Total synthesis of biologically active lipoteichoic acids

Christian M. Pedersen,[a]* Mikael Bols,[a] and Yan Qiao [b]

[a]Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark
[b] State Key Laboratory of Coal Conversion, Institute of Coal Chemistry, Chinese Academy of Sciences, Taiyuan, Shanxi 030 001, PR China
E-mail: cmp@chem.ku.dk

Dedicated to Professor Richard R. Schmidt on the occasion of his 78th anniversary

DOI: http://dx.doi.org/10.3998/ark.5550190.0014.220

Abstract

Lipoteichoic acids (LTAs) are found in the Gram positive bacteria cell wall and consist of a lipid anchor connected to a repeating unit linked together with phosphate esters. Due to the resemblance with the immune active lipopolysaccharides (LPSs) found in Gram negative bacteria, there has been interest in studying their biological role. Isolation from bacteria is complicated, and has led to inconsistent conclusions. The best way to avoid biologically active impurities in the LTA is therefore chemical synthesis, but it was not until the total synthesis of *Staphylococcus aureus* LTA that a clear biological activity of synthetic material could be observed. This successful synthesis was followed up by the synthesis of derivatives as well as the preparation of two other LTAs, from *Streptococcus pneumoniae* and *Streptococcus* species DSM 8747, respectively.

Keywords: Lipoteichoic acid, total synthesis, immune activity, glycosylation, glycoconjugates

Table of Contents

1. Introduction

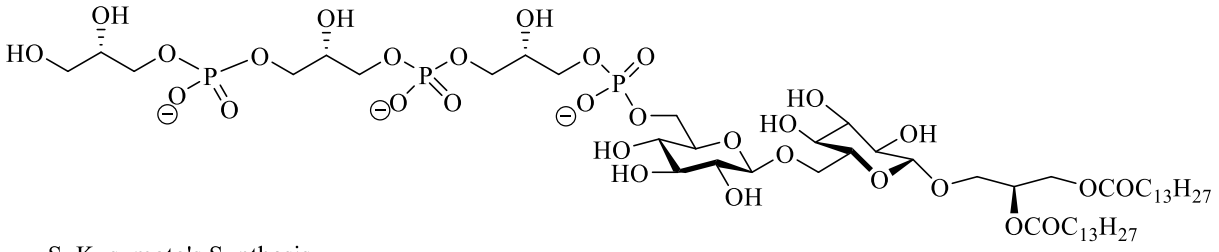
Living organisms are at constant war fighting pathogenic invasion from bacteria, viruses and fungi. Bacteria are the most common pathogens causing infection in mammalian organisms, and are responsible for well-known diseases such as tuberculosis, pneumonia and anthrax. Continuously society is faced with infections caused by bacteria in our drinking water, food, hospitals etc. Fortunately most bacterial infection can be treated by antibiotics. The intense use of antibiotics has however resulted in an increasing number of multi resistant bacteria, which makes simple infections deadly. New antibiotics and vaccines are therefore urgently needed and the importance of understanding the bacteria interaction with the immune system cannot be overemphasized.

Bacteria are divided into two main groups by the Gram stain method - Gram negative and Gram positive bacteria. Gram negative bacteria have a relative complex and thinner cell wall in contrast to the simpler and thicker cell wall in Gram positive bacteria. When the bacteria enter the organism the innate immune system is activated by a receptor recognition followed by release of cytokines. For Gram negative bacteria the biological pathway has been well understood for more than 50 years[1] and the most important actors in the cytokine release are lipopolysaccharides (LPS), which are structures found in the cell wall consisting of a lipid anchor connected to a polymeric repeating oligosaccharide. The biological mechanism of LPS has been studied in detail and it has been found that the innate immune system is trigged by binding of these structures to the toll-like receptor 4 (TLR 4), which then results in the release of cytokines.

Even though the structural counterpart of LPS in Gram positive bacteria, called lipoteichoic acid (LTA), has been known for some time, its biological role is still debated.[2] Despite intensive work in the field no clear connection between innate immune stimulatory response and LTA could be consistently proved. One of the main problems in determining the biological role of LTA has been its isolation from the cell walls. Firstly, it is very difficult to purify LTA in order

to avoid highly active components such as lipoproteins and LPS.[3] Secondly, the fragility of some LTAs has resulted in partly decomposition in the purification leading to inactive compounds.[3] Due to the obvious problems with isolating pure and active compounds from the bacteria as well as the, from a chemist point of view, very interesting structures, there have been a significant interest in synthesizing LTA during the past decades. LTA consist of a lipid anchor part, also called the core structure, which is bound to the cell membrane. The lipid anchor is connected to a repeating unit, which consists of monomers linked together with phosphate esters (ex. poly glycerol phosphates). The repeating unit is also referred to as the backbone. The first synthetic LTA derivative appeared from van Boom's group in the early 1980's with the synthesis of *Staphylococcus aureus* LTA fragment (Type I LTA).[4] More structures followed,[5,6] but no biological activity was reported.

J. H. Van Boom's Synthesis



S. Kusumoto's Synthesis

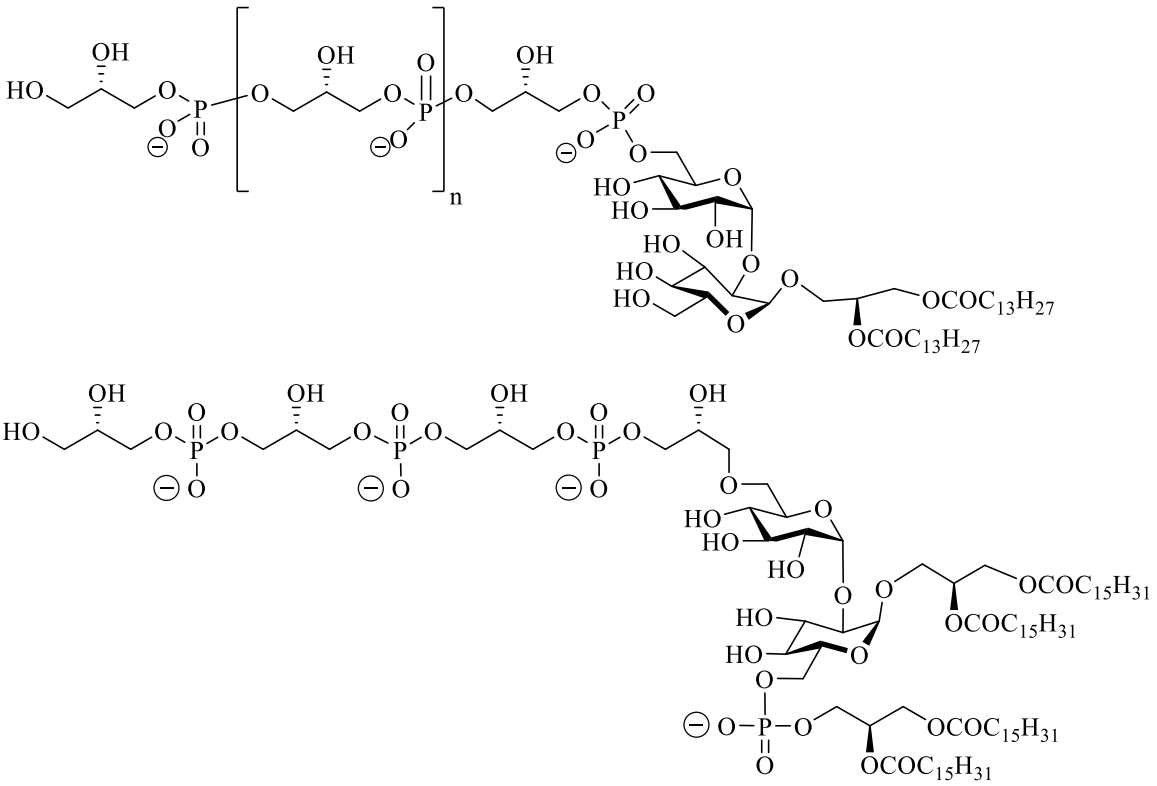


Figure 1. LTA derivatives synthesized by van Boom's and Kusumoto's groups.

In the mid 1990s Kusumoto took up the synthesis of LTA from *Streptococcus pyogenes*[7] and *Enterococcus hirae*,[8] both of which have been characterized by Fischer and Koch.[9] These LTA structures are similar to that of LTA from *S. aureus* (Type I), with a disaccharide lipid anchor connected to a repeating unit consisting of poly-glycerophosphates. The structures are very similar and mainly distinguished by an additional glycerolipid part on the lipid anchor in *E. hirae*. Despite reported anti-tumor activity of the native LTA[10] none of the synthesized LTA derivatives showed any biological activity.[11,12]

The synthesis of LTAs was again put to rest for another decade until the Schmidt group became interested in the synthesis of LTA from *S. aureus*. The renewed interest in LTA arose from biological studies performed by Hartung and coworkers in Konstanz, where it was shown that LTA induced cytokine release in a whole cell assay.[13] The biological activity was dependent on a new and milder isolation method which provided very pure and intact LTA. With renewed interest in the area and a clear target for the total synthesis, Schmidt successfully entered this area with the synthesis of *S. aureus* LTA[14] containing the *O*-alanyl substitution in the glycerophosphate repeating unit, which turned out to be crucial for biological activity.

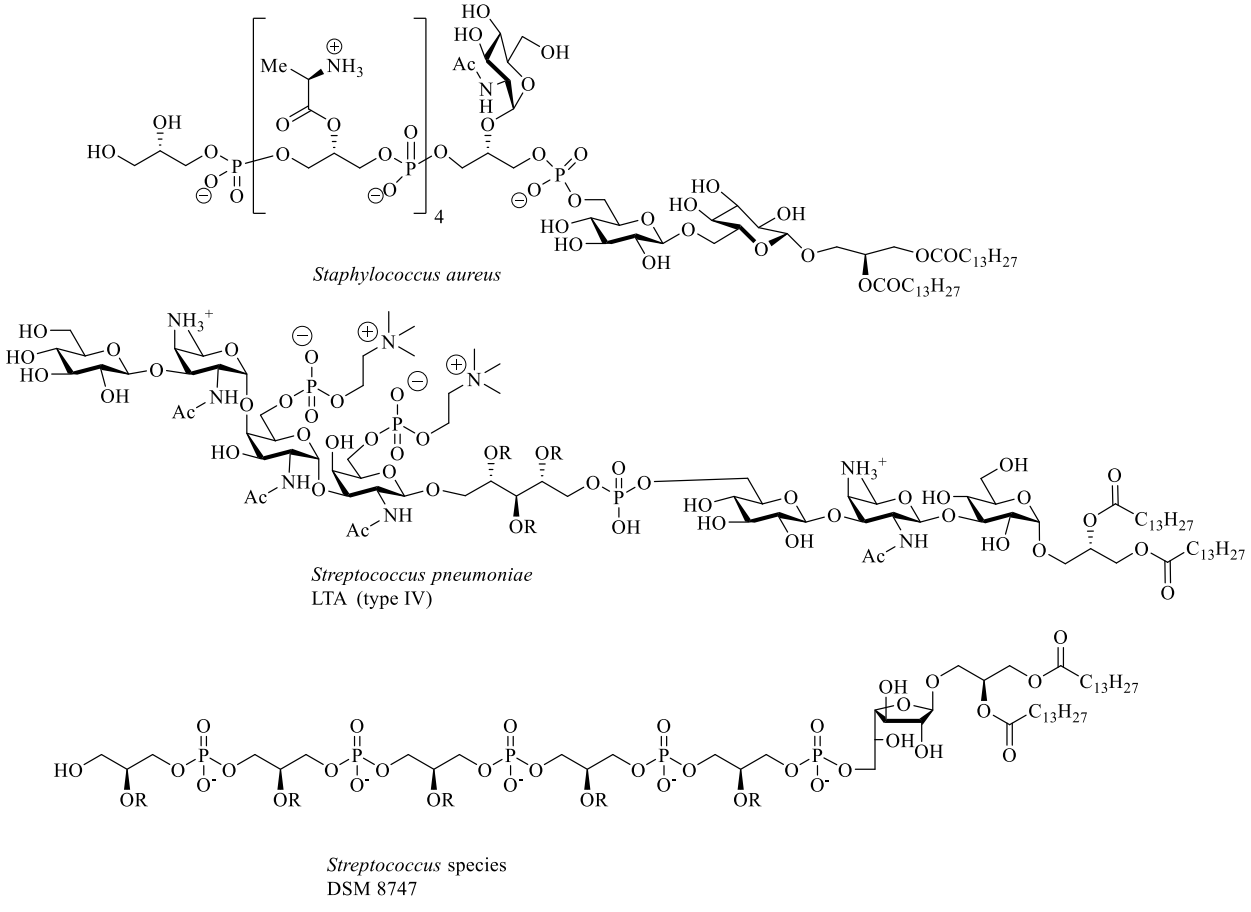


Figure 2. LTA structures synthesized by the Schmidt group.

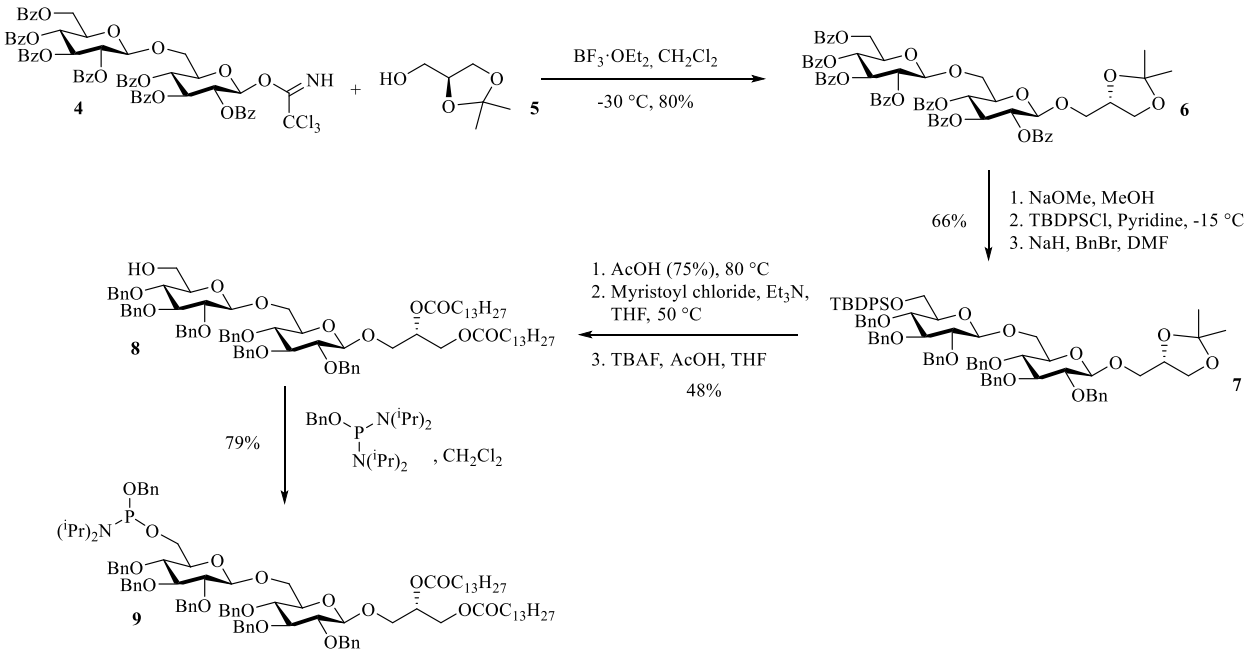
The synthesis was followed by derivatives to find the smallest structure giving activity[15-17] and the synthesis of LTA from other bacteria, where *S. pneumoniae* is a milestone. In this account the synthesis of LTA from *S. aureus*,[14] *S. pneumoniae*[18] and *Streptococcus* sp. DSM 8747[19] (Figure 2) will be discussed together with their biological activities.

2. Total synthesis of *S. aureus* LTA and derivatives

From the new preparations of LTA by the milder extraction methods and the analysis of the structures it seemed likely that the D-alanyl residues were important for biological activity, and it was therefore decided to synthesize the LTA having a partially alanylated glycerophosphate backbone (70% in the native), which had not been considered in the earlier synthesis by van Boom. From the work of the biologists it was clear that the D-alanyl residues significantly complicated the synthesis due to their instability (readily cleaved at pH 8.5) and a late installation of the groups was essential. Furthermore, a α-D-GlcNAc also had to be introduced as well as an unsubstituted hydroxyl group. This requested a new orthogonal protective group strategy for the glycerol backbone. The benzyl ether was chosen as a persistent protective group, whereas PMP and TBDPS ethers were orthogonal protective groups allowing chain extension and a late state oxidative deprotection of the PMP ethers to allow introduction of the D-alanyl residues. In the lipid anchor part control of anomeric selectivity was needed and hence benzoyl esters were used for neighboring group participation. As a temporary protective group for the 6′-OH (the linkage site to the repeating unit) TBDPS was preferred and again benzyl groups as permanent protective groups for the remaining hydroxyl groups.

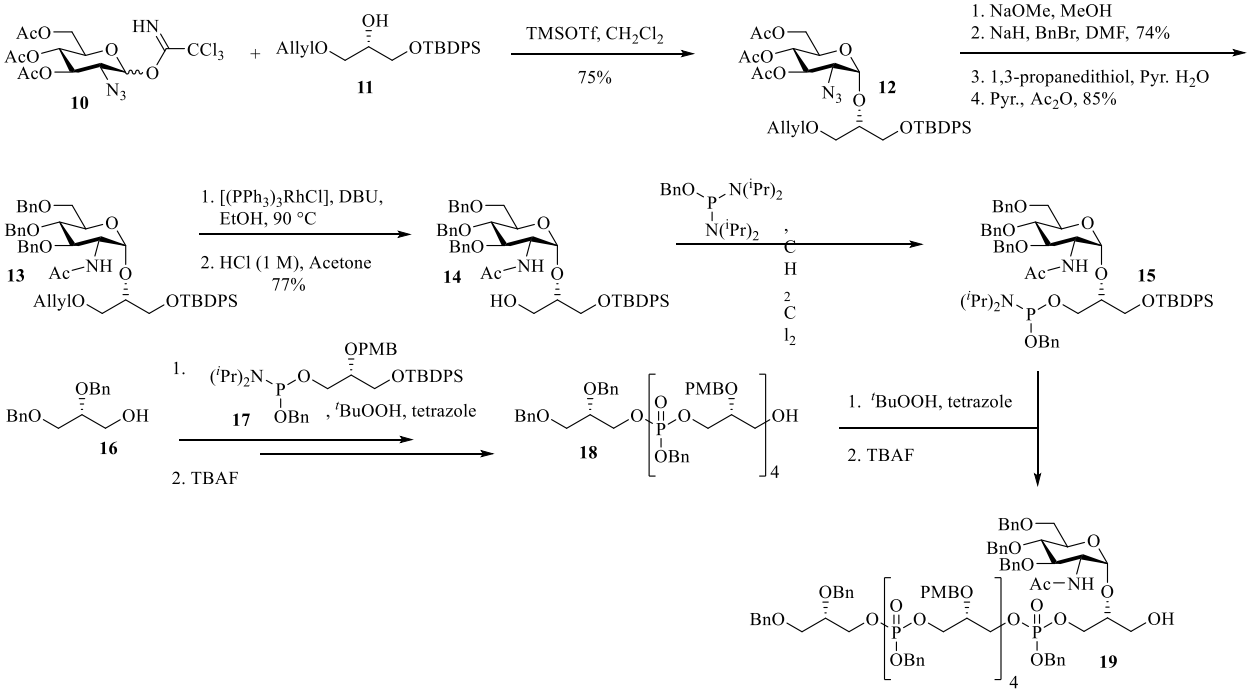
2.1 The lipid anchor

The disaccharide donor **4** was readily obtained from gentiobiose by perbenzoylation followed by anomeric ester hydrolysis. The reducing sugar was then transformed into the trichloroacetimidate **4** donor ready for $BF_3{\cdot}OEt_2$ promoted glycosylation with the commercial available 1,2-*O*-isopropylidene-*sn*-glycerol **5** to give exclusively the β-product **6** due to neighboring group participation. Debenzoylation and selective protection of the primary 6′-OH followed by benzylation of the remaining secondary hydroxyl group gave **7**. Acid mediated removal of the isopropylidene and acylation using myristoyl chloride and triethylamine in THF led to the fully protected lipid anchor. Selective removal of the TBDPS group using TBAF gave the mono-ol **8** after reaction with benzyl-oxy-bis(diisopropylamino)-phosphane furnished **9** ready for attaching the repeating unit.



Scheme 1. Synthesis of the lipid anchor from *S. aureus* LTA.

2.2 Synthesis of the poly-glycerophosphate backbone

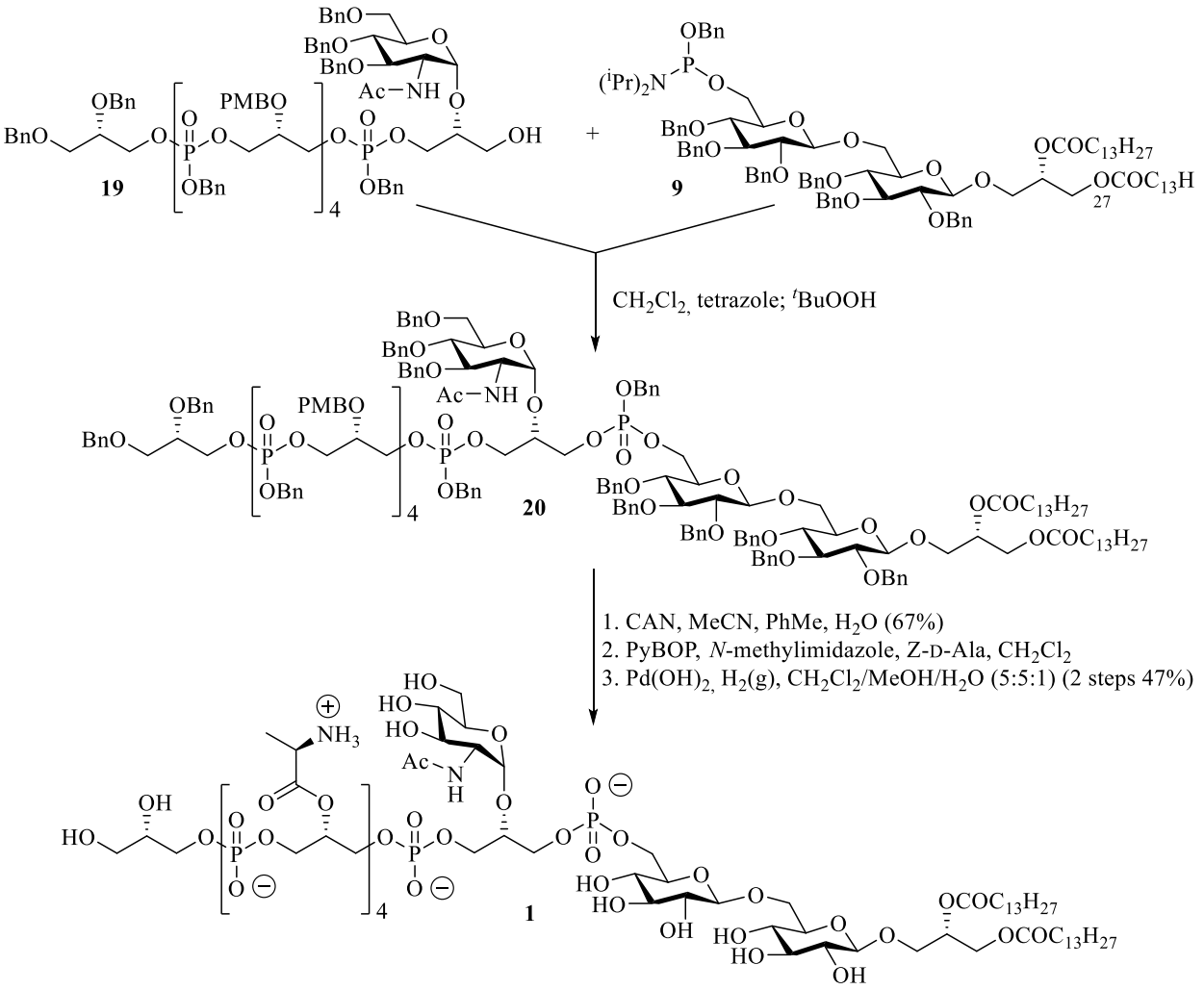


Scheme 2. Synthesis of the repeating unit of *S. aureus* LTA.

In LTA isolated from *S. aureus* the ratio between 2-*O*-residues in the repeating unit was known to be approximately 70% D-alanyl, 15% α-D-GlcNAc and 15% hydrogen i.e. approximately a ratio of 4:1:1. The smallest fragment containing this ratio is a hexamer of glycerophophates(GroPs). Since the *O*-D-alanyl residues are labile at pH 8.5 and above, a late introduction was necessary and as temporary orthogonal protective groups the *p*-methoxybenzyl group (PMB) was chosen. The unsubstituted 2-OH should be protected with the permanent protective group, and the α-D-GlcNAc should be introduced early in the synthesis to avoid late stage protective group manipulations. Three different glycerol building blocks were therefore needed; one terminal with permanent protection (**16**), one having 2-*O*-PMB protection (**17**) for late D-alanyl introduction and one having 2-*O*-α-D-GlcNAc substitution (**15**) (Scheme 2).

In order to be able to extend the GroP chain TBDPS groups were used to protect the extension site. The ligation site was equipped with benzyloxybis(diisopropylamino)phosphane, ready for tetrazole catalyzed ligation, followed by oxidation to the phosphates. The glycerol building blocks were prepared from commercial available 1,2-*O*-isopropylidene-*sn*-glycerol via allylation, deisopropylation and regioselective introduction of a TBDPS group at the primary hydroxyl group to give **11**. The free *O*-2 of **11** could be glycosylated using the triacetylated trichloracetimidate **10** having the amine masked as an azide, which secures α-selectivity under the reaction conditions. Exchange of the acetyl groups with the permanent benzyl group and reduction/acetylation of the azide provided the building block **13** ready for deallylation and phosphitylation to give **15**.

PMB protection of the 2-OH in **11**, deallylation and phophitylation gave building block **17** which was ligated to the terminal glycerol **16** using tetrazole followed by oxidation to the phosphorus triester using *tert*-butyl peroxide. The obtained dimer was desilylated followed by another cycle to give the trimer and so on. The pentamer **18** was finally ligated to the α-D-GlcNAc substituted glycerol building block **15** to give the hexamer, which after desilylation, to give **19**, was ligated to the lipid anchor part **9** using the standard procedure to give **20** in 75% yield. Selective oxidative removal of the PMB groups, using cerium(IV)ammonium nitrate liberated five hydroxyl groups in the repeating unit ready for D-alanylation using the Cbz protect amino acid and PyBOP activation. The fully protected LTA was then globally deprotected using Pearlman's catalyst ($Pd(OH)_2$) / hydrogen in CH_2Cl_2/MeOH/H_2O (5:5:1). The solvent mixture is crucial due to problems with insolubility of the partly benzylated intermediates and the formation of micelles in polar solvents. Hydrophobic interaction chromatography (HIC) provided the pure LTA **1** ready for biological studies



Scheme 3. Assembly of the LTA and global deprotection.

2.3 Derivatives

From the initial biological studies on the synthetic LTA (which will be discussed later) further investigation and hence more structural modification of the LTA were requested. In the following years a number of structures were synthesized by the Schmidt group. Most of the chemistry is closely related to the total synthesis with some protective group manipulations. Since it was clear that the D-alanyl groups played a central role in the recognition of this LTA by the immune system, the first derivative synthesized was a repeating unit having two lipid anchors (Figure 3, **24**). The hypothesis was that **24** would lead to a better exposure of the glycerophosphate (GroP) backbone and thereby better immune recognition. The synthesis again depended on the late introduction of the alanyl residues. One new orthogonal protective group was however needed in the previous mentioned terminal unit **16**. Monomethyltrityl (MMTr) was found to meet the demands since it can be introduced regioselectively on the primary hydroxyl group, is stable under the glycerophosphate chain extension cycles and can be easily removed

under mildly acidic conditions. Building up the glycerophosphate with the same substitution pattern as previous proceeded smoothly (not shown). Selective deprotection of the terminal protective groups, TBPDS and MMTr was carried out in 2 steps, both in excellent yields. Attachment of the two lipid anchors, removal of the PMB groups and D-alanylation followed the methods developed in the total synthesis **1**. Global deprotection of the benzyl groups gave the desired bis-amphiphilic compound **24** in a modest yield (Figure 3).

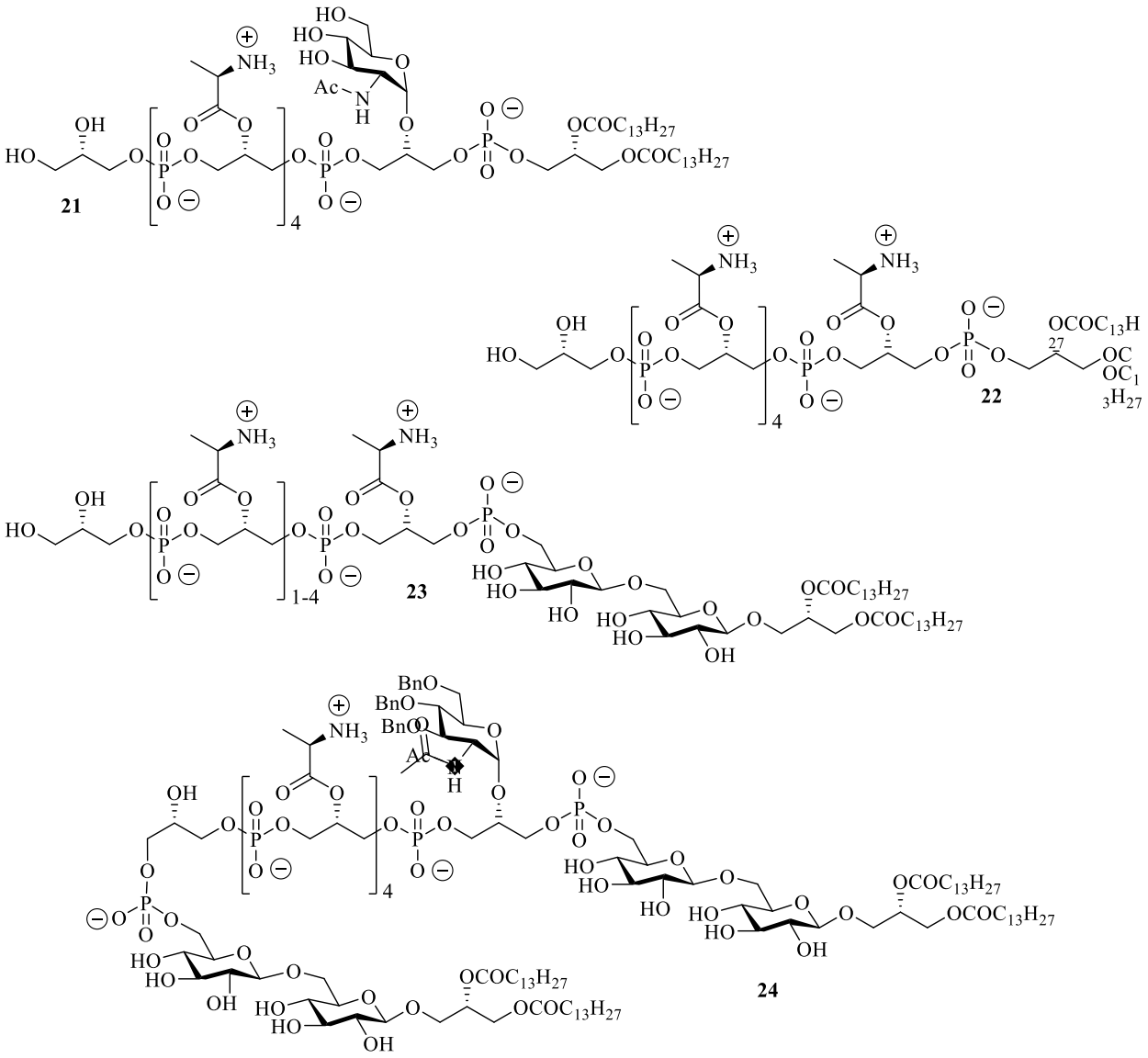
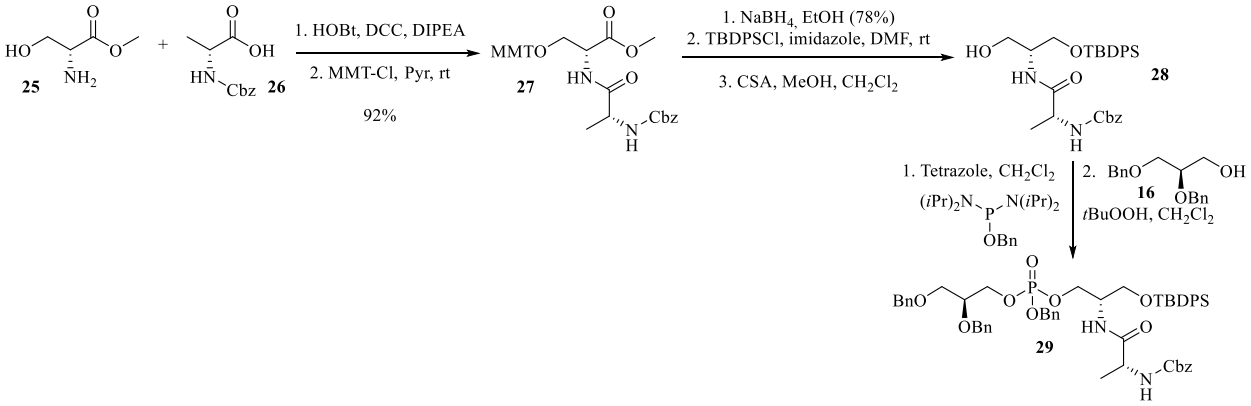


Figure 3. *S. aureus* LTA derivatives.

To elucidate the structural requirements for an immune response several other derivatives lacking one or more of the functionalities from the natural product were synthesized.[15] The chemistry was again based on the methods from the total synthesis of **1** and the building blocks

obtained there. Glycerophosphate backbones with different lengths (3-6 units, compound **23**, see Figure 3) were prepared and the α-D-GlcNAc was also left out. The lipid anchor part was minimized to contain only the diacylglycerol part directly connected to the backbone (**23**). A more drastic alteration of the LTA structure was the replacement of the ester linked D-alanyl residues with *N*-linked (i.e. amides).[15] This would enhance the stability of the alanyl residues, but require a new synthesis of the building block. Hence, starting from commercial available D-serine methyl ester **25**, which was coupled to Cbz protected alanine **26** using DCC conditions (Scheme 4); then monomethyltritylation of the hydroxyl group (to keep the chirality) followed by reduction of the ester **27** and subsequent hydroxyl group silylation (TBDPS) and MMTr removal gave the building block **28** ready for the earlier described linkage cycles.



Scheme 4. Synthesis of *N*-linked D-alanyl residues in the glycerophosphate backbone

2.4 Biological results on *S. aureus* LTA derivatives

The biological studies of the first synthetic *S. aureus* LTA were carried out by Hartung and coworkers, who were strongly engaged in the isolation of biologically active LTA from bacteria and therefore had an instrumental setup for the final purification of the amphiphilic products and study of their activity in inducing cytokine release in human whole blood cells. The biological results on synthetic LTA underlined the importance of D-alanyl residues in the glycerophosphate back-bone, which was practically as active as the native LTA.[20] Changing the residues to L-alanine decreased the activity with up to 100 times. The α-D-GlcNAc had practically no influence on activity and the lipid anchor alone did not induce cytokine release. Having two lipid anchors (**24**) on the same back-bone, however, enhanced the activity with a 10 fold.[15] Removing the gentiobiose core from the lipid anchor as well as connecting the D-alanyl residues with amide bonds having the opposite glycerol stereo chemistry, did not influence the activity significantly. Hence the D-alanyl decoration of the backbone, with five or more repeating units, together with a diacyl glycerol lipid anchor is the minimal structure for a LTA having specific biological activity.

3. Total synthesis of *S. pneumoniae* LTA

3.1 Introduction

With the total synthesis of LTA from *S. aureus* there was no more doubt about the biological activity of LTA from this species, but is this general to all LTA (as with LPS)? The structure of LTA varies a lot, even within the same species, and it became very exciting to synthesize LTA from one of the other main groups (according to Fischer[3]). LTA from *S. pneumoniae* was chosen as the next target in the Schmidt group. From a chemical point of view this LTA is a very demanding target, since in contains several rare sugar residues, phophocholine residues, a trisaccharide lipid anchor part and a repeating pseudo pentasaccharide back-bone. With these structural characteristics it represent not only one of the most complex LTAs known, but it is also very different from the other main types (I-III). As with other LTAs it is difficult to get clear answers from isolated natural material and only a synthetic compound would provide evidence for a biological activity and thereby provide insight into the activation of the immune-system by *S. pneumoniae* bacteria.

S. pneumoniae is one of the most common Gram positive pathogens and causes life threatening diseases such as pneumonia, bacteremia and meningitis.[21-23] The structure of this LTA was originally reported by Fisher and coworkers[24-26] and confirmed by other studies[27] during the years. There are minor alterations in the structures between different strains; the R6 was chosen as the target. As shown in Figure 2 the ribitol part, which is the linkage between the lipid anchor and the backbone, can contain a D-alanyl residue (not found in the R6 strain) or a α-D-GalNAc (app. 40% in this strain). The backbone has an average of two units in this strain, but up to seven in general. To meet the demands for eventual substructures for SAR studies a versatile block synthesis was developed, which allowed modifications on specific positions at a late stage in the assembly of the structure.

3.2 Lipid anchor synthesis

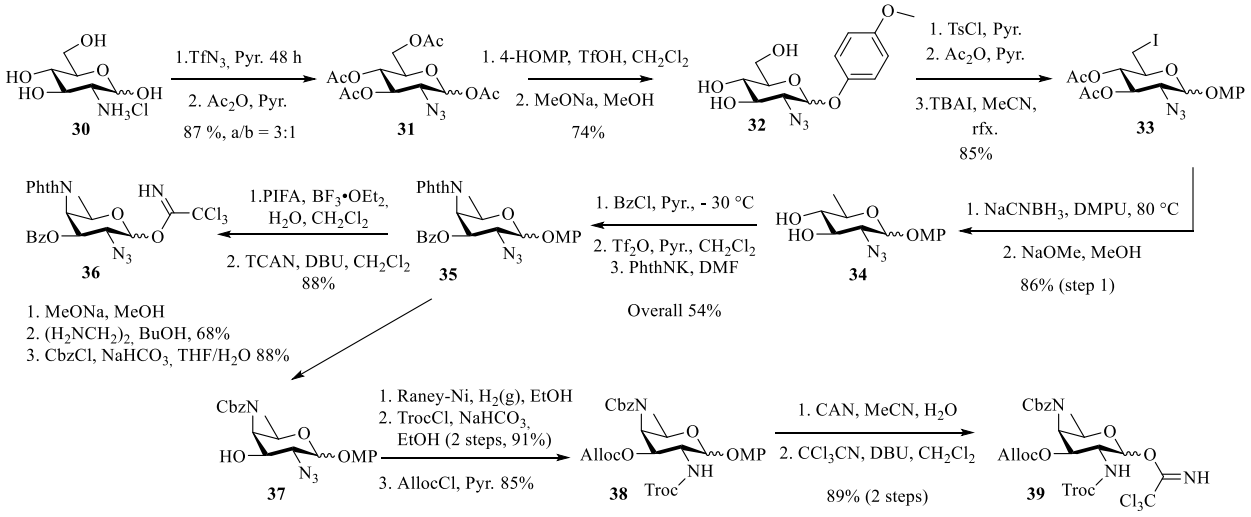
As with the total synthesis of *S. aureus* LTA benzyl groups were used as the permanent protection of hydroxyl groups and Cbz of the amino groups, which secure a one step global deprotection. Temporary protective groups in the lipid anchor were optimized during the synthesis; especially the second glycosylation (between **39** and **40** in Scheme 6) caused problems. Allyl groups were preferred as an orthogonal acid/base stabile protective group, which can be selectively removed by palladium[28] or rhodium[29] mediated enol-ether formation followed by acidic cleavage.

Four carbohydrate based building blocks were needed for the construction of the trisaccharide lipid anchor (core structure). The diacylglycerol part could be obtained from the commercial available 1,2-*O*-cyclohexylidene-*sn*-glycerol, which should be α-selectively glycosylated with a 3-*O*-allylated perbenzylated trichloroacetimidate donor (not shown). Deallylation would led to the acceptor **40**, glycosylation of this with the seldom fully protected donor **39**, 3´-*O*-deprotection and glucosylation with 6-*O*-allylated perbenzylated

trichloroacetimidate donor **43** would give the fully protected core structure **44** (Scheme 6). The 2 glucose building blocks were prepared uneventfully by standard protective group manipulations i.e. for building block **40** (Synthesis not shown**)**: 3-*O*-allylation of diacetone-glucose, acid mediated ketal cleavage, acetylation, introduction of 4-methoxy phenol (MP) in the anomeric position followed by deacetylation and benzylation. Removal of the MP group by oxidation (CAN) and treatment with trichloroacetonitrile, DBU in dichloromethane gave the desired donor. Glycosylation with 1,2-*O*-cyclohexylidene glycerol relied on anomeric control by the anomeric effect, but high selectivities were not obtained. Epimerization of the glycerol aglycon was however not observed when using the trichloracetimidate donor.[30-32] Removal of the cyclohexylidene and myristorylation provided the lipomonosaccharide, which by $PdCl_2$ mediated deallylation gave the acceptor **40** ready for glycosylation with the donor **39**.

3.3 Synthesis of the rare sugar building block AATDgal

By far the biggest challenge in the building block synthesis was the synthesis of the donors **37** and **39** in the required amounts. The 2-acetamino-4-amino-2,4,6-trideoxygalactose (AATDgal) is found both in the lipid anchor and in the repeating unit; both 1,3-linked but having different anomeric stereochemistry. In the original retrosynthesis it was proposed that the stereo chemistry could be controlled by either the anomeric effect (giving α) or by the nitrile effect[33] (β) and it would therefore be possible to use more or less the same building block in both the repeating unit and the lipid anchor. Azide was chosen as the "masking" group for the 2-amino group and this combination gave excellent α-stereo control in the back-bone synthesis, but problems in the lipid anchor synthesis.

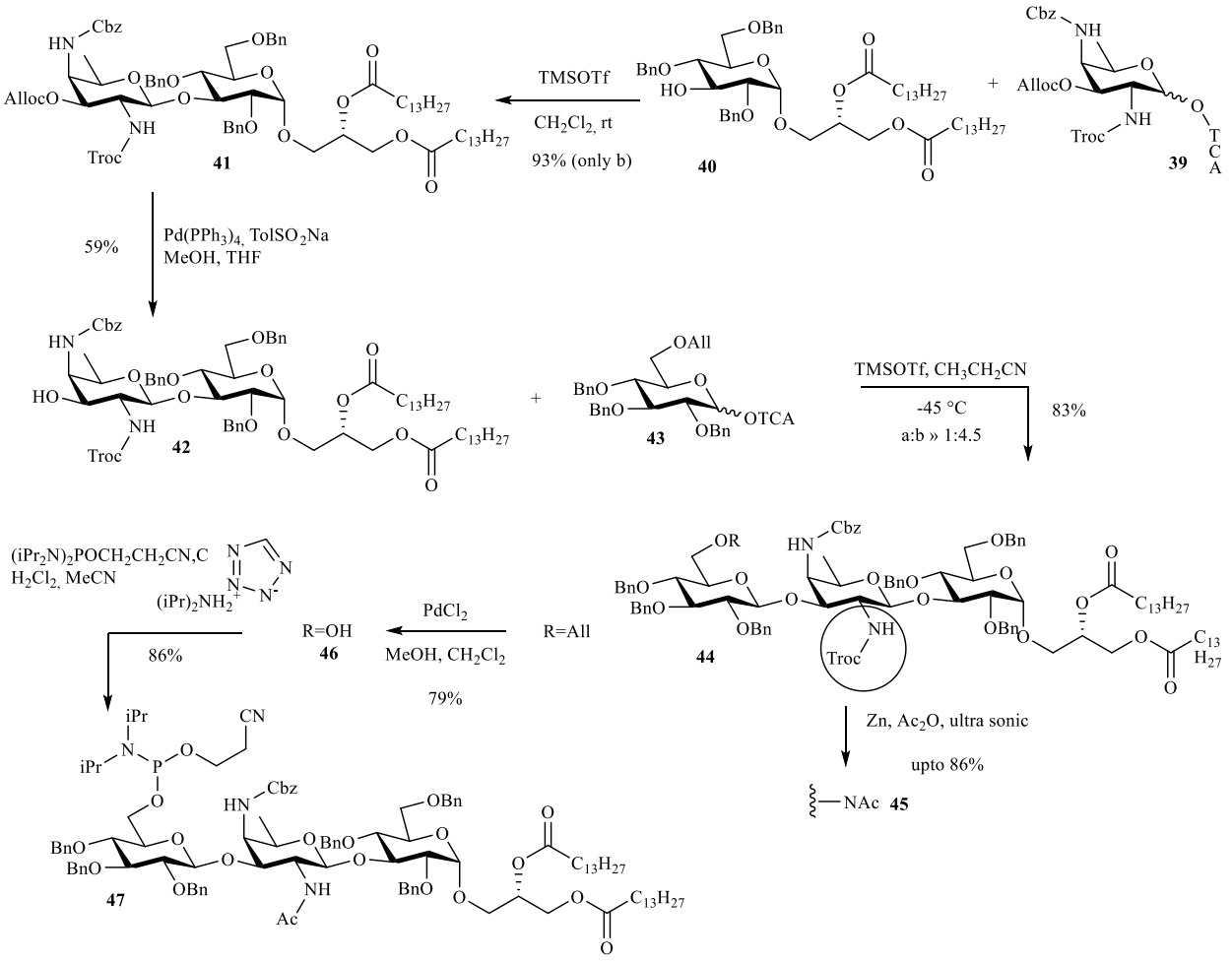


Scheme 5. Synthesis of AATDgal trichloroacetimidate donors with different protective group patterns.

After an extensive study of glycosylation conditions with limited success it was decided to redesign the donor. Starting from glucosamine **30**, which was treated with triflyl azide in water containing $CuSO_4$, per acetylated, treated with *p*-methoxyphenol together with TfOH and finally deacetylated to get the azide **32**. Two alternative routes to **36** was used, the most effective being selective 6-*O* tosylation and acetylation of the remaining hydroxyl groups. Substitution of the tosylate with iodide, to give **33**, was performed in MeCN using TBAI as the iodide source, the following reduction to the 6-deoxy, proceeded in DMPU at 80 °C using excess of $NaCNBH_3$ as the reductant. Deacetylation to give the diol **34** followed by a selective mono benzoylation of *O*-3, where starting material and side products easily could be recycled and thereby giving essentially quantitative yields. Triflation of the 4-OH and subsequent substitution with phthalimide in DMF gave **35** (Scheme 5).

Since a bulky phthalimido group at the 4 position could properly diminish β-selectivity, and be difficult to remove from the lipodisaccharide **45**, it was transformed into the Cbz protected amine **37**, by a three-step two-pot procedure. Removal of the benzoyl group, before applying the Hindsgaul conditions[34] for phthalimido deprotection (1,2-diamino ethane), turned out to be crucial for the reaction. Leaving the benzoyl group led to an intramolecular migration. The free amine was then Cbz protected and the 2-azido group reduced followed by a Troc protection and thereby securing anomeric β-selectivity. The remaining 3-OH was protected with Alloc to give **38**, which is orthogonal to Cbz, Troc and OMP.

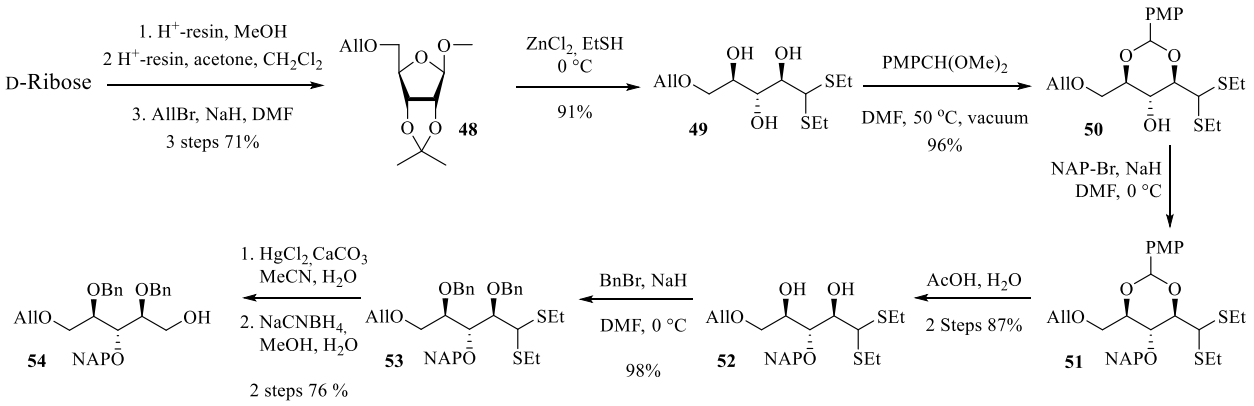
The CAN mediated oxidative deprotection of the anomeric OMP group followed by trichloroacetimidate formation provided the donor **39** for the chain elongation. Glycosylation with the lipomonosaccharide acceptor **40** provided the β-product in 93% yields (66% when using the 2-azido donor). Alloc removal using [Pd-$(Ph_3)_4$] and sodium toluenesulfinate as nucleophile gave the acceptor **42**, which upon glycosylation with the trichloroacetimidate donor **43** in nitrile solvents afforded the fully protected lipotrisaccharide **44**. The *N*-Troc protective group was exchanged with *N*-acetyl (**45**) in a one pot reaction involving Zn reduction in Ac_2O containing Et_3N.[35] The *O*-All was removed by *cat.* $PdCl_2$ in $MeOH/CH_2Cl_2$ and then treated with bis-diisopropylaminocyanoethoxyphosphine and diisopropyl ammonium tetrazolide to give the phosphite **47** ready for linkage to the pseudo-pentasaccharide repeating unit (Scheme 6).



Scheme 6. Synthesis of the lipid anchor part of *S. pneumoniae* LTA.

3.4 Synthesis of the repeating pseudo pentasaccharide

The pseudo pentasaccharide consists of five different building blocks: a ribitol part, which is connected through a phosphate to the lipid anchor part. The ribitol can furthermore contain an additional α-D-GalNAc side chain in the *S. pneumoniae* R6 strain (and D-alanyl residue in other strains). The ribitol is followed by two *N*-acetyl galactosides, the first being 1,3-β and the following 1,4-α. Both sugars contain a 6-*O*-phosphocholine residue, which would have to be installed late in the synthesis due to its high polarity. The α-GalNAc is followed by the rare sugar 2-acetamino-2-amino-2,4,6-trideoxygalactose (AATDgal), which is α–1-3 linked. The terminal sugar is a β-glucoside, which eventually need to contain an orthogonal protective group on *O*-6 in case of chain elongation.

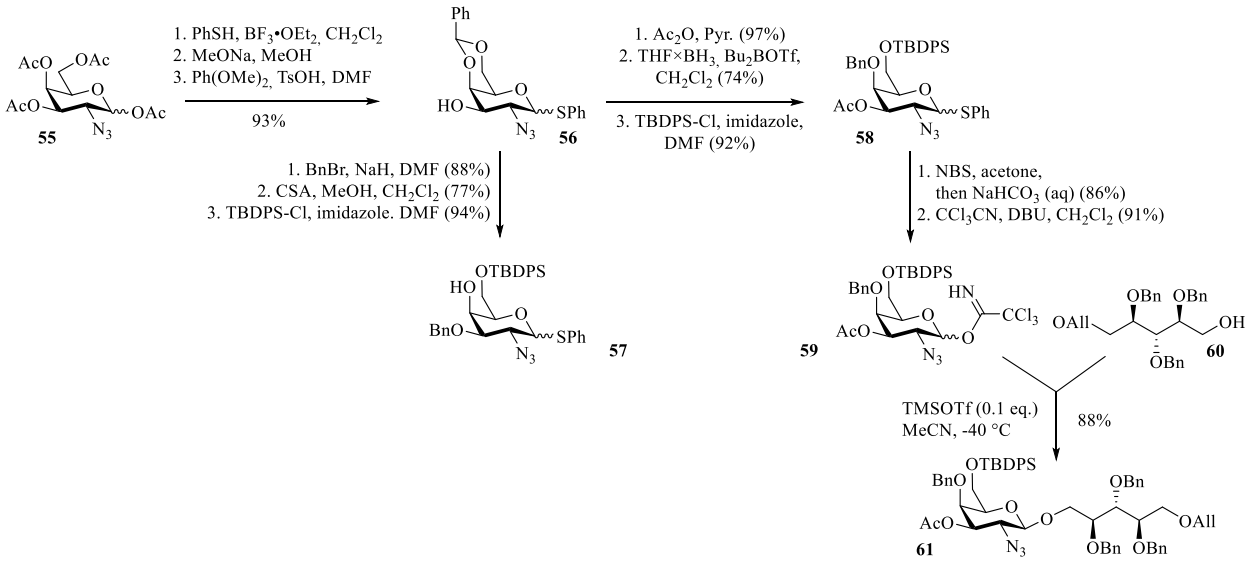


Scheme 7. Synthesis of orthogonally protected ribitol.

The ribitol part **60** had already been described in the literature[36] and was readily synthesized. A route to the building block **54** having an orthogonal protective group in the 3 position was also investigated and it was found that the naphthylmethyl (NAP) protection fulfilled the demands and could be prepared from the intermediate triol **49**, which was 2,4 tethered by acetal formation leaving the 3-OH free (**50**) for introduction of NAP. Mild acidic removal of the anisaldehyde acetal and benzylation of the diol gave **53**, which was transformed first to the aldehyde then reduced to give the acceptor **54** (Scheme 7).

Due to the different substitution pattern in the two GalNAc units, **57** and **59** (Scheme 8), different protective group strategies had to be employed. Both were prepared from the known[37,38] peracetylated 2-azido galactoside **55**, which was reacted with thiophenol under $BF_3{\cdot}OEt_2$ catalysis giving an anomeric mixture of thio-galactosides. Deacetylation and benzylidenation, leaving the 3-OH free, provided the key intermediate **56** from which building block **57** and **59** were prepared. Acetylation of 3-OH, regioselective reductive opening of the benzylidene using $BH_3{\cdot}THF$ and Bu_2BOTf in dichloromethane followed by silylation using TBDPSCl gave **58**. NBS mediated oxidative removal of the thiophenol followed by treatment with trichloroacetonitrile and DBU gave the donor **59**, which was glycosylated with the ribitol acceptor **60** taking advantage of the nitrile effect to obtain β-selectivity. Deacetylation provided the pseudo-disaccharide acceptor **61** (Scheme 8).

Benzylation of **56**, benzylidene removal and regioselective 6-*O*-silylation gave the 4-OH acceptor **57** ready for glycosylation.

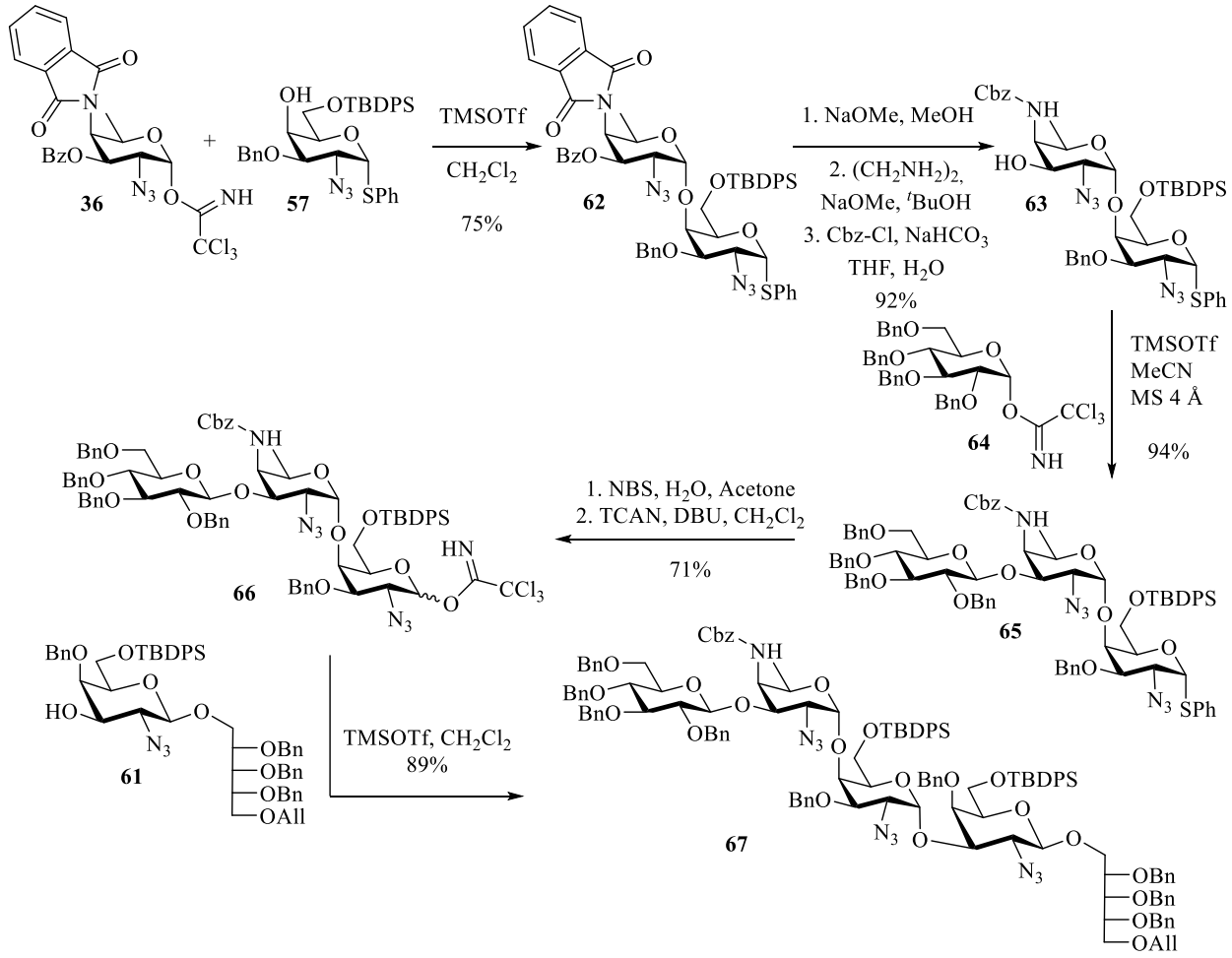


Scheme 8. Synthesis of galactosamine derived building blocks and glycosylation with the ribitol acceptor.

The donor **36** (Scheme 5) was prepared from the previous described building block **35**. The standard CAN mediated oxidative MP removal, however, turned out to be unpredictable and often low yielding. After numerous attempts to optimize the conditions it was clear that the problem had to be solved by other means. A new method was therefore developed i.e. (bis(triflouroacetoxy)iodo)benzene (PIFA) together with $BF_3{\cdot}OEt_2$ in wet dichloromethane smoothly removed the MP group without significant side reactions. The obtained reducing sugar was transformed into the trichloroacetimidate **36** and upon glycosylation with **57** in dichloromethane, an excellent α-selectivity was obtained due to the anomeric effect. Debenzoylation under Zemplén conditions, phthalimide removal and Cbz protection afforded the acceptor **63**. β-Glucosylation using the perbenzylated glucoside donor **64** taking advantage of the nitrile effect[33] gave the trisaccharide **65** in an excellent yield of 93% (Scheme 9).

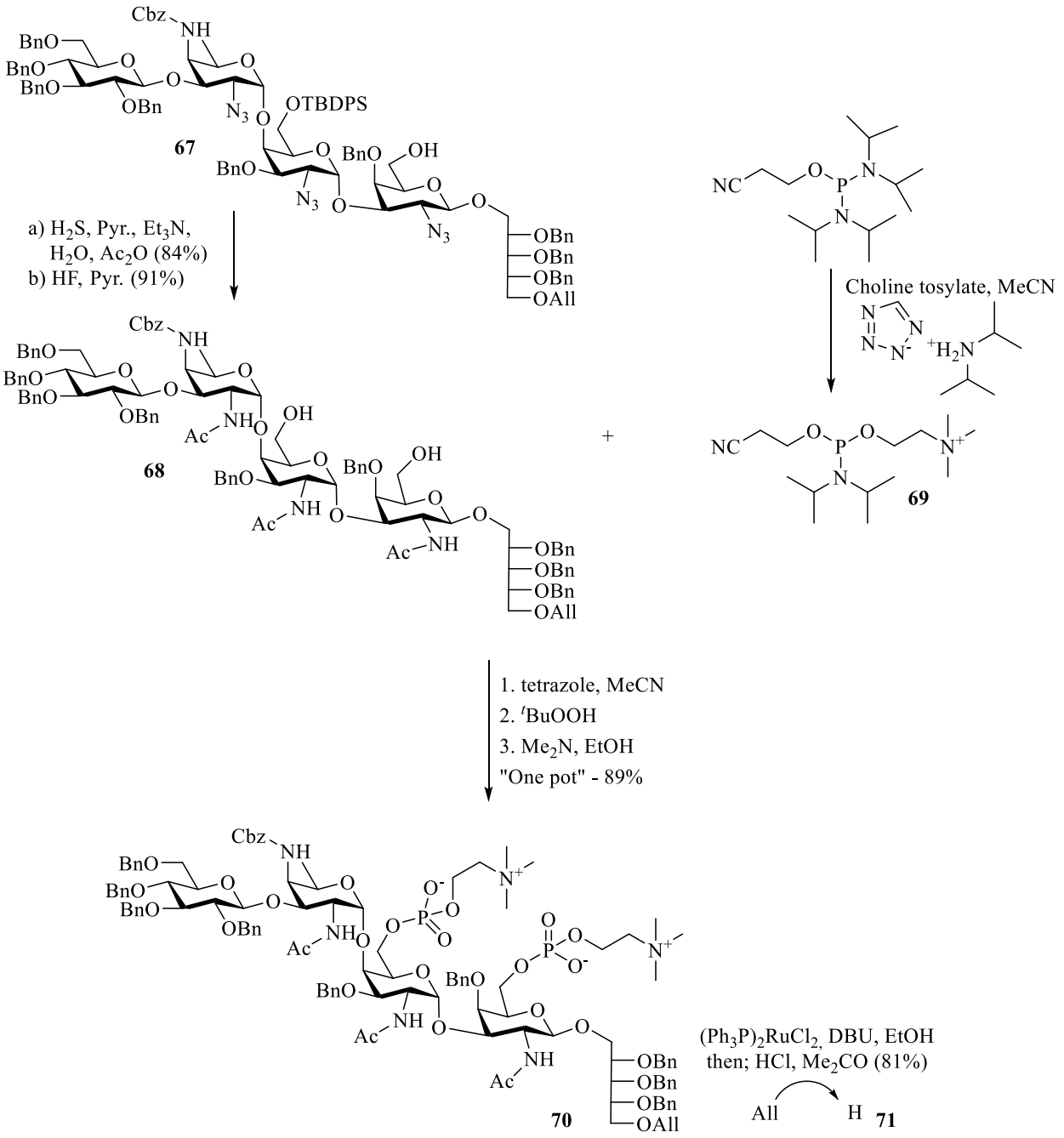
This thioglycoside was transformed into the trichloroacetimidate **66** and coupled to the pseudo disaccharide acceptor **61** taken advantage of the anomeric effect to obtain the α-linkage in an excellent yield. With the fully protected pseudo-pentasaccharide **67** in hand the last modifications before assembly of the LTA could be carried out. Transforming the azido moieties into *N*-acetyl groups was troublesome and some experimentation had to be carried out. Hydrogen sulfide in aqueous pyridine, followed by acetylation, finally provided the *N*-acetylated compound in high yields. HF/pyridine mediated TBDPS removal liberated two hydroxyl groups (**68**, Scheme 10) for introduction of the phosphocholine residues. Attempts to introduce phosphate esters or phosphites on the pseudo pentasaccharide followed by addition of choline tosylated were not fruitful and it seemed likely that macrocyclic phosphate esters were formed instead. This was overcome by preparing the phosphite reagent **69**, which together with tetrazole as catalyst provided the desired diphosphite. This was immediately oxidized to the phosphate and

the cyanoethyl group removed under basic condition to give the dicholinephosphate **70**. Deallylation using $[(Ph_3P)_2RuCl_2]$ complex as the catalyst followed by hydrolysis of the enol ether of the ribitol unit provided the desired pseudopentasaccharide **71** intermediate ready for the assembly (Scheme 10).



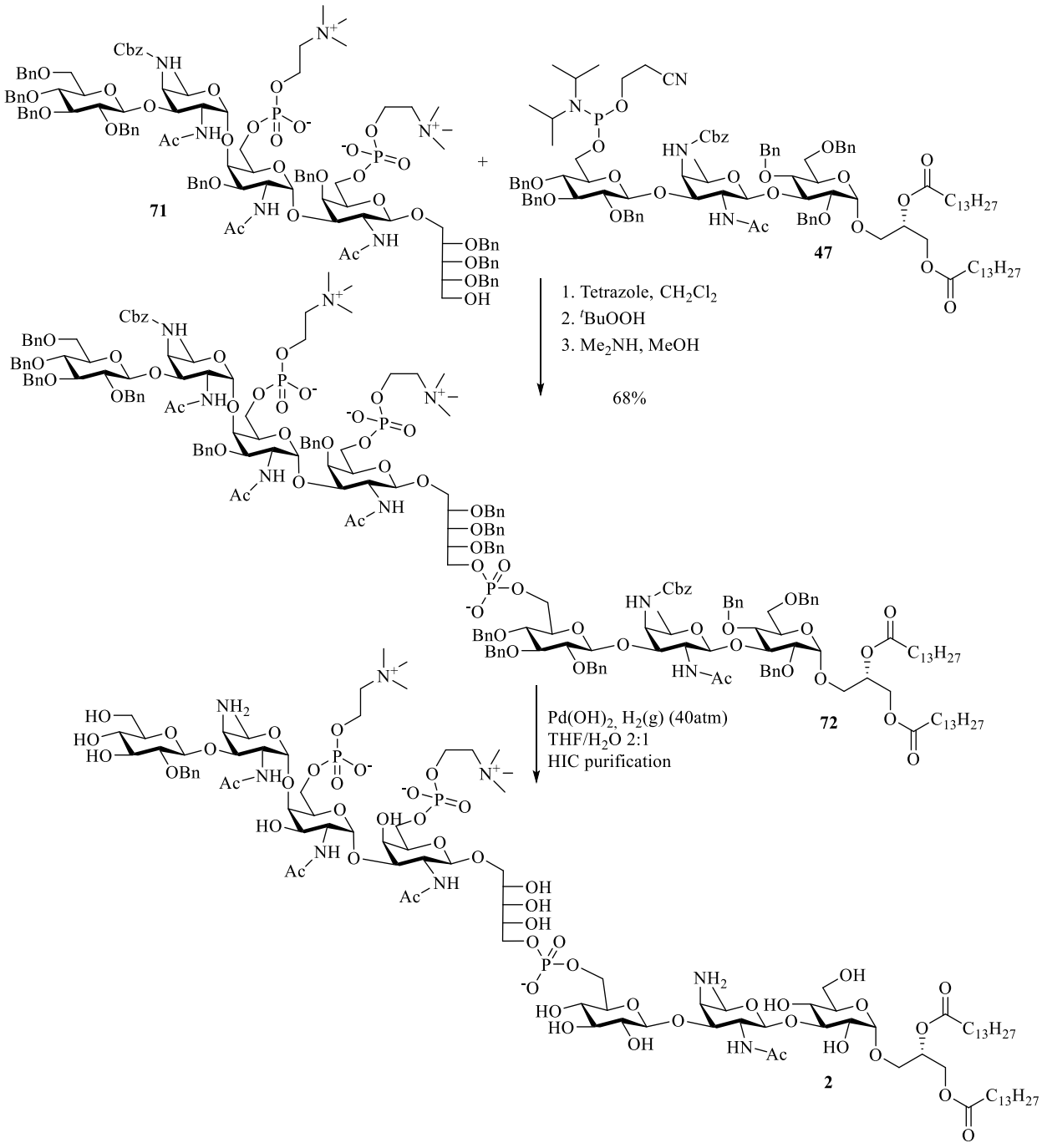
Scheme 9. Synthesis of the fully protected pseudo-pentasaccharide backbone.

The ligation between **71** and **47** was carried out using tetrazole as the catalyst, when estimated finished from LC-MS the phosphitetriester was oxidized to the phosphate and the cyanoethyl group was removed under basic conditions to give the protected **72** (Scheme 11).



Scheme 10. Synthesis of the protected pseudo-pentasaccharide backbone.

The final step – global deprotection of seventeen benzyl and Cbz groups, was carried out using Pearlman's catalyst and hydrogen under high pressure in THF/water 2:1. The crude product was isolated by hydrophobic interaction chromatography (HIC) with and ammonium acetate/ *n*-propanol gradient. NMR and ESI FT ICR MS spectroscopy were in perfect agreement with the target structure **2**. The stereochemistry of all anomeric centers could be confirmed and all essential peaks assigned.



Scheme 11. Final assembly of *S. pneumoniae* LTA and global deprotection.

3.5 Derivatives

During the total synthesis relevant substructures, i.e. the lipid anchor and the repeating unit, were deprotected and submitted for biological tests. The debenzylation of the repeating unit proceeded smoothly giving substantial amounts for biological studies. The debenzylation of the lipid anchor

part however was somewhat more troublesome, probably due to its amphiphilic nature, but it was however possible to prepare enough for biological studies.

3.6 Biological studies on *Streptococcus pneumoniae* LTA

All biological studies on *S. pneumoniae* were carried out by Prof. Zähringer and his team at Research Center Borstel. The complete as well as the sub-structures were tested for interleukin-8 (IL-8) induction by stimulation of human mononuclear cells (hMNCs) and in a whole blood assay.[2,18,45,46] Both assays revealed that the fully synthetic LTA **2** as well as the lipid anchor stimulated release of pro-inflammatory cytokines such as IL-8. The stimulation was however significant weaker the stimulation exhibited by lipopolysaccharides (LPS) and synthetic lipopeptide (Pam_3CSK_4). Neither of the preparations sensed TLR2 or TLR4/MD2/CD14 indicating that they all were free of contaminations of LPS and lipopeptides. The results clearly state that TLR2 (or TLR4) is not the receptor for this LTA. Since the activity of the lipid anchor is about as high as that of the complete LTA, this part of the molecule must be responsible for the interaction with a receptor causing the immune response. The most likely receptor so far is the lectin pathway of the complement system. The findings were supported with the fact that the pseudo pentasaccharide alone does not induce cytokines in the assays; hence the lipid anchor is crucial for recognition and thereby activity. The earlier suggested TLR-2 related activation in native LTA isolates was probably due to small impurities of highly active lipopeptides or LPS, which were not present in the synthetic preparations.

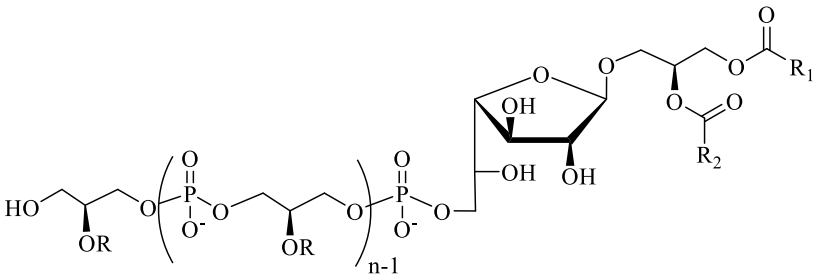
4. Total synthesis of *Streptococcus* Species DSM 8747 LTA

4.1 Introduction

The *Streptococcus* species DSM 8747 is homologous with *S. pneumoniae*; but contains a structurally totally different LTA, also referred to as LTA-T, in its cell wall.[39,40] LTA-T (Figure 4) comprises a linear (1–3)-linked poly(glycerophosphate) chain which is partly substituted with 2-*O*-D-alanyl residues; this part is phosphodiester linked to the 6-hydroxy group of 3-*O*-(β-D-galactofuranosyl)-1,2-di-*O*-acyl-*sn*-glycerol; thus, LTA-T belongs to the type I LTA structures. LTA-T is interesting in two aspects: firstly it only contains ten glycerophosphates (GroP) in length with about three D-alanyl residues attached on average, which is the shortest hydrophilic GroP chains known by now; secondly the core of the lipid anchor contains a rare *mono*-hexosyl-1,2-diacyl-*sn*-glycerol glycolipid in Gram-positive bacteria.

Previous studies of *Staphylococcus aureus* LTA revealed that a backbone length of about five to six residues is sufficient for biological activity. Hence, LTA-T having five glycerophosphate moieties with up to four D-alanyl residues attached before final deprotection was synthesized. Thus, the hydrolytic lability of the D-alanyl residues and their importance for biological activity has been taken into account and after workup of the target compound at least

the average number of D-alanyl residues should be available (~30%, n ≈ 1.5 D-Ala residues). For the acyl chain, myristoyl (C14) residues should be introduced.

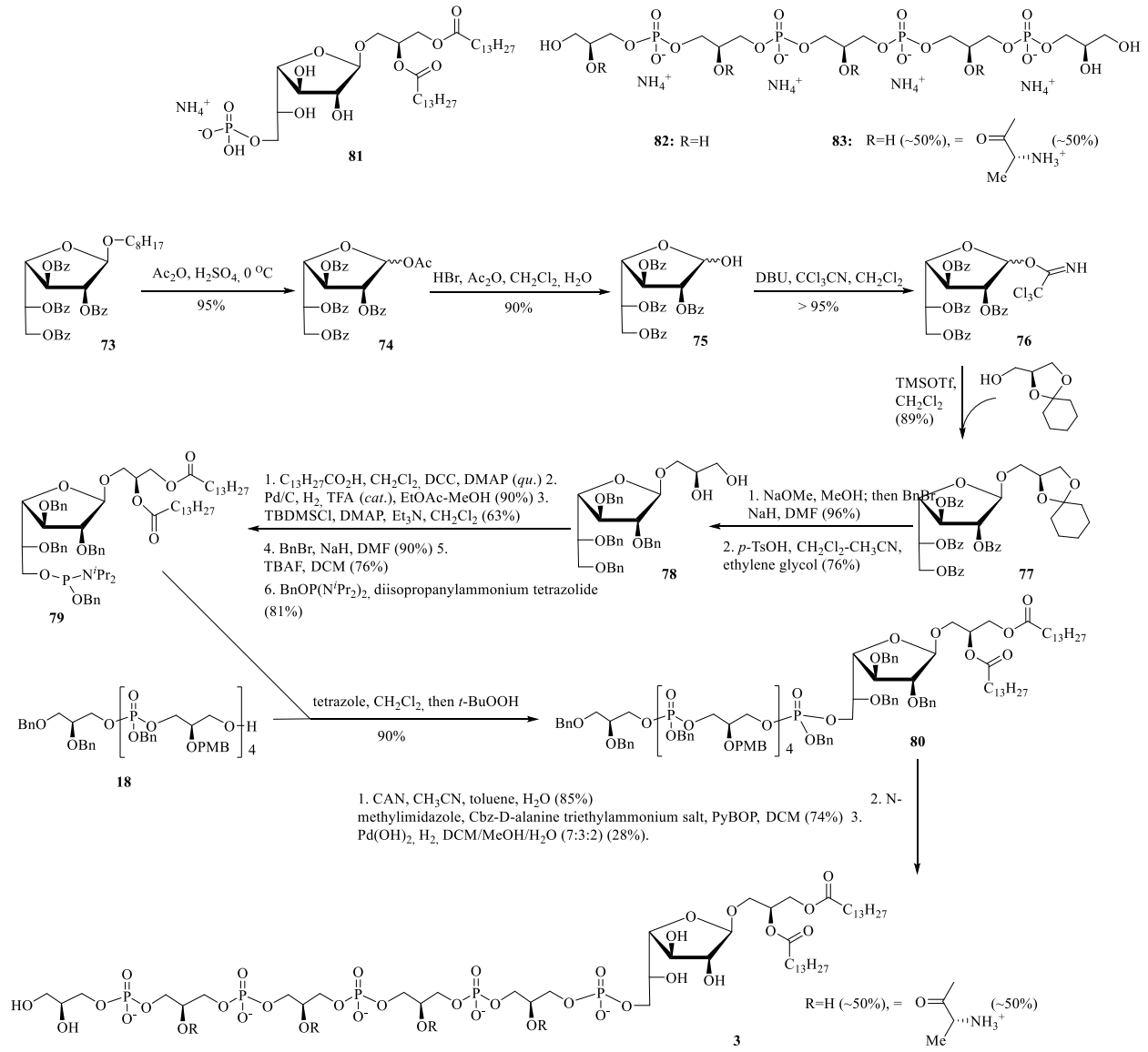


n ~ 7-17, average ~ 10
R: ~ 30% D-Ala, ~ 70% H
$R_{1,}$ R_2: C_{11} (~ 6%), C_{13} (~ 23%), C_{15} (~ 47%), C_{17} (~ 23%) including unsaturation

Figure 4. Structure of LTA-T.

4.2 Synthesis

The preparation of LTA-T consists of two parts (Scheme 12): a rare *mono*-hexosyl-1,2-diacyl-*sn*-glycerol lipid anchor **79** and GroP repeating unit **18**. The crucial step of lipid anchor synthesis is to achieve 2,3,5,6-tetra-*O*-benzoyl-α,β-D-galactofuranosyl acetate **74**. Acetolysis of octyl β-D-galactofuranoside **73** with acetic anhydride/sulfuric acid has earlier been investigated and found unsuccessful,[41] in our hands, however, the reaction was successful and a short and efficient synthesis was developed. **74** was then transformed to an 1-bromide derivative with HBr, followed by hydrolysis to yield 1-*O*-unproteced **75** in one pot. With Schmidt glycosylation, desired β-D-galactofuranoside **77** was obtained in high yield. In order to avoid migration in this molecule, the benzoyl groups were replaced by other protective groups and then the lipid anchor **79** was synthesized with standard procedure. GroP repeating unit **18** was obtained from intermediates **16** and **17** by the same procedure showed in Scheme 2. Lipid anchor **79** was coupled with **18** in the presence of tetrazole as catalyst afforded the phosphite triester intermediate that was oxidized with *tert*-butyl hydroperoxide to the corresponding phosphate **80** and obtained as a mixture of diastereomers. Oxidative cleavage of the PMB groups with CAN in an acetonitrile/toluene/water mixture liberated four hydroxy groups of the GroP backbone to allow the introduction of four D-alanyl residues in the beginning. Hydrogenolytic cleavage of the six *O*-benzyl and four Cbz groups was performed with Pearlman's catalyst in a mixture of dichloromethane/methanol/water, and after HIC with an ammonium acetate/*n*-propanol gradient (from 85:15 to 40:60) pure target compound **3** was obtained. However, NMR and MS data showed that only about two *O*-alanyl residues attached in the final product **3**. This means two D-alanyl were lost in the deprotection and/or purification procedure, LTA-T seems to be more sensitive to *O*-de-alanylation than the LTA of *Staphylococcus aureus*. Until now no better solution has been found to avoid this de-alanylation problem.



Scheme 12. Synthesis of LTA-T.

LTA-T is postulated to have cytokine-inducing, antitumor and antigenic activities.[10,42-44] Therefore, LTA-T and its analogues (Figure 5**)** were synthesized for LUNAMed AG, Switzerland to run the biological tests in *vivo*, including induce cytokine production, therapy or control malignant pleural effusion (pleurodesis). So far no biological data have been published.

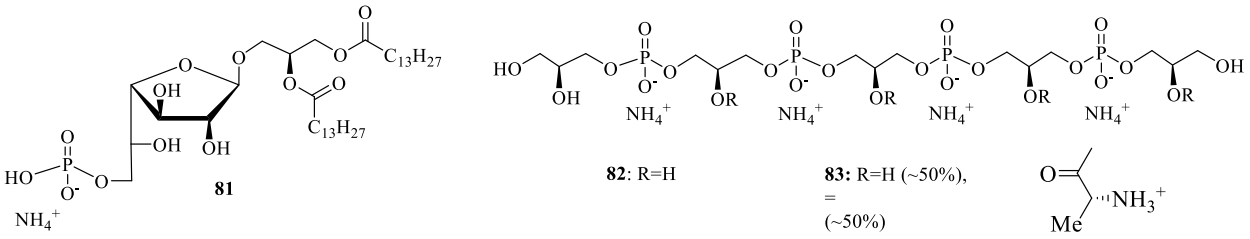


Figure 5. Analogues of LTA-T.

5. Conclusions

Total syntheses of LTAs from three bacteria species (*S. aureus*, *S. pneumoniae* and *Streptococcus* DSM 8747) have been accomplished and thereby demonstrated that access to highly pure material without any biological contamination is possible. The synthetic LTA from *S. aureus* and *S. pneumoniae* confirmed the structures of the natural isolates and were both studied in several biological assays. The importance of D-alanyl residues, in *S. aureus* LTA backbone, for the biological activity was underlined. Examination of *S. pneumoniae* LTA revealed that TLR2 cannot account for the pro-inflammatory effect of this LTA and from the studies the lectin pathway of the complement system seems to be the receptor. The work on LTA synthesis from the Schmidt group has opened the field and further studies are urgently needed in order to meet the demands for new treatments of bacterial infections caused by Gram positive bacteria.

Acknowledgements

The Danish Agency for Science, Technology and Innovation is kindly acknowledged for their support.

References

1. Westphan, O.; Lüderitz, O.; Keiderling, W. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.* **1952**, 152.
2. For discussion of the biological activity see: Schmidt, R. R.; Pedersen, C. M.; Qiao, Y.; Zähringer, U. *Org. Biomol. Chem.* **2011**, *9*, 2040.
3. Fischer, W.; Koch, U. In *Chemistry and Biological properties of Bacterial Surface Amphiphiles*; Shockman, G. D.; Wicken, A. J. Eds., Academic Press, New York, 1981, pp 181-194.
4. Oltvoort, J. J.; van Boeckel, C. A. A.; de Koning, J. H.; van Boom, J. H. *Synthesis* **1981**, 305.

5. Oltvoort, J. J.; van Boeckel, C. A. A.; de Koning, J. H.; van Boom, J. H. *Recl. Trav. Chim. Pays-Bas* **1982**, *101*, 87.
6. Oltvoort, J. J.; Klosterman, M.; van Boeckel, C. A. A.; van Boom, J. H. *Carbohydr. Res*. **1984**, *130*, 147.
7. Fukase, K.; Matsumoto, T.; Ito, N.; Yoihimura, T.; Kotani S.; Kusumoto, S. *Bull. Chem. Soc. Jpn*. **1992**, *65*, 2643.
8. Fukase, K.; Yoshimura, T.; Kotani, S.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 473.
9. Fischer, W. In *Handbook of Lipid Research*, Vol. 6, Kates, M. Ed. Plenum Press: New York, 1990, 123-234.
10. Tsutsui, O.; Kokeguchi, S.; Matsumura, T.; Kato, K. *FEMS Microbio. Immun*. **1991**, *76*, 211.
11. Suda, Y.; Tochio, H.; Kawano, K.; Takada H.; Yoshida T.; Kotani S.; Kusumoto S. *FEMS Immunol. Microbiol.* **1995**, *12*, 97.
12. Takada, H.; Kawabata, Y.; Arakaki, R.; Kusumoto, S.; Fukase, K.; Suda, Y.; Yoshimura, T.; Kokeguchi, S.; Kato, K.; Komuro, T.; *Infect. Immun.* **1995**, *63*, 57.
13. Morath, S.; Geyer, A.; Hartung, T.; *J. Exp. Med*. **2001**, *193*, 393.
14. Stadelmaier, A.; Morath, S.; Hartung, T.; Schmidt, R. R. *Angew. Chem. Int. Ed.* **2003**, *42*, 916.
15. Figueroa-Perez, I.; Stadelmaier, A.; Deininger, S.; von Aulock, S.; Hartung, T.; Schmidt, R. R. *Carbohydr. Res*. **2006**, *341*, 2901.
16. Stadelmaier, A.; Figueroa-Perez, I.; Deininger, S.; von Aulock, S.; Hartung, T.; Schmidt, R. R. *Bioorg. Med. Chem.* **2006**, *14*, 6239.
17. Figueroa-Perez, I.; Stadelmaier, A.; Morath, S.; Hartung, T.; Schmidt, R. R. *Tetrahedron: Asymmetry* **2005**, *16*, 493.
18. Pedersen, C. M.; Figueroa-Perez, I.; Lindner, B.; Ulmer, A. J.; Zähringer, U.; Schmidt, R. R. *Angew. Chem. Int. Ed.* **2010**, *49*, 2585.
19. Qiao, Y.; Lindner, B.; Zähringer, U.; Truog, P.; Schmidt, R. R. *Bioorg. Med. Chem*. **2010**, *18*, 3696.
20. Morath, S.; Stadelmaier, A.; Geyer, A.; Schmidt, R. R.; Hartung, T. *J. Exp. Med.* **2002**, *195*, 1635.
21. Zangwill, K. M.; Vadheim, C. M.; Vannier, A. M.; Hemenway, L. S.; Greenberg, P. D.; Ward, J. I. J. *Infect. Dis.* **1996**, *174*, 752.
22. Jedrzejas, M. J. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 187.
23. Lowy, F. D. *N. Engl. J. Med.* **1998**, *339*, 520.
24. Fischer, W.; Behr, T.; Hartmann, R.; Peter-Katalinic, J.; Egge, H. *Eur. J. Biochem*. **1993**, *215*, 851.
25. Greenberg, J. W.; Fischer, W.; Jonier, K. A. *Infect. Immun.* **1996**, *64*, 3318.
26. Fischer, W. *Microb. Drug Resist.* **1997**, *3*, 309.
27. Draing, C.; Pfitzenmaier, M.; Zummo, S.; Mancuso, G.; Geyer, A.; Hartung, T.; von Aulock, S. *J. Biol. Chem.* **2006**, *281*, 33849.
28. Codée, J. D.; Hossain, L. H.; Seeberger, P. H. *Org. Lett.* **2005**, *7*, 3251.

29. Corey, E. J.; Suggs, J. W. *J. Org. Chem.* **1973**, *38*, 3224.
30. For 2-epimerization of glycerol during glycosylation see: Imai, H.; Oishi, T.; Kikuchi, T.; Hirama, M. *Tetrahedron* **2000**, *56*, 8451-8459, and also references 31 and 32.
31. Shingu, Y.; Nishida, Y.; Dohi, H.; Matsuda, K.; Kobayashi, K. *J. Carbohydr. Chem.* **2002**, *21*, 605.
32. Shingu, Y.; Nishida, Y.; Dohi, H.; Kobayashi, K. *Org. Biomol. Chem.* **2003**, *1*, 2518.
33. Schmidt, R. R.; Behrendt, M.; Toepfer, A. *Synlett* **1990**, 694.
34. Kanie, O.; Crawley, S. C.; Palcic, M.; Hindsgaul, O. *Carbohydr. Res*. **1993**, *243*, 139.
35. Zhu, X.; Schmidt, R. R. *Synthesis* **2003**, 1262.
36. Qui, H.; Grindley, T. B. *Can. J. Chem.* **1999**, *77*, 481.
37. Alper, P. B; Hung, S.-C; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029.
38. Yan, R.; Wang, F.; Wu, Y.; Zhang, L.-H.; Ye, X. S. *Tetrahedron Lett*. **2005**, *46*, 8993.
39. Roethlisberger, P. PhD Thesis, ETH Zurich, Switzerland, 1995.
40. Roethlisberger, P.; Iida-Tanaka, N.; Hollemeyer, K.; Heinzle, E.; Ishizuka, I.; Fischer, W. *Eur. J. Biochem.* **2000**, *267*, 5520.
41. Ferrières, V.; Roussel, M.; Gelin, M.; Plusquellec, D. J. *Carbohydr. Chem.* **2001**, *20*, 855.
42. Takada, H.; Kawabata, Y.; Arakaki, R.; Kusumoto, S.; Fukase, K.; Suda, Y.; Yoshimura, T.; Kokeguchi, S.; Kato, K.; Komuro, T. *Infect. Immun*. **1995**, *63*, 57.
43. Truog, P.; Roethlisberger, P. U.S. Patent 6 214 978, 2001.
44. Rahman, N. M.; Davies, H. E.; Salzberg, M.; Truog, P.; Midgely, R.; Kerr, D.; Clelland, C.; Hedley, L.; Lee, Y. C. G.; Davies, R. J. O. *Lancet. Oncol.* **2008**, *9*, 946.
45. Pedersen, C. M.; Figueroa-Perez, I.; Boruwa, J.; Lindner, B.; Ulmer, A. J.;Zähringer, U.;Schmidt, R. R. *Chem. Eur. J.* **2010**, *16*, 12627.
46. Pedersen, C. M.; Figueroa-Perez, I.; Ulmer, A. J.; Zähringer, U.; Schmidt, R. R. *Tetrahedron* **2012**, *68*, 1052.

Authors' Biographies

Christian Marcus Pedersen studied chemistry at the University of Aarhus from where he received his PhD in 2007 under the supervision of Professor Mikael Bols. During the PhD studies he was a visiting scientist at University of Illinois at Chicago performing research with Professor David Crich. Postdoctoral studies were carried out at University of Konstanz in the laboratories of Professor Richard R. Schmidt working on the total synthesis of lipoteichoic acid from *Streptococcus pneumoniae*. He is currently assistant professor at the University of Copenhagen, where he is working on the total syntheses of lipoteichoic acids and in various fields of carbohydrate chemistry.

Mikael Bols was born in 1961 in Copenhagen, Denmark. He received his M.Sc. (1985) and Ph.D. (1988) from the Technical University of Denmark under the Supervision of Professor Inge Lundt. After a Postdoctoral stay in 1988-9 at Queen's University, Canada with Professor Walter Szarek he joined Leo Pharmaceutical Products, where he was a research chemist from 1989-91. He then returned to an assistant professorship at the Technical University of Denmark . In 1994 he did a sabbatical stay at Columbia University in Professor Gilbert Stork's group. In 1995 he went to Aarhus University where he in 2000 became a full Professor. Finally in 2007 he assumed his present position as head of the chemistry department of University of Copenhagen. His research interests are medicinal chemistry, carbohydrates and artificial enzymes.

Yan Qiao received her Ph.D. degree (2007) in organic chemistry from the Institute of Chemistry (Beijing), Chinese Academy of Sciences (CAS). Her PhD work focused on the synthesis of lipid A. From July 2007 to June 2009, she worked in Professor Richard Schmidt's team as a Postdoctoral research fellow and her project was involved in the synthesis of lipoteichoic acid. During July 2009 to February 2011, she was Postdoctoral Scholar in LCMD-ESPCI (Paris) and worked on the preparation of amphiphilic polysaccharide and their applications in surface modification. Now she is an associate Professor of the Institute of Coal Chemistry, CAS. Her research interests mainly include two parts; one about the solid CO_2 absorbents and the other about the application of NMR techniques in mechanism studies for biomass conversion procedures.

