Synthesis of ribitol phosphate based wall teichoic acids

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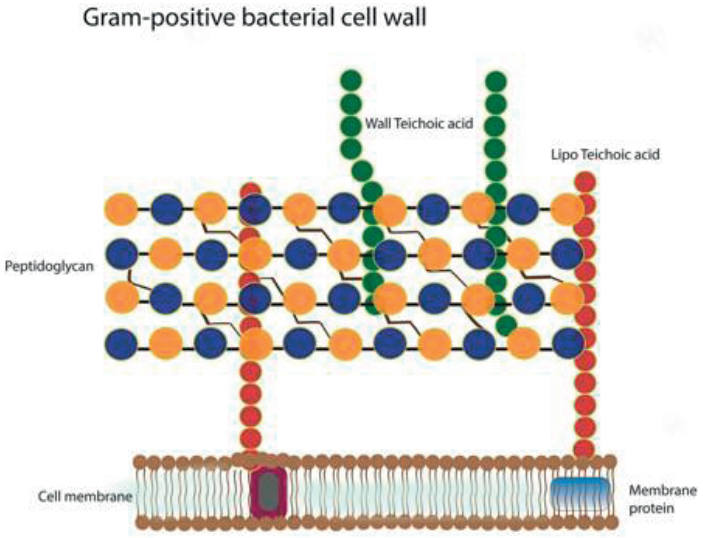
Synthetic teichoic acid chemistry for vaccine applications

INTRODUCTION

The cell wall of virtually all Gram-positive bacteria contains characteristic anionic carbohydrate-based polymers, called teichoic acids (from the Greek word τεῖχος, fortified wall). Teichoic acids (TAs) are alditol phosphate (predominantly glycerol or ribitol phosphate) based polymers that can be either covalently connected to the peptidoglycan or linked to the cell membrane through a glycolipid anchor. The first class of TAs is referred to as wall teichoic acids (WTAs), while the latter is called lipoteichoic acids (LTAs). Both classes can be further subdivided in different WTA- and LTA-subclasses, depending on the position of pyranosyl or furanosyl carbohydrate moieties, in or on the alditol phosphate chain, and the presence or absence of an anomeric phosphodiester linkage. Figure 1 presents a schematic drawing of the Gram-positive cell wall composition and the different subclasses of TAs.[1-5] The roles of TAs in the bacterial cell wall are equally diverse and important, as they are involved in the protection of the bacteria against the environment (for example against antimicrobial peptides), nutrient uptake, cation homeostasis and cell wall enzyme regulation as well as binding to receptors and surfaces.[6] Thus, TAs are crucial cell wall components for bacterial fitness and virulence. Protruding from the cell wall towards the environment, they represent anchor points for host cells through binding of cell surface lectins[7-8] for example, antibodies of the host immune system and they serve as recognition motifs for phage binding [9-10] and entry. TAs can be substituted with different carbohydrate and D-alanine [11] (D-Ala) appendages, generating micro-heterogeneous structures and the exact substitution patterns are important for the interactions of the TAs with the outside world. For example, phage binding has been shown to be dependent on the type of alditol phosphate polymers and glycosyl substituents [12-13], while D-alanylation is important for blocking the binding of cationic antimicrobials. [14-15] The micro-heterogeneity of TAs represents a major challenge if one aims to study the interaction of these molecules at the molecular level and therefore synthetic organic chemistry has been called upon to generate well-defined single TA molecules bearing various substitution patterns. This Chapter will describe the synthetic efforts reported to date to generate TA fragments for vaccine purposes. For a complete overview of synthetic methods to generate TAs, the reader is referred to recently published reviews on the subject. [16-18] The Chapter is divided in three subsections, each dealing with specific bacterial species, *Staphylococcus aureus*, *Enterococci faecalis* and *faecium* and *Clostridium difficile*, for which synthetic TAs have been used in the generation of conjugate vaccine modalities or diagnostic tools.

Sara Ali, Francesca Berni, Jacopo Enotarpi, Gijs A. van der Marel, Jeroen D.C. Codée, Synthetic teichoic acid chemistry for vaccine applications, *Recent Trends in Carbohydrate Chemistry*, Elsevier, Ed. Amelia Pilar Rauter, Bjorn Christensen, Laszlo Somsak, Paul Kosma, Roberto Adamo 10.1016/B978-0-12-820954-7.00006-2, (207-238), (**2020**).



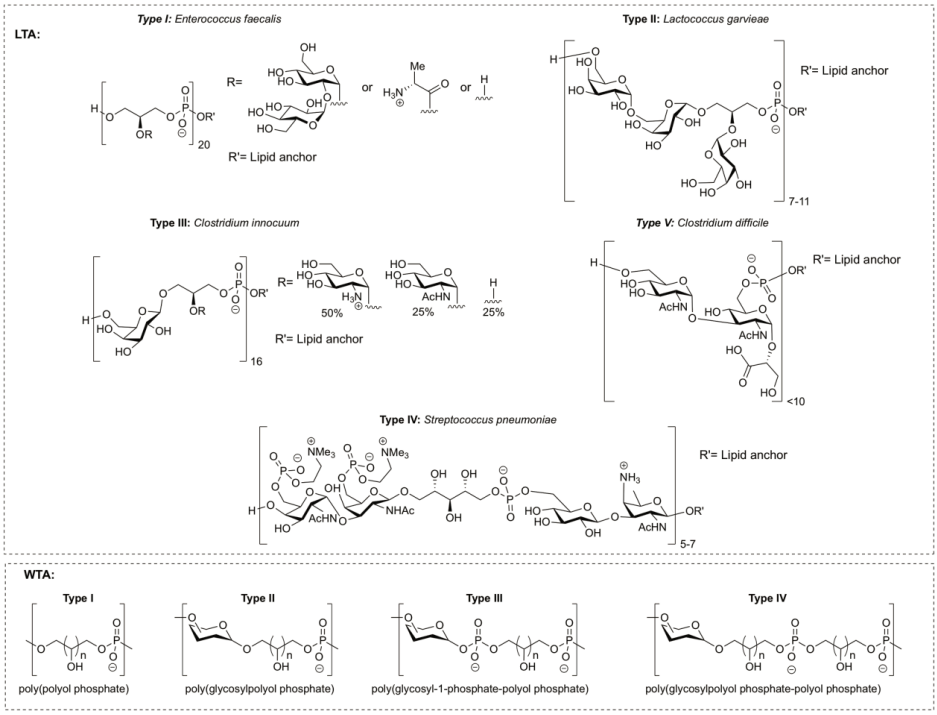


Figure 1. Schematic representation of the Gram-positive cell wall and different types of lipo- and wall teichoic acids.

SYNTHETIC TEICHOIC ACIDS

S. aureus TAs

S. aureus is an opportunistic pathogen, colonizing our skin, gastrointestinal tract, throat and anterior nares. While healthy people are commonly not at risk for *S. aureus* infections, hospitalized immunocompromised subjects are vulnerable and the bacterium can cause infections of the skin/soft tissue as well as respiratory and blood infections. Methicillin-resistant *S. aureus* (MRSA) is one of the major sources of fatal hospital acquired infections and the rise of antibiotic resistant strains represents a major challenge. Below the approaches are reviewed that have been directed at the use of synthetic LTA- and WTA-fragments of *S. aureus* in the development of vaccine modalities.

S. aureus LTA

The most common type of LTA is characterized by a glycerol phosphate (GroP) backbone randomly decorated at the C2 position of the glycerol unit with D-Ala or glycosyl moieties. Type I LTA is present in the cell wall of various Gram-positive bacteria, including *Bacillus subtilis*, *Listeria monocytogenes*, *Streptomyces hygroscopicus* and the important human pathogens *S. aureus*, *S. epidermidis*, *Enterococcus faecalis* and *E. faecium*. *S. aureus* type I LTA carries D-Ala and α-D-GlcNAc substituents, as depicted in Figure 2.

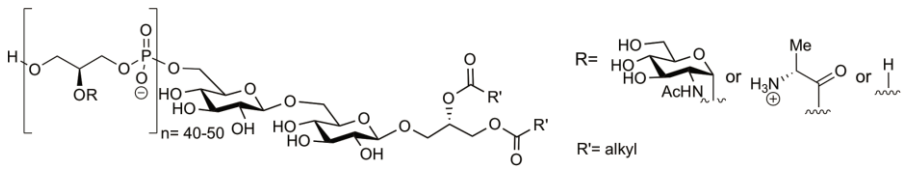
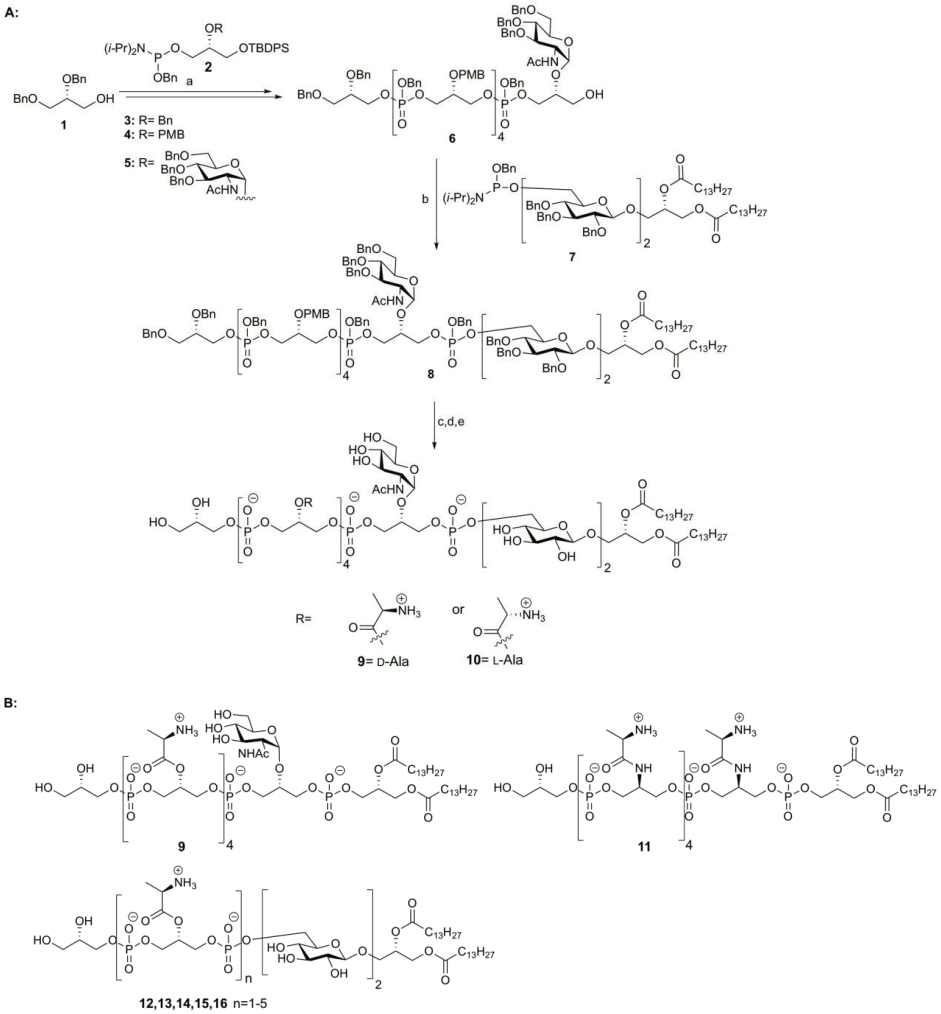


Figure 2. *S. aureus* LTA

A significant amount of synthetic work towards this type of LTA has been reported by Schmidt and co-workers who assembled a large set of *S. aureus* LTA oligomers to probe their innate immune-stimulating activity and establish the molecular basis of the "Gram-positive equivalent of the Gram-negative lipopolysaccharide (LPS)".[19] Notably, they reported not only on the total synthesis of LTA fragments including the glycolipid anchor, they also managed to install the labile D-Ala substituents on their synthetic fragments. As a representative example, Scheme 1A shows the synthetic strategy used to build compounds **9** and **10**, which relies on the use of benzyl phosphoramidite building blocks **4** and **5** in which the *tert*-butyldiphenylsilyl (TBDPS) group was used as a temporary protecting group for the primary alcohol. Since the required D-alanine moieties are base labile, they were introduced in a late stage of the synthesis. A *para*-methoxybenzyl (PMB) group was chosen to mask the C2-alcohols that had to be esterified, during the construction of the glycerol phosphate oligomers. The building blocks were united through coupling

cycles employing tetrazole as the activating agent for the phosphoramidites, *t*-BuOOH for the oxidation of the phosphites to the phosphotriesters and TBAF for the removal of the TBDPS groups (See Scheme 1A). After completion of the LTA chain, it was connected to the gentiobiose diacyl glycerol lipid anchor. After oxidative removal of the PMB ethers, the alanine residues were introduced before general hydrogenolysis to deliver the target LTAs **9** and **10**. Using a broad set of synthetic fragments (examples shown in Scheme 1B), the following structure-activity relationships could be established: the (glyco)lipid anchor, and the presence of positively charged alanine esters on the GroP repeating units were both required for full innate immune-stimulating activity (as assessed by cytokine production in a whole blood assay).[20] While the chirality of the alanine substituents proved to be important for activity (fragment **10**, bearing L-Ala esters proved to be 100 times less active than D-Ala LTA **9**), the labile ester linkage could be replaced by the more stable amide linkage (as in **11**). It was also shown that the diacyl glycerol anchor was indispensable for activity but that the gentiobiose core could be removed without having a large effect on the activity of the fragments. The fragments generated by Schmidt and co-workers have not been investigated for their potential effect in an adaptive immune response setting.

Snapper and co-workers have reported on the development of a synthetic *S. aureus* vaccine, employing synthetic GroP-LTA chains.[21] They used an automated solid phase synthesis strategy for the generation of the GroP oligomers as depicted in Scheme 2. Phosphoramidite building block **19** was used to build the oligomers. In line with contemporary nucleic acid chemistry, a dimethoxytrityl (DMTr) group was used for the protection of the primary alcohol to be elongated. Different from other approaches (*vide infra*), a benzoyl (Bz) group was chosen for protection of the secondary alcohol, even though this group is known to easily migrate from a secondary to a primary alcohol. Using an amino spacer functionalized glycerol controlled pore glass (CPG)-resin, a GroP-decamer was synthesized using 35-40 equivalents of the phosphoramidite per coupling cycle. Ammonia treatment released the product from the resin and cleaved all protecting groups (benzoates, cyanoethyl groups and the trifluoroacetyl (TFA) group on the amino group). Of note, the product was not purified and no spectroscopic data of the so-obtained product have been provided. The crude product was desalted before conjugation to the tetanus toxoid (TT) carrier protein using a formylbenzoate hydrazinonicotinamide conjugation couple. In order to establish whether the conjugate **24** was able to elicit a T-cell mediated immune response, mice were immunized with the TA oligomer **20** alone or conjugate **24** using a CPG-ODN (a TLR9 agonist) as adjuvant. A high IgG titer was detected when mice were immunized with the TA-conjugate and the serum raised against the conjugate was able to enhance opsonophagocytic killing of S. *aureus in vitro* and mediate protection in a bacteremia model *in vivo*.

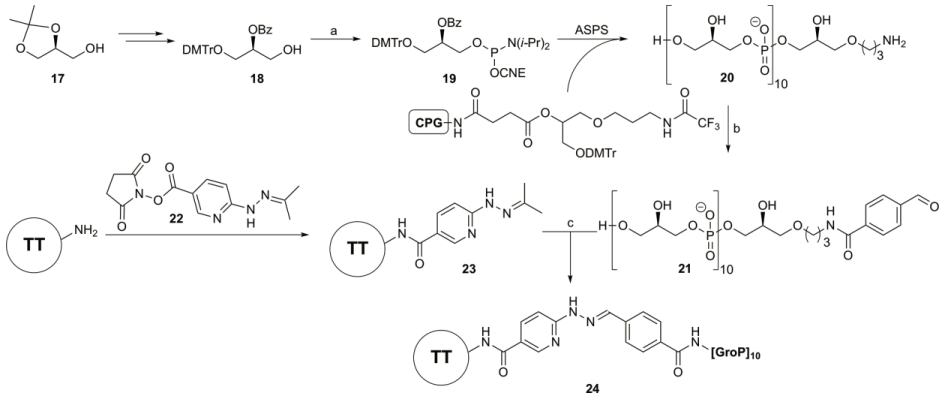


Scheme 1. A) Synthesis of *S. aureus* LTA fragments by Schmidt and co-workers;
Reagents and conditions: a) (i) **4** or **5**, tetrazole, DCM, (ii) *t*BuOOH; (iii) TBAF; b) (i) tetrazole, DCM; (ii) *t*BuOOH, 75%; c) CAN, ACN/toluene/H_2O, 67%; d) PyBOP, *N*-methylimidazole, Z-protected alanine, DCM; e) $Pd(OH)_2$-C, H_2, DCM/MeOH/H_2O, **9**: 25% over 2 steps, **10**: 33% over 2 steps. B) selection of generated LTA fragments for immunological evaluation.

S. aureus WTA

S. aureus produces a type I WTA, composed of 1→5-linked ribitol phosphate (RboP) repeats, that can be decorated with D-Ala residues at the C2 position and GlcNAc appendages at C3-β or C4-α/β, as depicted in Figure 3.

The glycosylation pattern has been shown to be critical for the fitness and virulence of the bacteria. The presence of β-GlcNAc residues, introduced on the WTA through the action of the glycosyl transferase TarS[22], has been related to β-lactam resistance[23] and



Scheme 2. Automated solid phase synthesis of a *S. aureus* LTA GroP oligomer for the development of a conjugate vaccine; *Reagents and conditions*: a) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite; b) 4FB-OSu; c) PBS, aniline.

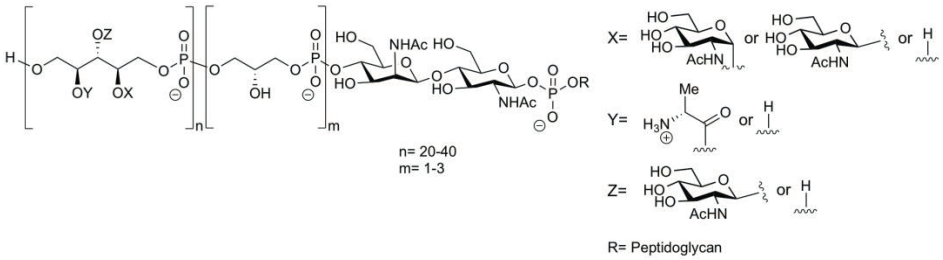


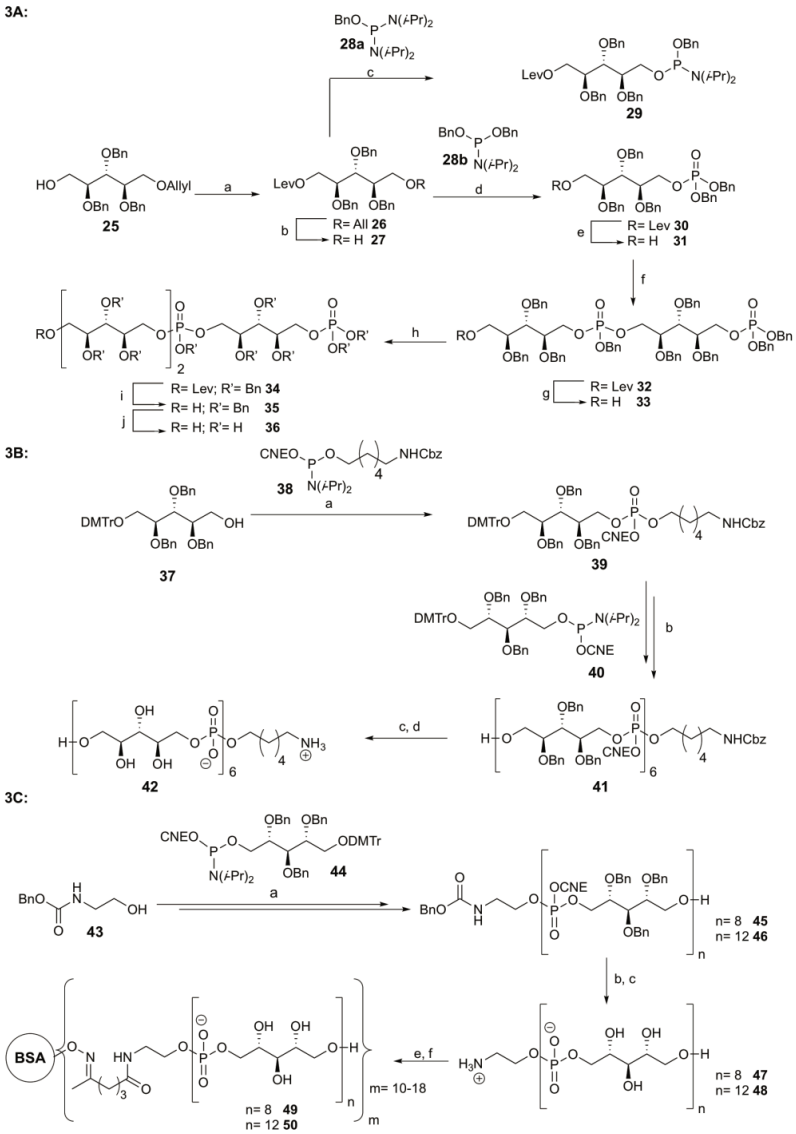
Figure 3. *S. aureus* WTA

the β-GlcNAc appendages also play a role in host colonization in the binding to human epithelial cells.[24-25] Because of the exposure to the bacterium, most humans have antibodies directed at the WTA of *S. aureus*, with C4 modified β-GlcNAc-decorated RboP WTA as the prime target. These antibodies have been shown to facilitate complement C3 deposition and subsequent opsonophagocytosis.[26] C4-β-GlcNAc-decorated RboP WTA is also recognized by human serum mannose binding lectin (MBL) to activate the lectin arm of the complement pathway.[7] Currently it is not clear why the other "carbotypes" are less virulent. Very recently, Peschel and Stehle and co-workers have shown that prominent healthcare associated (HA) MRSA-strains may escape from host immune surveillance by changing their WTA-glycosylation pattern.[27] These strains express, next to TarS, a second glycosyl transferase, TarP, that places a β-GlcNAc at the C3-OH of the RboP residues as opposed to the "normal" C4-position. Glycosylation by TarP was shown to be dominant over TarS glycosylation and this very subtle WTA modification was related to the ability of the bacterium to subvert the host immune system. Synthetic WTA fragments were used to probe the enzyme and solve the first crystal structure of a TarP glycosyl transferase in complex with a GlcNAc-UDP-pyrophosphate donor and an oligo-RboP-WTA acceptor, shedding light on the regiochemistry of the enzymatic

transformation. Two different WTA fragments were generated for this study: RboP trimer **36** (Scheme 3A) and RboP-hexamer **42** (Scheme 3B). Seeberger and co-workers generated trimer **36** using ribitol synthon **26**, featuring an allyl ether and a levulinoyl ester as a set of orthogonal protecting groups (Scheme 3A).[27] This building block was transformed into benzyl phoshoramidite **29** using reagent **28a**. Dibenzyl phosphate **31** was generated from the reaction of building block **27** and reagent **28b** after *t*BuOOH oxidation and delevulinoylation. Phosphoramidite **29** and building block **31** were coupled under the agency of tetrazole to provide, after *t*BuOOH oxidation, the levulinoyl protected RboP dimer **32**. Unmasking the primary alcohol and a subsequent coupling to building block **29** delivered the trimer **34**. Delevulinoylation and subsequent hydrogenolysis provided the desired RboP trimer **36.**

Our laboratory used an assembly strategy based on the use of cyanoethyl phosphoramidite building blocks, protected with a DMTr-group to mask the primary alcohol. This strategy, building on state-of- the-art nucleic acid chemistry and our previously described LTA-work (*vide infra*) has also been used by Pozsgay and co-workers for the assembly of longer RboP oligomers (See Scheme 3C).[28] In short, alcohol **37** was first coupled with spacer phosphoramidite **38** to provide spacer-equipped monomer **39**. Acidolysis of the DMTr group then delivered the primary alcohol for further elongation. Using phosphoramidite **40**, the hexamer **41** was obtained after 5 cycles of coupling/oxidation/deprotection. Standard deprotection conditions then provided the spacer-equipped hexamer **42** (Scheme 3B).

With the goal to investigate the role of the chain length in the immunological properties of ribitol phosphate oligomers, Pozsgay and co-workers set out to develop an automated solid phase assembly strategy using building block **44** (Scheme 3C). Initially the synthesis of a RboP-hexamer was explored. However, after six automated synthesis cycles and cleavage form the solid support, an intractable product mixture was obtained, necessitating the authors to revert to solution-phase chemistry. Starting with ethanol amine linker **43**, eight or twelve consecutive couplings led to the assembly of RboP-8-mer and RboP-12-mer **45** and **46**, which were deprotected using ammonia treatment followed by hydrogenolysis. Fragments **47** and **48** were attached to BSA through the use of a 5-ketohexanoic acid linker, the ketone functionality of which was used for conjugation to oxime groups that were installed onto the BSA carrier protein. On average 10–18 WTA oligomers were installed on the carrier protein. The immunogenicity of the conjugates has not been reported so far. Noteworthy, the aminospacer was installed on the side of the corresponding WTA fragments, to the side of the WTA that is not attached to the peptidoglycan.



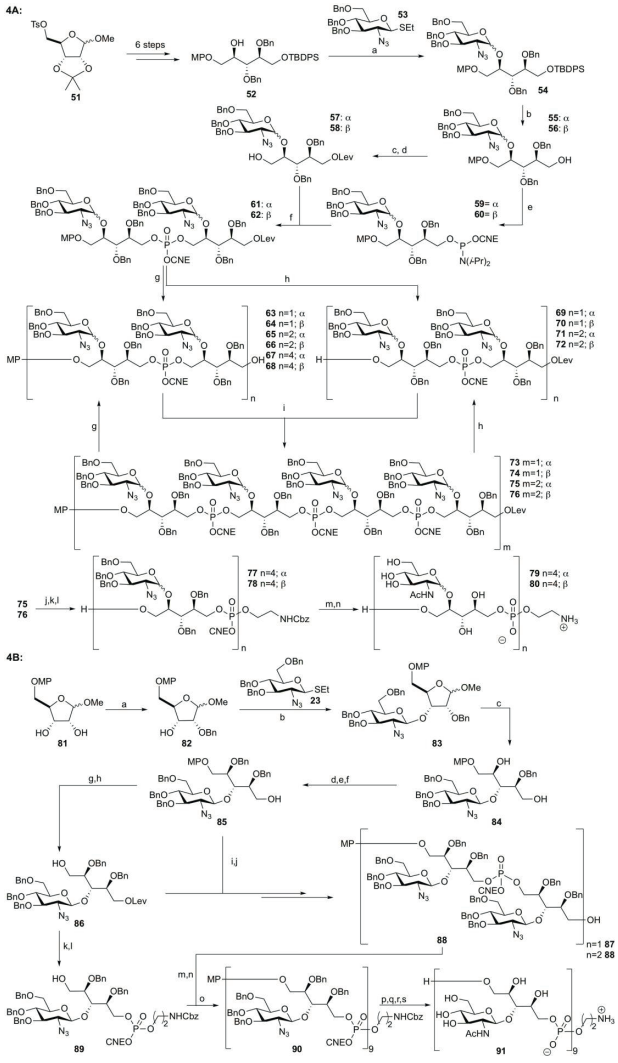
Scheme 3. A) Assembly of a *S. aureus* WTA RboP trimer; *Reagents and conditions*: a) LevOH, DMAP, DCC, DCM, 3 h, 98%; b) $Pd(PPh_3)_4$, 1,3-dimethylbarbituric acid, MeOH, 40°C, 24 h, 77%; c) diisopropylammonium tetrazolide, DCM, 2 h, 98%; d) (i) 1*H*-tetrazole, ACN, 2 h (ii) *t*BuOOH, 1 h, 85%; e) hydrazine hydrate, pyridine, AcOH, DCM, 4 h, 93%; f) (i) **29**, 1*H*-tetrazole, ACN, 2 h (ii) *t*BuOOH, 1 h, 86%; g) hydrazine hydrate, pyridine, AcOH, DCM, 4 h, 94%; h) (i) **29**, 1*H*-tetrazole, ACN, 2 h (ii) *t*BuOOH, 1 h, 94%; i) hydrazine hydrate, pyridine, AcOH, DCM, 4 h, 98%; j) Pd-C, H_2, EtOAc/MeOH/H_2O, 24 h, quant.

B) Assembly of an aminospacer functionalized RboP hexamer; *Reagents and conditions*: a) (i) DCI, ACN, **38**; (ii) CSO; (iii) 3% TCA in DCM, 85%; b) (i) DCI, ACN, **40**; (ii) CSO; (iii) 3% TCA in DCM, n=2, 74%, n=3, 88%, n=4, 80%, n=5, 76%, (**41**) n=6, 91%; c) NH_3 (30-33% aqueous solution), dioxane; d) Pd black, H_2, AcOH, H_2O/dioxane, 87% over 2 steps.

C) Pozsgay's synthesis of a RboP-12-mer-BSA conjugate; *Reagents and conditions*: a) (i) 10 eq. 0.45 M tetrazole in ACN, ACN, 23°C, 1 h; (ii) 0.5 M I_2 in 2:1 THF/water; (iii) 85:10:5 AcOH/DCM,H_2O n=1, 88%; b) (i) 10 eq. 0.45 M tetrazole in ACN, ACN, 23 °C, 1 h; (ii) 0.5 M I_2 in 2:1 THF/water, n=8; 9.9%, n=12; 2.4%; c) (i) MeOH, conc. NH_4OH, 50 °C, 8 h; (ii) H_2, 10% Pd/C, 2:1 *t*BuOOH/H_2O, n=8; 66%, n=12; 80%; e) 5-ketohexanoic anhydride, Et_3N, MeOH, H_2O; f) aminooxy-BSA, PBS (pH=7.4), EDTA, glycerol.

Glycosylated RboP-oligomers have recently been assembled and evaluated as potential antigens by a team at Sanofi Pasteur.[29] A panel of RboP-protein conjugates was assembled, in which the nature of the WTA fragments varied with respect to the glycosylation pattern and the manner in which it was generated (synthetic or isolated, see Scheme 5). Two different carrier proteins were probed: the detoxified α-hemolysin of *S. aureus* (HladM) or the detoxified recombinant exotoxin A from *Pseudomonas aeruginosa* (rEPA). Three different RboP-oligomers were synthesized: two RboP-octamers featuring either α-D-GlcNAc or β-D-GlcNAc substituents at all of the C4 hydroxyls, and one RboP-nonamer bearing β-D-GlcNAc residues on each of the C3-alcohols (See Scheme 4A and 4B). The building block required for the C4-glycosylated WTAs was generated from ribose **51** in six steps. This building block **52** was coupled with thiodonor **53** under activation of NIS/TfOH to yield **54** as a 52:48 α/β mixture in 94% yield. The diastereoisomers were separated after the TBDPS deprotection in the next step delivering far advanced intermediates for both the α- and the β-substituted RboP oligomers. Installing the levulinoyl ester (Lev) and removal of the methoxyphenol (MP) group provided alcohols **57** and **58**. The phosphoramidites **59** and **60** were formed *in situ* and used directly in the condensation with **57/58**, to give, after oxidation using pyridine/I_2/H_2O, dimers **61** and **62**. To facilitate a convergent synthesis approach, the Lev-group or the MP-ether of dimers **61, 62** were removed to give two dimer building blocks that were united using the *in situ* coupling strategy delivering the tetramers **65**, **66**. Following a similar approach, the so-obtained tetramers were transformed into two different alcohols, which were combined to give the desired fully protected octamers **67, 68**. The last steps in the synthesis of the octamers **67, 68** comprised coupling with the ethanolamine spacer phosphoramidite, followed by removal of the cyanoethyl groups. Of note, the spacer in these molecules is installed on the opposite site of the WTA fragment chain, with respect to the peptidoglycan binding site (See Figure 3). Next, the azides were transformed into the required acetamides using thioacetic acid in pyridine. Birch-type reduction of all benzyl groups then delivered the fully deprotected RboP-octamers **79** and **80**. Both native and synthetic WTAs were attached to the carrier proteins using adipic acid hydrazide as a conjugation handle (Scheme 5B).

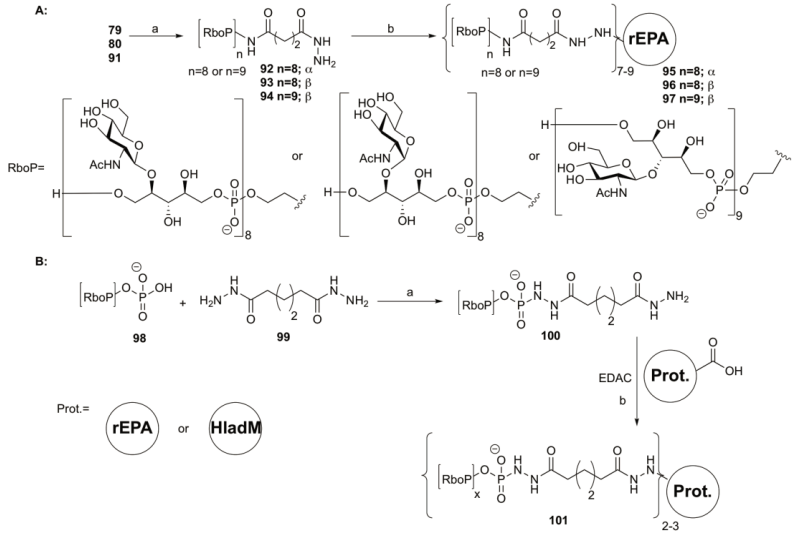
Scheme 4B depicts the synthesis of the C-3 β-GlcNAc nonamer. To regioselectively introduce the GlcNAc substituent on the ribitol chain, ribose **81** was first regioselectively benzylated at the C2-OH under phase-transfer conditions. Next the C-3 OH was glycosylated under NIS/TfOH conditions in a participating solvent mixture at low temperature (-70 °C) providing the desired β-stereoisomer **83** in excellent yield. Hydrolysis of the furanose linkage and reduction of the lactol delivered the ribitol chain. A series of protecting group manipulations then afforded the key intermediates **85** and **86**, featuring the methoxyphenol ether and levulinoyl ester protecting groups. Using the same



Scheme 4. A) Synthesis of C4-α- and β-GlcNAc RboP-octamers; *Reagents and conditions*: a) DCM/Et_2O, NIS and TfOH at -20°C for 10 min, 94%, (52:48 α/β); b) TBAF 1M/AcOH, THF, 0°C to rt 17h, 40% β, 46% α; c) Levulinic acid, DMAP, EDCI, DCM; d) CAN, ACN/H_2O, 0°C to rt 2h, 81% β; e) (i) Alcohol in ACN, cooled to 0°C, DIPEA and chloro-2-cyanoethyl-*N,N*-diisopropyl-amidite added at 0°C for 0.5 h (ii) cool to -10 °C, add alcohol then 5-ethiolthio-1*H*-tetrazole 1h at 0°C, m=1, β, 80%, m=2, β, 72%; f) step e, 69% β; g) hydrazine hydrate, pyridine/AcOH, 83% β; h) step d, 79% β; i) step e, **74** (80%), **76** (72%); j) hydrazine hydrate, pyridine/AcOH, 80% β; k) step e, benzyl *N*-(2-hydroxyethyl) carbamate, 67%; l) CAN, ACN/H_2O, 0°C to rt 2h, **78** (76%); m) thio acetic acid, pyridine, 3.5 days, β 97%; n) (i) Na, THF, NH_3, -78°C, 30 min, (ii) sat. aq. $NHCl_4$, -78°C, 1h, **80** (96%). **B) Synthesis of a C3-β-GlcNAc-RboP-nonamer;** ***Reagents and conditions*:** a) TBABr, BnBr, 10% aq. NaOH, DCM, 34%; b) ACN/propionitrile/DCM 2/1/1, NIS and TfOH, at -70°C 10 min, 84%; c) (i) 3M HCl/dioxane, reflux 7h, 73%; (ii) $NaBH_4$, MeOH, 0°C; d) TBDMSCl, DMAP, TEA, DCM; e) BnBr, DMF, NaH; f) THF, AcOH, TBAF, 0 °C to rt, 94%; g) Levulinic acid, DMAP, EDCI, DCM/dioxane 1/10 (0.08M); h) CAN, ACN/H_2O, 0°C to rt 2h, 71%; i) (i) Alcohol in ACN, cooled to 0°C, DIPEA and chloro-2-cyanoethyl-*N,N*-diisopropylamidite added at 0°C for 0.5 h (ii) cool to -10°C, add second alcohol then 5-ethiolthio-1H-tetrazole 1h at 0°C, n=1 (82%), n=2 (79%); j) hydrazine hydrate, pyridine/AcOH, **87** (86%), **88** (82%); k) step i, benzyl *N*-(2-hydroxyethyl) carbamate, 65%; l) CAN, ACN/H_2O, 0°C to rt 2h, 85%; m) step i, n=5, 77%, n) CAN, ACN/H_2O, 0°C to rt, 2h, n=5, (74%); o) step i, n=9, 27%; p) CAN, ACN/H_2O, 0°C to rt 2h, n=9, (80%); q) thio acetic acid, pyridine, 3.5 days, 90%; r) NH_4OH, MeOH, reflux 5h, 97%; s) (i) Na, THF, NH_3, -78°C, 30 min, (ii) sat. aq. $NHCl_4$, -78°C, 1h, 50%.

phosphoramidite approach used for the assembly of the C4-GlcNAc RboP-octamers, C-3 β-GlcNAc RboP tetramer **88** was generated. Building block **86** was also coupled to the ethanolamine phosphoramidite to give the spacer-functionalized monomer **89**. The union of tetramer **88** and monomer **89** then delivered the pentamer, which was coupled to a second copy of the tetramer to deliver the protected WTA nonamer **90**. Deprotection and linker installation as described above delivered the C-3 β-GlcNAc RboP-nonamer with a hydrazide linker (See Scheme 5).

The synthetic fragments as well as native TAs isolated from strains ATCC 10832 (carrying β-GlcNAc at C4), ATCC 25904 (having C4-α-GlcNAc substituents) and ATCC 55804 (with C3-β-GlcNAc monosaccharides) were conjugated to rEPA (Scheme 5A). Additionally, the ATCC 10832 TA was also conjugated to *S. aureus* alpha toxin (HladM) (Scheme 5B). To this end, the native WTAs were functionalized with a hydrazide linker using adipic acid dihydrazide (ADH) in a carbodiimide mediated condensation reaction. Using a similar coupling strategy, the hydrazide functionalized synthetic and native TA fragments were covalently linked to the carrier proteins. All conjugates, as well as the non-conjugated WTAs, were used to immunize mice, with or without adjuvant (AF04, a squalene emulsion containing the synthetic toll-like receptor 4 agonist, E6020, a hexa-acylated diphosphoryl urea), after which the IgG1 and IgG2-titers were determined after 0, 21, 35 and 42 days. It was revealed that the unconjugated WTAs were not able to induce a specific immune response against the WTA used for AF04 immunization, while immunization with the WTA-conjugates did lead to the production of IgG antibodies. The titers of the serum of the mice immunized with the adjuvant were significantly higher than those immunized without adjuvant. There was little difference in the immune response against the conjugates of the native WTAs or the synthetic fragments. The synthetic C4-β-GlcNAc WTA conjugate appeared to elicit a somewhat stronger immune response than the C4-α-GlcNAc conjugate. The cross reactivity of the sera was evaluated on 19 *S. aureus* strains and it was shown that the sera of mice immunized with the synthetic WTA-conjugates recognized homologous and heterologous strains better than sera from mice immunized with the native WTA-conjugates. The sera from mice immunized with the C4-β-GlcNAc WTA conjugate showed a higher cross reactivity than the sera raised against the C4-α-GlcNAc conjugate, while the C3-β-GlcNAc WTA conjugate serum did not appear to be cross reactive. The latter observation stands in contrast to the cross reactivity of the Nabi Pharmaceuticals PentaStaph vaccine, containing conjugates of the capsular polysaccharides 5 and 8 as well as a conjugate of antigen 336, the C3-β-GlcNAc WTA, that was shown to be cross reactive against different *S. aureus* strains as well as against *S. epidermidis*.[30] The results described by the Sanofi team, could suggest that the C4-β-GlcNAc WTA conjugate can be used as a vaccine modality rendering broad spectrum protection against a variety of *S. aureus* strains. Whether the vaccine can offer



Scheme 5. A) Conjugation chemistry and an overview of the WTA conjugates assembled by Sanofi pasteur of the synthetic WTAs; *Reagents and conditions*: a) (i) disuccinimidyl succinate, DIPEA, DMSO, 0.5 h rt (ii) hydrazine hydrate, **93**, (24%), **94**, (53%); b) rEPA, EDAC, 3h, pH= 5.7.
B) Conjugation of the native WTAs; *Reagents and conditions*: a) (i) aq. NaCl, aq. ADH, pH= 5.7, 1 M EDAC, 3h (ii) pH= 7.0 (iii) TA-AHs, rEPA or HladM, EDAC, pH= 5.7, 3h.

protection *in vivo* against different strains of *S. aureus* will have to be shown in future studies.

Enterococcal TAs

Enterococci are the third most common class of nosocomial pathogens and they can cause bacteremia, endocarditis, peritonitis, urinary tract infection and foreign-body infections.[31] Multiresistant enterococci, including Vancomycin-resistant enterococcus (VRE), represent a major health threat, especially to immunocompromised hosts. The rise of antibiotic resistance has been an important motivation for the development of alternative strategies, including active and passive immunization therapies. To this end, TAs have been investigated as potential antigens as they are prominently present in the cell wall of these bacteria.

E. faecalis and E. faecium ***LTA***

Figure 4 shows the type I LTA of *E. faecalis* and *faecium* with their characteristic glycosyl substituents. As described above poly-(1,3)-glycerol phosphate (GroP) LTA is present in the cell wall of various Gram-positive bacteria, and has therefore been probed as a universal antigen that could potentially be used in broad-spectrum vaccines targeting various Gram-positive species.[32] Initial studies conducted with isolated LTA of *E. faecalis* have shown that opsonophagocytic antibodies could be raised against type I LTA, that

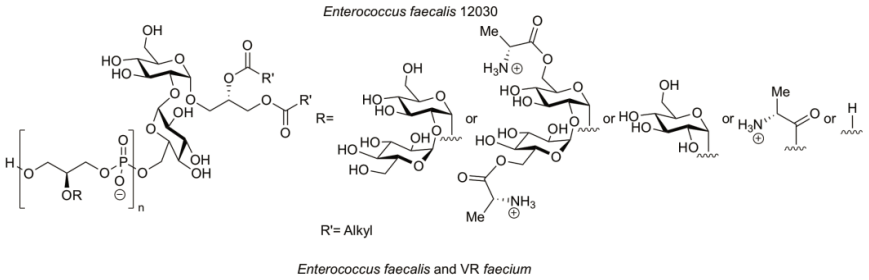


Figure 4. Structure of *E. faecalis and E. faecium* LTA.

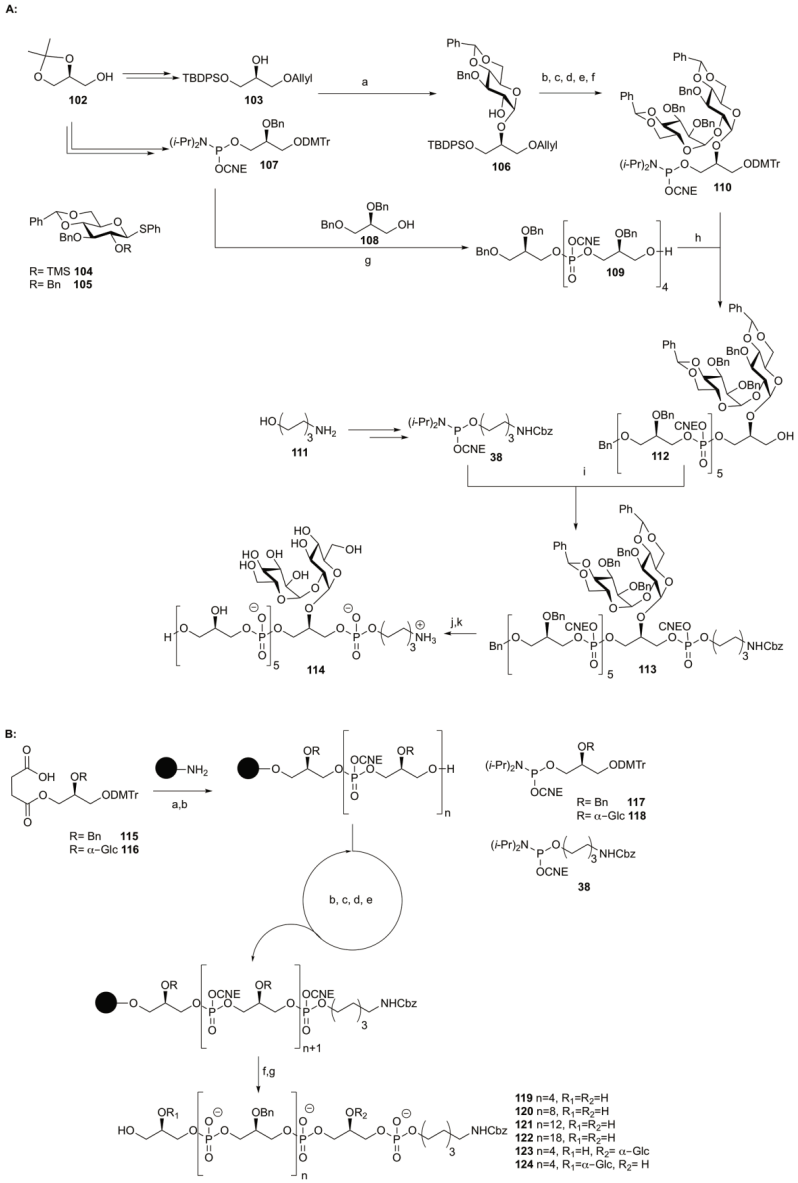
were cross reactive against different *E. faecalis* strains as well as *E. faecium* strains, including (VRE) and against *S. aureus* and *S. epidermidis*.[33]

The potential of (substituted) glycerol phosphate TA antigens in therapeutic and diagnostic applications has been an incentive to develop synthetic routes to assemble these molecules *de novo* through organic synthesis. Our group has reported several approaches to assemble *E.* faecalis LTA fragments with a special focus on the site-selective introduction of different carbohydrate moieties, the number of repeating units and the kind of functional moiety at the end of the chain, being either a hydroxyl or phosphate group. Initially, we developed 'traditional' solution-phase chemistry to assemble α-kojibiosyl GroP LTA hexamer **114** as depicted in Scheme 6A.[34] The required phosphodiesters were installed using phosphoramidite chemistry and the cyanoethyl phosphoramidite glycerol building blocks were generated starting from solketal **102**, which represents a convenient commercially available chiral starting material in which all hydroxyl groups can be addressed site specifically. Installation of the α-kojibiose (α-D-glucopyranosyl-(1→2)-α-D-glucose) moiety was achieved using alcohol **103** and a benzylidene-protected glucose donor. After condensation of donor **104** and acceptor **103** the TMS group was removed upon work-up to set the stage for the second glucosylation to complete the kojibiose moiety. A standard set of protecting group manipulations then installed the DMTr and the cyanoethyl phosphoramidite groups to give **110**. Assembly of the first target *E. faecalis* LTA fragment **114** started from dibenzyl glycerol **108**, which was elongated with four copies of building block **107** (using DCI as activating agent, I_2/H_2O/THF/pyridine for the P(III) to P(V)-oxidation, and dichloroacetic acid (DCA) and Et_3SiH for DMTr removal). Subsequently, the fully protected GroP-pentamer **109** and α-kojibiosyl-glycerol phosphoramidite **110** were coupled. DMTr cleavage form the resulting GroP-hexamer was performed using a pyridinium *para*-toluenesulphonate (PPTS)/MeOH mixture to avoid unwanted acidolysis of the benzylidene functionalities. Final coupling with spacer phosphoramidite **38** delivered the fully protected hexamer **113**. After removal of the cyanoethyl groups through ammonolysis, all the benzyl-type

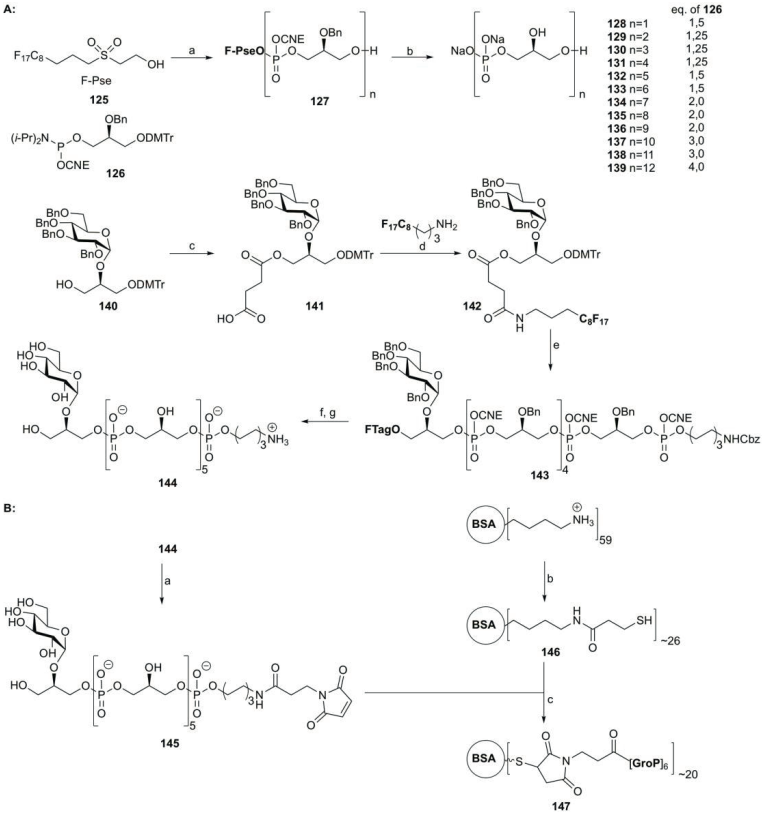
protecting groups were cleaved by hydrogenolysis, to afford the kojibiosyl TA-hexamer **114** in 76% yield.

To streamline the synthesis of substituted GroP oligomers, the established solution-phase chemistry was next translated to automated solid-phase methodology (Scheme 6B).[35] To this end, aminopropyl controlled pore glass (CPG) resin was coupled with succinyl linker building block **115** or **116**. With the first building block immobilized, the GroP chains were constructed in a fully automated manner using the commercially available ÄKTA™ oligopilot™ synthesizer. For each coupling cycle 5 equivalents of phosphoramidite **117** or **118** were used in combination with 5-benzylthiotetrazole (5-BTT) as activator. Oxidation by I_2 in pyridine/H_2O oxidized the phosphites to the phosphate triesters and was followed by a capping step of the unreacted alcohols using *N*-methylimidazole and acetic anhydride. Removal of the DMTr was performed using DCA in DCM, generally showing coupling efficiencies higher than 98% (automatic DMTr-count). Final coupling of the fragments with spacer amidite **38**, followed by oxidation, capping and detritylation afforded the complete resin-bound fragments. The target compounds were cleaved from the resin by aqueous ammonia treatment, which simultaneously removed the cyanoethyl groups. Purification of the semi-protected fragments was done by anion exchange chromatography and the final target compounds were obtained by hydrogenolysis. Using this assembly method, a small set of GroP oligomers was assembled varying in length between 6-mers and 20-mers and with different substitution pattern.

Although the latter methodology proved to be efficient in terms of synthesis time and labor, it does require the use of large amounts of phosphoramidite building blocks and can only be done on a limited scale. An alternative synthetic strategy is represented by the application of soluble supports, which combine a more rapid and effective intermediate isolation procedure (compared to solution phase synthesis) with the use of a relatively small excess of reagents (compared to solid phase chemistry) and the possibility to scale up. Based on light fluorous synthesis techniques, Hogendorf *et al.* applied two different fluorous scaffolds as soluble supports: a (perfluorooctyl)propylsulfonylethyl (F-Pse) linker for the assembly of TA fragments with a terminal phosphate monoester and a (perfluorooctyl)succinyl spacer delivering TA oligomers featuring a terminal hydroxyl group (Scheme 7A).[36] Using building blocks **126** and **127** GroP-oligomers were made up to the dodecamer level (n = 12). It was noted that a larger excess of reagents was required to push the coupling reactions to completion with growing length of the oligomers. Standard deprotection chemistry delivered the desired GroP-oligomers **128-139**. The light fluorous approach was used to scale up the synthesis of hexamer **144** using the (perfluorooctyl)succinyl linker.



Scheme 6. A) Solution phase synthesis of a kojibiosyl decorated GroP LTA fragment; *Reagents and conditions*: a) (i) **104**, Tf_2O, Ph_2SO, TTBP, DCM (α / β = 6:1) (ii) Na_2CO_3, MeOH, 46%; b) **105**, Tf_2O, Ph_2SO, TTBP, DCM, 82%; c) (i) Ir(COD)$(Ph_2MeP)_2$, THF (ii) I_2, aq $NaHCO_3$, 67%; d) DMTr-Cl, TEA, DCM, 98%; e) TBAF, THF, 91%; f) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, DIPEA, DCM, 72%; g) **107** (i) DCI, ACN (ii) I_2, THF, H_2O, pyridine (iii) DCA, TES-H, DCM, n=1 (80%), n=2 (82%), n=3 (81%), n=4 (62%); h) (i) **110**, DCI, ACN (ii) I_2, THF, H_2O, pyridine, 80%, (iii) pyridium-*p*-toluenesulphonate, MeOH, DCM, 70%; i) **38**, DCI, ACN (ii) I_2, THF, H_2O, pyridine, 77%; j) NH_3, H_2O, dioxane, 100%; k) Pd black, H_2, H_2O, dioxane, 76%.
B) Automated solid phase synthesis of a set of GroP oligomers; *Reagents and conditions*: a) **115** or **116**, DIC, ACN; b) 3% DCA, toluene; c) **117**, **118** or **38**, 5-BTT, ACN; d) I_2, pyridine, H_2O/ACN; e) Ac_2O, *N*-methylimidazole, 2,6-lutidine, ACN; f) conc. NH_4OH; g) Pd black, H_2, H_2O/dioxane, AcOH; **119** (65%), **120** (78%), **121** (84%), **122** (95%), **123** (68%), **124** (86%).



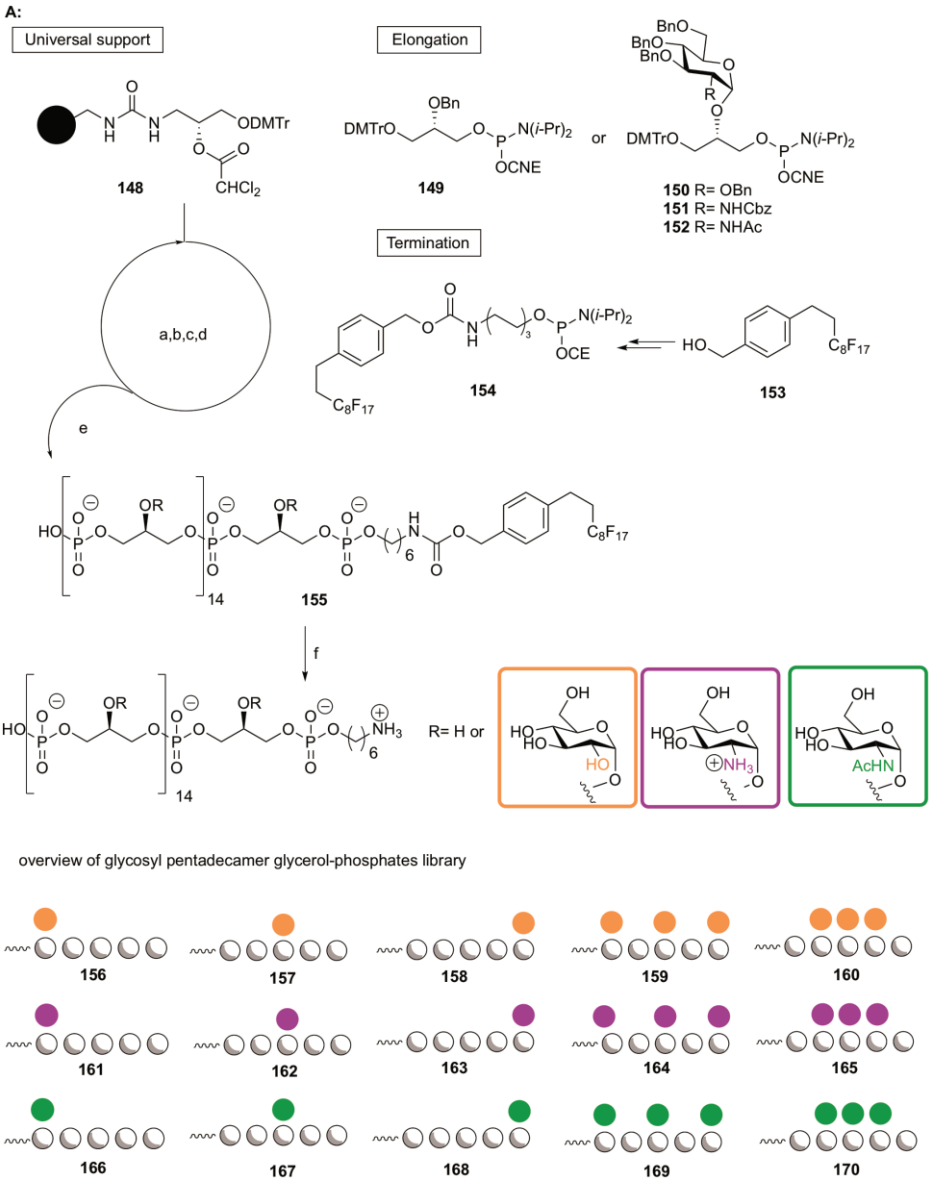
Scheme 7. A) Light fluorous assembly strategy; *Reagents and conditions*: a) (i) **126**, DCI, ACN (ii) I_2, pyridine, THF (iii) DCA, TES-H, DCM; b) (i) **126**, DCI, ACN (ii) I_2, pyridine, THF (iii) DCA, TES-H, DCM; c) succinic anhydride, TEA, DCM, 96%; d) (i) BOP, DIPEA, DMF, DCM (ii) DCA, TES-H, DCM (iii) F-SPE, 90% 3 steps; e) repetition (i) **126** or **38**, DCI, ACN (ii) I_2, pyridine, THF (iii) DCA, TES-H, DCM; f) conc. NH_4OH, 40°C; g) Pd black, H_2, H_2O, AcOH, 76-98% 2 steps. **B) Generation of a GroP$_6$-LTA BSA conjugate;** *Reagents and conditions*: a) *N*-succinimidyl-3 maleimidopropionate, aq. $NaHCO_3$, **145** (67%); b) (i) *N*-succinimidyl-3-thio-propionatehomodisulfide, PBS (pH= 8.0), DMF; (ii) dithiothreitol; c) PBS (pH= 7.2).

An opsonophagocytic killing inhibition assay (OPIA) was used to evaluate the synthetic fragments for their potency in binding to LTA-binding antibodies, present in rabbit serum raised against *E. faecalis* 12030-LTA.[35] These studies indicated a clear length dependency for the unsubstituted fragments, with better inhibition for the longer fragments. Surprisingly the fragment bearing the naturally occurring kojibiose substituent (**114**), proved to be less active than the structurally simpler mono-glucose hexamer **144**, which appeared to be the most potent synthetic antigen in the series.[37] Structures bearing the phosphate at the end of the GroP-chain all proved less active. From these studies, GroP-hexamer **144** was selected as a lead antigen to be used in the generation of a TA-protein conjugate vaccine modality for follow-up studies (Scheme 7B). Based on the approach[38] used by Verez-Bencomo and co-workers for the development of the Quimihib®-vaccine, the maleimide derivative **145** was generated and linked to thiofunctionalized BSA, to deliver the conjugate **147** with a TA-protein ratio of 20:1.[37] Rabbit serum obtained by

immunization with **147** proved to be highly opsonic not only towards *E. faecalis* strain but also *E. faecium* and S. *aureus*. While the activity against the former pathogen can be readily understood, because *E. faecium* LTA carries mono-α-D-glucose appendages, the reactivity against *S. aureus* is somewhat surprising, as the LTA of this bacterium has not been shown to carry α-D-glucose substituents. Perhaps the lead antigen **144** mimics well the naturally occurring α-D-*N*-acetyl glucosamine-GroP LTA or the α-D-*N*-acetyl galactosamine-bearing GroP-WTA of *S. aureus*. In all, these results support the idea that compound **144** is a good TA mimic and that LTAs can be used as antigen candidate for vaccine development with a broad spectrum of action.

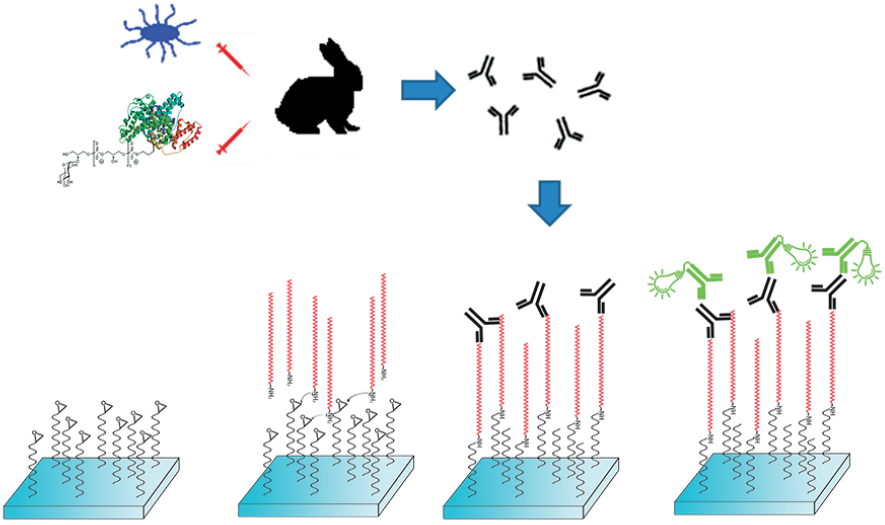
To further streamline the assembly of synthetic TAs, allowing for longer fragments, carrying more diverse substitution patterns, van der Es *et al.* developed a 'second generation' automated solid phase synthesis approach introducing a universal support, which obviates the need for linker functionalized building blocks, and the use of a fluorous tagging technique, to facilitate purification of the target structures from generated deletion sequences (See Scheme 8).[39] To this end the fluorous aminospacer phosphoramidite **154** was developed and it was shown that the fluorous tag facilitated the purification of the long fragments using a conventional reversed phase HPLC. A large GroP-15-mer library was generated bearing one or three α-D-glucose, α-D-*N*-acetyl glucosamine or α-D-glucosamine substituents, evenly distributed along the GroP chain or clustered together.

With the library of synthetic TA-fragments a TA microarray was developed to allow for the rapid screening of interactions with biomolecules using a minimal amount of analyte (Figure 5). The array was used to compare the binding specificities of antibodies and sera raised against different antigens, *i.e* purified LTA from *E. faecalis* 12030 or the synthetic BSA-mono-glucose GroP-hexamer conjugate **147**. As shown in Figure 5, the binding specificity of the sera and antibodies differed tremendously. The commercially available monoclonal antibody raised against *S. epidermidis* recognized many different GroP oligomers, with little specificity for length or substitution pattern, although the multiple substituted GroP oligomers showed least binding. This indicates that the antibody most likely binds to the GroP-backbone, providing an explanation for the cross reactivity of this antibody towards TAs derived from different bacterial species. The serum raised against the purified LTA contained both IgM and IgG-isotypes and the IgM-type antibodies showed no specificity for any substitution pattern, again indicating primarily binding to the GroP-backbone. The IgGs showed a clear preference for substituted TAs but were non-discriminative for the type of monosaccharides. Finally, the serum raised against the well-defined GroP-hexamer antigen showed highly specific binding towards mono-glucosylated structures, resembling the structure of the WTA-fragment



Scheme 8. 'Second-generation' automated solid phase assembly of long GroP-TA fragments; *Reagents and conditions*: a) 3% DCA, toluene; b) 5-BTT, ACN; c) I_2, pyridine, H_2O/ACN; d) Ac_2O, *N*-methylimidazole, 2,6-lutidine, ACN; e) (i) NH_3, MeOH; (ii) NH_4OH, H_2O; f) H_2, Pd^0, H_2O.

against which the serum was raised. These results have shown that the TA-microarray is a powerful analytical tool to map binding specificity for TA-interaction partners and it is expected that the technique will be very useful to study the interaction of TAs with other biomolecules, such as lectins and phage binding proteins. The results have also clearly highlighted that a very selective response against a well-defined TA antigen can



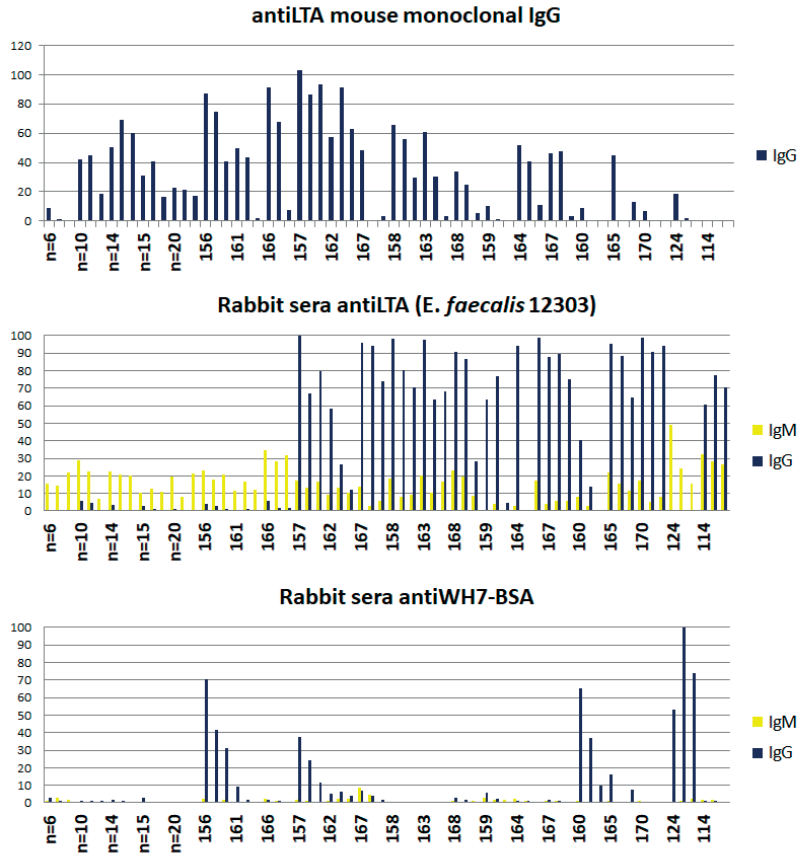


Figure 5: Evaluation of monoclonal antibodies and sera using microarray technology.

be achieved, indicating the possibility to selectively target a bacterial (sub)population if the proper antigen and vaccination method are used. Clearly such a response can not be expected from isolated LTA preparations as these are very heterogeneous highlighting a clear advantage of synthetic material over naturally sourced material.

E. faecium WTA

Besides LTA, *Enterococci* can express different types of WTA. In *E. faecalis* several type-II WTAs have been described that are involved in evasion of complement mediated phagocytosis (See Figure 6 for structures of different enterococcal WTAs).

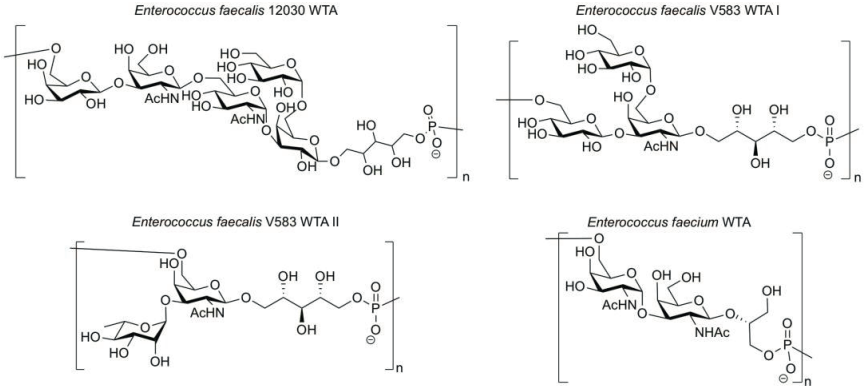


Figure 6: Structures of *E. faecalis* and *E. faecium* WTA.

E. faecium strain U0317 produces a WTA that can shield the LTA from opsonophogocytic antibodies. This WTA was shown to be built up from [→ 6-(α-D-GalNAc-(1→ 3)-β-D-GalNAc-(1→ 2)-GroP-(3→]-repeating units. As the stereochemistry of the GroP moiety was not revealed, van der Es *et al.* synthesized two sets of oligomers of the repeating units featuring either the *sn*-glycerol-1-phosphate or the *sn*-glycerol-3-phosphate constituents.[40] Scheme 9 describes the syntheses towards these oligomers. In a stereoselective glycosylation reaction, imidate donor **175** was condensed with azidogalactose **174** to give the α-linked disaccharide. Transformation of the selenoglycoside **176** into an imidate donor and subsequent coupling to either of the enantiomeric glycerol acceptors, using a solvent system of acetonitrile, propionitrile and DCM to control the stereoselectivity of the reaction, delivered the β-linked pseudo-trisaccharides **177** and **178** in high yield and as a single diastereoisomer. These were transformed into the required phosphoramidite building blocks for oligomerization **181** and **182**. First a spacer was installed for future conjugation purposes giving **183** and **184**. The employed coupling cycles used 4,5-dicyanoimidazole (DCI) as activator, (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) as oxidant and trichloroacetic acid (TCA) to unmask the DMTr protected alcohols. The elongation proceeded uneventfully to provide the oligomers of both GroP-epimers to

eventually provide structures encompassing three repeating units. Standard deprotection provided the set of target compounds. NMR analysis of the generated compounds and comparison to the spectra of the naturally occurring WTA revealed the stereochemistry of the *E. faecium* WTA GroP to be *sn*-3-glycerol phosphate. The biosynthesis of GroP-containing WTAs[41-42] and LTAs[5, 43] generally employs different glycerolphosphate donors. While LTA is assembled using phosphatidyl glycerol, having the *sn*-1-glycerol phosphate stereochemistry, as a source of the GroP units, WTA is generated through the use of cytidine *sn*-3-glycerol phosphate. The stereochemical assignment based on the synthetic compounds described above is thus supported by biosynthesis precedent.

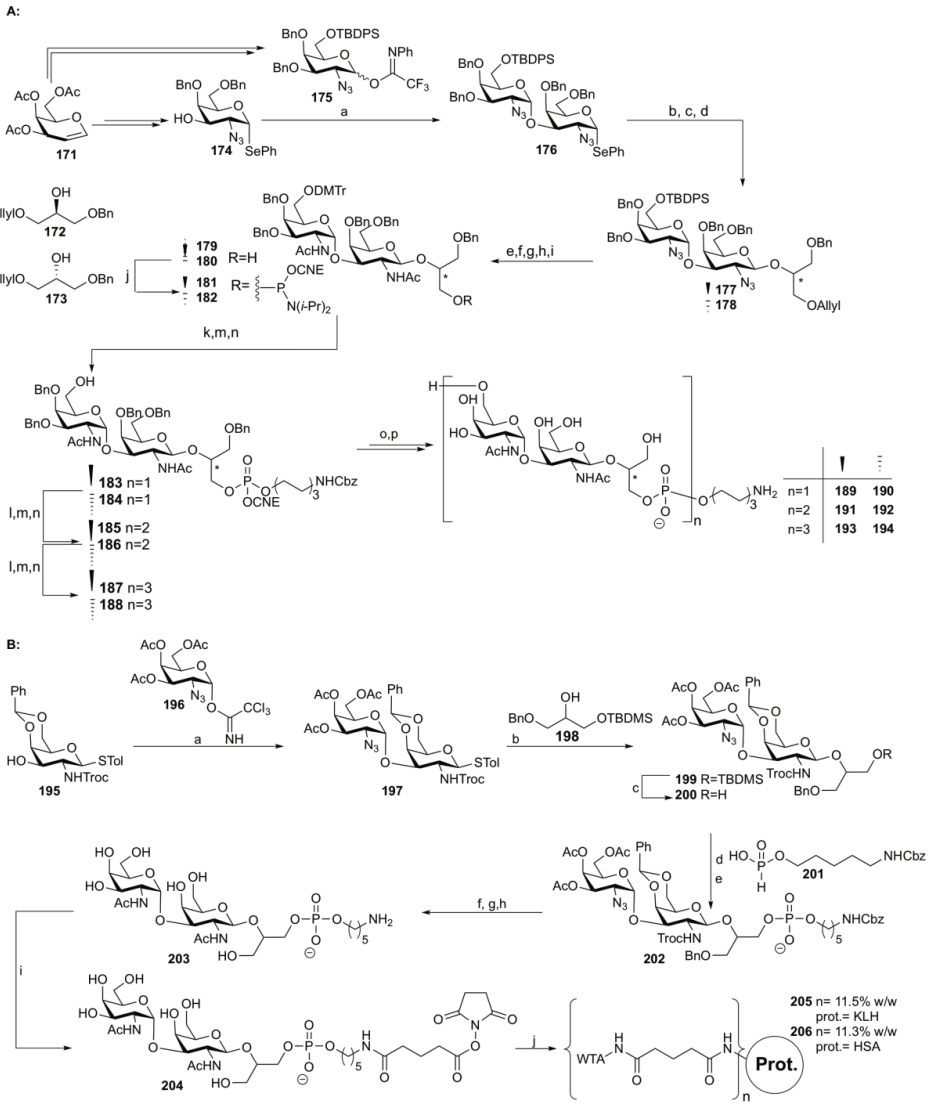
Wu and co-workers also reported on the synthesis of the *E. faecium* U0317 WTA monomer, although the stereochemistry of the glycerol moiety was not specified (See Scheme 9B).[44] The digalactosamine glycerol building block **200** was coupled to spacer **201** using H-phosphonate chemistry employing pivaloyl chloride to activate the H-phosphonate. Compound **202** was subjected to Zn/AcOH/Ac_2O to transform the azido and Troc protecting group into an acetamide, followed by deprotection of the acetyl groups using Zempl**é**n conditions and a hydrogenolysis reaction then yielded final compound **203**. The generated monomer was conjugated to KLH and HSA as carrier proteins using a bifunctional glutaryl ester method. The KLH conjugate was used to immunize mice and the serum raised against the conjugate was evaluated by ELISA to probe recognition of the HSA conjugate and the non-conjugated monomer. High levels of IgG were observed in the sera raised against the conjugate and the antibodies were able to recognise both **205**, **206** proving that they are antigen specific. The sera will have to be further evaluated for recognition of the naturally occurring WTA and opsonic properties towards *E. faecium* bacteria.

Clostridium difficile

Clostridium difficile is a Gram-positive anaerobic bacterium, frequently found in the environment as spores that can infect humans and other animals.[45-46] The spores can survive in the stomach and intestine of the host and colonize the gastrointestinal tract. *C. difficile* infections (CDI) are responsible for nosocomial diarrhea and antibiotic-associated colitis.[47] Cell surface-associated antigen-based vaccines can protect both the colonization by *C. difficile* and the symptoms of the infection.[48] The surface-associated antigens can be divided in two main classes: surface proteins (SLP, FliD and Cwp84) and surface polysaccharide antigens (called PSI, PSII and PSIII).

Clostridium difficile LTA (PSIII)

The structure of the cell-surface glycans has been elucidated and it has been shown that PSIII is an LTA that features a triglucoside diacylglycerol (DAG) lipid anchor and a



Scheme 9. A) Synthesis of *E. faecium* WTAs; *Reagents and conditions*: a) TfOH, DCM, 0°C, 58%; b) NIS, THF/H_2O; c) CF_3(=NPh)Cl, K_2CO_3, acetone, quant; d) **172** or **173**, TfOH, ACN/EtCN/DCM, -40°C, **177** (90%), **178** (80%); e) PMe_3, dioxane/H_2O; f) Ac_2O, TEA, DCM; g) TBAF, THF; h) DMTr-Cl, TEA, DCM; i) (i). Ir(COD)(Ph_2MeP)$_2$, THF (ii). I_2, $NaHCO_3$, H_2O/THF; **179** (28% 5 steps), **180** (46% 5 steps); j) DIPEA, *N,N*-diisopropylamino-2-cyanoethyl-chlorophosphite, DCM **181** (85%), **182** (62%); k) **38**, DCI, **179**, or **180**, ACN; l) **181**, or **182**, DCI, ACN; m) CSO, ACN; n) 3% TCA, DCM, **183** (72%), **184** (85%), **185** (62%), **186** (64%), **187** (69%), **188** (67%); o) conc. NH_3, dioxane; p) Pd^0, H_2, AcOH, H_2O, **189** (73%), **190** (57%), **191** (44%), **192** (45%), **193** (49%), **194** (56%). **B) Assembly of an *E. faecium* WTA-BSA conjugate;** *Reagents and conditions:* a) DCM/diethyl ether (2:1), TMSOTf, 4Å MS, -78°C, 78% (α:β = 5:1); b) DCM, TfOH, NIS, 4Å MS, -20°C, 61%; c) TBAF:AcOH = 1:1, THF, 75%; d) pyridine, pivaloyl chloride; e) Iodine, pyridine:H_2O = 9:1, 75%; f) Zn/AcOH/Ac_2O, 67%; g) NaOMe, MeOH; h) H_2, Pd(OH)$_2$/C, 41% over 2 steps; i) DSG, DMF and PBS buffer (4:1), rt, 4h, quantitative; j) KLH or HSA, PBS buffer, rt, 3 days.

di-α-glucosamine glyceric acid repeating unit interconnected through phosphodiesters bridging the primary alcohols of the glucosamine moieties (See Figure 7).

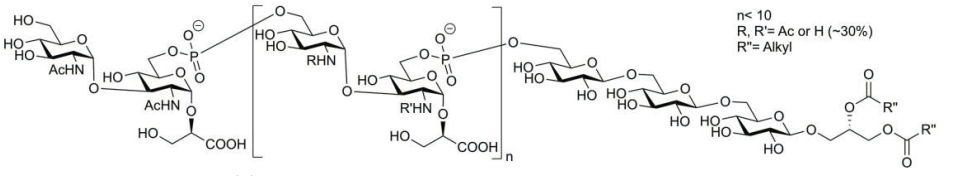
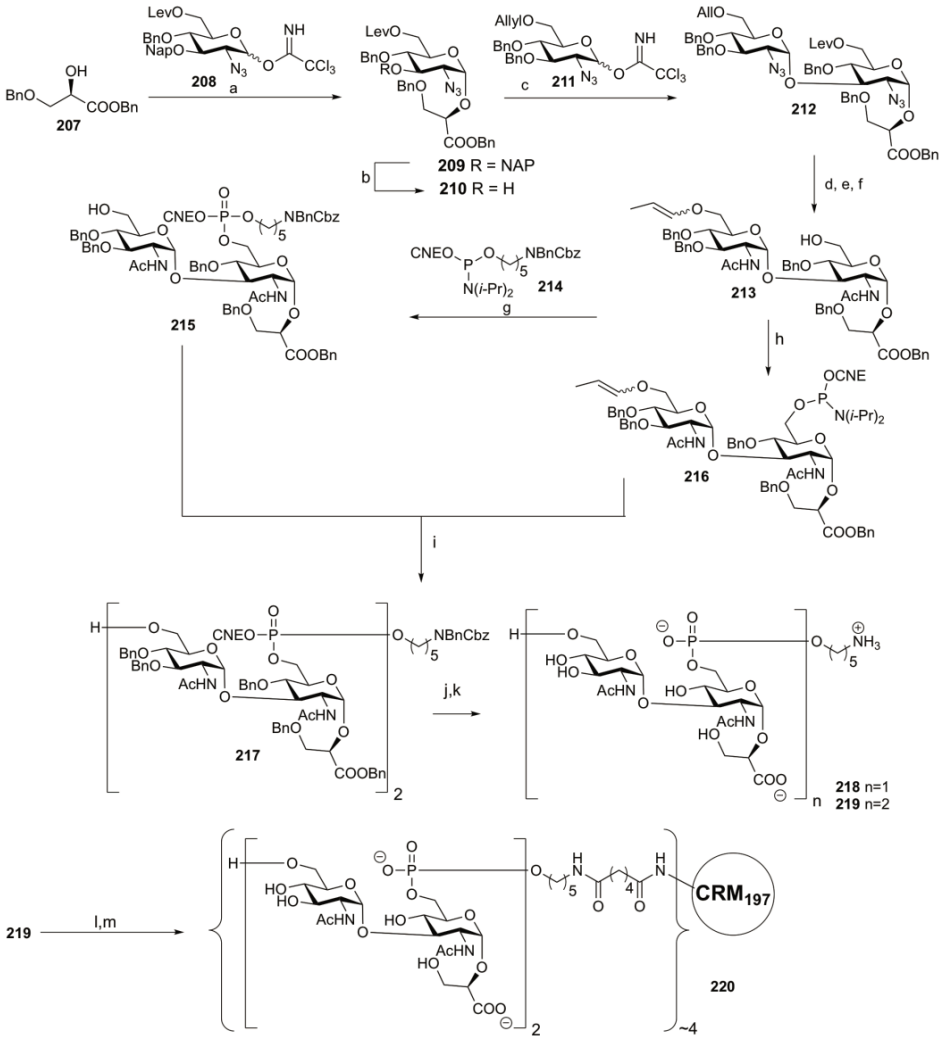


Figure 7. ***C. difficile*** **LTA**

Different synthetic approaches have been reported to generate well-defined fragments of the *C. difficile* LTA. Seeberger and co-workers successfully synthesized a monomer and a dimer of the repeating unit as depicted in scheme 10.[49] The key pseudo-trisaccharide **212** was obtained by the glycosylation of the benzyl protected (2*R*)-glyceric acid and the trichloroacetimidate **208**. Removal of the C3′-*O*-naphthyl ether and subsequent glycosylation with building block **211** then gave diglucosamine **212**. In both glycosylation reactions the desired α-selectivity was achieved through the use of low-temperature glycosylations in a dichloromethane-diethylether mixture. The azides were next transformed into the corresponding acetamides, after which the C6″-allyl ether was isomerized into the enol ether. Delevulinoylation provided the C6-OH (**213**), which was connected with the phosphoramidite aminopentanol spacer **214** using 5-ethylthio-1H-tetrazole (ETT) as the coupling agent. Notably, in the next step, iodine was not only used as oxidizing agent but also to cleave the C-6 enol ether protecting group. The monomer **215** was then elongated using phosphoramidite **216**. The monomer and dimer were then treated with triethylamine to cleave the cyanoethyl groups followed by hydrogenation to remove the remaining protecting groups to deliver the final targets **218** and **219**. The dimer repeat **219** was immobilized on a glycan microarray together with synthetic structures representing PSI and PSII and screened with sera of 12 patients with CDI. Serum IgG against PSI and PSII was detected in 10 and 11 out of 12 samples, respectively, while antibodies, recognizing LTA **219** were detected in half of the samples, indicating that *C. difficile* LTA can be a suitable antigen for a vaccine development against CDI. Encouraged by these preliminary results compound **219** was conjugated to carrier protein CRM_{197} using an adipic acid linker (Scheme 10).[50] With this glycoconjugate three different vaccination formulations were investigated. The first one using Freund's adjuvant (FA), the second one adsorbed on alum and the third without any adjuvant. Sera raised against the non-adjuvated conjugate **220** showed good opsonic killing of *C. difficile*, suggesting that the synthetic glycans provide an intrinsic adjuvant activity, while the conjugate with FA showed a weaker response. The most robust response was obtained with the alum formulation and this vaccine was used in a mouse-infection

protection model. It was shown that vaccination with the alum formulation offered protection and inhibited colonization, proving that surface antigen-based vaccines are indeed effective in reducing the colonization by *C. difficile*.

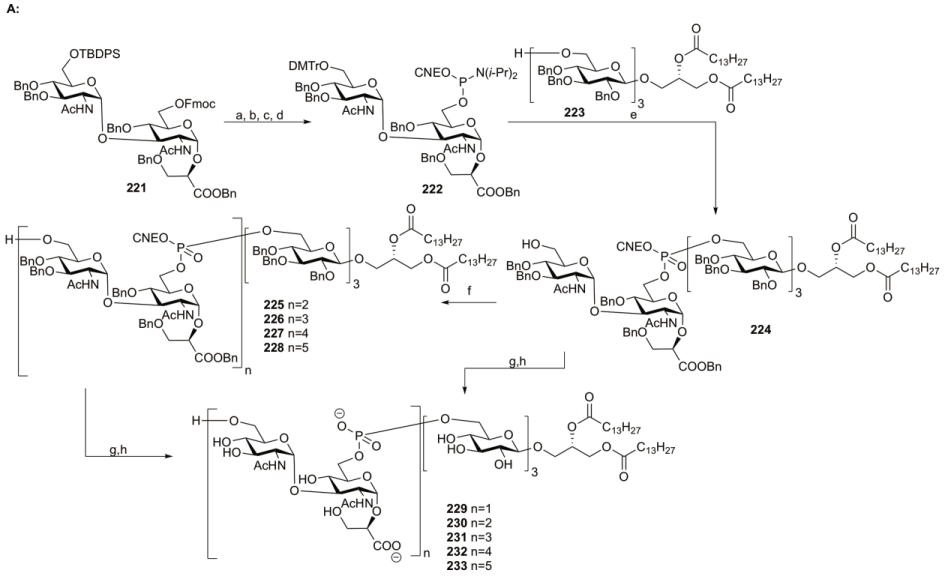


Scheme 10. Assembly of *C. difficile* LTA-conjugates;

Reagents and conditions: a) TMSOTf, DCM, Et_2O, -20°C to -10°C, 81% α/β = (9:1); b) DDQ, DCM, phosphate buffer pH= 7.2, 0°C to rt, 80%; c) TMSOTf, DCM, Et_2O, -20°C to -10°C, 69%, α/β = (8:1); d) AcSH, pyridine, 67%; e) $Ir(COD)(Ph_2MeP)_2$,THF; f) hydrazine hydrate, AcOH, pyridine, DCM, 78% over 2 steps; g) (i) 5(ethylthio)tetrazole, ACN; (ii) I_2, H_2O, THF, 98%; h) 2-cyano-ethyl bis(*N,N*-diisopropylamino)phosphoramidite, tetrazole, diisopropylamine, DCM, ACN, 82%; i) (i) 5(ethylthio)tetrazole, ACN; (ii) I_2, H_2O, THF, 78%; j) TEA; k) H_2 (4 bar), Pd/C, H_2O, AcOH; l) di-*N*-succinimidyl adipate, TEA, DMSO; m) CRM_{197}, 100 mM sodiumphosphate, pH=7.4.

Longer LTA fragments with the glycolipid anchor attached have been assembled by the group of Pedersen (see Scheme 11A).[51] Following a roughly similar approach as described

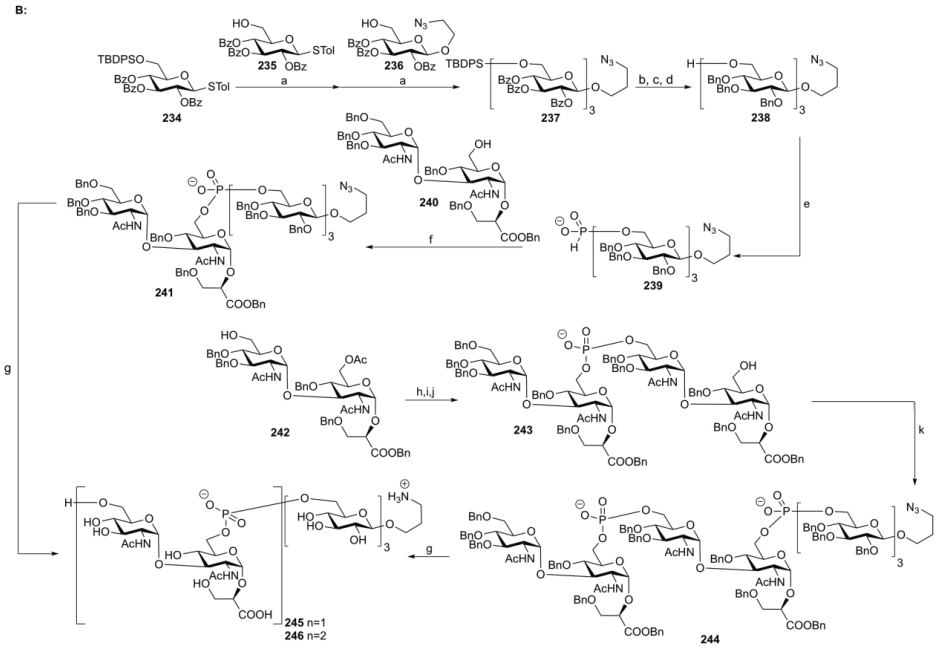
above, they assembled the di-glucosamine glyceric acid building block **221** with a C6-*O*-fluorenylmethylcarbonate and a C6'-*O*-TBDPS ether as an orthogonal set of protecting groups. This intermediate was transformed into the required LTA repeating unit synthon **222**, bearing a cyanoethyl protected phosphoramidite and a DMTr-ether as a temporary protecting group for the alcohol groups that were to be elongated. The lipid anchor **223** was first coupled to **222** using 4,5-dicyanoimidazole (DCI) as activating agent and the generated phosphite was oxidized using iodine followed by the liberation of the DMTr group using a dichloroacetic acid solution. Alcohol **224** was then further elongated with **222** to give oligomers up to 5 repeating units. It was observed that the yield of the elongation cycles dropped with growing chain length. The fragments were then treated with DBU to liberate the phosphodiesters and a final hydrogenolysis reaction cleaved all benzyl groups to deliver the target compounds **229**-**233**. The immunomodulatory properties of the LTA-fragments were investigated in human mononuclear cells (hMNCs) and in a whole blood assay but no innate immune system activation was observed.



Scheme 11. A) Total synthesis of *C. difficile* LTA; *Reagents and conditions:* a) HF, pyridine, THF, 95%; b) DMTr-Cl, pyridine, 91%; c) DBU, DCM, 94%; d) 2-cyanoethyl bis(*N,N*-diisopropylamino)phosphoramidite, tetrazole, DIPEA, DCM, 84%; e) (i) DCI, ACN; (ii) I_2, H_2O, pyridine, THF; (iii) DCA, TES-H, DCM 62%; f) (i) **222**, DCI, ACN (ii) I_2, H_2O, pyridine, THF; (iii) DCA, TES-H, DCM, n=2 (74%), n=3 (66%), n=4 (56%), n=5 (42%); g) DBU, DCM, n=1 (83%), n=2 (76%), n=3 (82%), n=4 (97%), n=5 (77%); h) Pd black, H_2, THF/H_2O, AcOH, **229** (56%), **230** (53%), **231** (mixture of LTAs, 59% product calculated, after purification by RP18 HPLC, 60%), **232** (mixture of LTAs, 26% product calculated, after purification by RP18 HPLC, 51%), **233** (mixture of LTAs, 45% product calculated).

Recently, Gu *et al.* reported an alternative strategy for the assembly of *C. difficile* LTA fragments based on H-phosphonate chemistry (see Scheme 11B).[52] They generated two target molecules, containing one or two LTA repeating unit and the lipid trisaccharide

core equipped with a conjugation handle instead of the diacyl glycerol moiety. The triglucosyl lipid anchor core was assembled in a one-pot procedure using conditions developed by Huang *et al.*[53], while the LTA repeating unit was assembled by comparable means as previously described. To conjugate the triglucoside to the repeating unit, it was transformed into H-phosphonate **239** which was then coupled to diglucosamine **240** using pivaloyl chloride (PivCl). Subsequent oxidation then generated the phosphodiester. In similar vein two repeating units were combined, to provide dimer **243**. The C6-acetate of this building block was removed to set the stage for a second coupling to H-phosphonate **239**. A single hydrogenation event transformed **241** and **244** into target compounds **245** and **246**. Conjugation to a carrier protein has been foreseen by the authors but not reported yet.



Scheme 11. B) H-phosphonate chemistry to assemble *C. difficile* LTA; *Reagents and conditions:* a) (i) *p*-TolSCl, AgOTf, TTBP, -78°C to rt (ii) **235** or **236**, 62% overall yield; b) sat NH_3, MeOH; c) BnBr, NaH, DMF; d) TBAF, THF, 98%; e) (i) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyridine/dioxane (v/v 2:3); (ii) H_2O, 87%; f) (i) PivCl, pyridine; (ii) I_2, H_2O, 78%; g) H_2, 10% Pd/C, DCM/MeOH/H_2O (v/v/v 10:10:1) n=1 (75%), n=2 (69%); h) (i) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyridine/dioxane (v/v 2:3); (ii) H_2O, 89%; i) **240**, (i) PivCl, pyridine (ii) I_2, H_2O, 76%; j) AcCl (cat), MeOH/DCM (1:1); k) (i) **239**, PivCl, pyridine; (ii) I_2, H_2O, 69%.

CONCLUSIONS

Teichoic acids are abundantly present in Gram-positive bacterial cell-walls and they play an all-important role in host-pathogen interactions. As such they represent attractive structures for vaccine development. Unfortunately, all attempts at using isolated TAs for vaccine purposes have failed. One of the reasons behind these failures may be the relatively ill-defined material used for generation of the vaccines. Organic synthesis can provide well-defined TA-fragments, that can be modified at will to, for example, attach conjugation handles at pre-determined sites in the molecule. It thus provides an excellent platform to tackle the problems that naturally sourced micro-heterogeneous TAs present. Over the years, several important advances have been reported regarding the synthesis of both LTA and WTA structures. To deal with the structural variety, automated synthesis techniques have been outlined that can allow for the rapid generation of libraries of TAs. Building block chemistry is now at a level that the required glycosylated building blocks can be reliably obtained through innovative stereoselective glycosylation methodology in combination with effective protecting group chemistry. Total syntheses have been reported of large and complex TAs, requiring the union of large building blocks. Labile moieties, such as the crucial D-alanine esters and functional lipid tails have been successfully incorporated. The large majority of approaches for the assembly of TAs hinges on the use of phosphoramidite building blocks to construct the phosphotriester linkages. This methodology has proven to be extremely reliable and will undoubtedly be used for the assembly of many TA targets in the future. In the future synthetic methods will further mature, to allow for the more rapid assembly of more complex and varied TAs as well as expand the library of available TAs. Interaction studies at the atomic level will unravel how TA-substitution patterns govern host-pathogen interactions and how they impact the fitness and virulence of important human pathogens. This will open up possibilities to use these molecules in vaccine formulations to neutralize the ever-growing threat of multidrug resistant super bugs.

OUTLINE OF THIS THESIS

Chapter 2 describes the synthesis of ribitol wall teichoic acid (WTA) fragments, both in solution, and on solid phase. These WTA fragments were used to screen for binding to human IgG sera and human langerin. The hexamer was used as a substrate for the enzyme TarP in crystallization studies probing the binding mode. The hexamer was enzymatically glycosylated and this product was coupled to magnetic beads and used to detect WTA-specific IgG in human serum.

Chapter 3 reports the synthesis of C-4 glycosylated WTAs using α- and β–linked C4-GlcNAc ribitol phosphoramidite building blocks. The binding affinity between human langerin and both glycosylated and non-glycosylated WTA fragments was evaluated on the micro array. This showed selective binding of C-type lectin to the WTA fragments bearing a β–GlcNAc. In addition, spacer-free trimers were synthesized for crystallization studies to probe the interaction in the active site of langerin.

Chapter 4 describes the synthesis of C-3 glycosylated WTAs. Building on the successful synthesis of unsubstituted WTAs on solid phase as described in Chapter 2, here the automated solid phase synthesis was used to assemble glycosylated WTA fragments. NMR data of the glycosylated WTA fragments were presented for structure elucidation of newly identified bacterial WTA species. The antibody binding of the C-4- and C-3 glycosylated WTAs was probed in the bead assay. This showed specific binding of the α-mAb toward α-GlcNAc WTAs, whereas β–mAb showed cross-reactive binding to both the C-3 and C-4 β–GlcNAc WTAs.

Chapter 5 describes an approach to synthesize a ribitol phosphate heptamer bearing D-alanine esters on the C-2 position. This ester modification is found in *S. aureus* WTAs, and the degree of this esterification seems to influence the susceptibility toward Vancomycin and other glycopeptide antibiotics. Isolation of these fragments is very difficult due to high lability of the esters.

Well-defined WTA fragments are required to evaluate the role of the D-alanine modification at the molecular level in structure activity studies. Due to the high hydrolytic and labile nature of the esters, benzyl protected phosphoramidites were used instead of the common cyanoethyl protected phosphoramidite which require a basic step in the deprotection stage. At the final deprotection stage it was necessary to keep the conditions acidic to protect the D-alanine esters from hydrolysis.

Chapter 6 reports a synthesis route for *E. faecalis* V583 WTA fragments composed of *N*-acetyl-β-D-galactosaminyl ribitol phosphate residues connected to an α-L-rhamnose branch at the C-3 of the galactosamine residue. The WTA repeating unit was assembled through the regioselective coupling between a rhamnose donor with a participating benzoyl group at the C-2 for α-selectivity and a C-3, C-4-diol GalNAc acceptor. Subsequent coupling with a ribitol acceptor yielded the pseudo-trissacharide.
This pseudo-trisaccharide was the key intermediate for further elongations. The intermediate was converted into an alcohol and into a phosphoramidite building block. Condensation of the alcohol with a phosphoramidite spacer yielded the monomer target

compound. This monomer was further coupled to the phosphoramidite building block to deliver the dimer target compound.

Chapter 7 provides a summary of this thesis and future prospects including crystallizations of WTA fragments with langerin and monoclonal antibodies, and the assembly of WTAs featuring both GlcNAc and D-alanine esters using a cleavable silyl linker on solid phase.

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