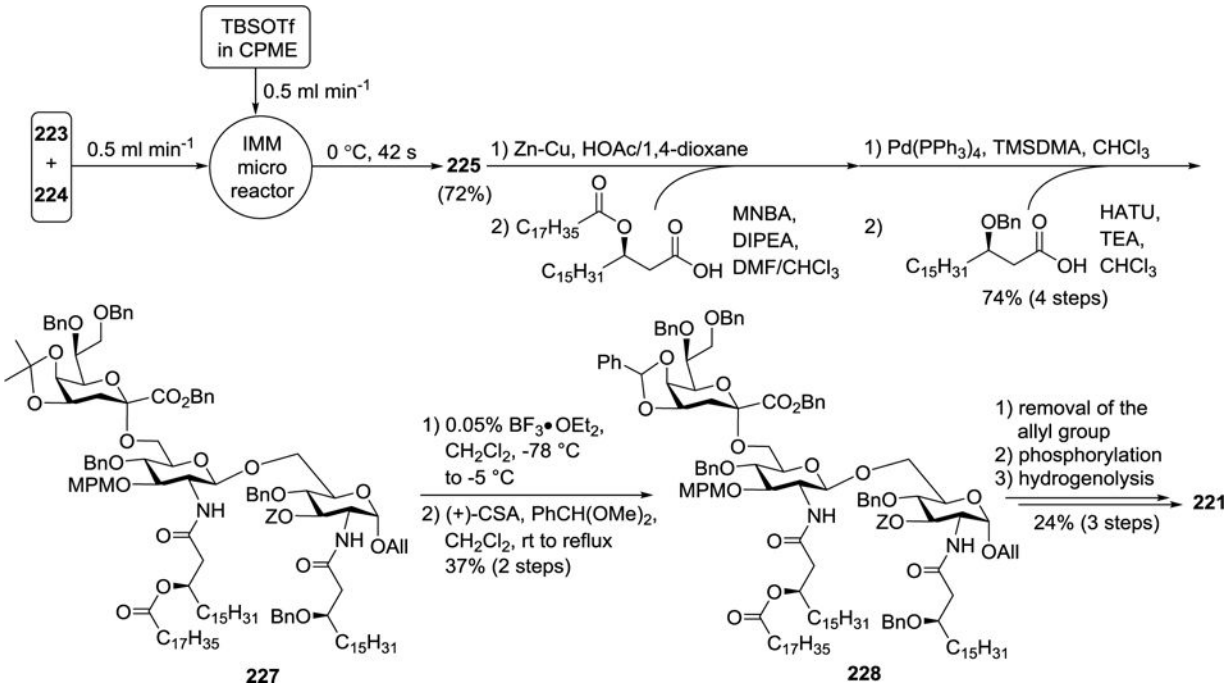
Scheme 23.

Synthesis of *H. pylori* Kdo-lipid A conjugate **212**.



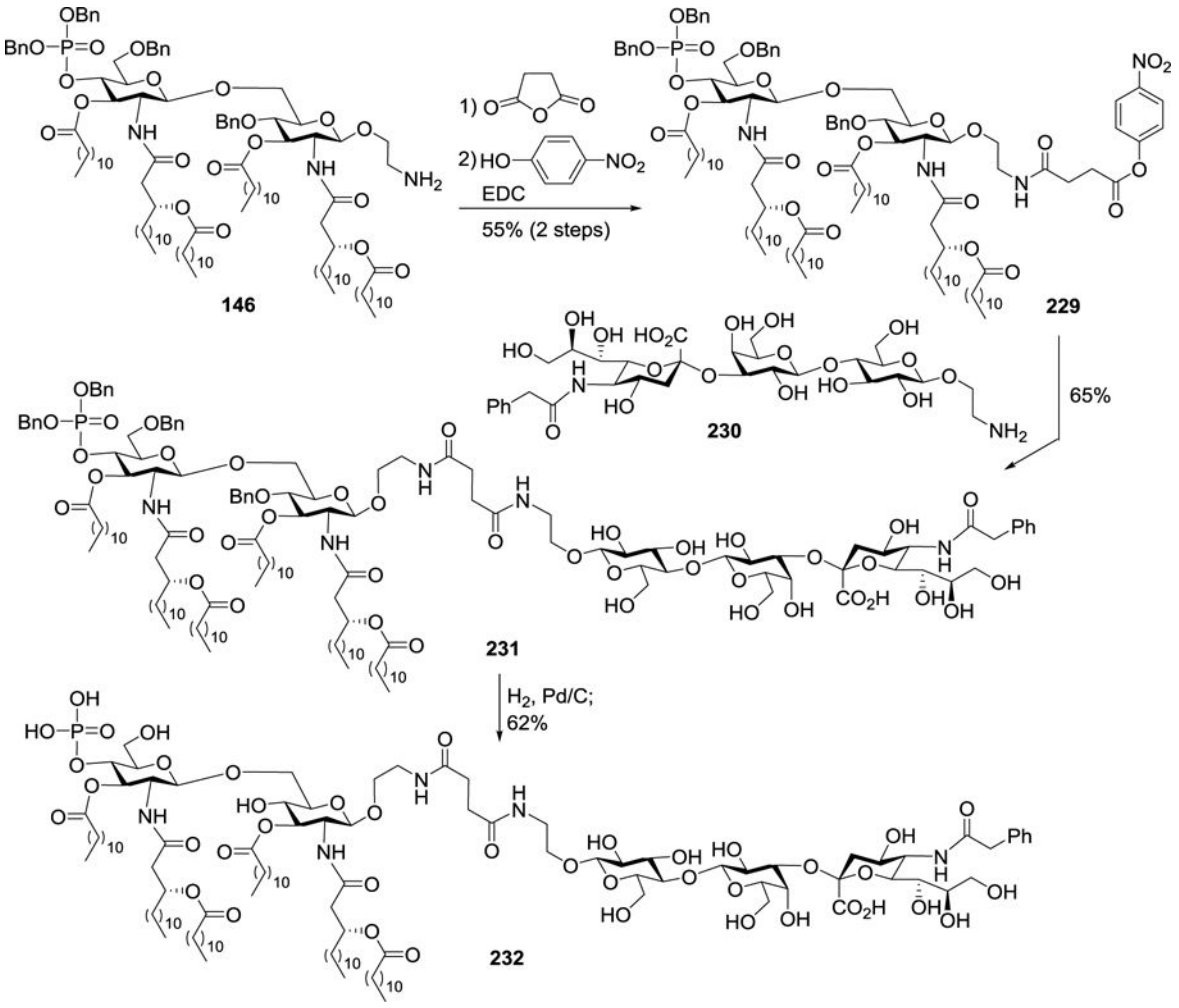
Scheme 24.

Glycosylation of **223** and **224** in the presence of Lewis acid.



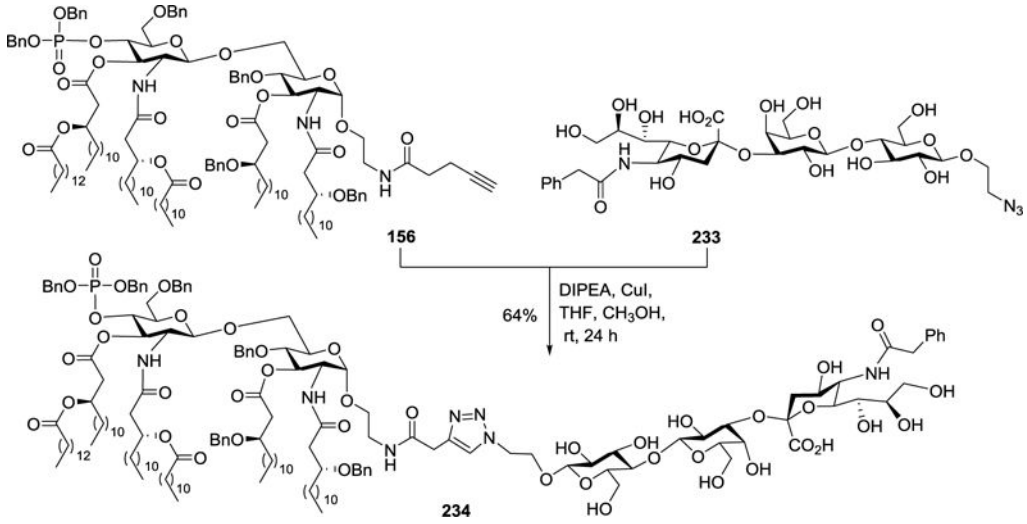
Scheme 25.

Synthesis of *H. pylori* lipid A-Kdo conjugate **221**.



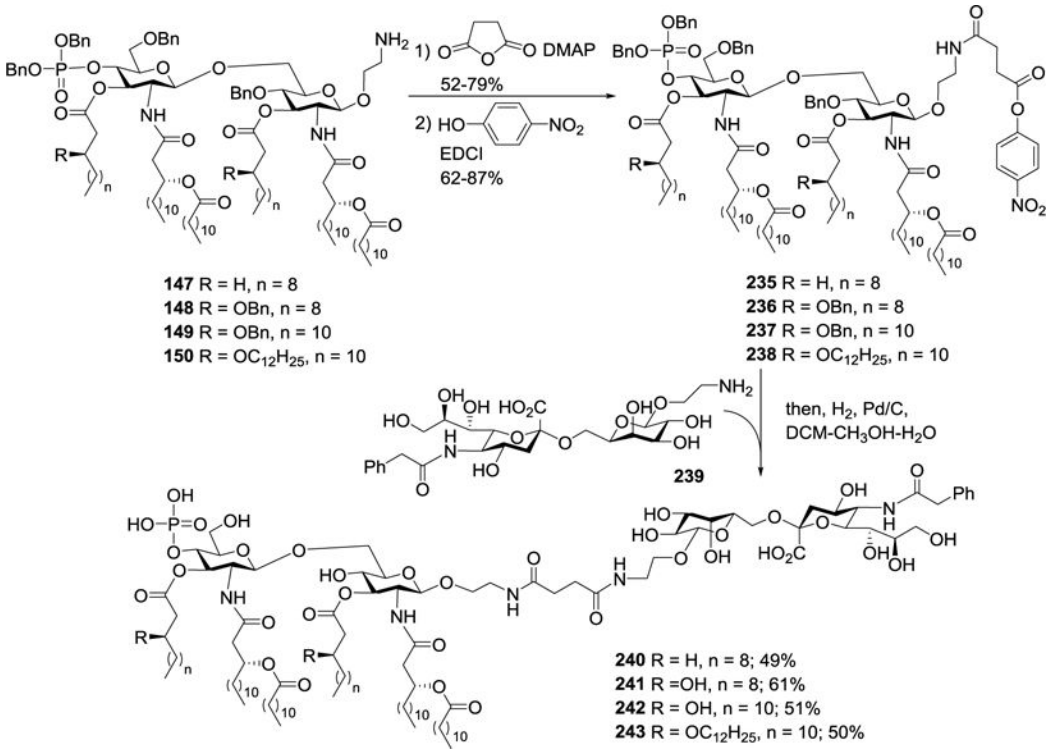
Scheme 26.

Synthesis of *N. meningitidis* MPLA–NPhAcGM3 conjugate **232**.



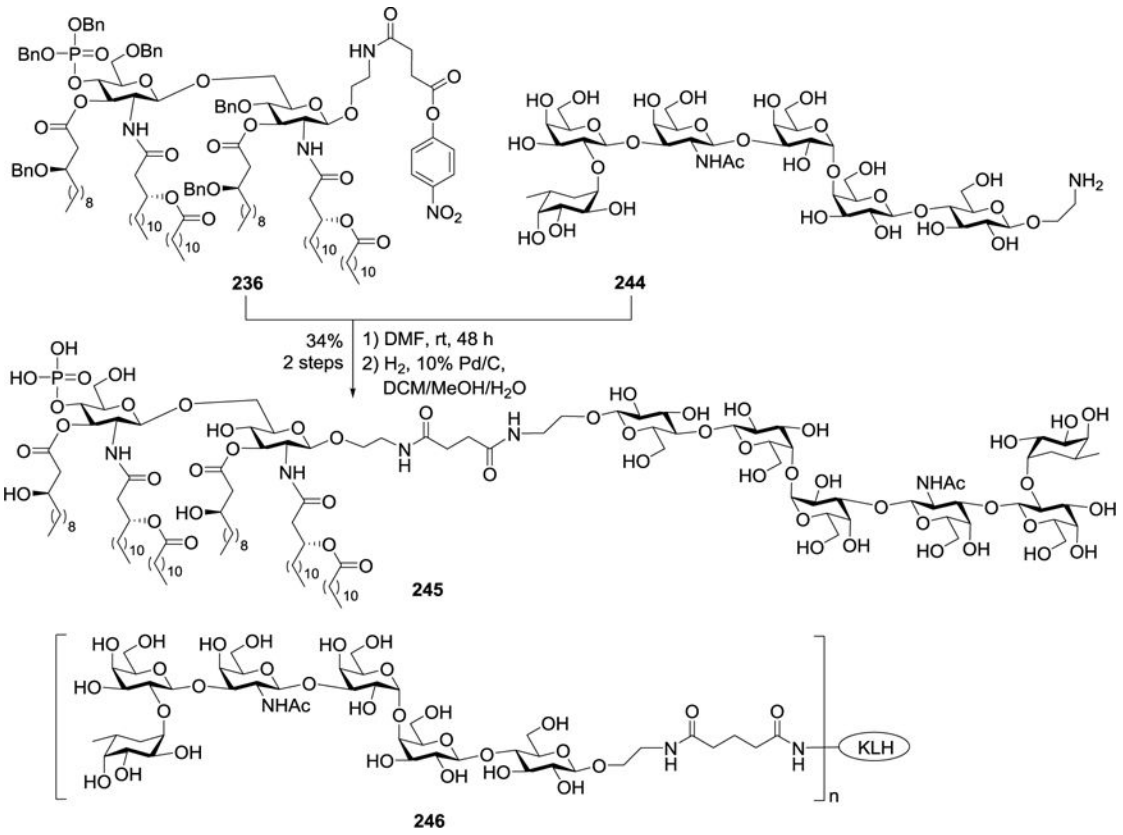
Scheme 27.

Synthesis of *E. coli* MPLA–NPhAcGM3 conjugate **234**.



Scheme 28.

Synthesis of *N. meningitidis* MPLA–NPhAcsTn conjugates **240–243**.

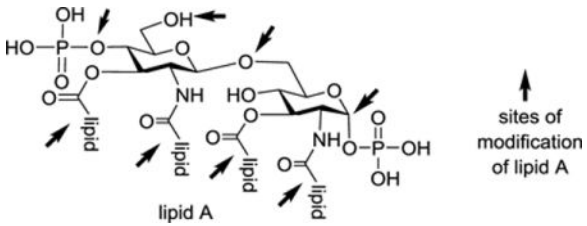


Scheme 29.

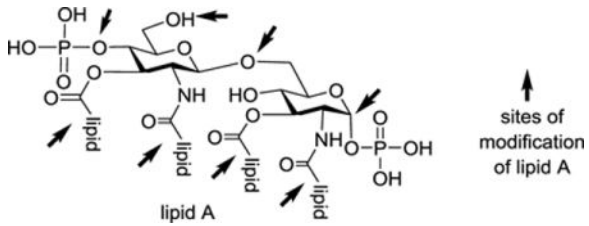
Synthesis of *N. meningitidis* MPLA-globo H conjugate **245** and the structure of KLH-globo H conjugate **246**.

Table 1

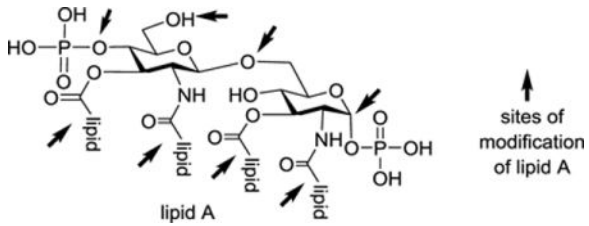
Natural lipid As and various analogs and derivatives of lipid As achieved by chemical synthesis



Compound	Bacterial origin	Group that realized the synthesis	Year	Reference	Key structural and synthetic features	Biological outcomes
Natural lipid As and lipid A derivatives bearing saturated lipids and both phosphate groups						
1	*E. coli*	Boons	2007	[43]	Natural lipid A with six acyl chains; synthesis based on an orthogonally protected disaccharide	Lower potency to induce cytokine production than *E. coli* LPS, suggesting the role of Kdo residues in LPS
10	*S. typhimurium*	Boons	2007	[43]	Natural lipid A with seven acyl chains.	Significantly lower potency to induce cytokine production than **1**
11	*E. coli*	Boons	2007	[43]	A lipid A derivative with shortened lipids	Higher potency to induce cytokine production than **1**
12	*S. typhimurium*	Boons	2007	[43]	A lipid A derivative with shortened lipids	Higher potency to induce cytokine production than **10**
13	*S. typhimurium*	Boons	2007	[43]	A lipid A derivative devoid of the anomeric phosphate group	Lower potency to induce cytokine production than **12**
		1 and **10**–**13** were synthesized by a similar strategy; their difference in biological activities indicated the importance of the number and length of the fatty acid lipid chains in lipid A				
26	*Ru. gelatinosus*	Fukase	2008	[45]	Natural lipid A with six acyl chains; synthesis by using lapidated monosaccharide blocks	Potent ability to antagonize LPS endotoxic activity.
27, **28**	*C. trachomatis*	Kosma	2004	[54]	*C. trachomatis* lipid A with tetra- or pentaacyl lipid chains.	
Natural lipid As and lipid A derivatives bearing unsaturated lipids						
34a,**b**	*R. sphaeroides*	Christ	1994	[37, 55, 56]	A derivative of natural lipid A; use of Alloc and All groups to protect the hydroxyl and phosphate groups	Devoid of LPS agonistic properties; strong *in vitro* antagonistic activity; suppress TNF-α production induced by LPS
40 (E5531)	*R. sphaeroides*	Yamatsu	1995	[57–59]	A derivative of natural lipid A with 3,3′-*O*-acyl groups substituted for hydrolytically stable ether	Strong antagonist of cytokine release of induced by LPSs; completely devoid of LPS agonistic activity; protecting BCG-primed

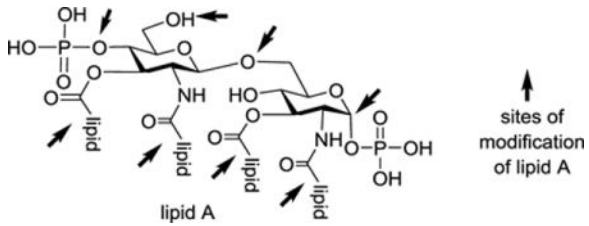


Compound	Bacterial origin	Group that realized the synthesis	Year	Reference	Key structural and synthetic features	Biological outcomes
					linkages and C-6′-hydroxyl group blocked as methyl ether	mice from LPS-induced lethality and *E. coli* infection-caused death.
41 (E5564)	*R. sphaeroides*	Christ	2002	60–62	Second generation of the above lipid derivatives	Excellent antagonist activity; clinical use in the trade name of *Eritoran* for the treatment of severe sepsis
Unnatural lipid A derivatives bearing two phosphate groups						
42–44	artificial	Savage	2003	40	Containing four lipid chains with incrementally shortened length	Different solubility in water; used to study the interaction of lipid A with polymyxin B
54–56	*E. coli*	Kusama	1990	69	Lipid A derivatives containing anomeric α/β-phosphonooxyethyl group instead of labile α-glycosylphosphate.	α-glycosides had comparable antitumor activity as natural lipid A; β-glycoside was less active
64, **65**	*E. coli*	Kusama	1991	74	Derivatives of lipid A biosynthetic precursor; containing anomeric α-phosphonooxyethyl	The first generation of potent antitumor lipid A derivatives with enhanced stability and reduced toxicity.
66, **67**	*E. coli*	Fukase	2001	75, 76	Lipid A derivatives with tritium-labeled α-phosphonooxyethyl	Useful for the study of the interactions between lipid A and their acceptors
Lipid A analogs carrying an anomeric carboxylic acid						
74a–c, **75a–c**	*E. coli*	Shiozaki	2000	79, 80	Lipid A derivatives with a carboxylic acid moiety in place of the glycosyl phosphate; **74a–c** and **75a–c** had six and four lipid chains; different 6′-*O*-substituents	**74a–c** was a LPS agonist but **75a–c** was a LPS antagonist, indicating the importance of the number of lipid chains; the carboxyl group and the 6′-*O*-substituent did not have a significant impact
92a–c, **93a–c**, **94a–c**	artificial	Shiozaki	2001	81	Anomeric carboxylic acid; unnatural 3,3′-*O*-ether linkage; different 6′-*C*-substituents; **92** and **94** had four lipid chains; **93** had six	Improved stability; **92** and **94** were strong LPS antagonists; **93b** was inactive; **93a**,**c** were moderate LPS agonists; ether linkage and anomeric carboxylic acid did not have a major impact
104a–c	artificial	Shiozaki	2003	51	Anomeric carboxymethyl group and 3,3′-*O*-ether linkages.	Improved stability; LPS antagonists; ether linkage and anomeric carboxylic acid did not have a major impact



lipid A

Compound	Bacterial origin	Group that realized the synthesis	Year	Reference	Key structural and synthetic features	Biological outcomes
111a,b–116a,b	artificial	Fukase	2006	84	Monosaccharide analogs of lipid A with aspartic acid or phosphoserine in place of non-reducing end phosphorylated glucosamine unit	Most compounds except **115a,b** showed antagonistic activity
Monophosphoryl lipid A and related derivatives						
119–121	*S. minnesota*	Jiang	2007	50	*S. minnesota* R595 MPLA derivatives; different substituents at the 3-*O*-position	All were strong adjuvants; **121** was not more toxic than natural R595 MPLA **118** that devoid of 3-*O*-acyl chain
127a-f	*S. minnesota*	Johnson	1999	52	*S. minnesota* R595 MPLA derivatives with variant fatty acids	Fatty acid chain length was important for inducing proinflammatory cytokines
133–140	*E. coli* K4	Corsaro	2016	94	Semi-synthetic lipid A analogs derived from selective modification of the C-6′-OH, lipid pattern and phosphate	Some of them, especially **136**, showed promising immunoadjuvant activity
146	*N. meningitidis*	Guo	2009	95	MPLA derivative with an amino group at the reducing end, readied for coupling with other carbohydrate antigens	Useful as a vaccine carrier for the development of self-adjuvant conjugate vaccines
147–150	*N. meningitidis*	Guo	2014	96	MPLA derivative with varied acyl chains with an amino group at the reducing end.	Used to discover new and optimized vaccine carriers and adjuvants.
156	*E. coli*	Guo	2010	46	MPLA derivative with an alkyne group at the reducing end for click reaction	Useful as a vaccine carrier for the development of self-adjuvant conjugate vaccines
157–160	*P. gingivalis*	Ogawa, Boons	2007, 2008	99, 100	Tetra-acylated MPLAs with branched lipid chains and devoid of 4′-*O*-phosphate	**159** and **160** showed potent antagonist activity, which was determined by their acylation pattern.
Non-phosphorylated lipid As and derivatives						
161–164	*R. sin*-1	Carlson, Vandenplas	2002	101, 102	Lack of both phosphate groups; aminogluconate in place of the reducing end glucosamine unit	Inhibited TNF-α release
165, 166	*R. sin*-1	Boons	2003	41	Reducing end anomeric carbon had different oxidation states	The gluconolactone unit in **166** was important for its antagonistic activity.
173, 174	*R. sin*-1	Boons	2004	42	Devoid of 3-*O*-acylation	Antagonists of LPS but less potent than **166**, indicating the biological importance of 3-*O*-acyl chain



Compound	Bacterial origin	Group that realized the synthesis	Year	Reference	Key structural and synthetic features	Biological outcomes
188, **189**	*R. sin*-1	Boons	2007	44	**189** contained the 27-hydroxyoctacosanoic group at its 2′-*N*-position	Lipid chain length, not the OH group on it, was critical for its antagonistic activity
LPS analogs and Kdo-lipid A conjugates						
199 (ReLPS)	*E. coli mutant*	Kusumoto	2001	53, 110	First chemical synthesis of a short LPS with two Kdo units linked to lipid A; Kdo fluorides with bulky 4,5-*O*-protecting groups were used for α-glycosylation	**199** showed slightly less potent cytokine-inducing activity than that of the natural ReLPS
212	*H. pylori*	Fukase.	2007	107	Lipid A-Kdo conjugate with fewer but longer acyl chains; devoid of 4-*O*′-phosphate; Kdo fluorides was used for α-glycosylation	TLR-4 antagonists; Kdo residue could enhance the inhibitory activity
221	*H. pylori*	Fukase, Fujimoto	2011	114	Lipid A-Kdo conjugate with an ethanolamine unit in its structure; Kdo *N*-phenyltrifluoroacetimidate and a microfluidic glycosylation protocol was developed and used for α-glycosylation	Inhibition of cytokine production; the presence of a Kdo unit in the structure reversed the bioactivity of its parent lipid A **220**