Synthesis of ribitol phosphate based wall teichoic acids

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Summary and future prospects

The Gram-positive bacterial peptidoglycan is densely functionalized with carbohydrate-based anionic cell wall polymers, known as wall teichoic acids (WTAs). These WTAs are composed of repeating ribitol phosphates (RboP), that can be randomly modified with carbohydrate appendages and/or D-alanine (D-ala) substituents. WTAs fulfil important roles in cell shape, cell division, biofilm formation, phage infectivity, and pathogenesis as well as resistance to cationic antimicrobial peptides.

This Thesis describes the synthesis of various WTA fragments of *Staphylococcus aureus* and *Enterococcus faecalis*. Multi-drug-resistant strains of both bacteria have developed, including the 'hospital bugs' Methicillin-resistant *S. aureus* (MRSA) and Vancomycin-resistant enterococci (VRE). Novel strategies to combat these bacteria are urgently required and passive or active vaccines may contribute to protection against these opportunistic pathogens. This Thesis describes the development of synthetic methodologies to generate both non-substituted and substituted RboP oligomers. The pursued substitution patterns include α- and β-*N*-acetyl glucosamine (GlcNAc) residues on the RboP C-4, β-*N*-acetyl glucosamine on the RboP C-3 and D-alanine (D-ala) at the RboP C-2, as found in the WTA of *S. aureus*. The synthetic strategy followed relies on the use of phosphoramidite chemistry both in solution and on an automated solid phase synthesizer. The WTA of *E. faecalis* consists of *N*-acetyl-β-D-galactosaminyl ribitol phosphate residues having a α-L-rhamnose branch at the C3 of the galactosamine residue and a ribitol phosphate linkage to the C-1 of the galactosamine. The isolation of these WTA structures from bacterial sources presents problems with contaminations and obtaining mixtures of structures with different lengths and substitution patterns. Organic synthesis on the other hand can deliver the target compounds with the length and substitution pattern of choice and both in higher purity and in larger amounts, allowing detailed immunological studies that can aid in future vaccine development.

Chapter 1 reviews the synthetic efforts in the field of teichoic acid (TA) chemistry to generate well-defined TAs for vaccine development. This Chapter describes the synthesis of WTA fragments of *S. aureus*, *E. faecalis* and *faecium,* and *Clostidrium difficile*. Besides these syntheses, this Chapter also introduces TA micro-arrays as a diagnostic tool to study the interaction between TAs and relevant molecules, like human sera or phage proteins. Synthetic TAs can also serve as substrates for bacterial biosynthesis enzymes.[1] The manipulation of these processes will enable studies unravelling the function of WTAs at the cellular level.

Chapter 2 describes the synthesis of non-substituted ribitol phosphates in solution with a length ranging from a trimer to an octamer from readily available building blocks. The same building blocks were applied for the assembly of an octa- and dodecamer

using automated solid phase synthesis. The synthesized WTA hexamer has been used as a substrate to probe the enzyme glycosyltransferase TarP, which was recently discovered to be responsible for the β-GlcNAcylation on the C-3 position of the RboP chain. In another application, the WTA hexamer was coupled to magnetic beads to generate WTA-functionalized beads. The WTA-oligomers were equipped with a biotin affinity handle and subsequently enzymatically glycosylated using the glycosyltransferases TarM, which introduces α-GlcNAc residues, TarS to attach β-GlcNAc residues at the RboP C-4, and TarP. The glycosylated-WTAs could next be bound to streptavidin-coated magnetic beads and used as diagnostic tools to detect WTA-specific IgG antibodies in human serum. The beads represent rapid and clean diagnostic tools to measure antibody levels and provide the opportunity to study the interaction of WTAs with other relevant biological samples. The enzymatic glycosylation using TarS was performed on a larger scale to provide 0.5 mg enzymatically glycosylated WTA. Future research will be directed to further scaling up this process for all three glycosyl transferases for the rapid assembly of larger amounts glycosylated WTAs.

Chapter 3 describes the synthesis of a set of WTA hexamers, glycosylated at the C-4 of the RboP repeating units, with one or two α- or β-GlcNAc moieties on the RboP chain. A micro-array was used to investigate the binding of the β-GlcNAc WTAs with human langerin. In addition, a RboP hexamer- and dodecamer (Chapter 2) were enzymatically modified, bound to magnetic beads and interrogated for binding with human langerin. Langerin binding was observed for the β-GlcNAc functionalized WTA fragments using the micro array, this powerful tool can also be used to investigate WTA binding of (monoclonal) antibodies (mAbs) and human sera to discover immunogenic promising antigen candidates.
To enable crystallization studies with biological binding partners, such as antibodies and lectins two WTA trimers were synthesized bearing a single α-GlcNAc or β-GlcNAc at the second RboP repeat. Future studies will be directed to the crystallization of these fragments with langerin as well as monoclonal antibodies.

Chapter 4 describes the synthesis of C-3 β-GlcNAc-RboP hexamers. The introduction of C-3 β-GlcNAc residues, was recently discovered to be mediated by the glycosyltransferase TarP, and TarP modified WTA was found on clinically relevant *S. aureus* strains.[2-4] The fragments were synthesized both in solution and on solid phase. The multi-milligram quantities of material that were obtained made it possible to fully characterize the fragments using NMR spectroscopy, corroborating the structure determination of WTA-species isolated from bacterial strains. The WTAs fragments bearing two α-GlcNAc or β-GlcNAc residues were coupled to magnetic beads as previously described (Chapter 2). The beads were screened against monoclonal anti-α-1,4-GlcNAc WTA antibodies

(anti-α) and anti-1,4-β-GlcNAc-WTA antibodies (anti-β). The anti-α-GlcNAc WTA mAb selectively bound the α-1,4-GlcNAc WTA in a concentration-dependent manner, and the anti-β-GlcNAc WTA mAb bound both the 1,4-β-GlcNAc-WTA and 1,3-β-GlcNAc-WTA functionalized beads, with slightly better binding of the 1,4-β-GlcNAc-WTA. The mAbs were also screened against the unsubstituted WTAs (Chapter 2), and the substituted C-4 α- and β-glycosylated WTAs using the WTA micro-array. Figure 1 shows that the mAbs bind only to the WTA fragments bearing a GlcNAc appendage, showing that these mAbs do not bind to the RboP-backbone. The graph also shows that mAbs bind very specifically to the configurational epitope to which they are raised and that only a single GlcNAc moiety is required for effective binding. As expected, the WTA fragment that bears both an α- and β-GlcNAc binds to both mAbs.

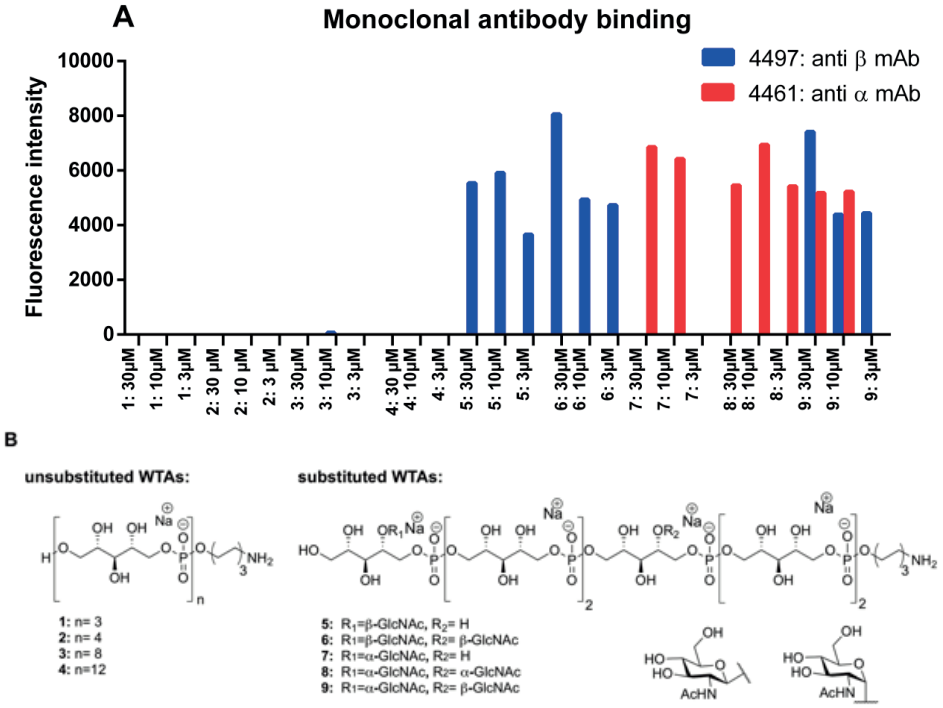


Figure 1. Binding specificity mAbs 4461 and 4497 (A) to synthetic WTAs on micro array (B).

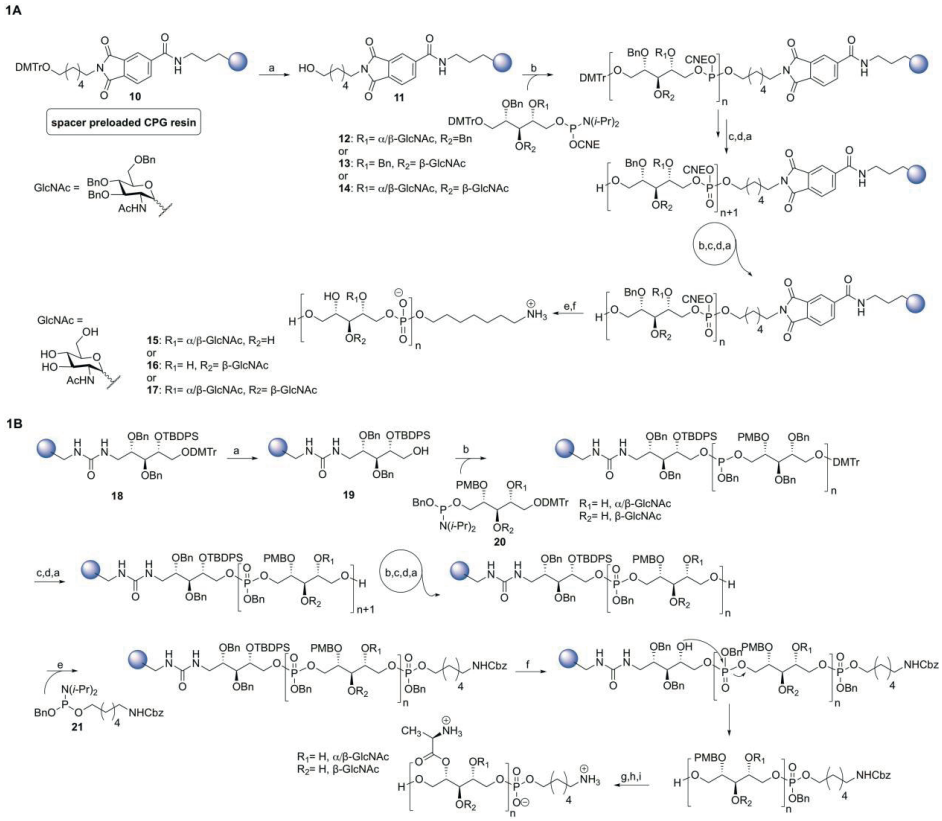
In an effort to further streamline the synthesis of GlcNAc WTAs, solid phase synthesis can be used to assemble these fragments of various length and featuring different substitution patterns, rapidly delivering a broad library of WTA fragments. An approach is depicted in Scheme 1A. Starting from commercially available spacer preloaded CPG resin **10**, an acid mediated step using DCA in toluene liberates the alcohol functionality. Condensation of alcohol **11** with either building blocks **12**, **13** or **14** under activation

of 5-(Benzylthio)-1*H*-tetrazole (BHT) will yield the phosphite intermediate, wich upon oxidation will be delivering the phosphate fragment. A capping step using Ac_2O, *N*-methylimidazole and 2,6-lutidine prevents any unreacted alcohol functionalities to react further in the upcoming cycles. Acidic detritylation then allows the next coupling cycle to start using one of the available phosphoramidite building blocks to assemble the library. Final deprotection steps include removal of the cyanoethyl groups by β-elimination using aqueous ammonia and the release from the CPG resin followed by palladium-catalyzed hydrogenolysis to afford the target fragments.

Chapter 5 has described an approach to synthesize an alanylated WTA oligomer. The presence of D-alanine esters in WTA fragments plays an important role in the biological activity of WTAs and it has been found that mutants lacking D-alanine esters are not only more sensitive to antimicrobial peptides like defensins but also show an increased susceptibility to Vancomycin and other glycopeptide antibiotics.[5-6] To explore the role of the D-alanine ester modification further, well-defined synthetic fragments are indispensable for biological activity studies. However, their synthesis poses a great challenge. Chapter 5 has described the synthesis of a heptamer carrying two D-alanine esters. It proved to be important to keep the final deprotection conditions slightly acidic because of the high lability of the D-alanine esters under basic conditions. A specific purification protocol was needed not to jeopardize the labile esters.

Scheme 1B presents a possible automated solid phase assembly procedure. Since most linkers require a basic step for cleavage, the novel silyl linker **18** is proposed, which requires mild fluoride-mediated conditions for cleavage. The phosphotriester group of the building blocks are protected with a benzyl group that is removable by hydrogenolysis, instead of the commonly used cyanoethyl group, which requires a basic step for removal. Starting from linker **18**, an acidic step removes the trityl group and the first coupling can take place. The first coupling cycle can start with building block **20**, which bears a PMB group that masks hydroxyl group that is to be functionalized with a D-alanine ester in the penultimate stage of the synthesis. Cleavage of the PBM ethers requires less harsh conditions than the removal of the NAP, described in Chapter 5. To allow for more variation in substitution patterns, the C-4 and C-3 positions of **20** can be modified with an α- or β-GlcNAc at the C-4 or β-GlcNAc on the C-3 yielding both an D-alanine and a glycoside substituent within the same RboP repeating unit. Oxidation, capping and DMTr cleavage complete the coupling cycle. Repetition of these steps will deliver the protected resin-bound fragments, which can be functionalized with a spacer using phosphoramidite **21**. In the next step the TBDPS group is cleaved off using TBAF leading to cleavage of the target fragment form the resin. Next the PMB groups can

be removed using DDQ, after which the coupling with D-alanine can take place. A final hydrogenation step can then deliver the target compounds.



Scheme 1A. Assembly of glycosylated WTAs using ASPS approach; *Reagents and conditions:* a) 3% DCA, toluene; b) phosphoramidite **12, 13,** or **14**, 5-(Benzylthio)-1*H*-tetrazole, ACN; c) I_2, pyridine, H_2O, ACN; d) Ac_2O, *N*-methylimidazole, 2,6-lutidine, ACN; e) i. 3% DCA, toluene; ii. 25% NH_3 (aq); f) Pd black, H_2, dioxane H_2O, AcOH. **Scheme 1B.** Assembly of alanylated WTAs using cleavable linker **18** based on ASPS approach; *Reagents and conditions:* a) 3% DCA, toluene; b) phosphoramidite **20**, 5-(Benzylthio)-1*H*-tetrazole, ACN; c) I_2, pyridine, H_2O, ACN; d) Ac_2O, *N*-methylimidazole, 2,6-lutidine, ACN; e) phosphoramidite **21**, 5-(Benzylthio)-1*H*-tetrazole, ACN; f) TBAF/THF; g) DDQ, β-pinene, DCM/H_2O/*t*-BuOH; h) Z-D-alanine, PyBOP, NMI, DCM; i) H_2, Pd black dioxane/H_2O, AcOH.

Chapter 6 describes the synthesis of Enterococcus faecalis (*E. faecalis)* WTA fragments. *E. faecalis,* a Gram-positive bacterium commonly found in human and animal intestines, is responsible for a wide range of infections like endocarditis, wound- and urinary tract infections. These infections are treated with antibiotics, however the widespread use of antibiotics has led to the emergence of resistant strains such as Vancoymicin Resistant Enterococcus (VRE), which poses a great danger for our healthcare system. Chapter 6 is focused on the synthesis of two well-defined WTA fragments belonging to *E. faecalis* which can serve for the development of a possible conjugate vaccine. The WTA is built

up from a -6-[((GalNAc)-α(3-1)-L-Rha)-β(1-1)-RboP]- repeating unit (Fig 2). The trisaccharide repeating unit was assembled using a regioselective glycosylation of a rhamnose donor, carrying a participating benzoyl group at the C-2 to ensure the stereoselective formation of the α-glycosidic linkage, and a C3, C4-diol GalNAc acceptor. The C-4 OH was masked with a benzoyl group at the dimer stage and the resulting building block was used in the next step in a glycosylation with the ribitol acceptor.

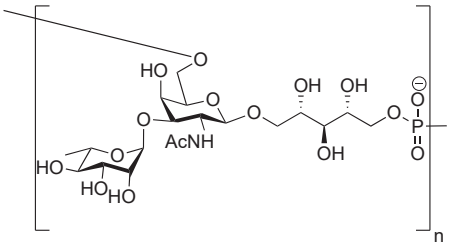


Figure 2. Structure of *E. faecalis* WTA.

Unfortunately, this coupling resulted only in 45% yield. Optimization was hampered by the low quantities of building blocks available. Converting the thiodonor into an imidate donor may increase the reactivity leading to a higher yield. Two repeating units were united through a phosphoramidite condensation to deliver the target hexasaccharide in 45% yield. This yield can possibly be increased by using more equivalents of the phosphoramidite. It was found that in the final hydrogenation step, the reduction of the trichloroacetyl groups led to the formation of HCl, which was responsible for partial cleavage of the rhamnosyl bonds. The hydrogenation steps therefore have to be optimized using non-acidic conditions. Currently, both fragments are available in sufficient quantities for use in biological studies. Whether they are suitable as components for a vaccine has yet to be determined using for example micro-array, competitive ELISA, and opsonophagocytic inhibition assays.

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