Synthesis and applications of cell wall glycopolymer fragments from Staphilococci and Enterococci

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Chapter 1

Synthetic carbohydrate-based cell wall components from *Staphylococcus aureus*

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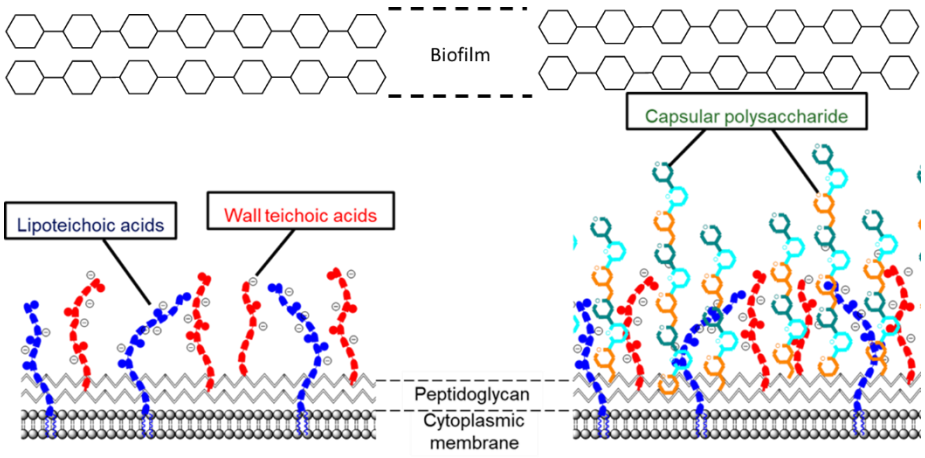
INTRODUCTION

Staphylococcus aureus is a commensal ubiquitous Gram-positive bacterium associated with a range of severe infections (skin and soft tissue infections, sepsis, arthritis, pneumonia, bacteraemia and others).[1] Although *S. aureus* can be present as asymptomatic colonizer, most of the infections are hospital acquired and affect mainly young children, elderly, immunocompromised and post-surgical patients, leading to high health-care costs and higher risk of in-hospital deaths.[2] The rise of highly antibiotic resistant strains, such as Methicillin resistant *S. aureus* (MRSA), urges the development of new treatments.[3] In the last decade much attention has been focused on the development of active or passive immunization strategies.[4]

The structurally complex cell envelope of *S. aureus* is composed of peptidoglycan, cell wall glycopolymers and proteins. [5] All of these are involved in several physiological processes and they play a key role in staphylococcal virulence, making them promising antigen candidates. Figure 1 shows a schematic representation of *S. aureus* cell wall, highlighting the three major classes of glyco-based cell wall components that have been found to be promising antigen candidates: 1) capsular polysaccharides[6], the structure of which varies between strain types and which may also be absent; 2) wall teichoic acids (WTAs) and lipoteichoic acids (LTAs),[7] which are anionic glycopolymers either covalently attached to the peptidoglycan or anchored to the lipid bilayer through hydrophobic interactions, respectively; 3) the thick peptidoglycan layer. Herein an overview is presented on the major *S. aureus* carbohydrate-based antigen candidates for which organic synthesis efforts have delivered well-defined fragments to delineate clear structure-activity relationships. These fragments are attractive tools not only for vaccine applications but also for diagnostics as well as other interaction studies (such as lectin binding) and biosynthesis studies.

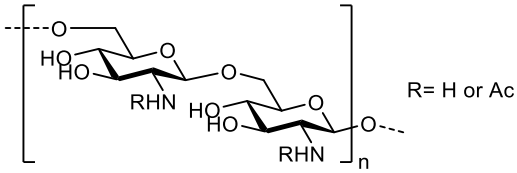
Figure 1: *Schematic representation of the cell wall from S. aureus*



PNAG

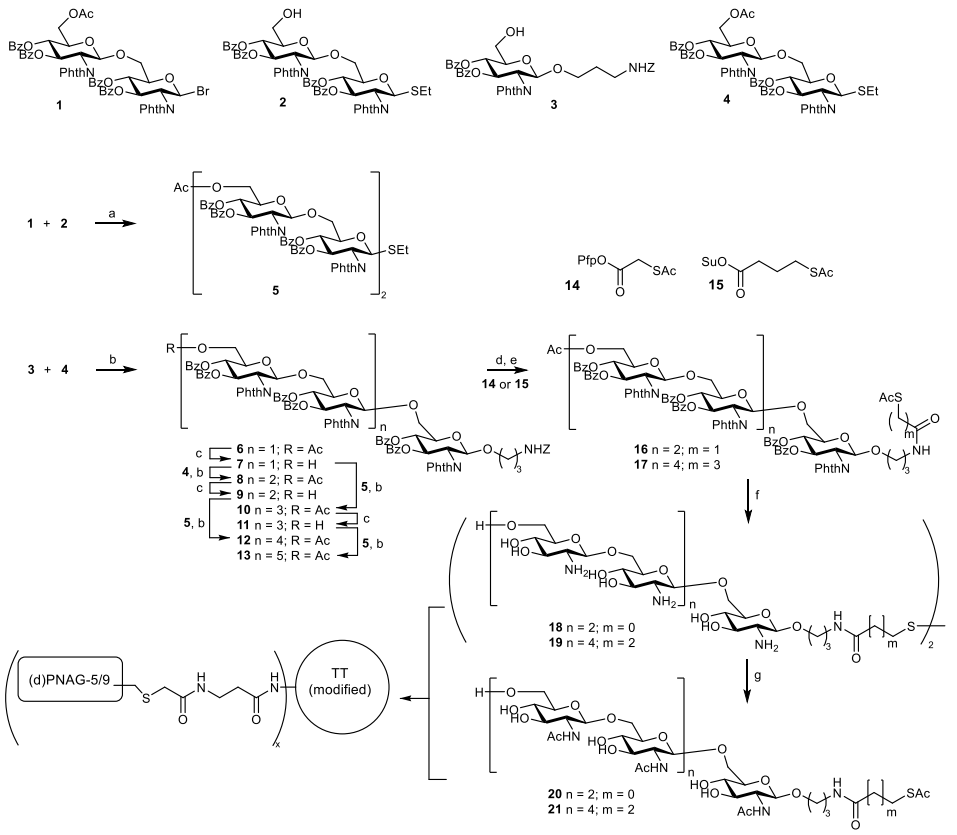
Biofilm formation represents one of the bacterial defence mechanisms against both host immune response and antibiotics. Poly-β(1-6)-*N*-acetylglucosamine (PNAG) is a cell surface polysaccharide produced not only by many bacterial species, including S. *aureus*, but also fungi and protozoal parasites.[8] Thus, in the last decades much attention has been addressed to the immunological properties of PNAG and its potential application in vaccine development.[9] The structure of PNAG is shown in Figure 2 and it has been reported that about 10-20% of the amino groups are not *N*-acetylated (dPNAG).[10]

Figure 2: *Structure of PNAG and dPNAG*



Native PNAG and chemically deacetylated PNAG (dPNAG) have been conjugated to diphtheria toxoid to generate model vaccines that were evaluated in different animal models and it was observed that antibodies against dPNAG were more effective in mediating opsonophagocytic killing than the ones raised against the PNAG conjugate.[11] Native polysaccharides are generally obtained as a heterogeneous mixture of oligosaccharides, varying in length and substitution pattern with different immunological activity. Chemically synthesized, well-defined fragments, on the other hand, can be used to define the structure-immunogenicity relationships. In 2007, Nifantiev and co-workers reported the synthesis of well-defined PNAG and dPNAG fragments up to the undecamer level, equipped with an aminopropyl linker for further functionalization.[12] The strategy relied on four key building blocks **1**, **2**, **3** and **4** (Scheme 1), the synthesis of which was reported earlier.[13] In their strategy, the C-6-OH was temporarily protected with an acetyl group, while the C-3 and C-4 hydroxyl groups were protected as benzoyl esters. The presence of a phthalimide group at the C-2-nitrogen allows to direct the glycosylation reactions stereoselectively via neighbouring participation group. Tetrasaccharide **5** was obtained in 76% yield by a coupling between disaccharide bromide donor **1** and disaccharide acceptor **2** under Helferich conditions. Coupling of disaccharide donor **4** with acceptor **3** using NIS and catalytic amount of TfOH afforded linker equipped trisaccharide **6** in 94%. Selective removal of the acetyl group using acetyl chloride in MeOH then afforded acceptor trisaccharide **7** in quantitative yield and the subsequent condensation with disaccharide donor **4** delivered pentasaccharide **8** in excellent yield. After acetyl cleavage, tetrasaccharide donor **5** was coupled with either trisaccharide acceptor **7** or pentasaccharide acceptor **9** to obtain an heptamer (**10**, 74%) and a nonamer (**12**, 60%) respectively. Final acetyl removal from the heptasaccharide gave **11** and subsequent coupling with tetrasaccharide donor **5** furnished fully protected undecamer **13** in 50% yield. In order to define structural requirements for

immunogenicity, pentasaccharide **8** and nonasaccharide **12** were selected to be further functionalized and conjugated to a carrier protein for immunization experiments.[14] Selective deprotection of the amino group of the spacer, followed by amide formation using **14** or **15**, afforded thiol functionalized pentasaccharide **16** and nonasaccharide **17**. Global deprotection was achieved using hydrazine hydrate in boiling EtOH to afford compounds **18** and **19**. *N*-acetylated derivatives **20** and **21** were generated using dithiothreitol and acetic anhydride. The oligosaccharide thiol derivatives were obtained by treatment of compounds **18**, **19**, **20** and **21** with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in a 7% solution of ammonia in water. The so-generated thiols were coupled to modified tetanus toxoid (TT) protein generating four glycoconjugates. Mice were immunized with the four synthetic glycoconjugates, after which the opsonic activity of the generated sera was evaluated. These studies confirmed that antibodies raised against the dPNAG conjugates from **18** or **19** had greater opsonic activity than the ones raised against PNAG conjugates derived from **20** or **21**. Interestingly, the former antibodies were cross-reactive towards native PNAG and dPNAG, while dPNAG was not recognized by antibodies raised against fully *N*-acetylated conjugates.



***Scheme 1**: Synthesis of well-defined PNAG, dPNAG fragments and its corresponding glycoconjugates. a) $HgBr_2$, $Hg(CN)_2$, CH_3CN, 76%. b) NIS, TfOH, MS 4Å, CH_2Cl_2, **6**, 94%; **8**, 90%; **10**, 74%; **12**, 60%; **13**, 51%. c) AcCl, CH_3OH, **7**, 95%; **9**, 93%; **11**, 96%. d) H_2, $Pd(OH)_2$, 1M HCl, CH_3OH*

/THF (1/2, v/v). e) ***14*** *or* ***15****, Et_3N, CH_2Cl_2/DMF (4/1, v/v),* ***16****, 95%;* ***17****, 90%. f) NH_2 NH_2•H_2O, EtOH,* ***18****, 80%;* ***19****, 86%. g) dithiothreitol (DTT), Ac_2O,* ***20****, 90%;* ***21****, 95%.*

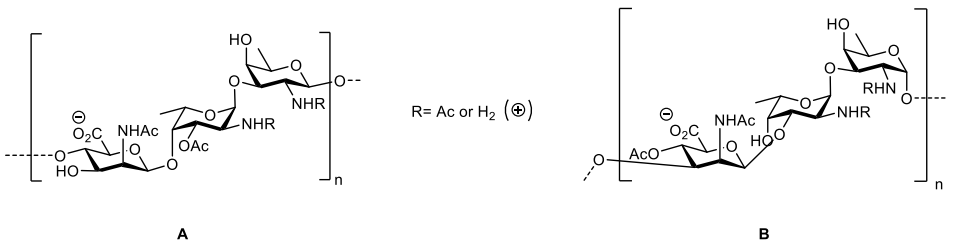
CAPSULAR POLYSACCHARIDES (CPs)

Capsular polysaccharides (CPs) are extracellular cell-wall components comprising long polysaccharide chains covalently attached to the peptidoglycan layer. They represent a first line of defence for bacteria by shielding important cell-wall constituents and providing a mechanism to evade phagocytic uptake and killing by the host immune system.[15] They also contribute to host colonization and biofilm formation and thereby in the progression of invasive diseases. To date, 13 serotypes have been identified among the clinical *S. aureus* isolates, carrying different CPs.[16]

CP5 and CP8

S. aureus CP5 and CP8 are the most abundant among the clinical isolates, and they account for the 25%-50% of clinically encountered MRSA.[17] Most of the vaccine candidates that reached an advanced stage in clinical trials, contained either of these two CPs as antigenic component.[18] CP5 and CP8 are structurally very similar.[19] The first is constituted by a trisaccharide repeating unit composed of an *N*-acetyl mannuronic acid, β-(1,4) linked to an *N*-acetyl-L-fucose moiety, bearing an acetyl group at the C-3-alcohol, which is α-(1,3)-linked to an *N*-acetyl-D-fucose. The repeating units are linked through an β-(1,4)-linkage between the latter fucose and the mannuronic of the next repeating unit (Figure 3A). The trisaccharide unit from CP8 is composed of the same monosaccharides, but the mannuronic acid is β-(1,3) linked to an *N*-acetyl-L-fucose and the acetyl substituent is placed on the C-4-hydroxy of the mannuronic acid unit and the *N*-acetyl-D-fucose is linked to the C-3 of the mannuronic acid through an α-linkage (Figure 3B). These structures have been shown to possess zwitterionic character originating from the presence of a negative charge from the mannuronic acid and a positive charge, resulting from (random) deacetylation of one of the two fucose residues. Currently it is not known how many positive charges are found in these structures and where exactly they reside in the polysaccharides.[20]

Figure 3: *Structures of trisaccharide repeating units of S. aureus CPs type 5 and type 8*



A **B**

The synthesis of these two CP trisaccharide units represents a great challenge due to the presence of several 1,2-*cis* glycosidic linkages, the anionic charges and the *O*-acetyl substituents. The first synthesis of the CP5 repeating unit was reported by Adamo and co-workers (Scheme 2A).[21] In their strategy, a benzyl glucuronate imidate donor **22** was used to introduce the β-linkage to the C-4 of L-fucosyl **23**, through a TMSOTf-mediated glycosylation affording the desired disaccharide **24** in 61% yield. The conversion to the *manno*-configured disaccharide derivative was achieved by removal of the levulinoyl protecting group, installation of the triflate and finally nucleophilic substitution using tetrabutylammonium azide. After deallylation and introduction of the imidate leaving group, disaccharide **26** was coupled with acceptor **27**, bearing an *N*-Cbz protected amino propyl spacer for ligation purposes, achieving trisaccharide **28** as a mixture of α/β anomers (2.4/1). Final hydrogenolysis and chemoselective *N*-acetylation with acetic anhydride in methanol deliver the final trisaccharide **29**. Several attempts were carried out to selectively reduce the azide groups over the Cbz protected amine of the linker, but unfortunately, either Staudinger conditions or treatment with H_2S led to (partial) lactamization of the mannuronic unit. From the immunological evaluation by competitive ELISA and immunodot blot experiments, it has become clear that longer fragments are needed to be sufficiently antigenic and effectively mimic the native CP structures.

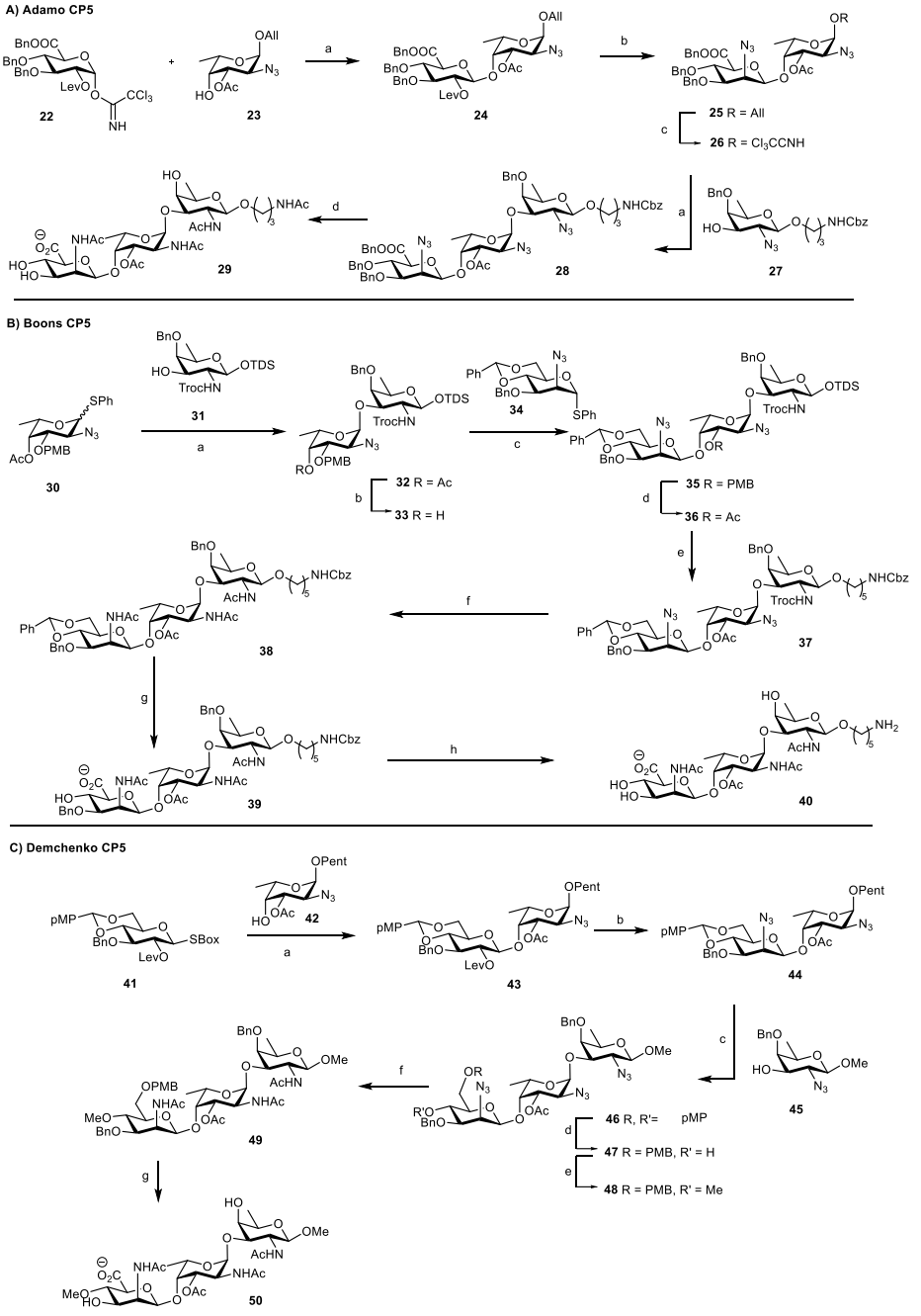
In 2015, Boons and co-workers reported a different synthetic strategy towards the synthesis of the CP5 trisaccharide unit (Scheme 2B).[22] First L-fucose donor **30** was coupled to D-fucose acceptor **31** and different glycosylation conditions were explored. Pre-activation of donor **30**, using either 1-benzene-sulfonyl piperidine (BSP) or diphenylsulfoxide (DPS) in the presence of Tf_2O and 2,4,6-tri-*tert*-butylpyrimidine as acid scavenger, stereoselectively afforded disaccharide **32** in 30% yield. Instead, activation of donor **30** with NIS in the presence of TMSOTf as promotor delivered the desired disaccharide **32** in much higher yield (72%) but as a 4:1 α/β-mixture. After treatment of the disaccharide with NaOMe in MeOH to effectuate the removal of the acetyl ester, disaccharide acceptor **33** was coupled with benzylidene protected azido-mannose donor **34**. Via formation of anomeric α- triflate at low temperature using DPS/Tf_2O as promoter, the β-mannoside trisaccharide **35** was formed as the major anomer and isolated in 72% yield. Subsequently, the PMB protecting group was replaced by an acetyl to deliver fully protected trisaccharide **36** in excellent yield over two steps. At the reducing end the TBS group was removed to introduce an *N*-Cbz protected aminopentanol spacer as ligation handle via the intermediate *N*-phenyl trifluoroacetimidate. In order to avoid the possible

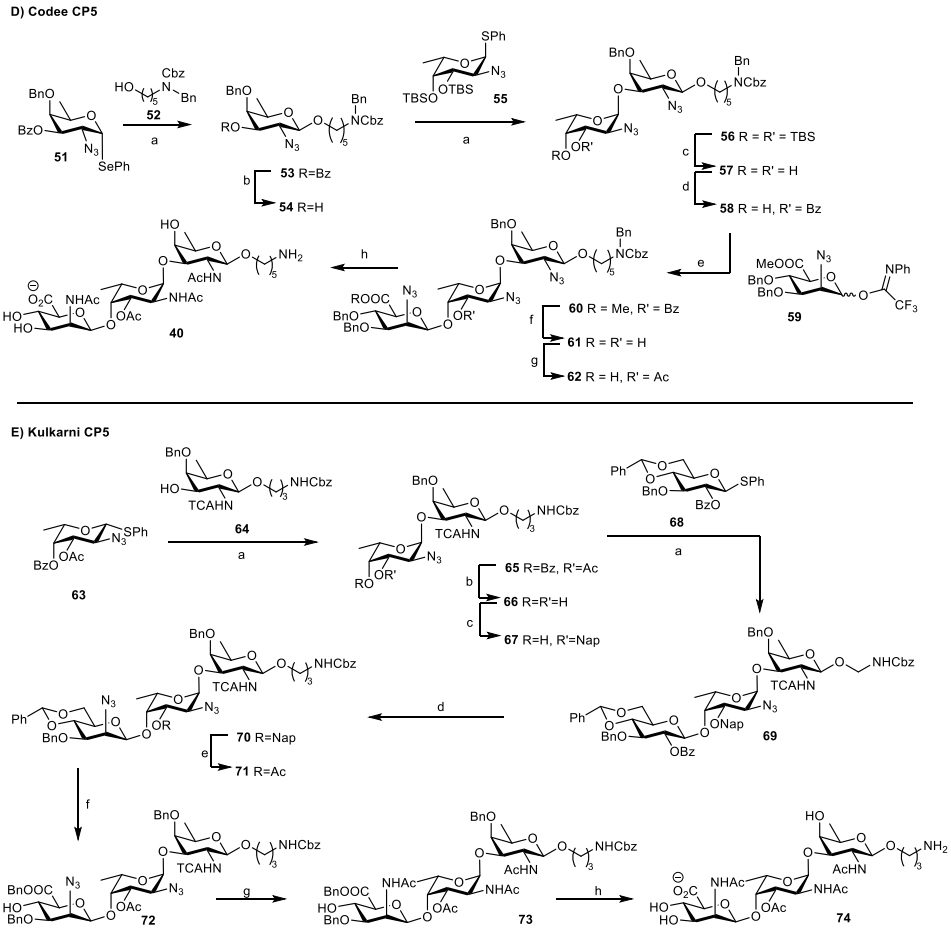
formation of the lactamized by-product, the azide and Troc-groups were first converted to the corresponding acetamide moieties using zinc-mediated reductions in the presence of AcOH and Ac_2O, affording compound **38** in 63% yield. After acidic hydrolysis of the benzylidene group, the primary alcohol of the mannose sugar was selectively oxidized using a one-pot TEMPO/NaOCl-$NaClO_2$ procedure yielding compound **39** in good yield over two steps. At last, all the benzyl type groups were removed by hydrogenolysis using $Pd(OH)_2$ in MeOH. Completely deprotected trisaccharide **40** was isolated in quantitative yield.

In 2016, Demchenko and co-workers described the synthesis of a CP5 trisaccharide unit bearing methyl groups at the sides of propagation of the polysaccharide sequences (Scheme 2C).[23] First glucosyl donor **41**, bearing a Lev-protecting group at the C-2-hydroxy was coupled by selective activation of the SBox leaving group using AgOTf to L-fucose acceptor **42** delivering disaccharide **43** in 78% yield as a single anomer. Epimerization of the glucosyl C-2 stereocenter was achieved by a three-step sequence, similar to the one described previously by Adamo, affording mannosyl disaccharide **44** in 70% yield overall. Subsequently, disaccharide **44** was coupled to D-fucose acceptor **45** by activation of the *O*-pentenyl group with NIS and TfOH, affording the fully protected trisaccharide **46** as a single anomer. The differences in stereochemical outcome of this glycosylation and the condensation between donor **26** and acceptor **27** as reported by Adamo, described above, are at present difficult to rationalize. Next the 4'',6''-*p*-methoxybenzylidene was regioselectively opened using $NaCNBH_3$ in the presence of 2M HCl after which the C-4''-hydoxy was capped with a methyl group affording **48** in 74% yield. All azide groups were reduced using propane 1,3-dithiol and triethylamine in pyridine, followed by acetylation, obtaining the derivative **49** in 91% yield. The last steps involved deprotection of the PMB group, followed by selective oxidation of the primary alcohol using TEMPO/BAIB and finally a hydrogenolysis event, affording the target compound **50** in 73% over the three steps.

One year later, another synthetic route towards the CP5 trisaccharide unit was published by Codée and co-workers (Scheme 2D).[24] Their strategy relied on a [1+2] coupling similar to the one applied by Boons. First D-fucosyl selenophenyl donor **51** was coupled with aminopentanol spacer derivative **52** under pre-activation conditions using Ph_2SO, Tf_2O and TTBP in a mixture of DCM and Et_2O. It was observed that the presence of Et_2O as co-solvent increased the stereoselectivity of this glycosylation reaction and product **53** was isolated in 80% yield as a 1:7 α/β-mixture. Removal of the benzoyl moiety under Zémplen conditions afforded acceptor **54** in 95% yield, which was next coupled to L-fucose donor **55**. In an extensive study on the reactivity of azidofucose donors, it was observed that 'arming' protecting groups (such as di-*tert*-butyldimethylsilyl ethers) on the C-3 and C-4-hydroxy groups enhances the α-stereoselectivity of the glycosylation reaction. Indeed, α-linked disaccharide **56** was isolated as a single anomer in 76% yield. Both silyl groups were next removed and the C-3'-OH was selectively benzoylated using Taylor's boron catalyst. Different than the strategies shown above, the synthesis of the fully protected trisaccharide **60** was achieved by employing a mannuronic acid donor (**59**), which enables the stereoselective formation of the required β-glycosidic linkage. The use of a pre-oxidized donor for the assembly of the trisaccharide unit reduces the functionalization and deprotection steps at a late stage of the synthesis. Using a large excess of donor **59**

and TBSOTf as promoter, β-linked trisaccharide **60** was isolated in 75% as a single anomer. Subsequently, the ester group were removed in a KOH/H_2O_2 mediated saponification, followed by the introduction of the acetyl group on the L-fucosyl C-3-alcohol and conversion of the azides into the corresponding acetamides using AcSH in pyridine. Final hydrogenolysis delivered target trisaccharide **40** in 37% overall yield.
Recently, one additional synthesis of the CP5 trisaccharide unit was reported by Kulkarni and co-workers.[25] First a condensation between L-fucose donor **63** and D-fucose acceptor **64** using NIS/TMSOTf as promotor delivered α-linked disaccharide **65** in 94% yield. Zémplen conditions were used to simultaneously remove all esters and subsequently the tin-mediated regioselective protection delivered disaccharide **67**. Compound **67** was used as acceptor for the glycosylation reaction with glucosyl donor **68** in the presence of NIS and TMSOTf delivering trisaccharide **69** in 84% yield. The mannosyl trisaccharide derivative **70** was then obtained by employing a similar epimerization strategy discussed previously. Functionalization and protecting group manipulations to deliver the final trisaccharide **74**, started with a selective oxidative cleavage of the Nap-ether, followed by acetylation, delivering intermediate **71**. Subsequent benzylidene hydrolysis, selective oxidation of the primary alcohol to the carboxylic acid and protection with a benzyl group afforded trisaccharide **72** in 69% overall yield. The azides were converted to acetamide groups by a zinc-mediated reduction, followed by acetylation and finally a hydrogenolysis reaction to deliver the target compound **74** in 72% over the three steps.


A) Adamo CP5
22
23
24
25 R = All
26 R = Cl3CCNH
27
28
29
B) Boons CP5
30
31
32 R = Ac
33 R = H
34
35 R = PMB
36 R = Ac
37
38
39
40
C) Demchenko CP5
41
42
43
44
45
46 R, R'= pMP
47 R = PMB, R' = H
48 R = PMB, R' = Me
49
50

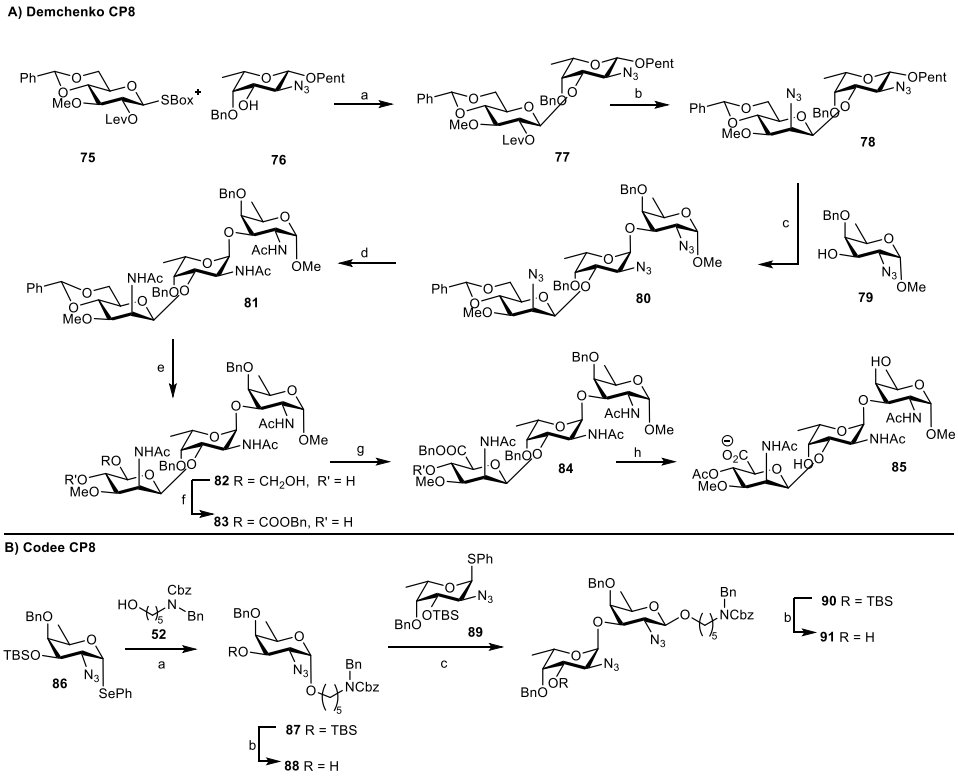


Scheme 2: *Chemical synthesis of trisaccharide repeating units of S. aureus CPS type 5. A) a) TMSOTf, CH_2Cl_2, -10 °C,* ***24****, 61%;* ***28****, 65%. b) (i) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH, 86%; (ii) Tf_2O, Py, CH_2Cl_2; $TBAN_3$, Toluene, 70%. c) (i) $PdCl_2$, CH_3OH, 78%; (ii) CCl_3CN, DBU, CH_2Cl_2, 98%. d) (i) 10% Pd/C, CH_3OH/H_2O, AcOH; (ii) Ac_2O, CH_3OH/H_2O, 40%. B) a) NIS, TMSOTf, CH_2Cl_2, -60 °C, 73%, α/β = 4/1. b) Na(cat.), CH_3OH, guanidine•HCl, rt, 18 h, quant. c) DSP, Tf_2O, TTBP, CH_2Cl_2, -60 °C to -30 °C, 1 h, 72%. d) (i) DDQ, CH_2Cl_2/H_2O (9/1, v/v), rt, 4 h; (ii) Ac_2O, Py, DMAP, rt, 2 h, 85% over 2 steps. e) (i) HF/Py, THF, rt, 18 h, 90%; (ii) CF_3(=NPh)CCl, Cs_2CO_3, CH_2Cl_2, rt, 2 h; (iii) $HO(CH_2)_5NHCbz$, TMSOTf, CH_2Cl_2/CH_3CN (1/1, v/v), -78 °C, 10 min, 72% over 2 steps. f) Zn, THF, AcOH, Ac_2O, rt, 1 h, 63%. g) (i) 80% aq. AcOH, 90 °C, 2 h; (ii) TEMPO, BAIB, CH_2Cl_2/H_2O (4/1, v/v), rt, 3 h, $NaClO_2$, 2-methyl-2-butene, t-BuOH, rt, 1 h, 61% over 2 steps. h) $Pd(OH)_2$, CH_3OH, AcOH, 2 h, quant. C) a) AgOTf, 3Å MS, 1, 2-dichloroethane, 1 h, 78%, β only. b) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH (20/1, v/v), rt, 16 h, 86%; Tf_2O, Py, CH_2Cl_2, 0 °C, 4 h; NaN_3, DMF, 60 °C, 3 h, 81%. c) NIS, TfOH, 3Å MS, 1, 2-dichloroethane, 0 °C, 1 h, 79%, α only. d) $NaCNBH_3$, 2 M HCl/ether, THF, rt, 1 h, 94%. e) MeI, Ag_2O, DMF, 16 h, rt, 79%. f) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt, 3 h; then Ac_2O, CH_3OH, rt, 2 h, 91% over 2 steps. g) (i) DDQ, H_2O, CH_2Cl_2, rt, 6 h, 84%; (ii) TEMPO, BAIB, aq. CH_2Cl_2, rt, 16 h; H_2, 10% Pd/C, aq. EtOH, rt, 16 h, 73% over 2 steps. D) a) Ph_2SO, Tf_2O, TTBP, 3Å MS, -80 °C to -40 °C, for* ***53:*** *acceptor* ***52****, CH_2Cl_2/Et_2O (1/1, v/v), 80%, α/β = 1/7; for* ***56:*** *donor* ***55****, CH_2Cl_2, 76%, α only. b) Na, CH_3OH, 95%. c) TBAF, THF, quant. d) Taylor's catalyst, BzCl, DIPEA, CH_3CN, 67%. e) TBSOTf, 3Å MS, CH_2Cl_2, -80 °C to -55 °C, 5 h, 75%. f)*

*H_2O_2, KOH, THF, H_2O. g) Ac_2O, Py. h) (i) AcSH, Py; (ii) $Pd(OH)_2/C$, H_2, AcOH, THF, t-BuOH, H_2O, 57% over 3 steps. E) a) NIS, TMSOTf, 3Å MS, CH_2Cl_2, for **65:** 0 °C, 30 min, 94%; for **69:** -60 °C to -10 °C, 4 h, 84%; b) CH_3ONa, CH_3OH, rt, 82%. c) Bu_2SnO, toluene, 110 °C, TBAB, NapBr, 60 °C, 79%. d) NaOMe, CH_3OH, rt, 2 h, 90%; Tf_2O, Py, CH_2Cl_2, 0 °C, 30 min; $TBAN_3$, toluene, 60 °C, 3 h, 71% over 2 steps. e) (i) DDQ, CH_2Cl_2/H_2O (9/1, v/v), rt, 1 h; (ii) AcCl, Py, CH_2Cl_2, 0 °C - rt, 4 h, 92% over 2 steps. f) (i) 80% aq. AcOH, 80 °C, 1 h; (ii) TEMPO, BAIB, CH_2Cl_2/H_2O (4/1, v/v), rt, 14 h, (iii) BnBr, $NaHCO_3$, DMF, 6 h, 69% over 3 steps. g) (i) Zn, AcOH, THF, rt, 12 h; (ii) Ac_2O, Et_3N, CH_3OH. h) $Pd(OH)_2/C$, EtOH, rt,10 h, 72% over 3 steps.*

Up to now only one synthetic route towards the CP8 trisaccharide unit has been described.[23] In 2015 Demchenko and co-workers reported the synthesis of the trisaccharide bearing methyl groups at the sites of propagation similar to the CP5 fragment **50** shown previously (Scheme 3A). First, coupling of glucosyl donor **75** to L-fucosyl acceptor **76** delivered disaccharide **77** in 73%. Subsequent C-2'-epimerization afforded mannosyl disaccharide derivative **78** in 65% overall yield. Glycosylation of donor **78** and acceptor **79** was performed using NIS and TfOH, delivering fully protected trisaccharide **80** in 87% yield as a single α-anomer. Subsequently, the azide groups were reduced using propane-1,3-dithiol and acetylation afforded the triacetamido derivative **81**. Hydrolysis of the benzylidene group, followed by TEMPO/BAIB mediated oxidation and subsequent protection of the newly formed carboxylic acid with a benzyl group delivered compound **83** in 44% overall yield. Finally, acetylation on the C-4''-OH of the mannuronate moiety and a last hydrogenolysis step afforded target compound **85** in 96% over the last two steps.

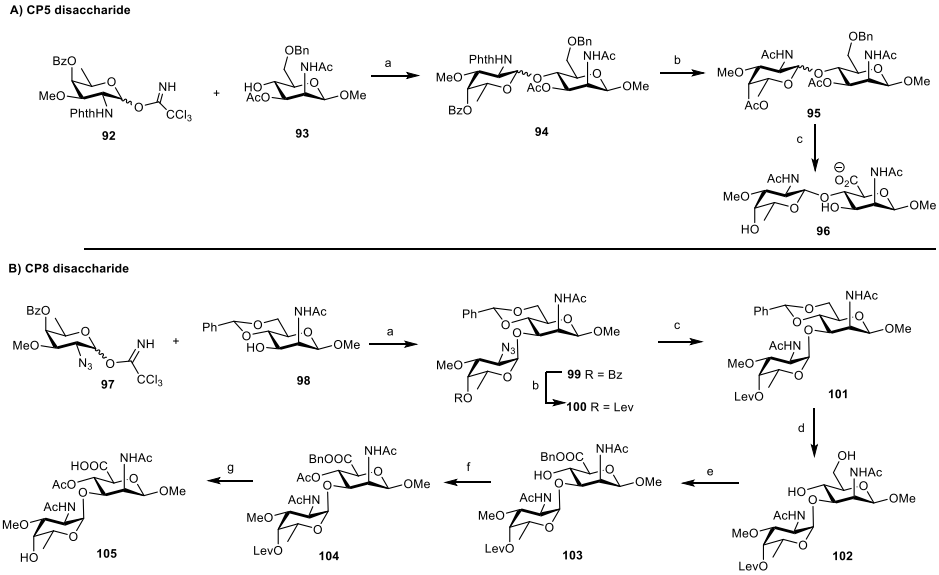
Together with the synthesis of the CP5 trisaccharide unit,[24] Codée and co-workers reported the synthesis of a semi-protected CP8-disaccharide core, built up from the two enantiomeric fucosyl moieties, that can be used to achieve the complete trisaccharide fragment in the future (Scheme 3B). First D-fucose donor **86** was coupled to aminopentanol linker **52**. In order to enhance the stereoselectivity a protocol, previously described by Bennett and co-workers, was explored. Donor **86** was pre-activated using Ph_2SO/Tf_2O at -78°C, followed by the addition of acceptor and tetrabutylammonium iodide (TBAI), to enable the *in situ* formation of the anomeric iodide, and *N*-methylmaleimide as an electrophilic scavenger. This protocol delivered D-fucose **87** in 85% yield as α/β mixture (7:1). After removal of the silyl-group, D-fucose acceptor **88** was coupled with L-fucose donor **89** delivering fully protected disaccharide **90** in 73% as α/β mixture (7/1). Subsequent deprotection of the silyl group with TBAF afforded the pure α-linked disaccharide **91** in 71% yield.



Scheme 3*: Synthesis of trisaccharide repeating units and disaccharide fragment of S. aureus CPS type 8. A) a) AgOTf, 3Å MS, 1, 2-dichloroethane, rt, 1 h, 73%, β only. b) (i) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH (20/1, v/v), rt, 3 h, 72%; (ii) Tf_2O, Py, CH_2Cl_2, 0 °C, 3 h; (iii) NaN_3, DMF, 60 °C, 16 h, 90%. c) NIS, TfOH, 3Å MS, 1, 2-dichloroethane, 0 °C, 1 h, 87%, α only. d) (i) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt, 6 h; (ii) Ac_2O, CH_3OH, rt, 16 h, 94% over 2 steps. e) aq. TFA, CH_2Cl_2, rt , 16 h, 92%. f) (i) TEMPO, BAIB, aq. CH_2Cl_2, rt, 4 h; (ii) BnBr, $NaHCO_3$, DMF, rt, 16 h, 61% over 2 steps. g) Ac_2O, Py, rt, 16 h, 99%. h) H_2, 10% Pd/C, aq. EtOH, rt, 24 h, 97%. B) a) Ph_2SO, Tf_2O, TTBP, N-methylmaleimide, 3Å MS, -80°C to -70°C; TBAI, -80°C to r.t., 1,4 dioxane,* ***52****, 85%, α/β = 7/1. b) TBAF, THF, 65% for* ***88****, 71% for* ***91****. c) Ph_2SO, Tf_2O, TTBP, CH_2Cl_2, -80°C to -70°C,* ***87****, -80°C to -50°C, 73%, α/β = 7/1.*

Recently Demchenko and co-workers reported the synthesis of two disaccharides (**96** and **105**) from CP5 and CP8 to develop chemistry that would allow the interconnection of the repeating units.[26] In the case of the CP5 fragment (Scheme 4A), D-fucose trichloroacetimidate donor **92** was coupled to mannose acceptor **93** using a catalytic amount of TMSOTf affording disaccharide **94** in 63% as an 1:5 α/β mixture. Treatment of the dimer with hydrazine monohydrate and subsequent acetylation provided disaccharide intermediate **95** in 81% yield. Hydrogenolysis, TEMPO/BAIB mediated oxidation to carboxylic acid of the primary alcohol and final hydrolysis of acetyl esters delivered disaccharide derivative **96** in 44% yield. In the case of the CP8 disaccharide fragment (Scheme 4B), D-fucose donor **97** was coupled with acceptor **98** using TfOH in DCM affording disaccharide **99** in 65% yield as a single anomer, likely as the result of the presence of the C-4 fucosyl benzoyl protecting group, capable of performing long-range

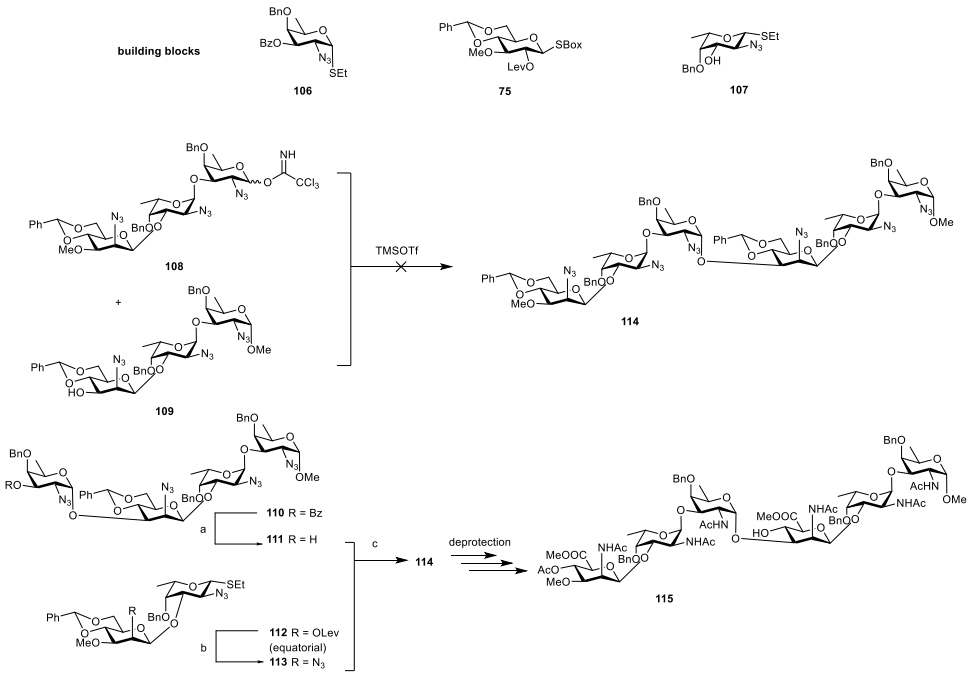
participation[27]. Next, the benzoyl group was replaced by a levulinoyl ester, obtaining disaccharide intermediate **100** in 89% over two steps. Reduction and subsequent acetylation of the azide delivered disaccharide derivative **101** in excellent yield, after which hydrolysis of the benzylidene acetal, selective oxidation of the primary alcohol, benzyl ester formation and acetylation delivered disaccharide **104** in 60% yield overall. Finally, the selective removal of the levulinoyl group with hydrazine acetate and hydrogenolysis afforded target disaccharide **105** in 93% over the two steps.



***Scheme 4**: Synthesis of interconnection disaccharides of S. aureus CPS type 5 and type 8. A) a) TMSOTf, 4Å MS, CH_2Cl_2, α/β = 1/5. b) (i) $NH_2NH_2•H_2O$, CH_3OH, 65 °C; (ii) Ac_2O, Py, 81%. c) (i) H_2, Pd/C, EtOH, H_2O, 75%; (ii) TEMPO, BAIB, CH_2Cl_2, H_2O, 70%; (iii) aq. NaOH, CH_3OH, 84%. B) a) TfOH, 4Å MS, CH_2Cl_2, 65%. b) (i) NaOMe, CH_3OH; (ii) LevOH, DIC, DMAP, CH_2Cl_2, 89%. c) (i) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt; (ii) Ac_2O, CH_3OH, rt, 96% over 2 steps. d) TFA, wet CH_2Cl_2, 81%. e) (i) TEMPO, BAIB, CH_2Cl_2, H_2O; (ii) BnBr, $NaHCO_3$, DMF, 76%. f) Ac_2O, Py, 99%. g) (i) $NH_2NH_2•AcOH$, CH_3OH, CH_2Cl_2; (ii) H_2, Pd/C, EtOH, H_2O, 93%.*

The first synthesis of a fully protective CP8 hexamer has been achieved recently by Demchenko's group (Scheme 5).[28] In line with their previous studies, they capped the propagation sites with methyl groups. At first a [3+3] strategy was explored and to this end trisaccharides **108** and **109** were generated via a synthetic route similar to the one described above. Unfortunately, the TMSOTf-mediated glycosylation didn't deliver any desired product and acceptor **109** was fully recovered. The authors attributed this failure to steric hindrance from both the benzylidene group of **109** and the bulkiness of donor **108**. Therefore, a different synthetic approach was adopted based on a [2+1+3] strategy. To this end, donor **106** was synthesized and coupled to trisaccharide acceptor **109** using NIS and TfOH as promoter. Tetrasaccharide **110** was isolated in 81% yield as a single anomer. After removal of the benzoyl protecting group using Zémplen conditions, tetrasaccharide acceptor **111** was coupled with disaccharide thioethyl donor **113**,

activated by the NIS/TfOH couple, affording completely protected hexamer **114** in 85%. Subsequently, this hexasaccharide was converted to the acetamide derivative using an unusual double reduction/acetylation protocol. First, 1,3-propandithiol and TEA were used in wet pyridine at 70°C, which was followed by acetylation, after which the product was treated with zinc dust in acetic acid and acetic anhydride. After deprotection of the benzylidene acetals, the primary alcohols were oxidized using Huang's one-pot TEMPO/NaOCl–$NaClO_2$ procedure. After protection of the carboxylic acids with benzyl bromide in the presence of NaH and DMF, the C-4-OH of the mannuronate sugars were acetylated. By NMR and mass spectroscopy, it was found, however, that compound **115** was formed due to probably a transesterification while quenching the acetylation with methanol. No further deprotection steps were reported.



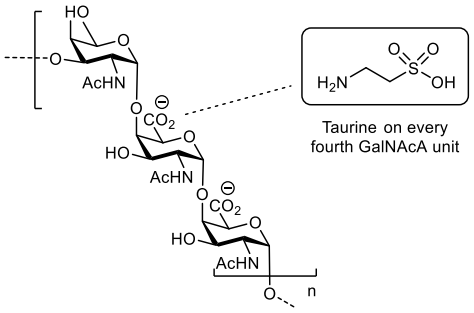
Scheme 5: *Synthesis of hexasaccharides of S. aureus CPS type 8. For synthesis of tetrasaccharide* ***110****: donor* ***106****, acceptor* ***109****, NIS, TfOH, CH_2Cl_2, 0 °C, 81%; For synthesis of disaccharide* ***112****: donor* ***75****, acceptor* ***107****, AgOTf, CH_2Cl_2, 0 °C, 85%. a) CH_3ONa, CH_3OH, 75%. b) (i) NH_2NH_2•AcOH, (ii) 85%; Tf_2O, Py 0 °C; (iii) NaN_3, DMF, 70 °C, 59%. c) NIS, TfOH, 1,2-dichloroethane, 0 °C, 4 h, 85%.*

Up to now, no extensive biological studies have been reported using the synthetic CP5 or CP8 trisaccharides. However, as described above, it can be concluded from the competitive ELISA assay and immunodot blot analysis performed by Adamo, that longer fragments might be necessary to understand the key structural immunogenic elements of these saccharides.

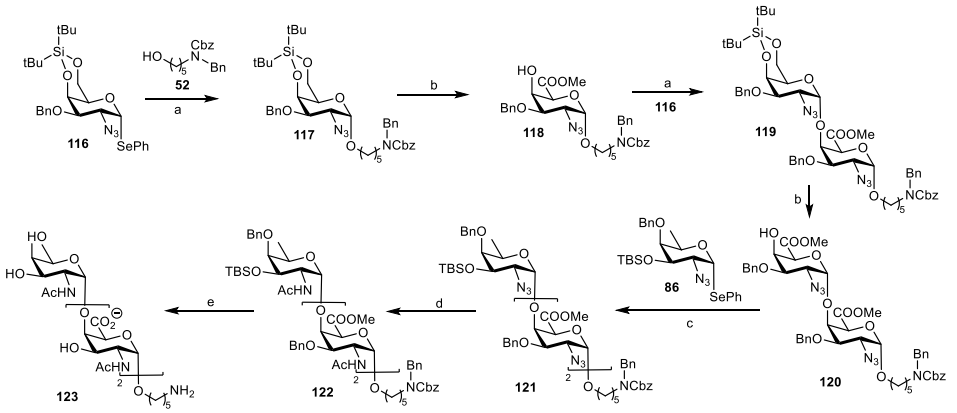
STRAIN M (CP1)

The isolation of the capsular polysaccharide from S. *aureus* stain M was reported in 1962 by Smith,[29] and the complete molecular structure was later elucidated by Murthy et al. in 1983 (Figure 4).[30] The repeating unit consists of an *N*-acetyl-D-fucose α(1,4)-linked to an *N*-acetyl-D-galacturonic acid, which is α(1,4) linked to a second *N*-acetyl-D-galacturonic acid, connecting to the next fucose moiety of the next trisaccharide repeating unit through an α-(1,3)-linkage. It has been described that a taurine unit is incorporated in one out of four GalNAcA sugars through an amide bond.

Figure 4: *Structure of the CPS from S. aureus strain M*



In 2017 Codée and co-workers reported the synthesis of the trisaccharide unit featuring an aminopentanyl linker for ligation purposes (Scheme 6).[31] The synthetic strategy relied on a [1+2] coupling strategy and a post-glycosylation oxidation procedure to introduce the galacturonic acid moieties Using galactosamine donors, bearing a di-*tert*-butylsilylidene ketal ensured the formation of the desired α-linkages, as shown originally by Kiso and co-workers.[32] Thus, donor **116** was first coupled to amino-protected pentanol spacer **52** affording product **117** in 82% yield. Subsequently, the silylidene protecting group was removed using HF*pyridine, after which the primary alcohol was selectively oxidized and methylated, delivering compound **118** in 85% yield. Acceptor **118** was coupled with donor **116** using NIS and catalytic amount of TMSOTf and disaccharide **119** was isolated in 88% as a single anomer. The same sequential steps of silyl deprotection, oxidation and methylation of the newly carboxylic acid afforded disaccharide acceptor **120** in 84% yield. A modification of the oxidation protocol was used since cleavage of glycosidic bond was observed when using the 'normal' TEMPO/BAIB conditions. In this new oxidation protocol, the aldehyde was first formed using TEMPO and BAIB under anhydrous conditions, after which a Pinnick oxidation was employed to provide the desired carboxylic acid. Final glycosylation between 'armed' D-fucose donor **86** and acceptor **120** was performed using Ph_2SO/Tf_2O activation system stereoselectively delivering fully protected trisaccharide **121** in 79% yield. Conversion of the azides into acetamides using AcSH in pyridine afforded intermediate **122** after which desilylation, saponification and hydrogenolysis delivered the target trisaccharide **123**.

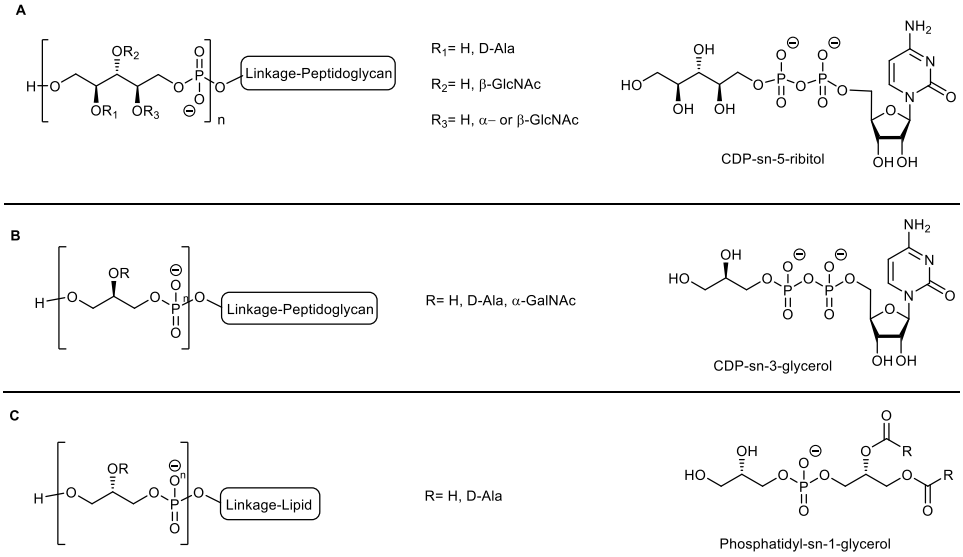


Scheme 6: *Synthesis of trisaccharides repeating unit of S. aureus CPS strain M. a) NIS, TMSOTf, CH_2Cl_2, 0 °C, 2 h, for* ***117****, 82%; for* ***119****, 88%. b) i) HF•Py, THF, rt, 2 h, quant; ii) then TEMPO, BAIB, AcOH, CH_2Cl_2, H_2O, 4 °C, 16 h; iii) MeI, K_2CO_3, DMF, rt, 16 h, for* ***118****, 85%; for* ***120****, 84%. c) i) Ph_2SO, TTBP, CH_2Cl_2, Tf_2O, -80 °C to -70 °C, ii)* ***86****, -80 °C to -40 °C, 6 h, 79%. d) AcSH, Py, 9 d, rt, 47%. e) HF•Py, THF, rt, 2 d; 30% aq. H_2O_2, KOH, THF, t-BuOH, H_2O, rt, 2 d; then H_2, $Pd(OH)_2$/C, AcOH, THF, t-BuOH, H_2O, 3 d, 34% over 3 steps.*

TEICHOIC ACIDS (TAs)

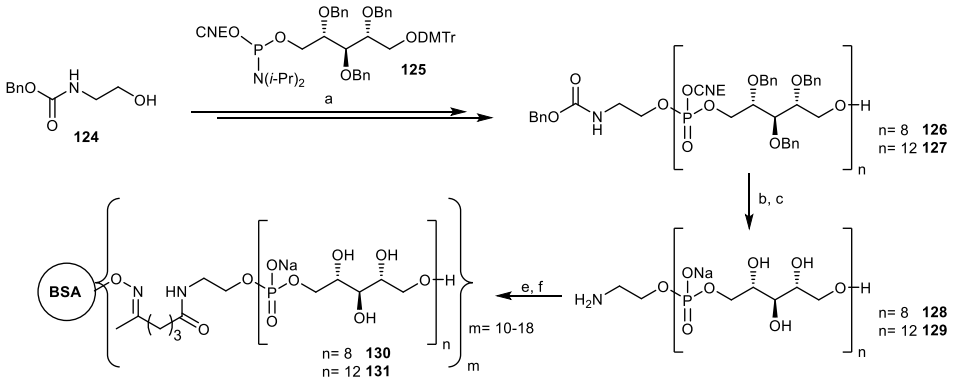
Teichoic acids (from the Greek word τείχος, fortified wall) are structurally diverse anionic carbohydrate-based polymers that can be found in the cell wall of the majority of Gram-positive bacteria. They are divided in two main classes: wall teichoic acids (WTAs), which are covalently attached to the peptidoglycan matrix, while lipoteichoic acids (LTAs) are inserted in the lipid bilayer through a diacyl glycerol lipid anchor. They are involved in several important biological processes contributing to bacterial fitness and virulence.[33] Since TAs are exposed to the extracellular milieu, they constitute possible recognition sites for host cell surface lectins, antibodies of the host immune system and phage binding.[34] TAs have been considered to be suitable antigen candidates for vaccine development.[35] The WTA from S. *aureus* is generally constituted by 1→5-linked ribitol phosphate (RboP) units, which can be modified with D-alanine substituents at the C-2 and α- or β-*N*-acetylglucosamine appendages at C3 or C4 (Figure 5A).[31] However, some strains carry structurally different WTAs. For example, the WTA from S. *aureus* ST395 is composed of 3→1 linked glycerol phosphate (GroP) chains, where the C-2 position can be decorated with either D-Ala or α-GalNAc substituents (Figure 5B).[36] In contrast, S. *aureus* LTAs feature 1→3 linked GroP-oligomers having D-Ala or α-GlcNAc substituents.[37] Notably, the GroP-backbone of WTA and LTA are enantiomeric structures, whose stereochemistry has been assigned based on their biosynthetic pathways. While GroP-WTA is built by oligomerization of CDP-*sn*-3-glycerol, LTAs are constructed using phosphatidyl-*sn*-1-glycerol (Figure 5C).[38]

Figure 5: *General structures and biosynthetic precursors A) RboP-WTA from S. aureus and CDP-sn-5-ribitol. B) GroP-WTA from S. aureus ST395 and CDP-sn-3-glycerol. C) GroP-LTA from S. aureus and phosphatidyl-sn-1-glycerol.*



WALL TEICHOIC ACIDS

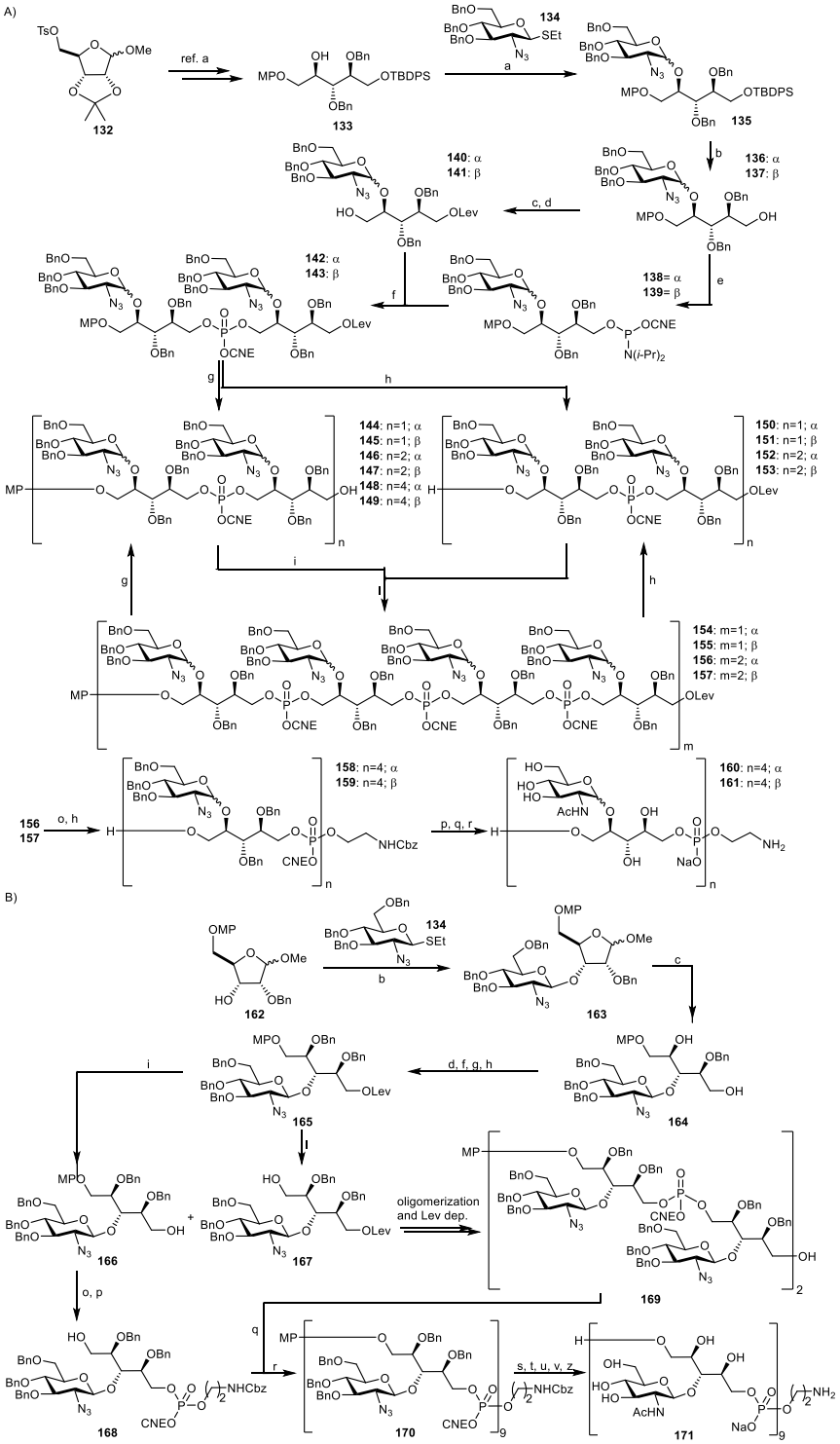
In 2006 Pozsgay and co-workers reported the synthesis of an RboP-octamer and dodecamer, equipped with an amino spacer for further conjugation to BSA as carrier protein.[39] Their synthetic strategy relied on the introduction of the phosphodiester linkage using the phosphoramidite approach, developed for solid-phase nucleotide assembly. In scheme 7 the synthetic route is shown, where phosphoramidite derivative **125** was used as key building block, bearing a dimethoxytrityl (DMTr) group at the hydroxyls to be elongated. Phosphoramidite **125** was coupled with amino ethanol derivative **124** using tetrazole in ACN, followed by oxidation using I_2 in a mixture of THF and water. The DMTr group was removed using AcOH in DCM and water. After 8 and 12 cycles of coupling/oxidation/deprotection, fully protected octamer **126** and dodecamer **127** were obtained, respectively. Final compounds **128** and **129** were obtained after treatment of **126** and **127** with ammonium hydroxide in MeOH to remove the cyanoethyl protecting group, followed by hydrogenolysis using Pd/C. Conjugation to BSA of compound **128** and **129** was performed using a procedure developed by Kubler-Kielb and Pozsgay, involving the functionalization of the oligomers with a levulinoyl group and connection to the carrier protein through the formation of an oxime linkage. No evaluation of the WTA-BSA conjugates **130** and **131** has been reported to date.



Scheme 7: *Pozsgay's synthesis of a ribitol 12-mer-BSA conjugate. a) (i) 10 eq. 0.45 M tetrazole in CH_3CN, CH_3CN, r.t., 1 h; (ii) 0.5 M I_2 in 2:1 THF/H_2O; (iii) 85:10:5 AcOH/CH_2Cl_2/H_2O n=1, 88%; b) (i) 10eq. 0.45 M tetrazole in CH_3CN, CH_3CN, r.t., 1 h; (ii) 0.5 M I_2 in 2:1 THF/H_2O, n=8; 9.9%, n=12; 2.4%; c) (i) CH_3OH, conc. NH_4OH, 50 °C, 8 h; (ii) H_2, 10% Pd/C, 2:1 tBuOOH/H_2O, n=8; 66%, n=12; 80%; e) 5-ketohexanoic anhydride, Et_3N, CH_3OH, H_2O; f) aminooxy-BSA, PBS (pH=7.4), EDTA, glycerol.*

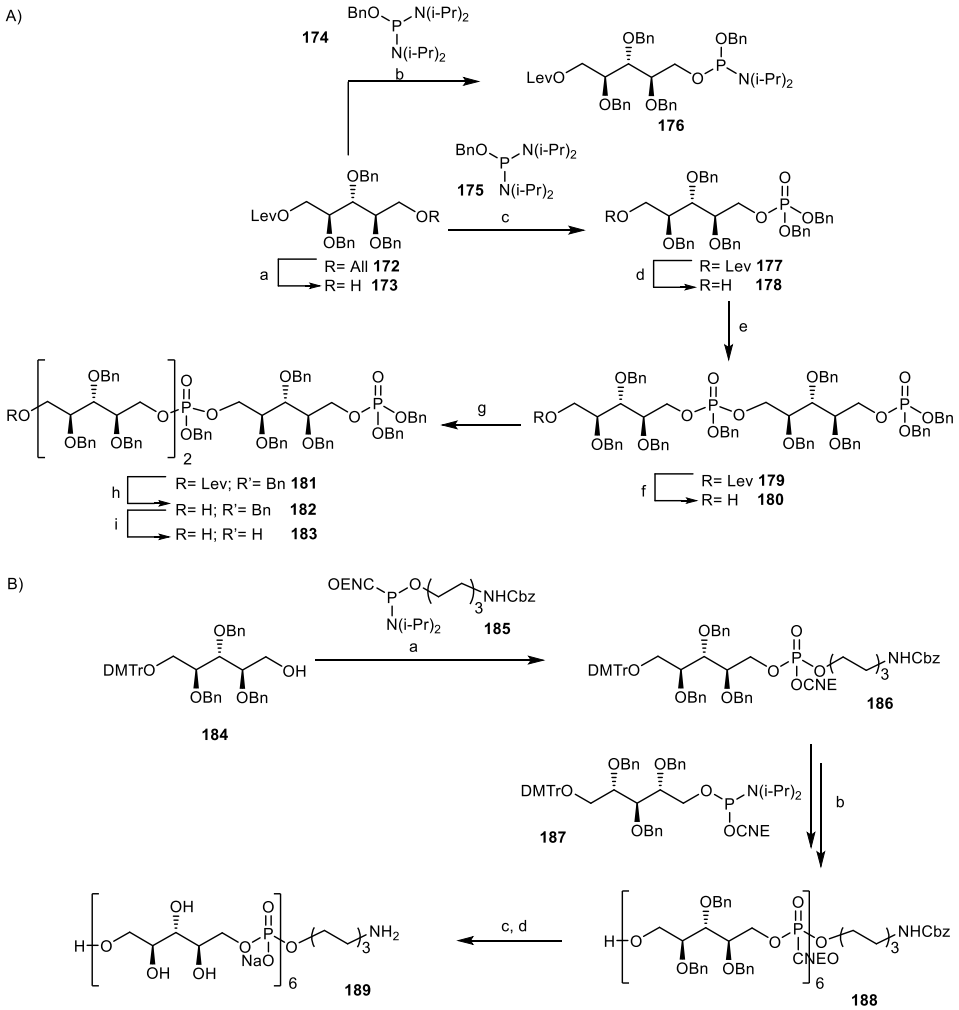
Three RboP oligomers, bearing different GlcNAc appendages, were recently synthesized by a team at Sanofi Pasteur, featuring either α-D-GlcNAc or β-D-GlcNAc at all C-4 hydroxyls of a RboP octamer (**160** and **161**) or a β-D-GlcNAc at all C-3 hydroxyls of a RboP nonamer (**171**).[40] In order to introduce a glucosyl moiety at the C4 of a ribitol synthon (Scheme 8A), acceptor **133** was synthesized in 6 steps from compound **132**. Glucosyl thiodonor **134** was then coupled to acceptor **133** under activation of NIS/TfOH yielding **135** in 94% as a 52/48 of α/β-mixture. The two anomers were separated after TBDPS removal. In order to access RboP dimers **142/143**, a phosphoramidite group was introduced and *in situ* coupled with compound **140/141**, obtained from **136**/**137** by protection of the primary alcohol with a levulinoyl group and subsequently removal of the methoxyphenyl (MP) group. The coupling step was performed using 5-ethiolthio-1H-tetrazole, while the oxidation was accomplished using I_2/pyridine/H_2O. Further elongation was accomplished by either removal of the Lev or MP group, delivering two tetramers **154** and **155** that were coupled using the same *in situ* condensation approach as described. The same strategy was then used to obtain octamers **156** and **157**. Next these octamers were functionalized with an alcohol spacer. Of note, the spacer was attached to these WTA oligomers at the opposite side of the peptidoglycan attachment. The final deprotection was accomplished by removal of the cyanoethyl groups, transformation of the azides into the corresponding acetamides and Birch-type reduction of all benzyl groups. In the case of 1,3-β-D-GlcNAc nonamer **171**, the glycosyl substituent was introduced by glycosylation of acceptor **162** and donor **134** using NIS/TfOH as activating system at low temperature (-70°C), affording disaccharide **163** in 84% yield (Scheme 8B). Subsequent hydrolysis and reduction delivered ribitol derivative **164** in 73% yield and after three protecting groups manipulation steps, compound **165** was obtained in excellent yield. Similar to the strategy described before, compounds **166** and **167** were used to build up a fully protected nonamer **170**. Deprotection of **170** using the same conditions as described above afforded target **171**.

In order to access the immunological properties of the different glycosylated RboP oligomers, synthetic fragments **160**, **161** and **171**, together with native RboP isolated from different strains, were conjugated to rEPA or S. *aureus* alpha toxin (Hla). The conjugation was performed via carbodiimide condensation reaction using hydrazide linker derivatives. Immunization in mice was performed using conjugated and as well nonconjugated fragments, with or without adjuvant. Subsequently IgG1 and IgG2 titers were determined after 0, 21, 35 and 42 days. A strong and robust immune response was elicited when the conjugates were used in combination with adjuvant. No differences were detected between synthetic and native TAs, but it was observed that C-4-β-GlcNAc RboP was able to induce antibodies that were cross-reactive towards different S. *aureus* strains, carrying either C-4-β-GlcNAc or C-3-β-GlcNAc substituents, suggesting that this antigen can be used for the development of a broad-spectrum S. *aureus* vaccine.



Scheme 8: *Synthesis of glycosylated RboP fragments by Sanofi Pasteur. A) a) CH_2Cl_2/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, CH_2Cl_2. d) CAN, CH_3CN/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-diisopropylamidite added at 0°C for 0.5 h, (ii) cool to -10 °C, add alcohol then 5-ethiolthio-1H-tetrazole 1h at 0°C, m=1, β, 80%, m=2, β, 72%. f) step e, 69% β. g) $NH_2NH_2•H_2O$, pyridine/AcOH, 83% β. h) step d, 79% β. i) step e,* ***150*** *(80%);* ***151*** *(72%). j) $NH_2NH_2•H_2O$, pyridine/AcOH, 80%β. k) step e, benzyl N-(2-hydroxyethyl) carbamate, 67%. l) CAN, CH_3CN/H_2O, 0°C to rt 2h,* ***159*** *(76%). m) thioacetic 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,* ***161*** *(96%). B) a) TBAB, BnBr, 10% aq. NaOH, CH_2Cl_2, 34%. b) CH_3CN/propionitrile/CH_2Cl_2 (2:1:1), NIS and TfOH, at -70°C 10 min, 84%. c) (i) 3M HCl/dioxane, reflux 7h, 73%; (ii) $NaBH_4$, CH_3OH, 0°C. d) TBDMSCl, DMAP, TEA, CH_2Cl_2. e) BnBr, DMF, NaH. f) THF, AcOH, TBAF, 0°C to rt, 94%. g) Levulinic acid, DMAP, EDCI, CH_2Cl_2/dioxane 1:10 (0.08M). h) CAN, CH_3CN/H_2O, 0°C to rt 2h, 71%. i) (i) Alcohol in CH_3CN, 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) $NH_2NH_2•H_2O$, pyridine/AcOH, n=1 (86%), n=2 (82%). k) step i, benzyl N-(2-hydroxyethyl) carbamate, 65%. l) CAN, CH_3CN/H_2O, 0°C to rt 2h, 85%. m) step I, n=5, 77%. n) CAN, CH_3CN/H_2O, 0°C to rt, 2h, n=5, (74%). o) step i, n=9, 27%; p) CAN, CH_3CN/H_2O, 0°C to rt 2h, n=9, (80%). q) thioacetic acid, pyridine, 3.5 days, 90%. r) NH_4OH, CH_3OH, reflux 5h, 97%. s) (i) Na, THF, NH_3, -78°C, 30 min; (ii) sat. aq. $NHCl_4$, -78°C, 1h, 50%.*

The role of the different glycosylation patterns on *S. aureus* WTA is still under investigation. Recently it has been suggested that changing Rbo-GlcNAc glycosylation might contribute to *S. aureus* escape from host immune surveillance.[41] In a study by Peschel and coworkers, two synthetic RboP fragments were used to unravel the activity of a glycosyl transferases that had unknown activity. For this study, a trimer and a hexamer were synthesized by Seeberger's and Codée's groups respectively. Trimer **181** was synthesized starting from key building block **173** (Scheme 9A). Compound **173** was coupled with either reagent **174**, using diisopropylammonium tetrazolide, or **175**, using 1H-tetrazole, delivering, after oxidation with *t*BuOOH, compound **176** and compound **177**. After removal of the Lev group from compound **177**, the coupling between **177** and **176** was performed using the same conditions as for the synthesis of **177**. After another cycle of Lev removal, coupling and oxidation, the fully protected trimer was generated. Final deprotection was performed by treating compound **181** with hydrazine acetate, followed by hydrogenolysis. For the synthesis of the hexamer **189** the strategy reported by Pozsgay was applied.

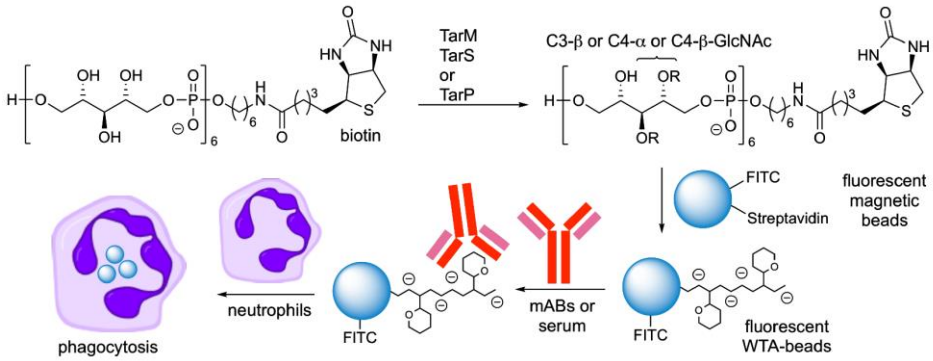


Scheme 9*: Assembly of a S. aureus WTA RboP trimer. a) LevOH, DMAP, DCC, CH_2Cl_2, 3 h, 98%. b) $Pd(PPh_3)4$, 1,3-dimethylbarbituric acid, CH_3OH, 40°C, 24 h, 77%. c) diisopropylammonium tetrazolide, CH_2Cl_2, 2 h, 98%. d) (i) 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 85%. e) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 93%. f) (i) 29, 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 86%. g) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 94%. h) (i) 29, 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 94%. i) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 98%. j) Pd-C, H_2, EtOAc/ CH_3OH/H_2O, 24 h, quant.*

In the study by Peschel's group,[38] a screen on S. *aureus* genomes was performed with the intend of identifying paralogues of WTA biosynthesis genes. It was found that three S. *aureus* prophages were able to encode a glycosyl transferase, TarP, which was shown to be 27% identical to the well-known TarS enzyme, that transfers the β-GlcNAc to the RboP units. While TarS places the carbohydrate on the C4 of the ribitol backbone, TarP was shown to place the β-GlcNAc on the C3 position, as deduced from NMR studies and crystal structures using synthetic trimer **183**. In order to probe the immunogenicity of these subtle differences in glycosylation activity, sera from healthy human donors were

evaluated for the binding towards the different β-GlcNAc-WTA types. The relatively low IgG titer towards TarP glycosylated Rbo-WTA as compared to the TarS homologue led to the hypothesis that the former enzyme can be used by *S. aureus* for immune evasion. Different results were obtained by van Sorge's group, where the synthetic hexamer **189** was used as precursor for the generation of semi-synthetic Rbo-WTA structures.[42] In this study compound **189** was first biotinylated and subsequently enzymatically glycosylated either with TarS (delivering C4-βGlcNAc), TarM (leading to C4-αGlcNAc) or TarP (yielding C3-GlcNAc). The semisynthetic fragments were immobilized on streptavidin coated beads and used to probe binding of IgG antibodies present in pooled sera of healthy human donors. It was observed that the titer of IgGs against the TarS glycosylated WTA was higher than the titer against the TarP glycosylated WTA, but the level of antibodies against the latter type were still significant. The designed protocol (Figure 6) proved to be much more sensitive than the assay used by Gerlach *et al.* The WTA-beads have also been used to probe antibody mediated phagocytosis, showing effective uptake of the TarS and TarP modified WTA-beads.

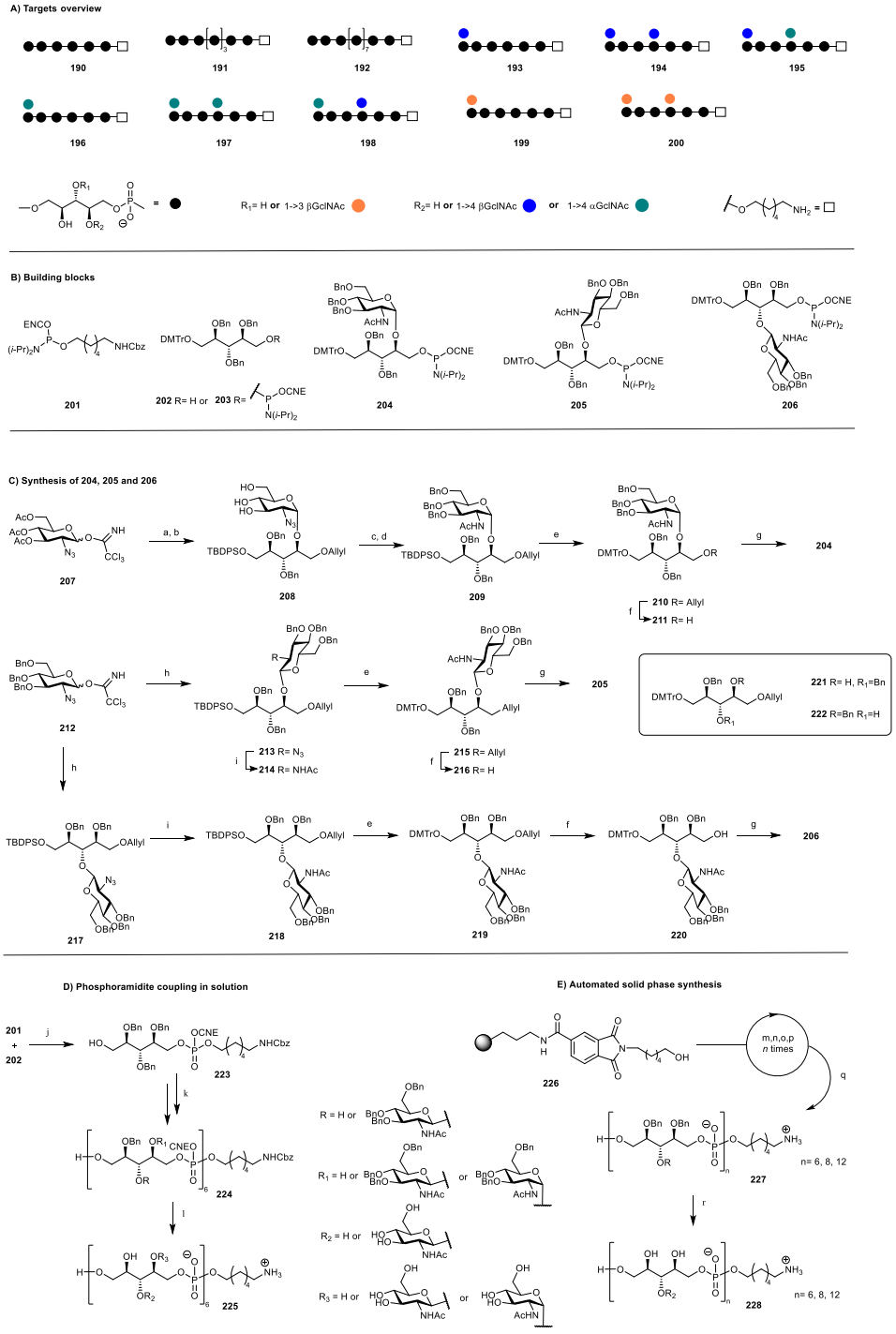
Figure 6: *Schematic overview of antibody mediated phagocytosis using enzymatically modified synthetic biotinylated (RboP) hexamer immobilized on streptavidin coated beads.*



More recently, both automated and in solid phase strategies were developed to deliver different unsubstituted or site-specific mono- or di- glycosylated RboP fragments (**190**-**200**, Scheme 10A).[43] Compare to the synthetic route undertaken by the team at Sanofi Pasteur, a stereoselective glycosylation methodology was employed by Codeé's group to deliver C-4-αGlcNAc RboP (**204**), C-4-βGlcNAc RboP (**205**) and C-3-βGlcNAc RboP (**206**) building blocks in a highly efficient manner (Scheme 10B). In order to introduce the α glycosylic bond (Scheme 10C), glucosazide donor **207** was coupled with acceptor **221** using TMSOTf as promoting agent, delivering glycosylated product in 92% as 7:1 α/β ratio. After deacetylation, the α isomer **208** was isolated in 70% yield and all free hydroxyl were protected with benzyl groups. Subsequently the azido moiety was reduced by Staudinger conditions and acetylated affording **209** in excellent yield. Final protecting groups manipulation delivered key phosphoramidite derivative **204**. For the assembly of building blocks **205** and **206**, glycosylation reaction between donor **212** and acceptors

221 or **222** respectively was performed using acetonitrile as β-directing solvent at low temperature to deliver compounds **213** and **217** in high yields. Similar protecting groups manipulation, as previously, was then followed to obtain phosphoramidite derivatives **205** and **206**. With all building blocks in hand (Scheme 10B), oligomerization was investigated via phosphoramidite approach at first in solution (Scheme 10D). Differently than Pozsgay's RboP synthesis, 4,5-dicyanoimidazole (DCI) was used as activating agent for the generation of a phosphite intermediate upon attack by primary alcohol and subsequently oxidized using (10-camphorsulfonyl)oxaziridine (CSO). The temporary DMTr group placed at the site of propagation was removed using dichloroacetic acid (DCA, 3% in DCM) and the elongated intermediate purified. All couplings proceeded with high efficiency and final targets **190-200** obtained after deprotection of the cyanoethyl protecting groups in basic conditions and hydrogenolysis to remove benzyl groups. Contrarily than Sanofi Pasteur RboP library, the aminohexanol linker was placed at the site where naturally occurring peptidoglycan is attached to the RboP-WTA. In order to streamline the oligomerization of RboP based fragments, automated solid phase synthesis was investigated (Scheme 10E). Previously, Pozsgay and co-workers attempted this approach for the synthesis of compounds **128** and **129** (Scheme 7). Unfortunately, after cleavage from the solid support, a complex mixture was obtained and the failure was attributed to the use of trichloroacetic acid for the DMTr removal. Using the same conditions as reported by Pozsgay but replacing TCA with the milder acid DCA, as proved in solution, Codeé and co-workers obtained semi-protected octamer and dodecamer in 15% and 11% yield. The same conditions were then applied to obtained final targets **199** and **200**.

The synthesized compounds were biotinylated in order to be evaluated for antibody binding using the aforementioned magnetic beads assay. Monoclonal antibodies directed towards α-1,4-GlcNAc WTA were able to recognized also monosubstituted fragments. In the case of the β isomers, two monoclonals were tested. Interestingly one of the antibodies could recognize both regioisomer 1,4 and 1,3 GlcNAc RboP hexamers with a preference for the former and as previously observed even one substituent was sufficient for antibody deposition. In the other case, the monoclonal was able to recognized only the fragment enzymatically glycosylated due to a higher degree of substitution or multiple displacement of the epitope recognized.



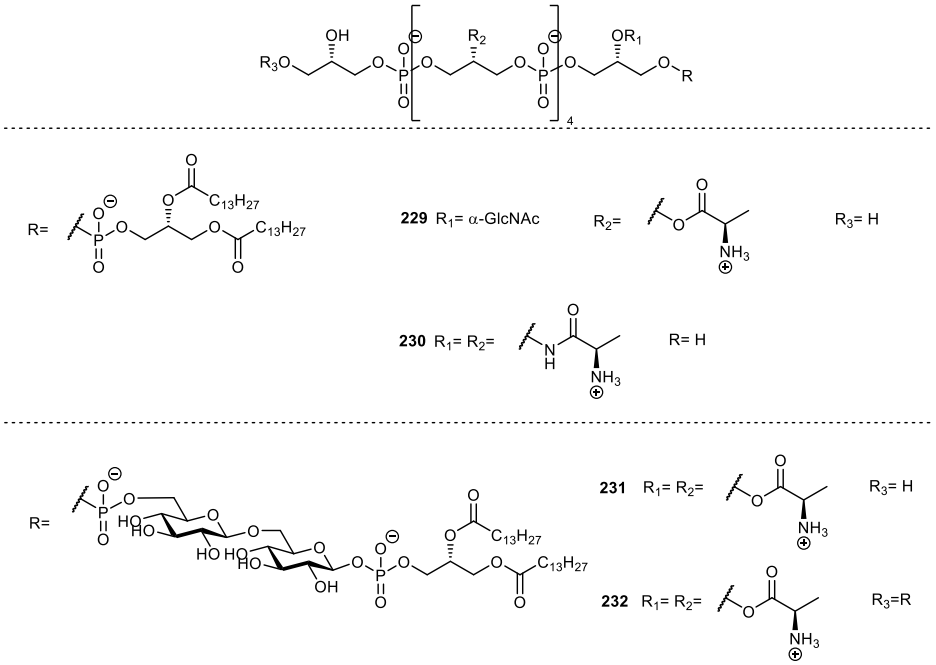
***Scheme 10**: (Automated) synthesis of unsubstituted and glycosylated RboP well-defined fragments. A) Schematic overview of the target compounds **190-200**; ribitol phosphate repeating unit = black*

circle, β-1,3-GlcNAc = orange circle, β-1,4-GlcNAc = blue circle, α-1,4-GlcNAc = green circle. B) Building blocks ***201-206****. C) Synthesis of building blocks* ***204, 205*** *and* ***206;*** *a)* ***221****, TMSOTf, CH_2Cl_2, rt, 92 %, 7:1 α/β; b) NaOMe, CH_3OH, rt, α-anomer* ***208*** *70%; c) BnBr, NaH, THF/DMF (7:1), 0°C to rt, 73 %; d) (i) PMe_3, KOH, THF; (ii) Ac_2O, pyridine,* ***209*** *89 % over 2 steps; e) (i). TBAF, THF, rt; (ii) DMTrCl, TEA, CH_2Cl_2,* ***210****: 67 %;* ***215****: 36 %;* ***219****: 60%; k) (i). Ir(COD)$(Ph_2MeP)_2PF_6$, H_2, THF, (ii). I_2, sat. aq. $NaHCO_3$, THF,* ***211****: 88 %;* ***216****: 79%;* ***220****: 94 %; g) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2,* ***204****: 81 %;* ***205****: 78%;* ***206****: 85 %; h)* ***222****, TMSOTf, CH_3CN, - 40°C to 0°C,* ***213****: 85 %;* ***217****: 80 %; i) (i) propanedithiol, pyridine, H_2O, TEA, rt; (ii). Ac_2O, pyridine,* ***214****: 59 %;* ***218****: 86 % 2 steps. D) Oligomerization in solution; j) (i) DCI, CH_3CN, phosphoramidite; (ii) CSO; (iii) 3 % DCA in DCM. 85%. k) (i) DCI, CH_3CN, phosphoramidite* ***203****,* ***204****,* ***205*** *or* ***206****; (ii) CSO; (iii) 3 % DCA in CH_2Cl_2. 53%-quant. l) (i) NH_3(30-33% aqueous solution), dioxane; (ii) Pd black, H_2, AcOH, H_2O/dioxane,* ***190****: 87 %;* ***193****: 96 %;* ***194****: 55%;* ***195****: 16 %;* ***196****: 68 %;* ***197****: 78%;* ***198****: 88 %;* ***200****: 70 %. E) Automated solid phase assembly. m) 3% DCA in toluene (3 min); n)* ***203*** *or* ***20,*** *BTT, CH_3CN (5 min); o) I_2, Pyridine/H_2O(1 min); p) Ac_2O, N-methylimidazole, 2,6-lutidine, CH_3CN (0.2 min); q) 25 % NH_3(aq) (60 min); 11%-20%; r) Pd black, H_2, dioxane H_2O,AcOH,* ***191****: 100 %;* ***192****: 100 %;* ***199****: 87%;* ***200****: 100%.*

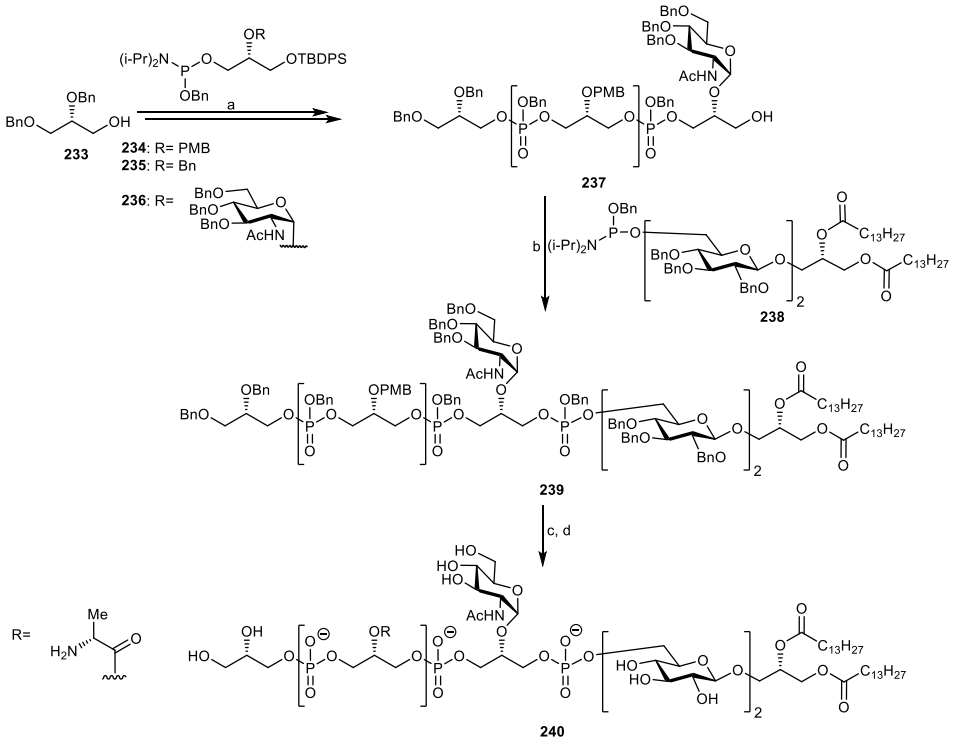
LIPOTEICHOIC ACIDS

A large set of S. *aureus* type LTA-oligomers has been synthesized by Schmidt and co-workers. Figure 7 shows a selection of these fragments, in which a lipid anchor as well as α-GlcNAc and D-Ala substituents were incorporated.

Figure 7: *A selection of GroP fragments from LTA of S. aureus synthesized by Schmidt's group*

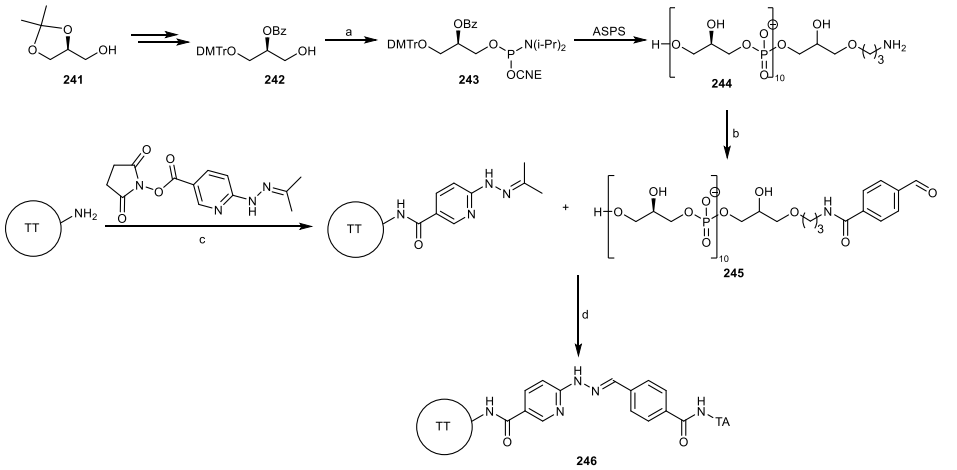


Scheme 10 shows a representative synthetic strategy used to build this kind of structures.[44] The strategy relied on a phosphoramidite approach, where glycerol derivatives **234**, **235** and **236** carried a temporarily TBDPS protecting group at the site of elongation. Notably, due to the lability of D-Ala moieties, glycerol **235** carried a PMB protecting group to allow the introduction of these labile substituents at a later stage. Thus, elongation proceeded using tetrazole as activating agent, *t*BuOOH for oxidation and subsequent TBDPS removal using TBAF. After 5 cycles, compound **237** was coupled to gentibiose diacyl lipid anchor derivative **238**. Before final hydrogenolysis, the PMB groups were removed by oxidative cleavage and D-Ala substituents were introduced. The set of oligomers were used to establish the structural requirements needed to elicit an innate immune response. As assessed by cytokine production in a whole blood assay, it was observed that both lipid anchor and positively charged D-Ala substituents were important structural features for the innate immune stimulating activity.[45]



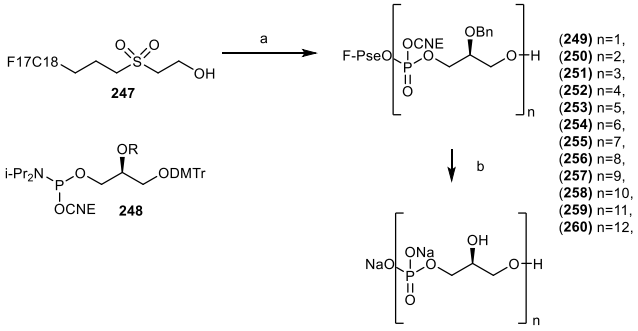
Scheme 11: *Synthesis of S. aureus LTA fragments by Schmidt and co-workers. a) (i)* ***234, 235*** *or* ***236****, tetrazole, CH_2Cl_2, (ii) tBuOOH; (iii) TBAF. b) (i) tetrazole, CH_2Cl_2; (ii) tBuOOH, 75%. c) CAN, CH_3CN/toluene/H_2O, 67%. d) PyBOP, N-methylimidazole, Z-protected alanine, CH_2Cl_2. e) $Pd(OH)_2$, H_2, CH_2Cl_2/ CH_3OH/H_2O, 9:1:1, 25% over 2 steps, 10: 33% over 2 steps.*

A solid phase approach for the construction of unsubstituted GroP oligomers was used by Snapper and co-workers (Scheme 11).[46] The key building block **243** was designed based on the contemporary nucleic acid chemistry, having a DMTr as orthogonal protecting group at the site of elongation. Different than other strategies (*vide infra*), the secondary alcohol was protected with a benzoyl group, even though it is known that migration to the primary position readily takes place. For the solid support amino spacer functionalised glycerol-controlled pore glass (CPG)-resin was used. A GroP decamer was synthesized using 30-40 equivalent of phosphoramidite building block **244** per cycle and upon treatment with ammonia, the final compound **245** was released from the resin and simultaneously all protecting groups were removed. After desalting, the crude product (no purification or characterisation of the material has been reported) was conjugated to tetanus toxoid (TT) via the formalbenzoate hydrazinonicotinamide conjugation couple. Mice serum raised against glycoconjugate **246** showed to mediate opsonophagocytic killing of S. *aureus in vitro* and protection in a bacteremia model *in vivo*.



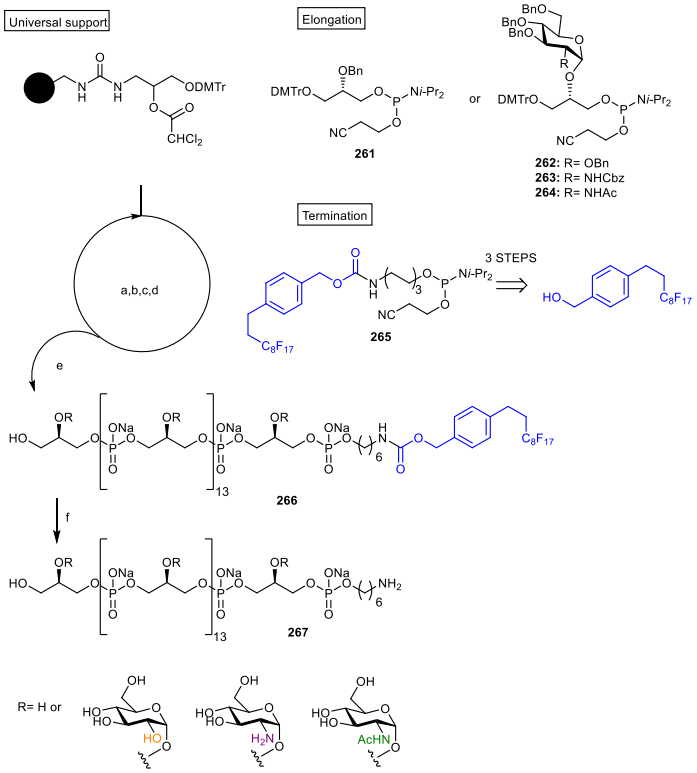
Scheme 12: *Automated solid phase synthesis of a S. aureus LTA GroP oligomer. a) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. b) 4FB-OSu; c) PBS, aniline.*

The automated solid phase approach is an efficient method that can reduce synthesis time and labour, but it also requires large amounts of building blocks per cycle and has scale limitations. As an alternative, Codée and co-workers reported the use of a fluorous soluble support for the construction of unsubstituted GroP oligomers up to the 12-mer (Scheme 12).[47] This methodology allows the rapid and efficient isolation of the synthetic intermediates, with the possibility to scale up and performing the coupling cycles using a relatively small excess of reagent. As shown in Scheme 13, (perfluorooctyl)succinyl spacer **247** was used to deliver TA oligomers featuring a terminal hydroxyl group. Based on the phosphoramidite approach, as previously described, building block **248** was used and it was noticed that a larger amount of phosphoramidite reagent was needed with growing chain length of the oligomers. The usual deprotection steps delivered target fragments **249-260**.



Scheme 13: *Automated solid phase synthesis of GroP based fragments. a) (i)* ***248****, DCI, ACN (ii),* I_2*, pyridine, THF (iii) DCA, TES-H,* CH_2Cl_2*. b) (i)* ***248****, DCI, ACN (ii)* I_2*, pyridine, THF (iii) DCA, TES-H,* CH_2Cl_2*.*

More recently, the same group reported the synthesis of a large set of GroP-based TA fragments using automated solid phase synthesis (Scheme 14).[48] A universal solid support was used in order to avoid the need of linker functionalized building blocks and at the end a fluorous aminospacer phosphoramidite **265** was used to facilitate purification of the long fragments. Notably, several pentadecamers were synthesized having different carbohydrate substituents, including α-GlcNAc from S. *aureus* LTA, in different position of the chain.



Scheme 14: *"Second-generation" automated solid-phase assembly of long GroP-TA fragments. (a) 3% DCA, toluene. (b) 5-BTT, ACN; (c) I_2, pyridine, H_2O/CAN. (d) Ac_2O, N-methylimidazole, 2,6-lutidine, CAN. (e) (i) NH_3, CH_3OH;(ii) NH_4OH, H_2O. (f) H_2, Pd_0, H_2O.*

CONCLUSION

Since the arising of *S. aureus* antibiotic resistant strains, over the last two decades, much more efforts have been focussed on the development of a vaccine against it. Although glyco-based active immunization strategies proved to be promising in preclinical trial settings, understanding on how this pathogen interacts with human immune system has to be still carried out. Therefore, structure activity/immunogenicity

relationship studies can represent a suitable methodology to unravel the basis for developing effective *S. aureus* treatments.
In the last decade, much more efforts have been focused on the achievement of well-defined glyco-based structures. A panoramic overview is here presented, dealings with synthetic strategies for the achievement of fragments, based on surface and capsular polysaccharides, as well different glycosyl substituted teichoic acids oligomers.
The achievement of carefully designed and variegate libraries can be applied to evaluate at molecular level not only the minimal structural elements required for the immunogenic properties, but also to deepen the in and out of their biological role. This review also stresses the need of a close collaboration among different scientific fields, from organic chemistry to immunobiology and medical expertise. By sharing the cutting-edge on different scientific skills, the hard challenge against *S. aureus* might be overcome in the near future.

OUTLINE OF THIS THESIS

The work described in this thesis aimed to understand the interaction between antibodies and glyco-based cell wall components from both staphylococci and enterococci species at molecular level. To this end, well defined fragments were synthesized and therefore new or already established organic chemical methodologies were applied. In **Chapter 2**, the first synthesis of CP5 hexamer is described and in particular a strategy was developed in order to selectively introduced the acetyl substituent on the C3 of the L-Fucosyl moiety. With the described approach both oligomers with and without substituents can be achieved in order to understand the role of this decoration in the future for both antibody binding and virulence.
As it has been described in this introduction, in Jeroen Codeé's lab a large library of GroP and RboP based fragments has been generated. In order to evaluate it in a rapid and efficient way, a TA-microarray tool was developed (**Chapter 3**). The assessment was performed using monoclonal antibodies, the specificity of which was already known. The tool was then applied for antibody profiling using polyclonal sera from immunized rabbits or even healthy human volunteers. All the GroP and RboP compounds differ for the length (number of repeating units), presence and degree of glycosyl substituent, the kind of glycosyl substituent as well the position of it along the chain. One structural feature that has not been evaluated was the stereochemistry of the backbone. In **Chapter 4** the synthesis of sn-1-GroP fragments differing for the position of a glucosyl substituent along the chain is described as diastereomeric counterpart of the already synthesized sn-3-GroP library. During the generation of this fragments, it has been observed that the stereoselectivity of the glycosylation reaction, when PPh_3O (triphenylphosphinoxide) was used as modulating agent in the presence of TMSI (trimethylsilyl iodide), was affected upon the GroP chirality especially if the acceptor was bearing a bulky protecting group. The polyclonal sera from rabbit immunized with either native LTA from *E. faecalis*

or a previously generated fully synthetic TA-conjugate showed binding preference for one or the other stereochemistry.
Based on previous results, where a GroP hexamer bearing a glucosyl substituent at the terminal part of the chain was able to induce protective and opsonic antibodies against enterococci and staphylococci species, the fully synthetic conjugate was used to generate a monoclonal antibody via hybridoma technology. The monoclonal was then employed to characterize the interaction with synthetic TA-fragments using a variety of techniques. In order to understand the structural feature of the epitope recognized, the synthetic fragments were evaluated using TA-microarray, ELISA, SPR and STD-NMR.
A summery of the overall work described in this thesis is provided in **Chapter 6**, addressing possible future prospects. The synthesis of GroP based fragments bearing *N*-acetyl-galactosamine at the C2 is provided as this kind of biomolecules have been found in other *S. aureus* species as well as an extended application of the TA-microarray for the evaluation of glycan binding protein such as DC-SIGN and Langerin.

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