Synthetic methods to glycerol teichoic acids

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Chapter 1

General introduction

Teichoic acids: An introduction

Teichoic acids (TAs, see Figure 1), a prominent class of phosphodiester containing compounds of bacterial origin are the topic of this thesis. TAs, the name of whom is derived from the Greek "Teichos", or city wall, are important components of the cell wall of Gram-positive bacteria. TAs comprise 30-70% of the walls of these bacteria and consist mostly of alditol (glycerol, ribitol) phosphates, non-stoichiometrically functionalized with D-alanyl and carbohydrate moieties.[1-6] TAs are involved in several functions necessary for survival of the bacterial species. In addition, TAs are known to interact with the host immune system, although the molecular mode of action of this process has yet to be established. Structure activity relationship studies could provide more insight in the immunological behavior of TAs but remain a difficult undertaking as TAs isolated from biological sources generally consist of a microheterogenous mixture of compounds varying in length, glycosylation/alanylation pattern, fatty esters, linkage units etc. Well-defined fragments which can be obtained by means of chemical synthesis can be a valuable tool in the elucidation of the immunological mode of action of TAs.

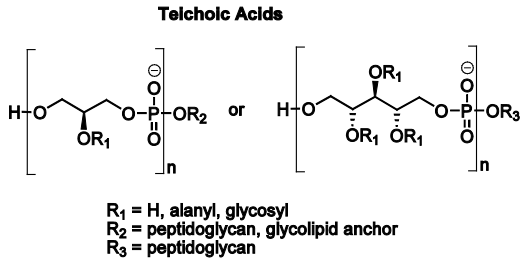


Figure 1. Chemical structure of TAs.

This introductory chapter presents the discovery, the structural classification, as well as physiological and immunological aspects of TAs. Additionally, it presents a selection of previously reported syntheses of TAs and an outline of the thesis.

Discovery of TA and its subclasses

The discovery of teichoic acid (TA) can be viewed as a consequence of combined evidence that was collected by research performed in the 1950s. In 1951, Leloir and coworkers identified uridine diphosphate (UDP) sugars as being the active ingredients in the biosynthesis of oligosaccharides.[7] With the finding of cytidine diphosphate glycerol (CDP-glycerol) and CDP-ribitol in the Gram-positive cell wall, the existence of a TA type of polymer was hypothesized.[8] Moreover, the total amount of phosphate present in the Gram-positive cell wall was much higher than could be attributed to the then known phosphate-containing biomolecules (nucleic acids, phospholipids etc.).[9] Finally, a polymer containing both ribitol phosphate and glycerol phosphate was isolated by Baddiley and coworkers from the cell wall of *Lactobacillus arabinosus*.[1] Further studies showed that the cell walls of Gram-positive species *Bacillus subtilis*, *Staphylococcus aureus* and *L. arabinosus* contained 30-70% of these polymers, which were named teichoic acids. In contrast, it was observed that in the Gram-negative *Escherichia coli* no TA was present at all.[10-13]

It was found that some TAs are connected to the peptidoglycan layer via a covalent linker that consists of a *N*-acetyl glucosamine moiety, often in combination with one or two other amino sugar residues.[10] This subclass of TAs is now known as wall teichoic acid (WTA) and an example of such a structure is given in Figure 2 (structure **1**). Ensuing studies on *Lactobacillus casei* showed the presence of a substantial amount of polyglycerol phosphates that shared many structural similarities with the WTA,

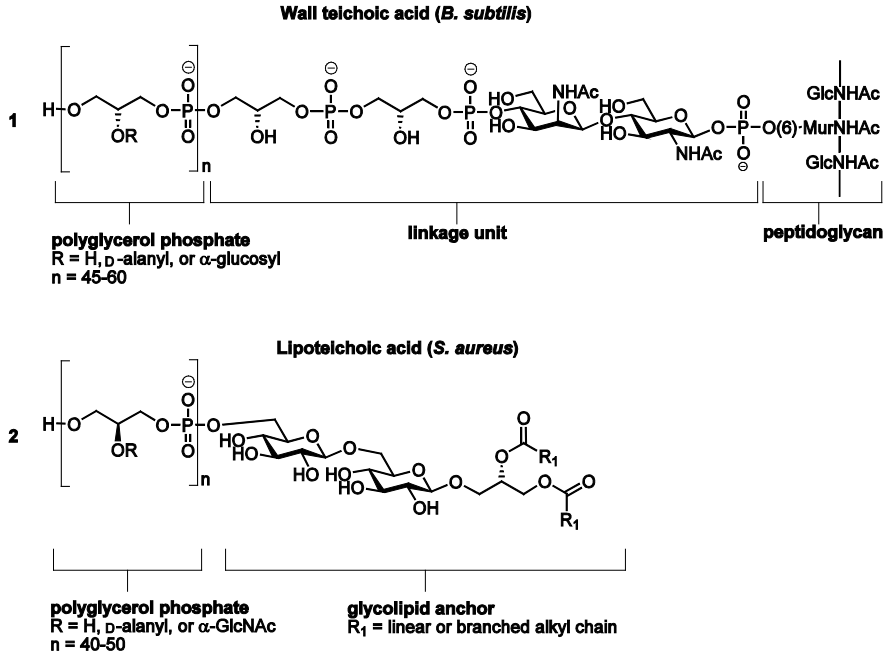


Figure 2. Examples of **1**: WTA (*Bacillus subtilis*)[4] and **2**: LTA (*Staphylococcus aureus*).[14]

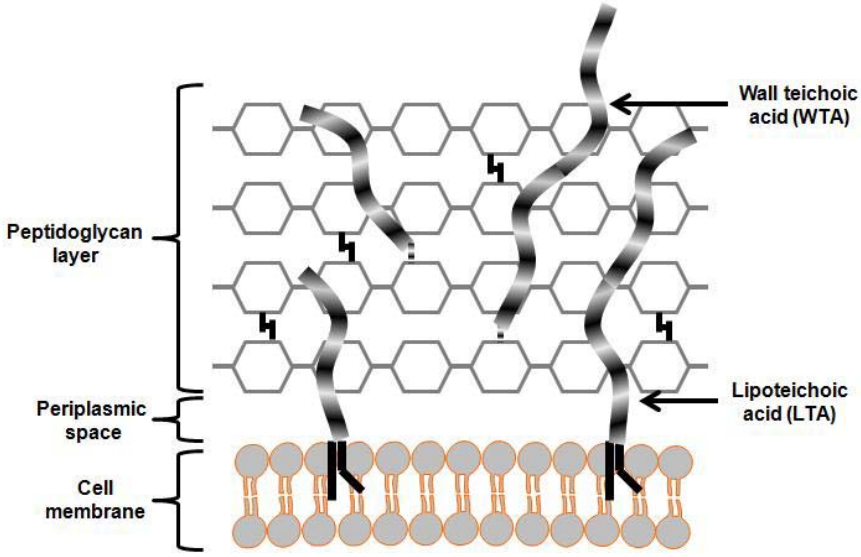


Figure 3. Schematic representation of LTA/WTA placed in the Gram-positive cell wall.

bearing D-alanyl and various glycosyl substituents. On the basis of their discriminative lipophilicity these polymers were initially named intracellular teichoic acids.[15] Later it became clear that the polyglycerol phosphate chain of these TAs is terminated with a glycolipid moiety (glycosyl diacylglycerol) that, presumably, intercalates in the lipid bilayer.[16] Therefore their name was changed into lipoteichoic acid (LTA), of which compound **2** is an example (Figure 2). A representation of WTA and LTA in the Gram-positive cell-envelope is given in Figure 3.

Over the years, various TA structures have been identified and they have been classified by Fischer into four distinctive types, differing in the nature of the repeating units.[2,17] Examples of type I-IV TA structures are portrayed in Figure 4. Type I TA is ubiquitous among Gram-positive species and consists of alditol phosphate repeating units; generally poly 1,3-glycerol phosphate or poly 1,5-ribitol phosphate (see also Figure 1). The general structure of type II TA consists of poly glycosylalditol phosphate.An example is the major TA from *Bacillus stearothermophilus*, consisting of poly α-glucosylglycerol phosphate, in which the glucosyl C-6 and the glycerol C-1 are linked via a phosphodiester.[18] Type III TA is built up from alternating repeating unit consisting of alditol phosphate and glycosyl phosphate, exemplified by the TA from *Staphylococcus hyicus* NCTC 10350.[19] Finally, type IV TA is characterized by alternating alditol phosphate and glycosylalditol phosphate moieties, such as the TA from several *Nocardiopsis* species.[20,21]

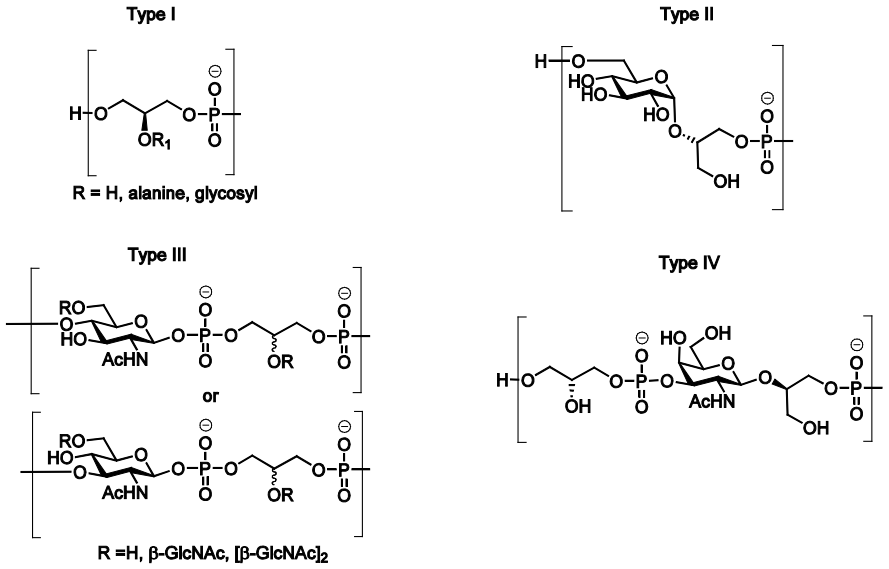


Figure 4. Examples of Type I-IV TA. Type I: ubiquitous in most Gram-positive species. Type II: glucosylglycerol phosphate as present in *Bacillus stearothermophilus* B65.[18] Type III TA from *Staphylococcus hyicus* NCTC 10350.[19] Type IV TA from several *Nocardiopsis* strains.[20,21]

TAs in bacterial physiology

TAs are situated in the Gram-positive cell-envelope, protruding through the peptidoglycan layer and are essential components for the well-being of the bacteria as explained in the coming section of this introduction.[2-6] Studies on LTA-deficient *Bacillus subtilis* and *Staphylococcus aureus* mutants showed that these bacteria were only able to proliferate under a narrow range of conditions. The LTA-deficient bacteria became more susceptible to temperature stress and showed significant impairment of growth.[22,23] A mutant of *B. subtilis* that was prevented from WTA expression showed similar behavior. Additionally, these WTA deficient mutants were affected by normal buffer components such as citrate and had the tendency to aggregate.[24-27] Because species, deficient in both WTA and LTA, showed no viability it was suggested that WTA and LTA have overlapping functions and they can compensate for each other to some extent.

TAs have the capability to bind cations, especially bivalent ones (e.g. Mg^{2+} and Ca^{2+}). Upon metal ion binding, the conformation of the TAs changes, leading to an alteration of the structure and shape of the peptidoglycan/TA matrix, thereby affecting the rigidity and porosity of the cell envelope.[28] TAs play a vital role in the bacteria's cation homeostasis. This is illustrated by the finding that *Bacillus subtilis* boosts up its TA production in a low Mg^{2+} environment. Additionally, when the bacteria are grown under phosphate deficient conditions a significant amount of teichuronic acids, a non-phosphorylated polymer built up from negatively charged sugar uronic acids, is

produced.[29] It is suggested that TAs control the activity of autolysins, an important class of cell-envelope based enzymes, which are responsible for the local break down of the peptidoglycan layer to allow the bacteria to grow or divide. It is suggested that autolysins are activated by bivalent cations present in the TA.[27,30]

Because of their polyanionic character TAs also bind to cationic antimicrobial peptides and glycopeptides based antibiotics, thereby playing an important role in bacterial defense. TAs are also considered as targets for bacteriophages.[31-33]

Most TAs are randomly substituted with D-alanine esters and glycosyl moieties and the exact substitution pattern differs per Gram-positive species and can change depending on the environment. Whereas the function of glycosyl moieties on the TA chain is unknown, the role of the alanine substitution is more clear.[5,6] The rate of D-alanylation strongly affects the ability of TA to bind metal ions. In this respect, the group of Baddiley found that non-alanylated TA binds up to 60% more Mg^{2+} when compared to the corresponding alanylated TA.[34] The degree of alanylation of the TA depends on the species, the growth medium, the pH and the temperature.[6] The TA chains undergo less electrostatic repulsion when alanylated to a higher degree (increased neutrality) and probably by forming stabilizing ion pairs that change the structure and porosity of the cell-envelope.[35] Mutants that lack alanine moieties on the TA and thus have a higher overall negative charge are known to be more susceptible to antimicrobial peptides, antibiotics and lytic enzymes.[36-40] These mutants are more easily targeted by the TLR-2 dependent host defense system and they can be killed using a significantly lower amount of vancomycin.[38] Additionally, these alanine deficient strains show impairment of their ability to bind to hosts or artificial surfaces and, surprisingly, a lower autolysine activity. The functions of glycosyl substitutions on TAs are poorly understood, but it was observed that a genetically altered *Staphylococcus aureus* mutant, lacking the glycosyl transferase necessary for this substitution was significantly less virulent.[41]

TAs and the immune system

Immunological studies have shown that TAs may activate both the innate as the adaptive immune system.[4-6] The effect on the innate immune system of TA and especially LTA has been studied quite extensively. It is thought that in combination with peptidoglycan TA is the Gram-positive counterpart of lipopolysaccharide (LPS), the most important cause of Gram-negative septic shock. However, whereas LPS activates the innate immune system through binding with Toll-like receptor 4 (TLR-4)[42], it is not clear how LTA activates the innate immune system.[43] Until recently it was presumed that LTA by virtue of its amphiphilic nature was recognized by TLR-2.[44,45] However, a clear connection between the immune response and LTA has not been established. This can be explained by the fact that the LTA preparations contained immunogenic impurities (lipoproteins, lipids, peptidoglycan) that interfered in the experiments, and/or that the LTA structures were (partly)

decomposed. The D-alanyl esters present on the TA backbone can be easily lost during the purification process. Additionally, TA preparations from biological sources are generally microheterogenenous, which makes it difficult, if not impossible, to perform structure activity relationship studies on these molecules. In 2002, the immunostimulatory activity of LTA was proven when Morath *et al.* observed and quantified the cytokine induction in human whole blood of a fully synthetic staphylococcal LTA (the synthesis of which is described later in this chapter).[46] The question on how the innate immune system is triggered by LTA remains unanswered. In 2006 Hashimoto and Götz impugned the hypothesis that LTA is a ligand of TLR-2 by the finding that a lipopeptide deficient mutant of *S. aureus* showed negative results when tested on TLR-2 dependent activation.[47] These findings were corroborated when in 2009 Kang *et al.* reported on the binding of pneumococcal LTA to TLR-2. Although these researchers were able to obtain a crystal structure from an LTA/TLR-2 complex, binding of LTA to the TLR-2/TLR-6 heterodimer, essential for initiation of the intracellular immune response was not observed.[48] Schmidt and coworkers suggested that innate immune recognition of LTA occurs via the complement system and, specifically, binding of the TA to a lectin.[43,49]

An effect of TAs on the adaptive immune system can be similar to that of bacterial capsular polysaccharides. Opsonophagocytic antibodies directed to these polysaccharides are at the basis of protective immunity against various encapsulated bacteria.[50-56] Like capsular polysaccharides, TAs can be recognized by the immune system of the host as they are exposed on the surface of many Gram-positive bacteria.[52]

Enterococcus faecalis, a normally harmless commensal bacterium has become a considerable threat in hospitals as growing antibiotic resistance makes this species difficult to treat. Currently, it is ranked second among the most prevalent Gram-positive nosocomial infection sources and accounts for 10% of all infections in intensive care unit patients, causing septic shock, urinary tract infections, peritonitis and endocarditis. Because of its reduced susceptibility against antibiotics such as vancomycin, the interest in finding alternative remedies is renewed.[57] In 1999 Wang *et al.* described that antiserum against a polysaccharide of *E. faecalis* strain 12030 was able to kill a homologous strain. In addition, the serum was capable of killing various other heterologous strains, including other *E. faecalis* strains and also *E. faecium* strains. Notably, also some vancomycin resistant strains were susceptible to killing by this serum. It was also shown that the purified capsular polysaccharide of *E. faecalis* could be used as an active vaccine against *E. faecalis* infections in mice.[58] In 2006, Theilacker *et al.* reported that the polysaccharide of *E. faecalis* 12030 against which the opsonic antibodies were directed, actually was a type I LTA structure. Notably, this LTA lacked D-alanyl modifications in the backbone.[59] In another study the group of Huebner reported the cross-reactivity of rabbit antibodies raised against *E. faecalis* 12030 LTA. These antibodies were capable of binding heterologous LTA from different enterococcal strains as well as the LTA of *S. aureus* and *S. epidermis* to some

extent. In addition, the antibodies opsonized *S. aureus* and *S. epidermis* and passive immunization with the rabbit serum led to clearance of *E. faecalis* and *S. epidermis* from the bloodstream in mice. These studies indicate that the raised opsonic antibodies were directed at a common structure in the LTA of all these Gram-positive bacteria. Because the LTA structures differ by the nature of the C-2 appendages of these species (different carbohydrate substituents are encountered in different species) the group of Huebner argued that the antibodies were directed against poly-1,3-glycerolphosphate, the backbone of all LTA structures.[60]

Synthetic strategies to TAs and applications thereof

This part of the introduction describes a selection of syntheses of TAs reported throughout the past 30 years. Since, the crucial linkages in TAs are phosphodiesters, the assembly of TAs has benefited from the phosphorylation procedures developed in the field of nucleic acid chemistry and the first part of this section will briefly sum up the main synthetic approaches to the introduction of phosphodiester bonds (see Figure 5).[61]

In the mid 1950s and the early 1960s the groups of Todd and Khorana were leading in the development of methods for the synthesis of DNA and RNA fragments. Although Todd and coworkers were the first to obtain a fully synthetic dinucleotide[62], Khorana and his coworkers advanced and refined the phosphodiester approach, the first common approach to oligo(deoxy)nucleotides.[63-65] In the key step of this approach a phosphomonoester is made suitable for nucleophilic attack by activation with a condensation agent (e.g. *N*,*N*-dicyclohexylcarbodiimide, tosyl chloride) to give a transient leaving group, which is displaced by a nucleophile (e.g. a nucleoside or an

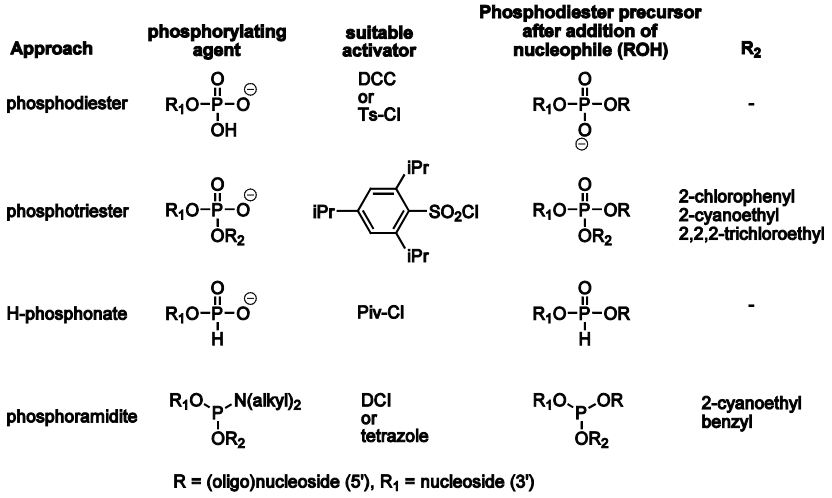


Figure 5. Synthetic approaches that (ultimately) lead to phosphodiesters.

oligonucleotide 5' hydroxyl). In this way an unprotected, charged phosphodiester is immediately obtained. The yields obtained by using this approach were generally reasonable (60-70%) in a linear approach, but significantly lower for block-couplings.[66] Several side reactions can occur. The phosphomonoester substrate can be activated twice and the resulting phosphodiesters, although less reactive, are still vulnerable to the activating agents, resulting in symmetric phosphotriesters and pyrophosphates. Notably, an increase in the chain length of the oligonucleotide synthesis is accompanied by an increase of the quantity of unwanted side products. Another major drawback of the phosphodiester approach is the purification procedure to separate the target compound from the often also charged side products. The required ion-exchange chromatography step is a slow and labor-intensive purification procedure. DNA fragments up to a tetramer have been made via this approach before it was abandoned in favor of the development of a more efficient method, the phosphotriester approach. The synthesis of TAs has not been explored with the aid of the phosphodiester approach.[66,67]

The phosphotriester approach is a common term for a wide variety of procedures developed throughout the early 1960s to the late 1980s, especially by the groups of Letsinger[68,69], Reese[70], Eckstein[71] and van Boom[72,73]. The conformity of nearly all the phosphotriester procedures is the use of a substituted phenol as phosphate protecting group. In the classical phosphotriester approach a phosphodiester, consisting of a suitably protected nucleoside and a phenol (e.g. 2-chlorophenol) protecting group is made suitable for nucleophilic attack by reaction with an activating agent (e.g. triisopropylphenylsulfonyl chloride) to give an activated intermediate that rapidly reacts with an alcohol of choice (e.g. a second suitably protected nucleoside) under basic conditions to produce the target compound as a phenol protected phosphotriester. Compared with the phosphodiester method, phosphotriester approaches generally have shorter reaction times, result in higher coupling yields (especially in block-couplings) and are purified by less laborious chromatographic procedures due to the uncharged phosphotriester intermediates. However, also the phosphotriester approach suffers from the occurrence of side reactions. For example, even the use of sterically compromised activating agents (e.g. triisopropyl-phenylsulfonyl chloride) does not prevent unwanted sulfonylation of the incoming alcohol in the condensation step. In the synthesis of oligonucleotides the reaction time increases and the yield decreases with the growing of the oligonucleotide chain. Finally, the removal of the phenol protecting groups in the final stage of the synthesis can be accompanied by unwanted phosphate cleavage reactions.[61]

Todd and co-workers[74] already reported on the synthesis of a dinucleotide using a H-phosphonate intermediate in the late 1950s and renewed interest in the H-phosphonate approach was initiated in 1985 by the group of Garegg which was continued by this and several other research groups for the next ten years.[75] In the condensation step of this approach, activation of an H-phosphonate monoester with a reagent such as pivaloyl chloride results in an activated intermediate, which reacts

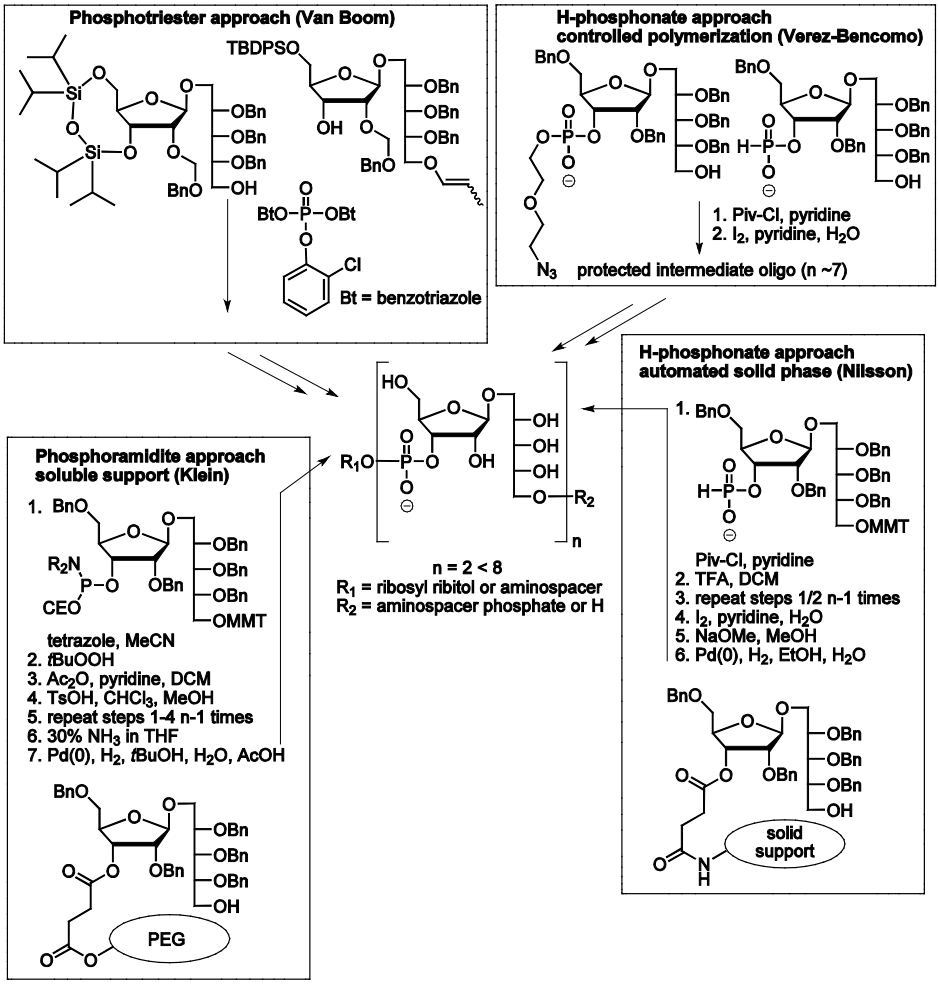
with an alcohol to give a H-phosphonate diester. Oxidation of this H-phosphonate diester directly leads to the target phosphodiester. The H-phosphonate approach is especially suitable for syntheses on solid support and the elongation sequence of an automated DNA synthesis protocol consists of only two steps: coupling and liberation of the 5' hydroxyl. At the final stage of the synthesis, global oxidation and deprotection affords the target oligonucleotide. The coupling yields are generally high (>90%) and oligonucleotides of a length of more than hundred residues have been successfully made.[76-78] Shortcomings of this method are that during the coupling step side reactions can occur by over-activation of the H-phosphonate mono- and diesters , and also that H-phosphonate diesters are prone to transesterification. These drawbacks and favorable properties of the phosphoramidite method (see below) are the reason for the limited use of the H-phosphonate approach in contemporary oligonucleotide synthesis.[79,80]

The most effective and widely used method to obtain phosphodiesters is the phosphoramidite approach, introduced by Beaucage and Caruthers in the early 1980s.[81] The decisive advantage of this approach is the optimal reactivity of the intermediates at room temperature and the selectivity of the reactions, leading to a minimum amount of by-products. This method was derived from the earlier developed, less widely used, phosphite triester approach, in which chloride is used as a leaving group. The main disadvantages of the phosphite triester approach are the practical difficulty (reaction temperatures around -80 ^{o}C) and the fact that it leads to a number of by-products.[82] In the phosphoramidite approach the chlorine as leaving group is replaced by a dialkyl amino moiety, which can be activated by mild and non-nucleophilic acids such as tetrazole (pK_a 4.9), to allow substitution by an alcohol. In the next step the obtained phosphite triester is oxidized to give the more stable phosphotriester. Soon after the introduction of this approach, it was found that the *N,N*-diisopropyl phosphoramidites are most suitable for oligonucleotide synthesis as these amidites are considerably more stable than the corresponding *N,N*-dimethyl derivatives and have a half-time of ~200 hrs in aqueous acetonitrile (at 25 ^{o}C).[83-85] The stability of the phosphoramidites in combination with the short reaction times (normally within seconds) with alcohols upon activation with a weak acid as well as the fact that no significant side-reactions take place during the elongation sequence are the main advantages of this approach.[61,86]

The past decades, besides oligo(deoxy)nucleotides, various phosphodiester containing (bio)molecules have been synthesized using the approaches described above. A salient example is the antigenic capsular polysaccharide from *Haemophilus influenza*.[87,88] In the framework of the development of a vaccine, several research groups explored the synthesis of functionalized fragments of this polysaccharide (see Scheme 1). Oligomers with ribosylribitol phosphate as repeating unit, have been prepared via a solution phase modified phosphotriester approach (Van Boom)[89,90], an automated solid phase H-phosphonate approach (Nilsson)[91], solution phase/soluble support phase phosphoramidite approaches (Van Boom, Klein)[92,93] and a controlled

polymerization approach using H-phosphonate chemistry (Verez-Bencomo)[94]. The finding that fragments of the capsular polysaccharide from *Haemophilus influenza* conjugated to an immunogenic protein carrier induce a specific immune response *in vivo* culminated in the world's first synthetic carbohydrate vaccine. In 2004 Verez-Bencomo and coworkers developed a tetanus toxoid (TTd) conjugate, which is now used in Cuba to prevent *H. influenza* infections in children (99.7% success rate).[94]

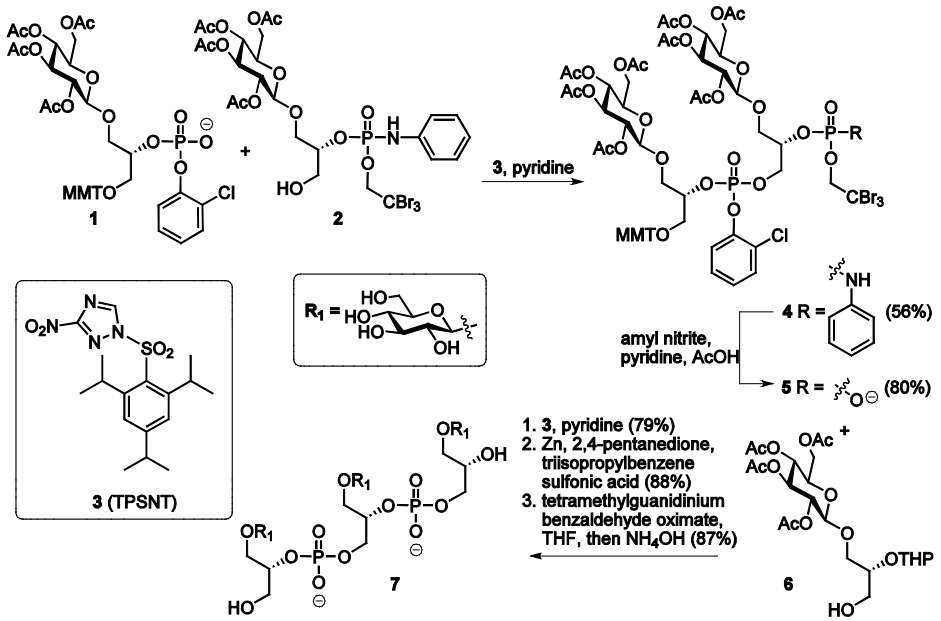
Scheme 1. Four synthetic approaches to (oligo) ribosylribitol phosphate, an antigenic capsular polysaccharide found in *Haemophilus influenza* which is structurally closely related to TA.



In 1983 the first successful synthesis of a TA (type I) fragment was reported by Van Boeckel *et al*. A trimer derived from the TA from *Bacillus subtilis var. niger* was assembled in solution using a modified phosphotriester approach.[95] Of primary concern in the construction of the oligo 1,2-glycerol phosphate backbone was

excluding the formation of a cyclic phosphate in the "middle" unit. Therefore, phosphodiester (**1**) was coupled to tribromoethylphosphor anilidate (**2**) using triisopropylphenylsulfonyl nitrotriazolate (TPSNT, **3**) as the condensating agent.[96] The phosphoramidate proved stable under the coupling conditions, the TPSNT reagent gave minimal unwanted alcohol sulfonylation and the dimer (**4**) was obtained in 56%. The aniline moiety was cleaved in a Sandmeyer type reaction using amyl nitrite in a mixture of AcOH and pyridine to give phosphodiester **5** in 80% yield.[97] Phosphodiester **5** was coupled with 2-*O*-THP protected glucosyl glycerol building block **6** again under influence of TPSNT to give the protected trimer in 79%. A four-step and two-pot deprotection procedure finally gave the deprotected TA trimer **7** in good yield (~75%).[95]

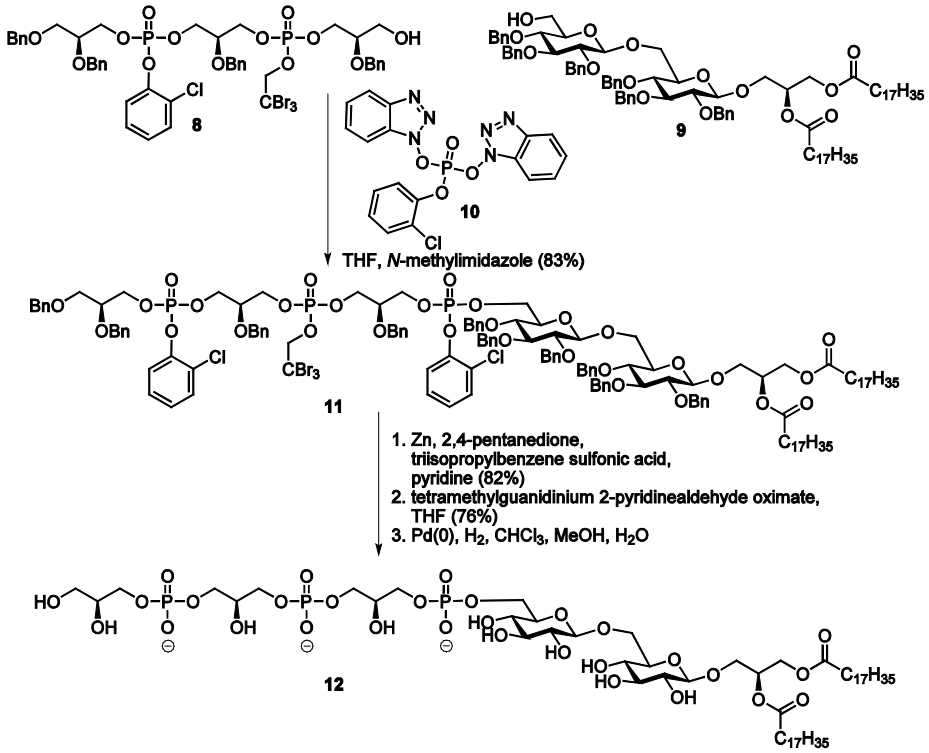
Scheme 2. Synthesis (phosphotriester approach) of van Boeckel *et al*. to a *B.subtilis* TA trimer.



In 1982 Oltvoort *et al.* reported on the synthesis of the gentiobiosyl diacylglycerol glycolipid anchor of *Staphylococcus aureus* LTA.[98] Two years later, the same group of researchers completed the convergent synthesis of an elongated fragment, consisting of the same glycolipid anchor connected to a glycerol phosphate trimer (see Scheme 3).[99] The protected glycerol phosphate trimer (**8**) was obtained via a multistep strategy in which the phosphotriester functions were introduced in high yield by the use of the bifunctional phosphorylating agents 2-chlorophenyl-*bis*-benzotriazole phosphate **10**[73] and the corresponding 2,2,2-tribromoethyl agent (not shown). Block coupling of trimer **8** with benzylated gentiobiosyl alcohol **9** using phosphorylating agent **10** gave the protected construct **11** in 83% yield. The deprotection procedure consisted of the following three steps: 1) removal of the tribromoethyl moiety using activated zinc, 2) hydrolysis of the 2-chlorophenylphosphate triesters using

tetramethylguanidinium 2-pyridinylaldehyde oximate, and 3) hydrogenolysis of the benzyl ethers, giving LTA fragment **12**. The first two steps proceeded uneventfully in ~60% while the yield of the latter step is not mentioned.

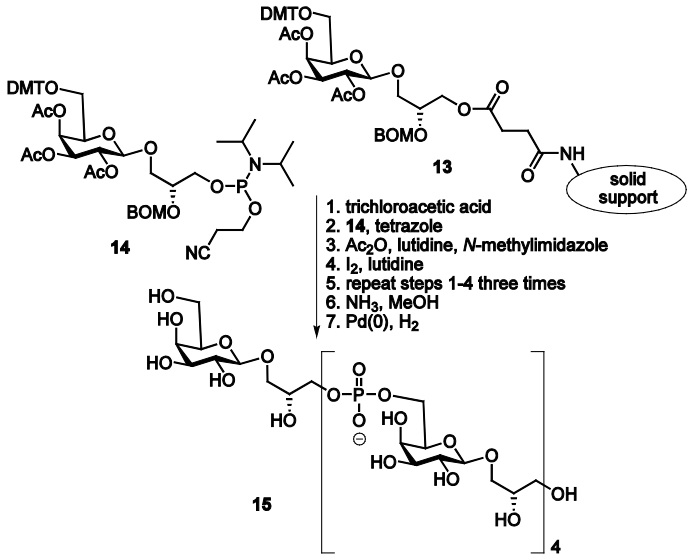
Scheme 3. Oltvoort's synthesis of *S. Aureus* lipoteichoic acid carrier fragment.



The third TA fragment synthesized by the group of Van Boom features the type II teichoic acid from *Bacillus licheniformis ATCC 9945*, which consists of a 1-O-(β-D-galactopyranosyl)glycerol repeating unit (see Scheme 4). A pentameric fragment of this TA was synthesized using an automated solid phase procedure in combination with phosphoramidite chemistry.[100] Immobilized **13** and phosphoramidite **14** were both obtained from the same orthogonally protected galactosyl glycerol building block. With the aid of a DNA/RNA synthesizer, resin **13** (200 mg, 10 μmol) was subjected to the following sequence of reactions: 1) 2% trichloroacetic acid in DCM (removal of the DMT ether), 2) phosphoramidite **14** and tetrazole in MeCN (elongation step), 3) Ac_2O, 2,6-lutidine and *N*-methylimidazole (capping of remaining unreacted alcohols), and 4) I_2 in 2,6-lutidine and 1,4-dioxane (oxidation of the phosphite intermediate). After a three-fold repetition of this elongation cycle, the resin was removed from the synthesizer and treated with methanolic ammonia to cleave the base labile acetyl and cyanoethyl groups and to release the TA fragment from the resin. Finally, purification using size exclusion chromatography and subsequent hydrogenolysis afforded target pentamer **15** in good yield (~25% from

immobilized **13**). It was described that for efficient couplings 50 equivalents of phosphoramidite **14** were needed. Nonetheless, this work presented a significant improvement compared to the previously described syntheses in terms of time and labour efficiency. Additionally, the chemistry (phosphoramidites) and methodology (DNA synthesizer) that were amended were at the time both still in its infancy. Surprisingly, no more TA fragments were synthesized in the following years using this seemingly suitable technique.

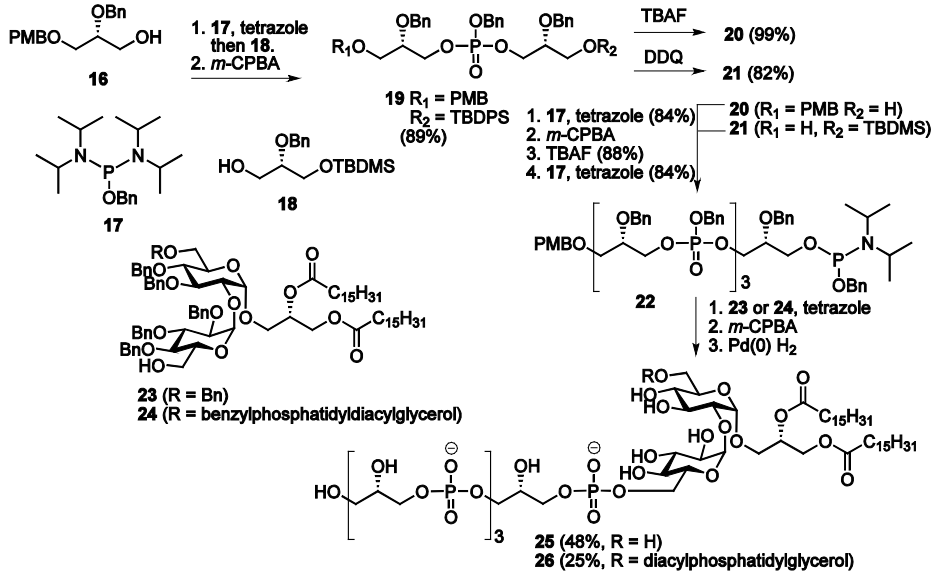
Scheme 4. Westerduin's automated solid phase approach to a *B. Licheniformis* TA pentamer (**15**).



In the early 1990s Fukase *et al.* reported on the syntheses of LTA fragments derived from *Streptococcus pyogenes* and *Enterococcus hirae*.[101,102] The closely related structures contain a 1-(diacylglycerol)-α-D-kojibiosyl (2-*O*-[α-glucosyl]-glucosyl) glycolipid with the polyglycerol phosphate backbone linked to the C-6 of the terminal glucosyl moiety. The LTA of *E. hirae* LTA contains an additional phosphatidyl diacylglycerol part, which is connected to C6 of the inner glucosyl (see Scheme 5). In the synthetic targets of Fukase *et al.* the naturally occurring oleoyl esters were replaced by palmitoyl tails and the glycerol phosphate backbone, normally comprising ~20 subunits was brought back to a tetrameric unit. As can be seen in Scheme 5 the glycerol phosphate part was assembled via a convergent approach. Starting from an enantiomerically pure glycerol derivative, two differently and orthogonally protected building blocks (**16** and **18**) were acquired and connected via phosphoramidite chemistry comprising phosphitylation, tetrazole mediated coupling and oxidation. The resulting dimer (**19**) was treated with either DDQ or TBAF, resulting in alcohols **20** and **21**, respectively, which were connected using the sequence of reactions mentioned above. The TBDMS in the tetrameric fragment was cleaved with TBAF and the resulting alcohol was converted into the benzylamidite (**22**) using benzyl bis-(*N*,*N*-

diisopropyl)phosphordiamidite and tetrazole. Finally, the glycolipid anchor (**23** or **24**) was coupled to the TA phosphoramidite (**22**) under the agency of tetrazole to give, after deprotection, the respective target TA fragments **25** and **26**. The coupling, oxidation and single step deprotection in case of *S. pyogenes* LTA (**25**) proceeded uneventfully in 48% yield over three steps. In the final coupling and oxidation of **22** and **24** the initial product was obtained in a significantly lower yield (27%). In the latter case a severely lowered reaction concentration (~65 mM for **23** vs ~5 mM for **24**) was employed, suggesting that the bis(diacylglycerol) glycolipid is less soluble. The final deprotection of the more complex **26** proceeded in high yield (93%). Both fragments were evaluated for their ability to induce cytokine production in muramyl dipeptide primed mice, but no activity was detected.[103]

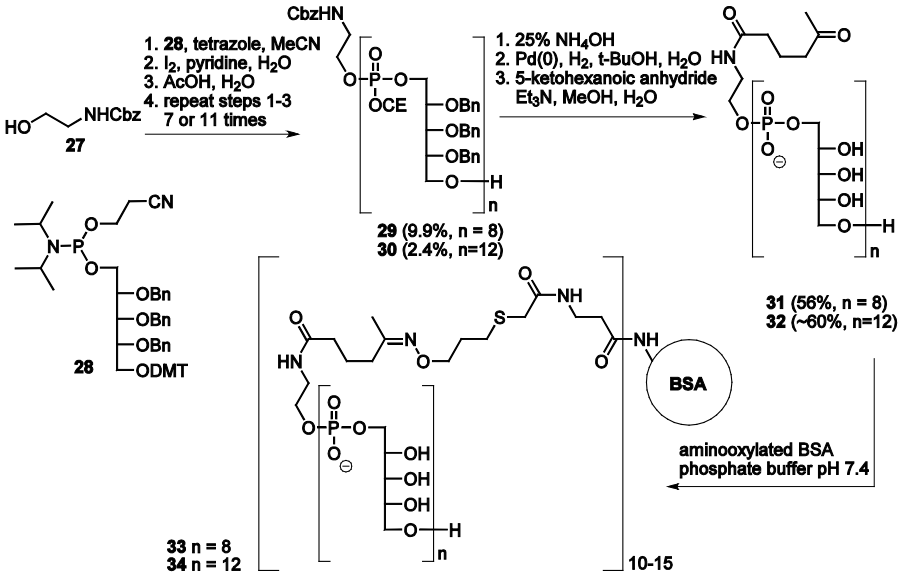
Scheme 5. Phosphoramidite approach by Fukase *et al.* to proposed LTA carrier structures of *S. pyogenes* (**25**) and *E. Hirae* (**26**).



Poly-(1,5-ribitol phosphate) is ubiquitously found in the WTA of Staphylococci and Bacilli. In 2006, the group of Pozsgay reported on the synthesis of an octa- and a dodecamer ribitol phosphate (**33** and **34**) and their subsequent conjugation to bovine serum albumin (BSA).[104] Their first attempt to make a hexameric fragment of these WTA oligomers comprised an automated solid phase approach using phosphoramidite **28**. However, an intractable mixture, in which a hexamer predominated, was obtained and the applied chemistry was reinvestigated in a solution phase approach using aminospacer alcohol **27** as starting compound. Coupling of **27** with phosphoramidite **28** under influence of tetrazole, subsequent oxidation of the phosphite, and detritylation with 2% trichloroacetic acid in DCM, reportedly, resulted in loss of the integrity of the phosphotriester moieties. Switching to the milder AcOH/water/DCM (85:10:5) detritylation cocktail resulted in selective

cleavage of the DMT without loss of the phosphotriesters. However, the prolonged reaction time (four hours instead of a few minutes for TCA/DCM) prompted to abandon the automated solid phase approach and continue their synthesis in solution (see Scheme 6). Using this approach fully protected octamer **29** was obtained in 9.9% yield over 8 elongation cycles/24 steps (~75% per cycle consisting of coupling, oxidation, detritylation) and dodecamer **30** was obtained in 2.4% yield over 12 elongation cycles/36 steps (~73 % per cycle). Release of the cyanoethyl groups was accomplished by β-elimination using ammonium hydroxide in a mixture of methanol and water. The subsequent hydrogenolysis proceeded uneventfully after the $EtOH/H_2O$ mixture was replaced by tBuOH/H_2O as the former combination resulted in partial *N*-ethylation. The fully deprotected oligomers were treated with 5-ketohexanoic anhydride and the resulting *N*-acylated octamer **31** (56% over three steps) and dodecamer **32** (~60%) reacted with aminooxylated BSA[105] forming a stable oxime linkage. The evaluation of the immunogenic properties of these conjugates, which contained 10-15 WTA fragments per molecule of BSA, has not been reported in the literature so far.

Scheme 6. Pozsgay's synthesis of two ribitol phosphate-BSA conjugates (**33**, **34**).



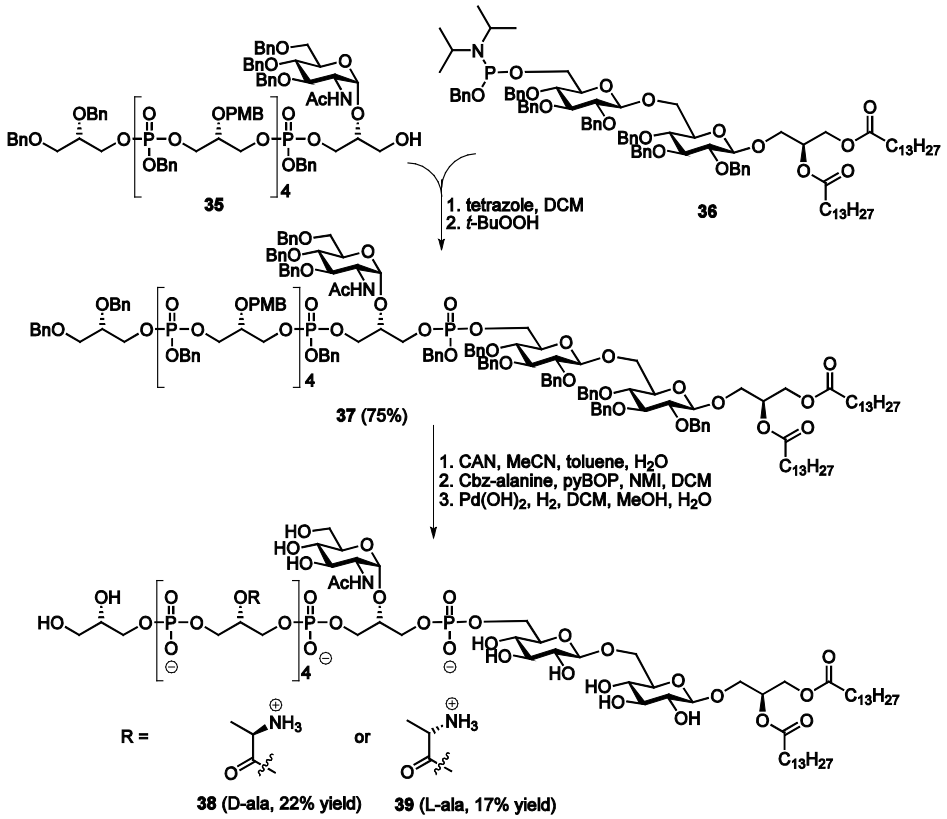
About a decade ago, the group of Hartung developed an improved method for the isolation and purification of LTA from *Staphylococcus aureus*, which involved a butanol extraction followed by hydrophobic interaction chromatography (HIC) using a slightly acidic buffer (NH_4OAc, pH 4.7). With this method it was ensured that the D-alanine esters present on the polyglycerol phosphate backbone were preserved and the structure of the full LTA could be revised: The gentiobiosyl glycerol glycolipid anchor, containing a mixture of saturated fatty acid tails (~C16), is linked via the C6 hydroxyl of the sugar to the glycerol phosphate backbone. The glycerol phosphate

part, averaging 45-50 residues in length was found substituted with D-alanine esters (~70%) and α-D-*N*-acetylglucosamine (~15%). A small part of the TA was found unsubstituted (~15%).[14]

In 2003, the group of Schmidt reported on the synthesis of this revised LTA structure. Their first aim was to make an LTA construct, containing a glycerolphosphate backbone with a glycerol C-2 substitution pattern similar to the native staphylococcal LTA. Target LTA **38** (see Scheme 7) consists of a glycolipid moiety connected to a hexaglycerol phosphate provided with four D-alanine esters and one *N*-acetylglucosaminyl residue.[106] To postpone the installation of the labile alanine esters to the penultimate stage of the synthesis the temporary *para*-methoxybenzyl (PMB) protecting group at the glycerol C-2 was applied. It was reasoned that the PMB groups could selectively be cleaved in the fully protected stage of the LTA construct, followed by introduction of the alanine esters. Starting from dibenzylglycerol the hexaglycerol phosphate backbone (**35**) was made in a linear, step-wise procedure (elongation, oxidation, desilylation) using either the 2-*O*-PMB or 2-*O*-(*N*-acetyl-α-glucosaminyl) glycerol benzylphosphoramidites. After a block coupling of hexameric alcohol (**35**) and the glycolipid anchor benzylphosphoramidite (**36**) under the agency of tetrazole followed by oxidation, fully protected **37** was obtained in 75% yield. The PMB ethers were replaced by protected alanine esters by treatment of **37** with cerium ammonium nitrate (67% yield) and esterfication with *N*-carbobenzyloxy-D-alanine using PyBOP as the condensing agent (70% yield). Finally, the protected precursor was deprotected in a single step ($Pd(OH)_2/H_2$) and purified with hydrophobic interaction chromatography giving LTA **38** in 47% yield. In addition, in an analogous manner LTA **39**, equipped with L-alanine esters was prepared (62% yield, Scheme 7). The immunostimulatory properties of both LTA fragments were assessed by an assay in which the response of human blood leukocytes was determined. This assay revealed that LTA **38** has a similar effect on cytokine production as the LTA which was isolated by the group of Hartung. Contrary, L-alanylated LTA **39** showed a strongly diminished activity (10-100 fold) compared to the D-alanylated fragment, indicating that alanine modifications play a critical role in recognition by the immune system of hosts.[14,46]

With the objective to determine the minimum structural properties required for innate immune response the group of Schmidt continued their research by synthesizing a number of *S. aureus* LTA derivatives. A convergent approach was devised that made it possible to prepare several constructs by minor alterations of the synthetic route. In 2005, the syntheses of fragments **40** and **41** were described (see Scheme 8).[107] Whereas LTA **40** only lacked the gentiobiosyl moiety, derivative **41** features more radical changes: both gentiobiosyl and GlcNAc moieties were omitted and the D-alanine esters were replaced by the more stable amide analogues on the C-2 glycerol residues of opposed stereochemistry. The effects of these modifications on the immunostimulatory properties were minimal, showing that the gentiobiosyl and

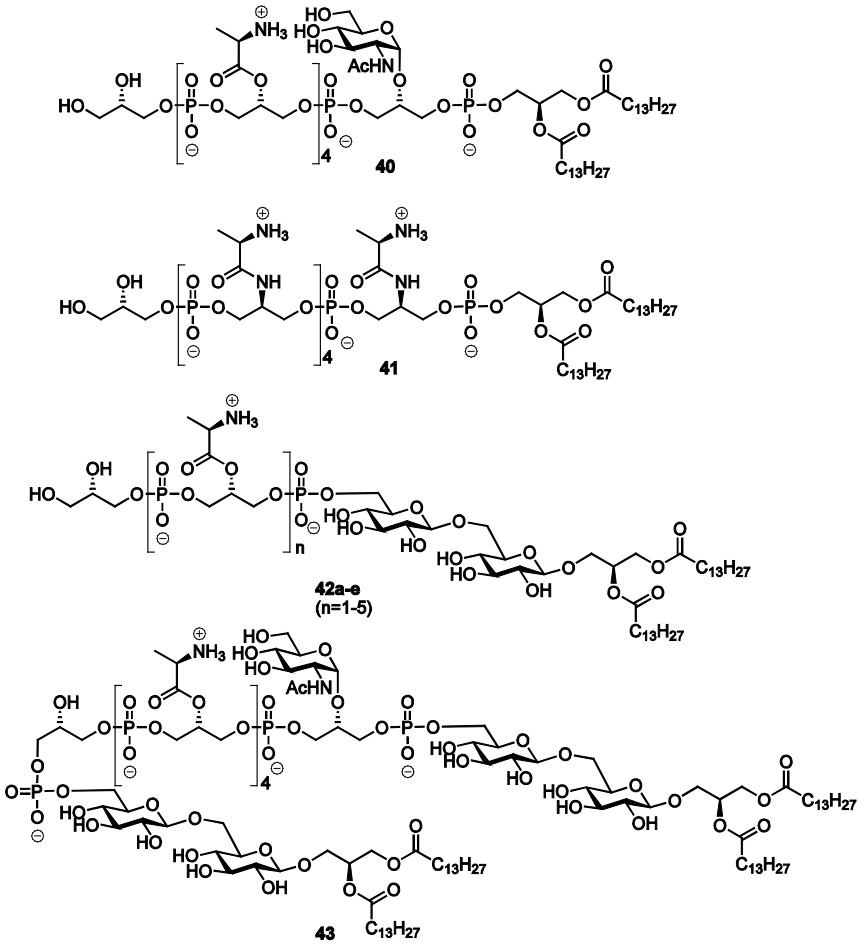
Scheme 7. Schmidt's synthesis of a complete staphylococcal LTA construct (**38**) and analogon (**39**).



GlcNAc were not necessary for cytokine (IL-8, TNF-α) production in human whole blood, peritoneal macrophages or TLR-transfected HEK-cells.[108] The presence of the alanylated glycerol phosphate backbone proved more important. This finding was confirmed shortly thereafter by the synthesis and evaluation of TA derivatives **42a-e** by the same authors. These compounds consist of the complete gentiobiosyl diacyl glycerol moiety connected to an oligoglycerol phosphate backbone ranging in length between two (**42a**) and six residues (**42e**) and containing between one and five alanine modifications (Scheme 8).[109] Biological evaluation showed that a minimum of two alanylated glycerol phosphates in the construct is required for significant activity (10-fold increase of cytokine induction compared to one alanylated residue). The gentiobiosyl diacyl glycerol anchor (structure not shown) alone did not induce any immune response. However, it was suggested that the presence of this moiety in LTA derivatives is important for innate immune activity. The group of Schmidt also reported the synthesis of a construct that contained all the natural substitutions and a second glycolipid moiety (structure **43**, Scheme 8).[110] It was shown that in an immune assay cytokine (IL-8, TNF-α) titers were significantly higher compared to the monoglycolipid **38** (see Scheme 7). The presentation of the crucial recognition

elements, such as the D-alanyl esters, could possibly be increased by this bis-amphiphilic construct.[43,110]

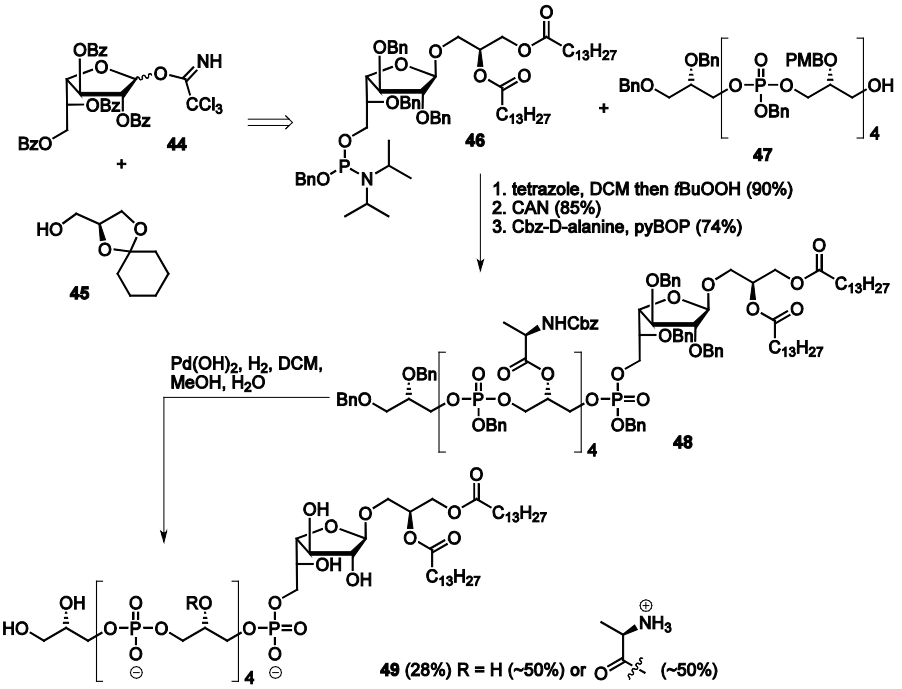
Scheme 8. Synthetic derivatives of *Staphylococcus aureus* LTA (**40**-**43**) made in the lab of Schmidt.



The type I LTA from *Streptococcus sp* DSM 8747, a mutant which is genetically related to *Streptococcus pneumonia*, consists of a galactofuranoside diacyl glycerol glycolipid with the oligoglycerol phosphate backbone connected to the C-6 of the galactofuranose residue. The backbone is substituted for about 30% at C-2 positions with D-alanine esters.[111] In 2010, Schmidt and coworkers reported on the synthesis of a fragment of this LTA.[112] The β-galactofuranoside diacylglycerol phosphoramidite **46** was constructed via the imidate glycosylation procedure using anchimeric assistance of a temporary 2'- benzoyl ester, followed by several consecutive (protecting group) manipulations (see Scheme 9). Block coupling of the previously reported PMB-protected pentamer **47** and glycolipid phosphoramidite **46** using tetrazole followed by oxidation yielded the fully protected intermediate in 90%. At this stage the PMB

ethers were selectively cleaved (85% yield) and the resulting tetraol was decorated with alanyl moieties using Cbz-D-alanine and PyBOP (74% yield). Global deprotection of the fully protected **48** (containing four alanine esters) followed by purification resulted in the target LTA fragment **49** provided with roughly two alanine esters. It is unclear why the alanine esters in this particular molecule were prone to hydrolysis, as this phenomenon was not observed in similar syntheses reported earlier by the group of Schmidt.[106,107,109,110]

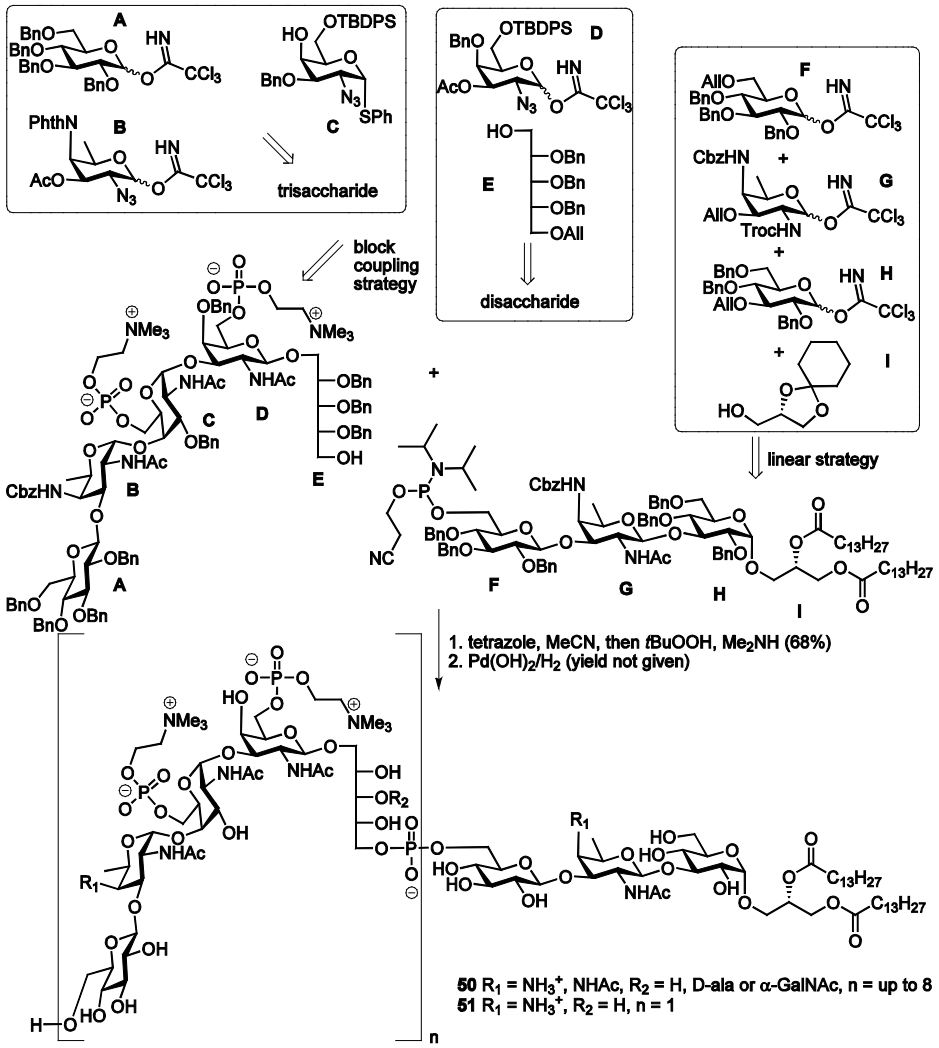
Scheme 9. Synthesis of the unusual LTA as found in *Streptococcus* species DSM 8747.



The complex structure of the type IV LTA found in *Streptococcus pneumonia* is made up from a glycolipid anchor, that consists of diacylglycerol linked to a trisaccharide (glucose-diaminofucose-glucose), connected to a repeating unit (n = 1-8, 2 on average) pentasaccharide phosphate (glucose-diaminofucose-[phosphatidylcholine]-galactosamine-[phosphatidylcholine]galactosamine-ribitol) (see Scheme 10, structure **50**).[113] In 2010, Pedersen *et al.* reported on the synthesis of the structural variant **51** in which the diaminofucose moieties were mono-(2')-*N*-acetylated and the ribitol unit was non-substituted.[114] The synthesis comprised 88 steps starting from the protected monosaccharide building-blocks. The pentasaccharide repeating unit was constructed by the coupling of trisaccharide **ABC** and disaccharide **DE**. Ensuing manipulations (reduction of the azides, acetylation and HF/pyridine treatment) was followed by decoration of the resulting alcohols on subunits **C**/**D** in the pentasaccharide with phosphatidylcholines. Next, the terminal alcohol of the ribitol (**E**) was selectively unmasked by isomerization of the allyl ether

and cleavage of the resulting 1-propenylether, giving pentasaccharide **ABCDE** (see Scheme 10). The glycolipid phosphoramidite (**FGHI**) was constructed via a linear glycosylation strategy. First alcohol **I** was glycosylated using imidate **H** and, subsequently, the cyclohexylidene group was removed. The resulting diol was myristoylated and after several protecting group manipulations, glycosylations and finally phosphitylation, the phosphoramidite was acquired. The crucial step was the coupling of pentasaccharide alcohol **ABCDE** (including zwitterionic phosphatidylcholine functionalities) and glycolipid phosphoramidite **FGHI**. Phosphitylation in acetonitrile under influence of the activator tetrazole and oxidation under basic conditions, by which the cyanoethyl phosphate protecting groups were

Scheme 10 *Streptococcus pneumonia* LTA (**50**). Structure **51** was synthesized by Pedersen *et al.*



concomitantly cleaved, the partially protected pseudononasaccharide triphosphate (**ABCDEFGHI**) was obtained in 68% yield. Finally, global deprotection ($Pd(OH)_2/H_2$) gave the target LTA construct (**51**).

At the same time the separate deprotection of the glycolipid[115] and repeating unit[116] moieties were undertaken. The resulting compounds (**51**, glycolipid and repeating unit) were evaluated on their potency to activate the innate immune system in human whole blood cells and isolated human mononuclear cells. It was found that both the sole glycolipid and construct **51** stimulated the release of the pro-inflammatory cytokine IL-8. The repeating unit alone did not result in any innate immune response. These results support the hypothesis that LTA fragments are not recognized by TLR-2 or 4. According to the authors these molecules most probably activate the innate immune system via the complement system and, more specifically, are recognized via the lectin pathway of the complement system.[114-116]

Conclusion

TAs are important Gram-positive bacteria cell wall components. Although TAs are structurally diverse and microheterogenous, their basic structure consists of alditol phosphates, randomly decorated with sugars and alanine esters. TAs are ligands for both the innate and the adaptive immune system. In this respect organic synthesis is indispensable to obtain well-defined TA fragments of desired length and substitution pattern. Subsequent structure activity relationship studies of these fragments may provide insights in mechanism of the immune system and help in the development of future vaccines.

Outline of this thesis

The work described in this thesis involves the development of synthetic methodologies that give access to glycerol TAs differing in length and composition. Solution, solid phase and fluorous phase chemistry methods are applied to obtain the desired (library of) TA structures, which have been evaluated for their ability to inhibit opsonic killing of *Enterococcus faecalis* by antibodies raised in rabbits against natural LTA of this species. The most active fragments have been coupled to an immunogenic carrier protein and the resulting conjugates evaluated on their immunogenicity and their potency to function as a (Gram-positive) vaccine.

Chapter two describes the solution phase synthesis of a glycerol phosphate hexamer, using phosphoramidite chemistry. First, the route towards the suitably protected glycerol phosphoramidite building blocks is presented after which the construction and deprotection of the target oligomer, containing an α-kojibiosyl (α-D-glucopyranosyl-(1→2)-D-glucose) substituent, is given.

Based on the chemistry presented in chapter two, the assembly of a small library of TAs is described in **chapter three**. These molecules were made with the use of an automated solid phase strategy and a DNA synthesizer. Four fragments ranging in

length from 6<20 glycerol phosphate subunits and two hexamers provided with an α-glucosyl substitution were synthesized and evaluated on their antigenicity by an opsonophagocytic inhibition assay (OPIA).

Chapter four deals with a fluorous phase protocol for the generation of several (aminoglucosylated) TAs that are found in a range of bacterial species. The protocol combines the advantages of both solution and solid phase strategies, especially through a rapid purification step, using a fluorous solid phase extraction (F-SPE) methodology after each elongation cycle. These TA fragments, featuring a terminal phosphate moiety are immunologically evaluated.

In **chapter five** the use of an alternative base-labile fluorous linker is described. The applied succinyl type of linker gives access to non-phosphorylated TA fragments. Additionally, an acid-stable tetrabenzylglucosyl glycerol phosphoramidite building block, that is suitable for incorporation in an earlier stage of the TA oligomer synthesis, is prepared.

Chapter six covers the conjugation to an immunogenic carrier protein of the most antigenic TA fragment, the synthesis and evaluation of which is described in chapter three and five. The conjugate is evaluated for its potential to function as a vaccine modality against *E. faecalis* and some other Gram-positive strains. The immunological experiments include: opsonophagocytic assays (OPAs) and passive immunization in a rat endocarditis model using rabbit anti serum raised against the synthetic conjugate.

The work described in **chapter six** has been carried out in cooperation with the Division of Infectious Diseases from the Department of Medicine of the University Medical Center Freiburg: A. Kropec-Huebner, D. Laverde Gomez, C. Theilacker and J. Huebner.

Chapter seven consists of a summary of the work described in this thesis. Several synthetic outlooks are given, including improved automated solid phase protocols using a photocleavable linker and a fluorous tag. Other future prospects include the synthesis of stable D-alanine ester analogues and the synthesis of an improved, clinically relevant, TA-tetanus toxoid conjugate.

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