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**PERSPECTIVE**

# Chemical synthesis of bacterial lipoteichoic acids: An insight on its biological significance

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During infections caused by Gram-negative bacteria, lipopolysaccharide (LPS, endotoxin) has a dominant role leading to fulminant pro-inflammatory reactions in the host. As there is no LPS in Gram-positive bacteria, other microbial cell wall components have been identified to be the causative agent for the pro-inflammatory activity since also Gram-positive bacterial infections lead to comparable clinical symptoms and reactions. On search for the "Gram-positive endotoxin" a widely accepted hypothesis has been raised in that the lipoteichoic acids (LTAs) serve as pathogen-associated molecular patterns (PAMPs) during Gram-positive sepsis, although the amount necessary for a pro-inflammatory *in vitro* response is several orders of magnitude higher than that for LPS. Therefore, LTA cannot be considered to be "the (*endo*)toxin of Gram-positive bacteria". Although LPS and LTA show structural relatedness (amphiphilic, negatively charged glycophospholipids), they are structurally quite different from each other and one might expect that they are also recognized by different receptors of the innate immune system, the so called toll-like receptors 4 and 2 (TLR4 and TLR2), respectively. Based on their chemical structure, the LTAs were classified into four types (type I–IV) of which we have carefully investigated the LTA of *Staphylococcus aureus* (type I), *Lactococcus garvieae* (type II) and *Streptococcus pneumoniae* (type IV). Hence, these LTAs have been synthesized in our group and biologically evaluated with respect to their potency to activate cytokines in transiently TLR2/CD14-transfected human endothelial kidney cells (HEK 293) or human macrophages and whole blood cells. Although LTA of type I and IV are structurally quite different, especially in their hydrophilic moiety, they originally were believed to interact with the same receptor (TLR2). Hence, the chemical syntheses leading to structurally defined, non-contaminated stimuli have a major impact on the outcome and interpretation of these biological studies of the innate immune system. With this material, it became evident that synthetic LTA from *S. aureus* and *S. pneumoniae* are not recognized by TLR2. Instead, another receptor of the innate immune system, the lectin pathway of the complement, known since many years to interact with LTA in quite a specific way, has gained increasing attractivity. With the help of synthetic LTA we obtained first evidences that this receptor is indeed the pathogen recognition receptor (PRR) for LTA.

## Introduction

During infection, the recognition of conserved bacterial structures called pathogen-associated molecular patterns (PAMPs) occurs *via* pattern recognition receptors (PRRs) on immune cells. This recognition triggers signalling pathways that activate transcription of pro-inflammatory cytokines which participate in the generation of an immune response against the microbes. A selected list of microbial compounds, serving as PAMPs derived from the cell wall of various organisms, are compiled in Table 1. The most important conserved bacterial structures of Gram-negative bacteria inducing cytokine release are the lipopolysaccharides (LPS) occurring in the outer leaflet of the outer cell membrane of the Gram-negative cell. Their immunological activity is extremely high and has been known for a long time.[1,2] Recognition by the immune system takes place by binding of LPS to MD-2 and the toll-like receptor 4 (TLR4), which has been crystallized recently;[3] the binding also involves other cofactors.[2,4–6]

The corresponding immunostimulatory component of Gram-positive bacteria, besides the peptidoglycan common to both types of bacteria, was not clear for a long time. Yet, a structural counterpart to LPS called lipoteichoic acid (LTA) was found in the cell wall of Gram-positive bacteria (Scheme 1). This LTA is also an amphiphilic molecule with a lipid anchor and a generally

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**Table 1** Bacterial pathogen-associated molecular patterns (PAMPs) located in the cell wall and being recognized by receptors of the innate immune system[2]

| Microorganisms | Typical structures: PAMPs derived from the bacterial cell wall that are recognized by pattern recognition receptors of the innate immune system |
|---|---|
| Bacteria | |
| Gram-negative bacteria | Peptidoglycan (PGN), Lipopeptides, Lipopolysaccharide (LPS), Lipid A |
| Gram-positive bacteria | Peptidoglycan (PGN), Lipopeptides, Lipoteichoic acid (LTA) |
| Mycobacteria | Lipoarabinomannan (LAM), Lipopeptides |
| Mycoplasma | Lipopeptides (MALP-2) and Glycolipids |
| Borreliae | Glycolipids (?) |
| Fungi | Glucans and Mannans |

negatively charged, hydrophilic glycerophosphate backbone. In some cases ribitolphosphate residues are constituents of LTA instead.[7,8] Recently, an improved preparation procedure applied to the isolation of LTA from *S. aureus* led to biologically active LTA[9–11] whose structure could be assigned by NMR and MS data.[9,12] The result of these studies is shown in Scheme 2.

**Prof. emeritus Dr Richard R. Schmidt**

*Richard R. Schmidt, since 1975 Professor of Chemistry at the University of Konstanz, Germany, has guided an active research group in the field of glycochemistry for many years. Education: PhD in organic chemistry, 1962, University of Stuttgart, Germany. After a postdoctoral fellowship in biochemistry at the Scripps Research Institute, La Jolla, USA, own research was initiated in various fields. He published over 600 refereed papers in international journals and received several calls and awards. The research group gained particularly high credit for the development of glycoside bond formation methodologies and for the synthesis of complex glycoconjugates.*

**Dr Christian Marcus Pedersen**

*Dr Christian Marcus Pedersen received his bachelor, master and PhD degrees from University of Aarhus, 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. Post doctoral 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 a postdoctoral scientist with Professor Mikael Bols at University of Copenhagen.*

**Yan Qiao**

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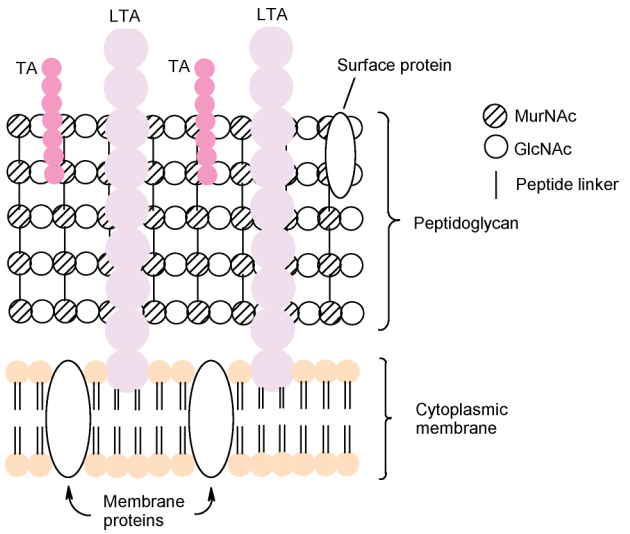
**Prof. Dr Ulrich Zähringer**

*Prof. Dr Ulrich Zähringer studied chemistry at the University of Freiburg, Germany from where he received his PhD in 1979. Since 1980 has been head of the* Division of Immunochemistry at the Research Center Borstel, Leibniz-Center for Medicine and Biosciences*. In April 1997 he received his habilitation and was nominated associate professor in 2002 by the Technisch-Naturwissenschaftliche Fakultät of the University of Lübeck. In 2002 he became a member of the editorial board of* Innate Immunity *and since 2008 he has also been member of the cluster of excellence "*Inflammation-at-Interfaces*" (Kiel-Borstel-Lübeck). Dr Zähringer has published 215 original papers and 53 reviews in peer-reviewed journals.*




**Scheme 1** Cell wall of Gram-positive bacteria consisting of a dense layer composed of numerous rows of peptidoglycan (also called "murein"; murus, Latin = wall), lipoteichoic acid (LTA; teichos, Greek = city wall), teichoic acid (TA), lipoprotein (not shown) and surface proteins.

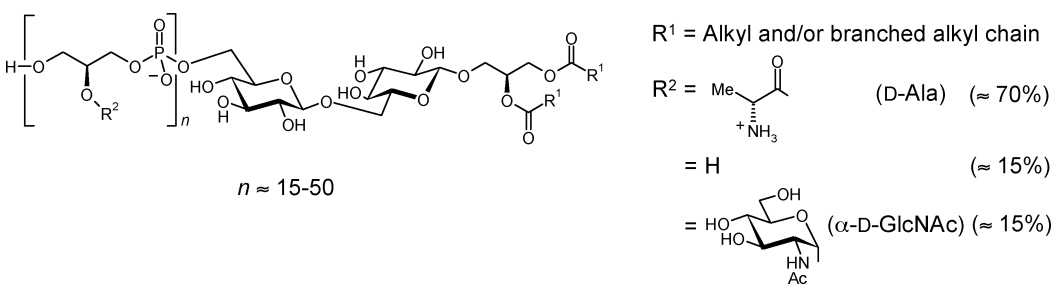
### *Staphylococcus aureus* LTA

The impact of *S. aureus* on human health is dramatic as *S. aureus* is still a major source of mortality in medical facilities.[13,14] The pathogen causes various infectious diseases, including sepsis, endocarditis and pneumonia. The resistance to most classes of antimicrobial agents (penicillins, macrolides, aminoglycosides, tetracyclins) is life-threatening. Widespread use of methicillin, a semisynthetic penicillin, led *via* acquisition of a penicillin-binding protein (PBP-2a) to methicillin-resistent *S. aureus* (MRSA) strains in many medical facilities. Currently, only glycopeptides, as for instance vancomycin, provide effective therapies against most *S. aureus* strains. Hence, further studies are urgently needed in order to fully understand the influence of its LTA on the innate immune system.

The biosynthesis of *S. aureus* LTA has been elucidated as shown in Scheme 3.[15,16] A decisive enzyme for the bacterial growth is the polyglycerophosphate synthase (LtaS) that tranfers from phosphatidylglycerol as source glycerophosphate residues to gentiobiosyl diacylglycerol and to the growing chain.[16] Thereafter alanyl and GlcNAc residues are randomly or possibly *via* a specific pattern attached to the glycerophosphate chain. It is evident that specific inhibitors of LtaS will serve as potent antibiotics for *S. aureus* infections.[16]

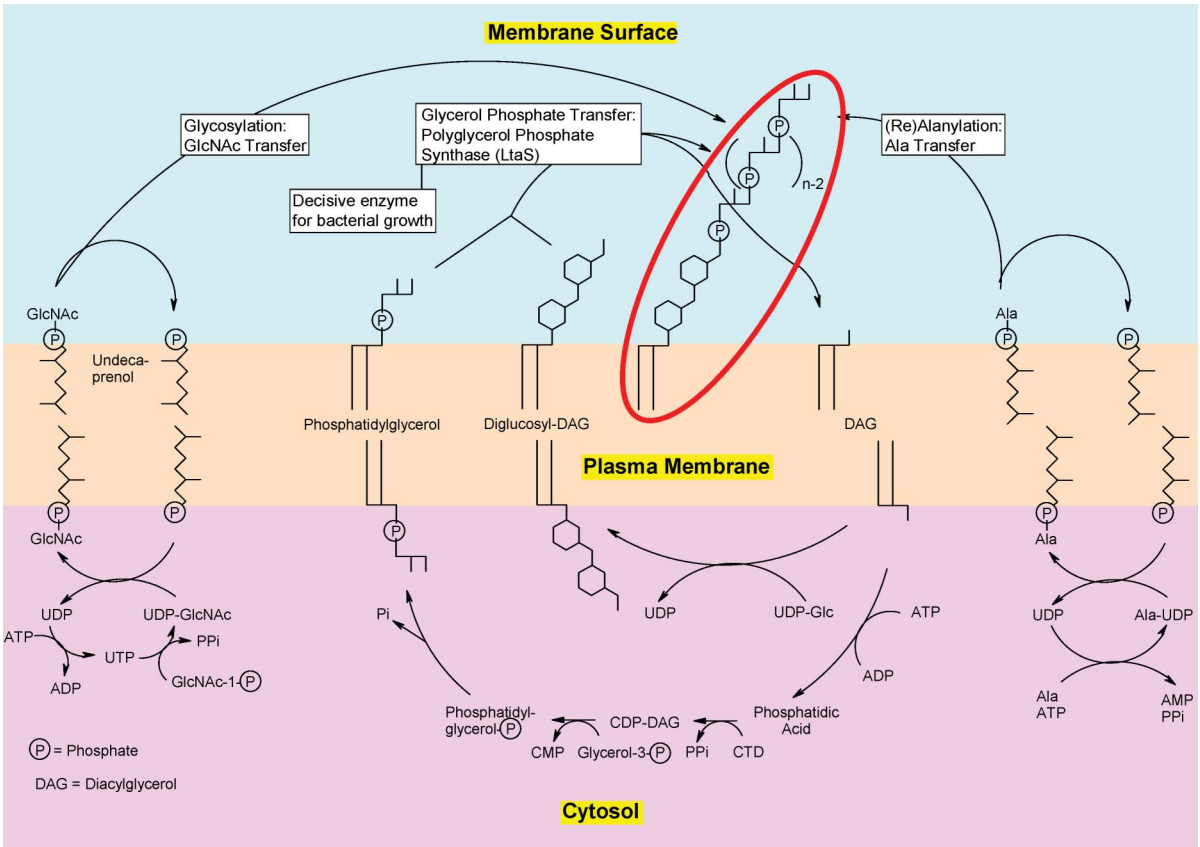
The biological studies with active *S. aureus* LTA, obtained by purification with hydrophobic interaction chromatography (HIC) originally introduced to LTA by W. Fischer,[15,17] led to the widely accepted view that the receptor for LTA recognition is TLR2[18] accompanied by cofactors TLR6,[11] CD14[19,20] and CD36.[21,22] Hence, LTA cell wall fragments released on *S. aureus* lysis bind to TLR2 and TLR6 that are found on body defence cells as the macrophages and claimed to be specific for LTA (Scheme 4). However, as structural variations of the LTA (the D-alanyl residues are already cleaved at pH 8.5 or above at a fast rate) or contaminations by lipids, lipoproteins, peptidoglycan, peptides and proteins cannot be fully excluded, chemical synthesis of structurally defined LTA is an important issue. In this way, differentiation between claims and real biological effects should be possible because the above mentioned contaminations can be avoided.

Our suspicion that, besides LTA, other contaminating bacterial PAMPs might cause the pro-inflammatory activity wrongly assigned to TLR2 was further supported by the elegant and successful crystallisation of the $Pam_3CSK_4$-TLR2/TLR1 complex by Lee and co-workers[23] who could show that indeed TLR2 signalling is quite specific for the chemical structure of the ligand and not as broad as widely anticipated.[2] By crystallization of the TLR2/TLR1 complex in the presence of the tri-acylated synthetic lipopeptide $Pam_3CSK_4$, specific binding of the heterodimer with lipopeptide was demonstrated. In tri-acylated synthetic lipopeptide the fatty acids were shown to act with the TLR2/TLR1 heterodimeric receptor in a quite specific way involving all three fatty acids in the terminal *N*-acylated cystein of the lipopeptide. The tri-acylated lipopeptide can act as a clamp forming the two "m-shaped" dimeric structures with the TLR2/TLR1 complex, which finally facilitates its dimerization. By contrast, one might expect that di-acylated $Pam_2CSK_4$ as well as the synthetic FSL-1 that are structurally more related to LTA, form the well known heterodimeric TLR2/TLR6 complexes, which are known to be specific for di-acylated lipopeptides.[24] The specificity of binding between the lipid and the leucine rich repeats (LRR) of the TLR6 is obtained *via* hydrogen bonds between the hydrophilic part of the di-acylated lipopeptides and selected amino acids of TLR6.[23,24] The role and importance of the "non-fatty acid part" of the TLR6 ligand was investigated by Kang *et al.*,[25] who published the crystal structure of LTA isolated from *S. pneumoniae* (pnLTA) bound to TLR2 aiming to investigate the specific binding of the polar head groups in lipids to TLR6 in more detail. Since the hydrogen bonds in the peptide part of lipopeptides are extremely different from those of LTAs, it is evident that LTA cannot mimic the peptide moiety in the putative LTA-TLR2/TLR6 complex. In addition,




**Scheme 2** General structure of lipoteichoic acid from *Staphylococcus aureus*.[7,9,12]



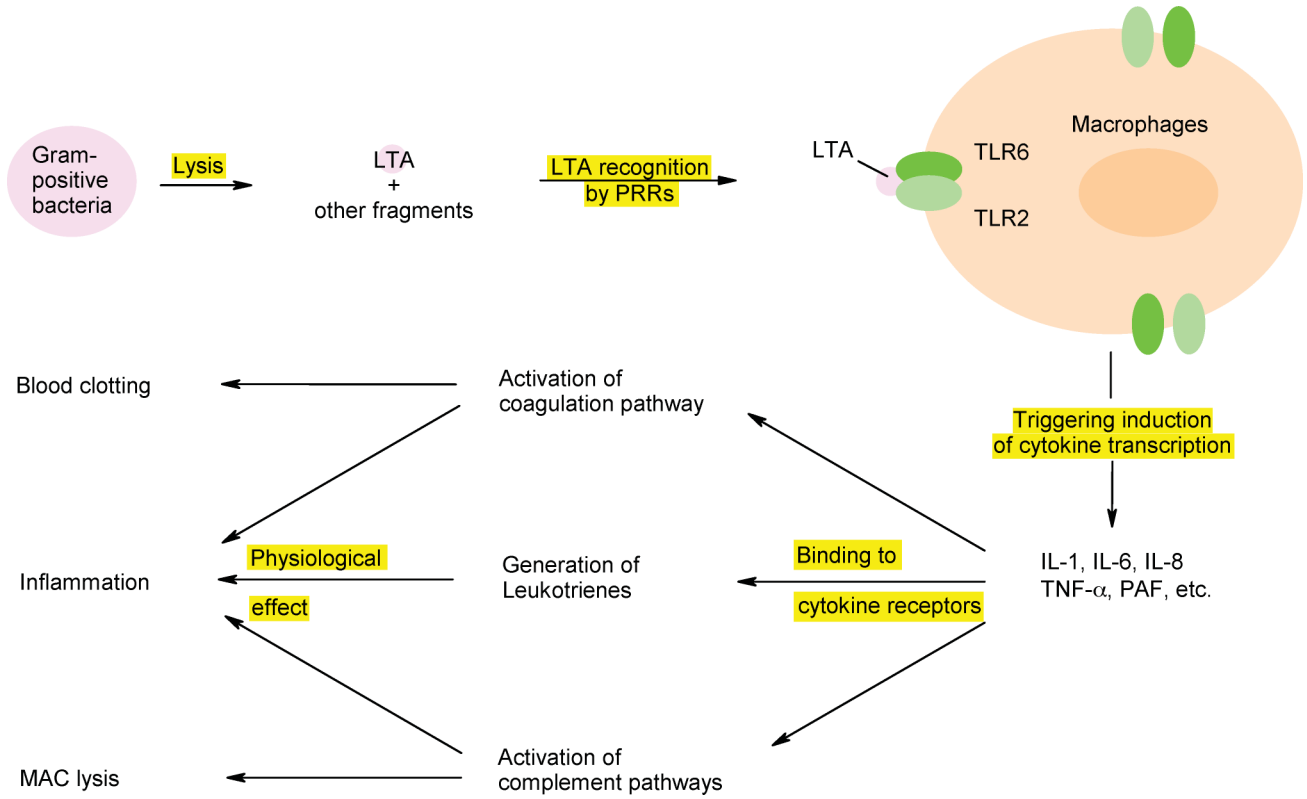

**Scheme 3** Biosynthesis of *Staphylococcus aureus* LTA[15,16] (drawing of structural mimics).

the role of the hydrophobic so called "sulfur site", turned out to be a critical element in the binding of the lipopeptide ligand to TLR6. However, as the thioether of lipopeptides, representing the "sulfur site", is lacking in LTA it cannot contribute to the specificity for TLR2 and TLR6, respectively, since pnLTA does not induce heterodimerization of the extracellular domains of TLR2 and TLR6.[25] Consequently the authors could not obtain a crystal structure from the pnLTA with the TLR2/TLR6 heterodimer as they only obtained the pnLTA-TLR2 complex as crystals. This further supports the view that LTAs might bind to TLR2, but this complex cannot form TLR2/TLR6 heterodimers, necessary for initiation of dimerization of the intracellular TLR domains, which then trigger the immune response *via* MyD88 to initiate intracellular signalling.[25]

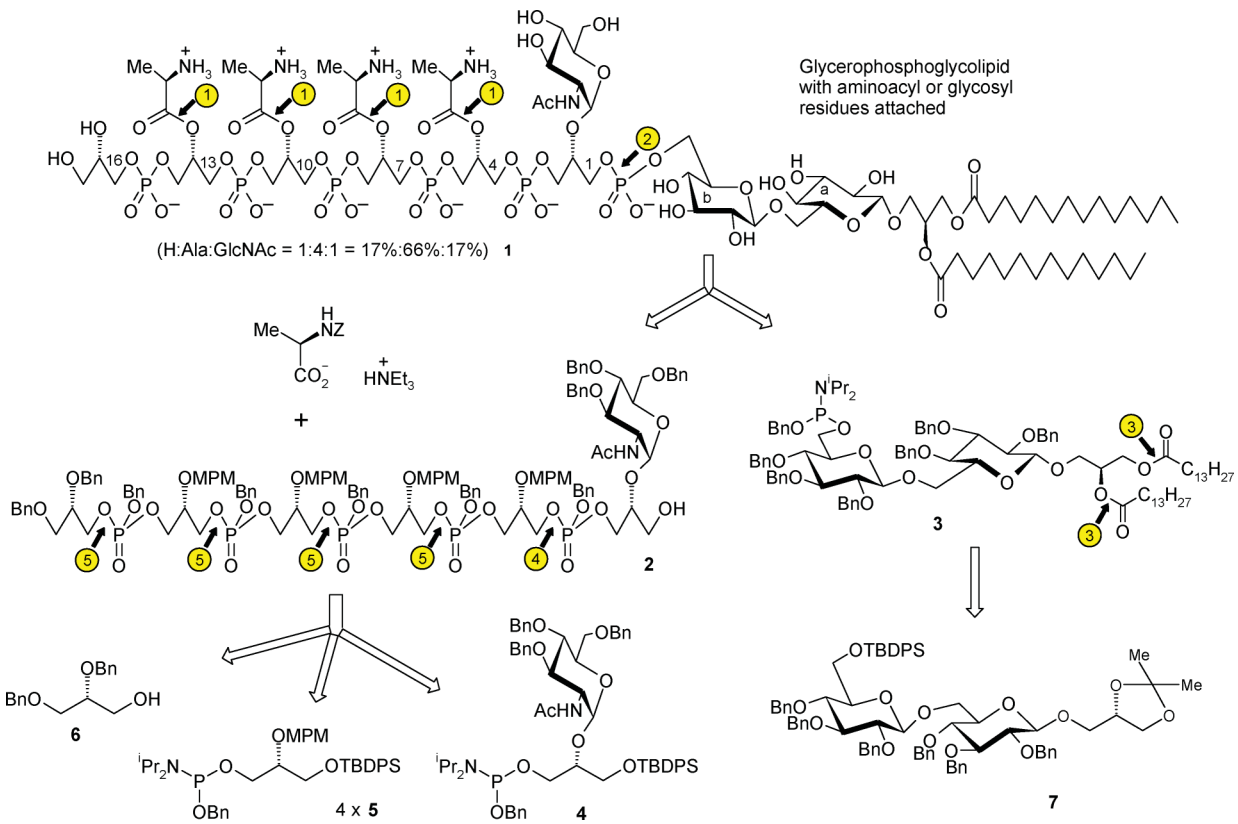
The finding that pro-inflammatory LTAs isolated from Gram-positive bacteria were probably contaminated with highly active lipopeptides was also corroborated by the work of Hashimoto and Götz who could show that the LTA isolated from a lipopeptide deficient Δ*lgt*-mutant of *S. aureus* SA 113 was almost inactive when tested for TLR2 activity, thus indicating that (contaminating) lipopeptide(s) but not LTA were responsible for the pro-inflammatory activity.[26] Taken together, a significant number of recent publications provide more and more evidence that LTA is not a PAMP for TLR2.[2] It has to be pointed out in this context that these more recent findings are also in contrast to elder publications,[27–30] including some from our groups.[20,21] As LTA is not recognized by TLR2, what else may be the PRR for LTA that is one of the best investigated representative of Gram-positive PAMPs? We will come back to answer this question at the end of this review.

When we began the chemical synthesis of the LTA from *S. aureus* (Scheme 5),[31–34] our working hypothesis that LTA and not contaminating lipopeptides are responsible for TLR2 activation, was guided by the fact that the LTA preparation isolated from *S. aureus*, after n-butanol/water extraction and HIC was "highly purified" (as deduced from MS and NMR data)[7,12,31] as also the degree in D-alanylation could be retained due to the improved extraction and purification protocol. At that time it was assumed that the alkali-labile degree of D-alanylation in LTA was correlated with its biological activity. We were guided by the hypothesis that the D-alanylation in the LTA of *S. aureus*, was the structural prerequisite being responsible for the biological activity of LTA. In order to further support or reject this working hypothesis, we started the chemical synthesis of *S. aureus* LTA.[31–33]

The required combination of glycolipid synthesis with glycerophosphate diester formation, having varying side chains, is particularly demanding as the ester bound D-alanyl residues are readily cleaved. Previous syntheses of LTA fragments lacked alanyl residues and since these preparations did not lead to biologically active materials,[34–37] thus further supporting our working hypothesis. The approximate ratio of the glycerophosphate 2-*O*-substituents of *S. aureus* LTA (see Scheme 2) of 70% D-Ala, 15% α-D-GlcNAc and 15% hydrogen[9] is practically attained with the target molecule **1** shown in Scheme 5 with a 4 : 1 : 1 ratio of these three 2-*O*-bound residues. Disconnection of the target molecule **1** between the glycerophosphate backbone and gentiobiosyl




**Scheme 4** Common explanation of the biological effects of LTA (and peptidoglycan fragments) released during Gram-positive bacterial infections. PRR = pattern recognition receptor; TLR = toll-like receptor; IL = interleukin; TNF = tumour necrosis factor; PAF = platelet activating factor; MAC = membrane attack complex.




**Scheme 5** Retrosynthesis of *Staphylococcus aureus* LTA.[32]

diacylglycerol (see arrows ① and ②) leading to intermediates **2** and **3** and *N*-benzyloxycarbonyl (Z)-protected D-alanine led to a successful route for the synthesis of the target molecule. Thus, **2** was accessible from glycerol derivatives **4–6** as building blocks (disconnections ④ and ⑤ in **2**) and **3** from the readily available gentiobiosyl diacylglycerol derivative **7**. The choice of permanent and temporary orthogonal protecting groups was decisive for the success: for chain extension of the glycerophosphate backbone (→ **2**) and the final ligation between intermediates **2** and **3** the *tert*-butyldiphenylsilyl (TBDPS) group was selected, and for the synthesis of intermediate **3** the isopropylidene group was chosen. The selective attachment of the alanyl residues was based on the mild oxidative removal of the 4-methoxyphenylmethyl (MPM) group, thus leading to the fully protected target molecule having only benzyl and Z-protection that could be cleaved by hydrogenolysis furnishing target molecule **1**. The variability of this concept in terms of different combinations of building blocks **4–6**, attachment of different amino acid residues and variation of the diacylglycerol moiety was successfully employed for SAR studies.[21,31–34,38–40]

For the biological activity of compound **1** the initiation of cytokine release by human blood leukocytes was investigated and found to be similar to that of natural LTA.[32,40] The available synthesis cassette consisting of six building blocks was also used for structure–activity relationship (SAR) studies. In this way, modifications of the LTA structure gave information on the prerequisites for the induction of cytokine release. For instance, omission of the D-alanyl residues or replacement by L-alanyl residues strongly reduced the immunostimulatory potency. Yet, neither the absence of the gentiobiose residue nor the absence of the α-linked *N*-acetyl-D-glucosamine residue altered the ability of LTA to induce cytokine release. However, the synthetic lipid anchor alone was not sufficient to induce cytokine release; the addition of three unsubstituted glycerophosphate units also exhibited low activity; however, the activity was amplified about ten-fold by 2-*O*-substitution of the glycerophosphate residues by D-alanyl residues. Hence, the minimal structural requirement for cytokine induction is (i) an LTA anchor with two fatty acids, (ii) a glycerophosphate backbone of about three moieties having (iii) two D-alanyl residues attached. Even a glycerophospholipid with deletion of the gentiobiose residue, deletion of the α-linked GlcNAc residue, amide linkage of the D-alanyl residues, and enantiomeric configuration of the 2-amino-1,3-propanediol residue led to cytokine release. Hence, several questions as to the importance of the other constituents of *S. aureus* LTA and functionalities remain to be elucidated.

However, none of the investigated structural modifications of *S. aureus* LTA led to an increased induction of cytokine release. For this to happen, an optimal presentation of the hydrophilic part of LTA to the receptor should be of utmost importance. Therefore, the bisamphiphilic compound **8** (Scheme 6) having two diacylglycerol gentiobioside residues at each end of the glycerophosphate backbone was designed and synthesized.[33] It was hypothesized that with two lipid anchors, possibly sticking into the same membrane (as shown in Scheme 6(a)), the epitope presentation should be ameliorated due to sterically improved accessibility to the D-alanyl and the α-*O*-linked GlcNAc residues.[41] As anticipated, bisamphiphilic compound **8** is more potent in terms of induction of cytokine release in human leukocytes than natural LTA and the shortened version **1** shown in Scheme 5.

Recently, another interesting biological effect of LTA was found after skin injury.[42] The normal microflora of the skin comprises *Staphylococcus* species that will induce inflammation as discussed above when present below the dermis, however they are tolerated on the epidermal surface without initiating inflammation. Hence, the question arises what happens after skin injury? Obviously, cytokine induced inflammation is an undesirable condition on skin but it is an important protective measure after skin injury. Prolonged and dysregulated production of inflammatory cytokines supports excessive neutrophil influx, resulting in extended inflammatory responses and poor healing, thus leading to extensive tissue damage.[43,44] In contrast, without a suitable inflammatory response wound healing is delayed, thus increasing the chance for microbial infection. The experimentally observed local modulation of the inflammatory response by natural and synthetic staphylococcal LTA at the site of an injury seems to be a beneficial therapeutic strategy for wound healing (or the control of other inflammatory skin disorders). Hence, the detrimental aspects of inflammation are reduced without increasing the risk of wound infection. These findings also display that the use of topical and systemic antibiotics leading to complete depletion of the skin microflora could have negative consequences.[42]

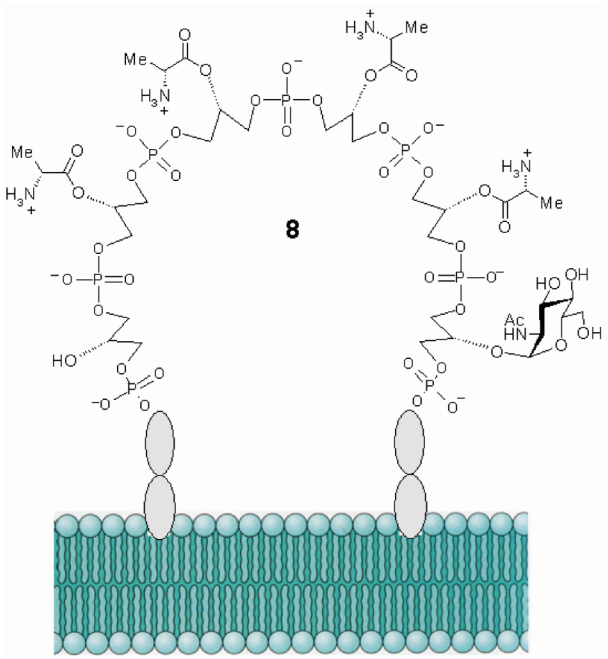
As discussed above, LTA induced cytokine release does not only lead to inflammatory responses but also to activation of the complement system, The complement system, which, besides the TLRs, is also part of the innate immune system, can be activated through three different pathways, the classical, the alternative and the lectin pathway. It may be considered as an irony of history that the first receptor for LTA identified in 1985 was complement, although at that time the alternative pathway was thought to be activated[45] Later a 28 kDa serum protein from normal mice was found to bind to LTAs from different bacteria, but also to various LPS. However the structural motif binding to this serum protein in both glycolipids remained unknown.[46]

The best investigated LTA binding proteins are L-ficolin and H-ficolin, which are plasma proteins in humans and structurally resemble to the mannose binding lectin (MBL). Ficolin was the first lectin identified to specifically interact with LTA, most likely *via* terminal hexosamines (GlcNAc and GalNAc)[47–49] present in the glycan part of *S. aureus* and *S. pneumoniae* LTA, respectively.[48,49] It is tempting to speculate that hexosamines (HexN, either *N*-acetylated or not[49]), are present in LPS as well as in LTA, thus explaining the before mentioned cross-reactivity of both glycolipids with the 28 kDa serum protein in the mouse.[46]

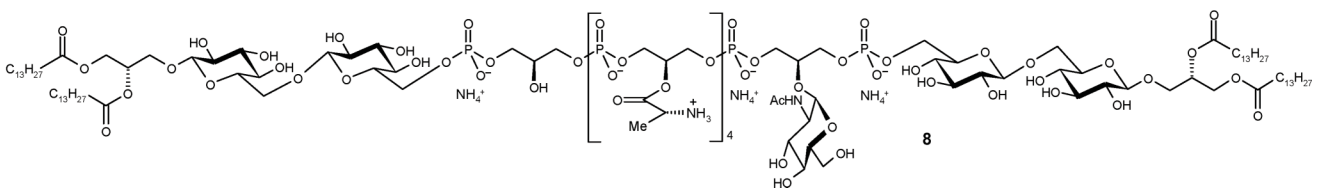
So far, only (very) minor induction of the complement system was observed with synthetic LTA **1** and its modifications. Even an increase of the number of α-linked GlcNAc residues had no effect; only LTA **8**, having two lipid chains, showed some activation of the complement system that is about 10% of that of natural *S. aureus* LTA.[51]

As already mentioned above, the role of LTA present in Gram-positive bacterial cell walls has led to controversial discussions in the last decade. The biological studies resulted in differing activities of LTA and due to contaminations in the isolated LTA some results of earlier studies are questionable. Hence, synthetic LTA derivatives lacking these contaminations have played a major role in the discussions in the following years. However, the early

(a)



(b)



**Scheme 6** (a) Proposed improved epitope presentation to the receptor by two lipid anchors; (b) Bisamphiphilic *Staphylococcus aureus* derived LTA **8**.[33]

approaches to LTA synthesis were lacking some fundamental structural moieties[34] and therefore the compounds were not biologically active. Yet the total synthesis of *S. aureus* LTA led to biologically active material and this work confirmed the crucial role of the ester bound D-alanyl residues for biological activity.[32] However, of all PRRs in innate immunity toll-like receptor 2 (TLR2) seems to recognise the broadest range of different bacterial compounds known as PAMPs.[2] Some of the most important agonists claimed to be recognised by TLR2 are compiled in Table 2. As these molecules are structurally quite diverse it seems unlikely that TLR2 has the capacity to interact with all these agonists to the same degree. Hence, based on the different sensitivity of the innate immune system to the various PAMPs it was concluded that the TLR2 receptor is more specific as one might conclude from the broad range of structurally non-related compounds that were published to serve as TLR2 ligands. Only lipopeptides and lipoproteins are sensed at physiological concentrations (picomolar level) by TLR2.[2,13,23,26,52] Consequently, it was stated: "In view of the fact that PRRs of the innate immune system need sensitivity and specificity, a promiscuous receptor appears to be a contradiction".[2] However, studies by S. von Aulock *et al.* showed that *S. aureus* LTA is a major immunostimulatory principle of Gram-positive bacteria,[53] yet the PRR for LTA remains so far unclear.

### *Lactococcus garvieae* LTA

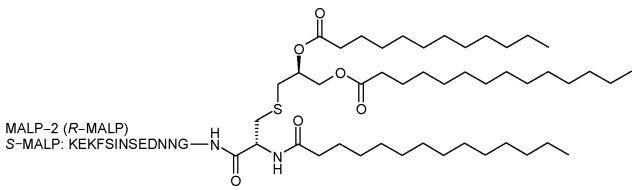
These discussions led us to look into other types of LTAs that were defined by W. Fischer already in the 1990s (Scheme 7) and we synthesized also the type I LTA of the Streptococcus species DSM 8747.[6,54] As the particularly active lipopeptide TLR2 ligands possess three fatty acyl chains, it appeared that the type II LTA from *L. garvieae* is a particularly important target molecule (Scheme 8).[7,55] Two fatty acyl chains are α-linked *via* a glycerol residue to the kojibiose part that in addition can carry a third fatty acyl residue linked to the 6-hydroxy group of the first glucose residue. Chain extension of this molecule is based on a α-D-Gal-(1→6)-α-D-Gal(1→3)-glycerolphosphate residue carrying at 2-*O* a further α-linked Gal residue. No D-alanyl residues are attached. The retrosynthetic synthesis design for constituent **9** with *n* being 1 exhibits that the kojibiosyl lipid anchor **10** requires a disaccharide donor having at 6- and 6′-position orthogonal temporary protecting groups permitting chain extension and fatty acyl group attachment, respectively. The repeating unit **11** requires at 6-*O* of the nonreducing end galactosyl residue permanent protection for chain termination (→ **11**$^{t}$) and temporary protection for chain extension (→ **11**$^{e}$). Hence, the target molecule should be accessible form the four building blocks **12–15** shown in Scheme 8. This could be confirmed for *n* being 1.[56]

**Table 2** Published Ligands of TLR2[2]

| | Type of ligand | Source |
|---|---|---|
| Glycolipids | Lipoteichoic acids (LTAs) | *Staphylococcus aureus*<br>*Streptococcus pneumoniae* |
| | Lipomannan (LM)<br>Lipoarabinomannan (LAM)<br>Glycosylphoshatidylinositol (GPI) anchors<br>Lipopolysaccharides (LPS) | |
| Peptidoglycan | From various bacterial origins | *S. aureus*<br>*S. pneumoniae*<br>*S. pyogenes* |
| Lipoproteins | From various microorganisms | |
| Lipopeptides[a] | Mycoplasma fermentans | |
| Proteins and glycoproteins | From various microorganisms | |
| Viruses | | |
| Whole bacteria | | |

[a] For instance:




Work on further chain extension is in progress and the results of biological studies will be reported in due course.

### *Streptococcus pneumoniae* LTA

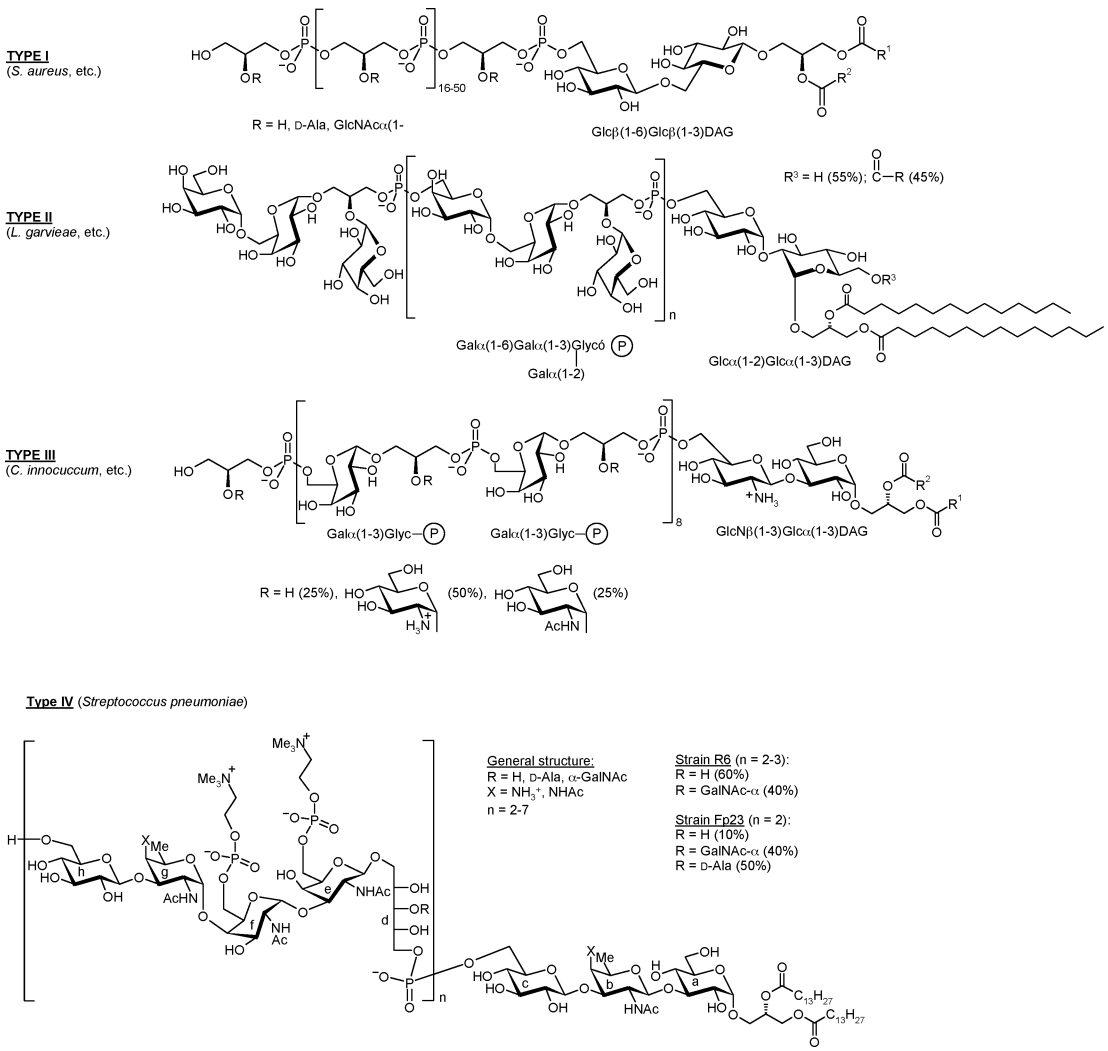
Structurally very different from type I to III LTAs is type IV LTA from *S. pneumoniae* (Scheme 7).[7] Hence, the question arose, does this LTA also signal *via* TLR2? The answer is of great importance for the biomedical impact as *S. pneumoniae* is one of the most common Gram-positive pathogens. By colonising the upper respiratory tract it causes severe infections; life-threatening diseases like pneumonia, bacteremia and meningitis are caused when it reaches the lower respiratory tract or the bloodstream,[57] thus resulting in a high mortality rate.[58,59] Structural analysis displayed that the polyglycerophosphate backbone of staphylococcal LTA is replaced by a pseudopentasaccharide repeating unit that consists of a ribitolphosphate, having eventually a GalNAc residue attached, and a tetrasaccharide moiety carrying two phosphocholine residues.[7,8] In order to investigate the impact of streptococcal LTA and its structural alterations on LTA function in general we developed the first and a modular synthesis of streptococcal LTA of the R6 strain, as depicted in Scheme 9.[60] Hence, the major structural isomer **16** having R = H, X = $NH_3^+$ and $n = 1$ (instead of about 2),[8,61] was selected as target molecule. This way, also the previous structural assignment could be confirmed.

The synthesis design of compound **16** is outlined in Scheme 9. For a convergent synthesis of this demanding molecule, disconnections at two glycosidic linkages (① and ③) and at the phosphate linkages (② and ④) were envisaged leading to intermediates **17–20**. Their final disintegration leads to the nine carbohydrate derived building blocks **21–29** of which **22** was required as precursor for the DAG moiety and **21**, **23–29** were the precursors for the sugar moieties in the target molecule. The glycosidic linkages were performed with *O*-glycosyl trichloroacetimidates as glycosyl donors[62,63] and the stereoselectivity was controlled by anchimeric assistance and solvent and/or stereoelectronic effects, respectively.[63] However, the final accomplishment of the total synthesis of this molecule turned out to be a demanding task as already the synthesis of suitable building blocks required about sixty steps. Their assembly to the target molecule **16** (*i.e.* ligation of **30** and **31** yielding **32** and final deprotection to afford **16**) required additional twenty five to thirty steps of which the final two steps are compiled in Scheme 10. This way, highly complex glycophospholipid **16** was successfully synthesized for the first time *via* totally eighty five to ninety steps from sugar precursors. The building blocks could either be highly stereoselectively linked or linked *via* phosphodiester generation, respectively, in a convergent manner. The structure of **16** could be fully confirmed and thus the previous structural assignment of natural material from *S. pneumoniae* R6 strain ascertained. Compound **16** also exhibits immunological activity that results in cytokine release, however, so far *via* an unknown mechanism, as TLR2 and TLR4 are not the signalling receptors for synthetic pneumococcal LTA.[60,64]

The structure of the LTA of *S. pneumoniae*, originally published by W. Fischer and colleagues,[65] was the basis for the synthetic LTA shown in Scheme 9. However, the structure of the repeating units has been recently revised by Nahm *et al.*[8] This structural revision did not concern the sequence of the sugars. Instead the pentameric repeating unit was "shifted", beginning at AATGalNAc (b) and ending at the 6-*O*-phosphocholine substituted α-D-GalNAc (f). The reason for this revision was the fact that the older structure of natural LTA (pnLTA) did not explain the Forssmann antigen properties of the pneumococcal LTA [terminal α-D-GalNAc-(1→3)-β-D-GalNAc-(1→].[8] On the other hand, the difference in structure could also be attributed to the different strains used in both studies, R6[65] and R36A.[7] Furthermore, the substitution of the ribitol-phosphate unit (d) with α-D-GalNAc as well as D-Ala residues, which had been carefully investigated by NMR[61] studies of natural pnLTA, was not considered in the synthetic LTA shown above.

In addition, we also synthesized the pseudopentasaccharide repeating unit **34** according to the structure published by Fischer and colleagues[65] and determined its biological activity. This work will be published in due course.[66]

Modification of these building blocks will also permit modular syntheses of *S. pneumoniae* LTAs of other strains. However, it seemed to be of interest to investigate the biological properties of the main constituents of **16**, namely the pseudopentasaccharide repeating unit **34** and the trisaccharide core structure **35**. To this end, intermediates **31** and **33** of the total synthesis were deprotected by hydrogenolysis, thus providing the desired compounds **34** and **35**, respectively (Scheme 11).[66,67] Not unexpected, pseudopentasaccharide **34**, lacking a lipid moiety, did not induce any immunological response.[64] However, the glycolipid core structure **35** stimulated cytokine release in human mononuclear cells (MNCs),[67] yet neither TLR2 nor TLR4 are the signalling receptors. This indicates that other receptors of the innate immune system, such as the lectin pathway of the complement system, might be the most likely PRR for **16** and **35**. The consequences of this finding for the previous biological studies will have to be elucidated.



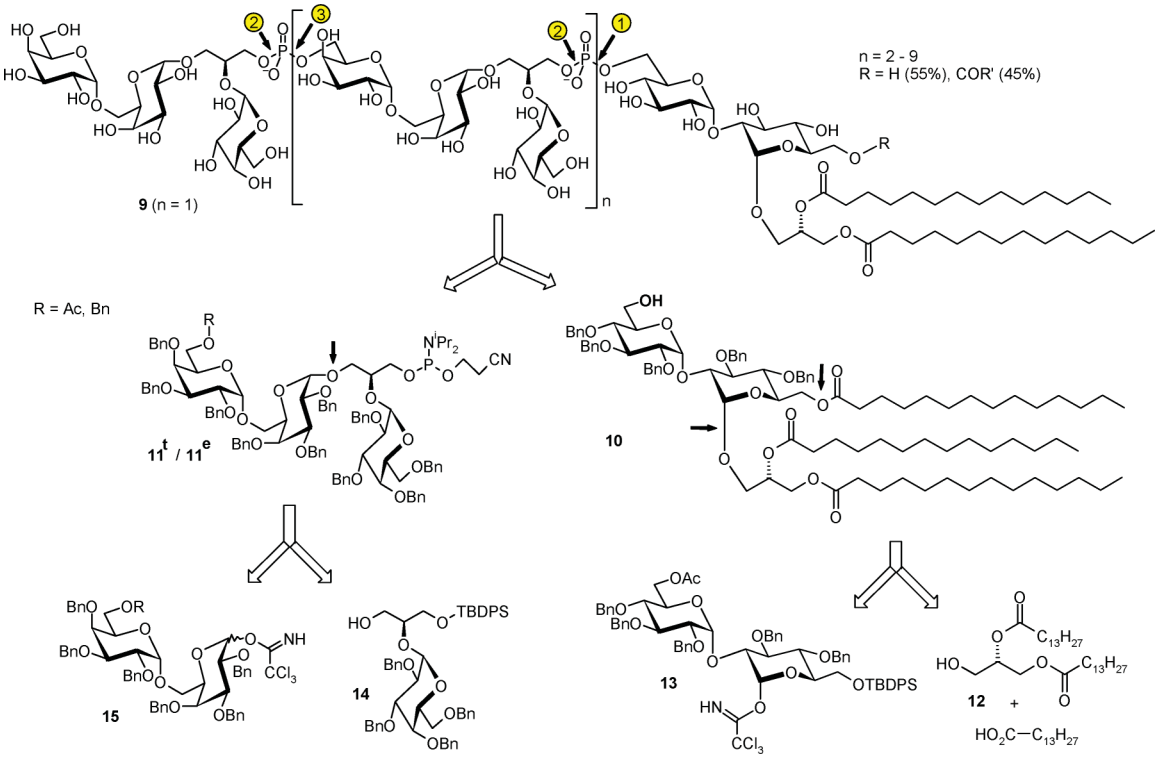

**Scheme 7** Lipoteichoic acids of type I–IV.[7]

## Contribution of the synthetic chemistry towards the identification of the receptor for LTA

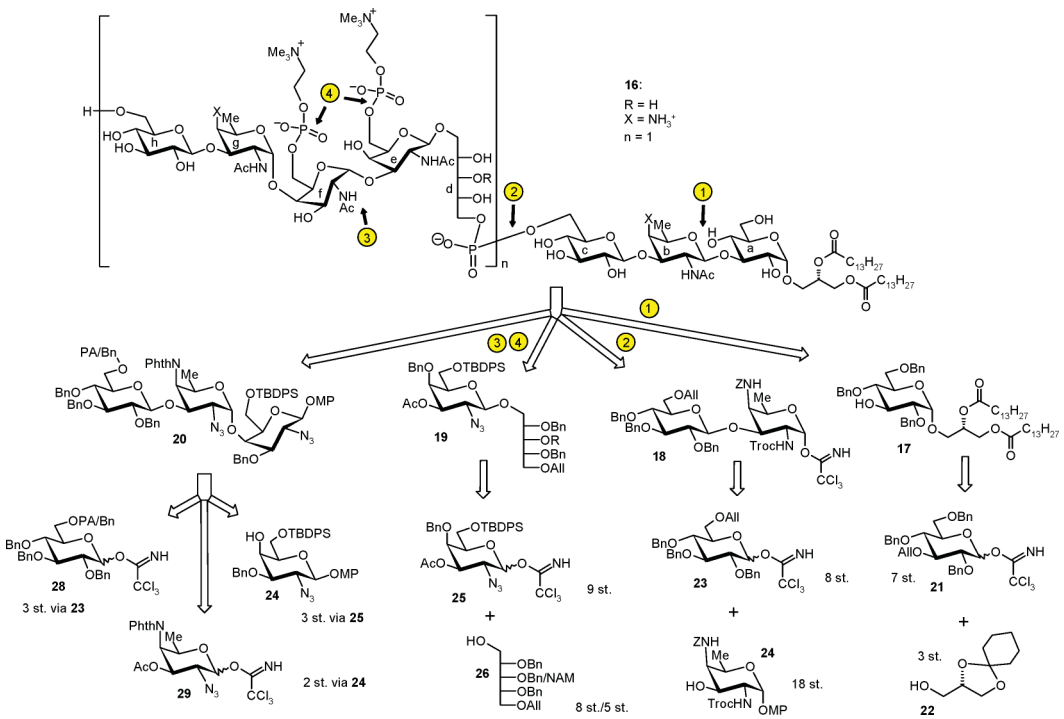
The perspective exhibits that the total synthesis of lipoteichoic acids (LTAs) is by now within reach and even an LTA being structurally as complex as that of *S. pneumoniae* could be successfully synthesized. In order to achieve these synthetic goals versatile building blocks have been designed that are also available for structural modifications. Thus, in a cassette approach structurally modified LTAs are accessible that are required for structure–activity relationship (SAR) studies.

The discussion about the PRRs which specifically interact with LTA has been controversial over the years[2] The fact that PRRs of the innate immune system use co-factors for lipophilic or amphiphilic PAMPs is well known for LPS (endotoxin). Such type of glycolipids need to be "solubilized" since they form high molecular aggregates (>1 000 000 Da), which cannot be recognized by PRRs of the innate immune system in a specific way.[2,68] Therefore, additional less specific co-receptors are necessary for the transport and correct presentation of amphiphilic PAMPs to the "real" receptor on the surface membrane of immunocompetent cells.[50] The physicochemical situation for LTA can be assumed to be quite analogous to that of LPS, although such biophysical studies for LTA as a membrane component are lacking so far. In LPS signalling co-receptors for endotoxin are LBP,[69] and CD14.[70] They are believed to enhance and strengthen the specificity for the interaction of LPS with the TLR4/MD-2 complex. Otherwise amphiphilic PAMPs tend to unspecifically adhere to each other to form higher aggregates or to unspecifically adsorb to proteins.[68] Consequently it was found that not only CD36 binds to LTA but also CD14,[20] indicating that these co-receptors have a broad specificity and assist to the receptor when interacting with lipids, glycolipids, and lipoproteins. Because of this it is tempting to speculate that, like LPS, LTA utilizes co-receptors for signalling, although such kind of studies are lacking till date. Some of such co-receptors have been identified quite recently as the scavenger receptors (SR) of liver endothelial and Kuppffer cells[71] or CD36.[21,22] Although quite speculative, for physicochemical reasons it makes sense that, as for TLR2/1/6 and TLR4, also the lectin-pathway of the complement uses co-factors for the presentation of amphiphilic PAMPs. This appears necessary to correctly discriminate "non-self" from "self" in a reliable way, which is the major issue of the innate immune system[2] It is noteworthy that in the literature these co-factors are often, if

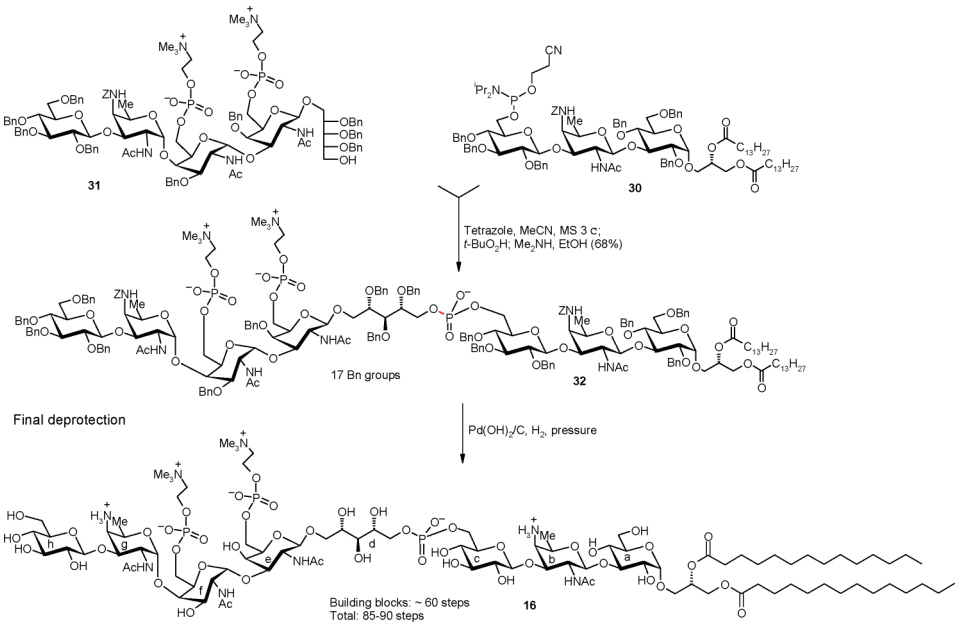



**Scheme 8** Retrosynthesis for *Lactococcus garvieae* LTA.[56]

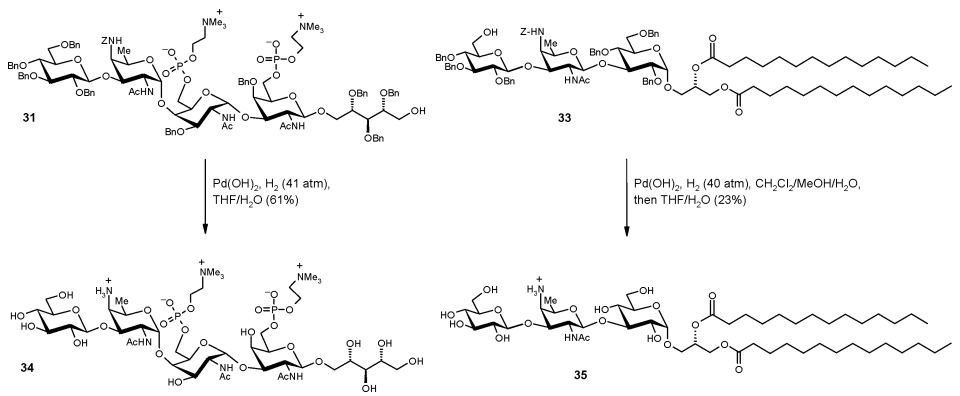



**Scheme 9** Retrosynthesis of *Streptococcus pneumoniae* LTA.[60] (st = required number of steps for the synthesis).

not always, termed to be receptors for LTA, which still needs to be proven. This way of changing the terminology has a historical counterpart in another co-receptor of LPS [membrane bound CD14 (mCD14)], which was considered for a long time to be the receptor for endotoxin, which later turned out to be wrong, since TLR4 was identified to represent the PRR for LPS. The reason for this revision was the fact that mCD14 represents a GPI-linked glycoprotein lacking the transmembrane domain necessary for signalling. Today the function of mCD14 to act as a co-factor for LPS signalling with MD-2/TLR4 heterodimer is well accepted.[50]




**Scheme 10** Completion of the total synthesis of *S. pneumoniae* LTA.[59]




**Scheme 11** Generation of deprotected **HGFED** and **CBA**-DAG fragments of *S. pneumoniae* LTA.[65,66]

In order to get more insight into the structure-function relationship of LTA, be it from *S. aureus* or *S. pneumoniae* or *L. garvieae*, we took advantage of the fact that synthetic LTAs were indeed helpful in order to clarify the question if contaminating bacterial compounds might have raised the activation, which will not be expected for synthetic LTAs. Therefore, we re-investigated the interaction of LTAs with its PRRs, as these synthetic compounds were the PAMPs of choice in order to clarify this point on a molecular based structure-function relationship. When testing the synthetic LTAs in various biological read-out systems our first results surprisingly revealed that TLR2 cannot account for the pro-inflammatory activity of LTA. Instead, another receptor, most likely identified as the lectin pathway of the complement,[66,67] was considered as a possible receptor candidate for LTA.

Today we are still at the very beginning to understand how LTA, representing (with the exception of mycobacteria) one of the major glycolipids in the membrane of Gram-positive bacteria, is recognized by (the) receptor(s) of the innate immune system. To this end, further studies with highly pure synthetic material are urgently needed in order to understand the innate immune response to these important constituents of the cell wall of Gram-positive bacteria with clinical relevance. This way, highly desirable new perspectives and treatments against Gram-positive bacterial infections should finally become available.

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