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Synthesis, structure and epitope mapping of well-defined *Staphylococcus aureus* capsular polysaccharides

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Chapter 6

Summary and future prospects

Summary

This Thesis presents the synthesis and evaluation of antibody recognition for various capsular polysaccharide (CP) fragments of *Staphylococcus aureus* (*S. aureus*). Previous glycoconjugate vaccine candidates to combat *S. aureus* infections, that made use of isolated CP5 and CP8, have all failed in late-stage clinical trials,¹⁻³ prompting the focus in this Thesis on well-defined synthetic materials. The synthesized CP fragments that have been studied include type 8, type 5, and type 1 (see Figure 1). To facilitate conjugation, all these saccharides were equipped with an amino-functionalized linker. All these CP-fragments are built up from rare monosaccharides, that were synthesized in effective multi-step routes from commercially available materials. The saccharides feature various functional groups, including carboxylic acids, acetamides, acetyl esters, and taurine amides, and are linked together in diverse configurations, through 1,2-*cis* and 1,2-*trans* linkages. The synthetic pathways were designed to produce these fragments as effective as possible, incorporating as little as possible modifications at the oligosaccharide stage and enabling the introduction of desired functionalities. For the 1,2-*trans* linkages, neighboring group participation was utilized, while for the 1,2-*cis* linkages, different factors such as donor protecting groups, donor/acceptor reactivity matching and solvent were employed to achieve the desired stereoselectivity in the formation of the linkages.

Chapter 1 introduces the capsular polysaccharides of *S. aureus* to provide the context for the research described in this Thesis. The biosynthesis routes of the two most clinically prevalent strains, CP5 and CP8 are presented and the working principles of glycoconjugate vaccines are introduced. Different modes for the generation of glycoconjugate vaccines are presented and synthetic precedents for the assembly of well-defined CP5 and CP8 trisaccharide fragments are described.

CP5 and 8 have previously been used as antigen candidates. Synthetic approaches in the past have so far only delivered the trisaccharide repeating unit⁴⁻⁶ and a protected hexasaccharide,⁷ highlighting the difficulties in the synthesis of these complex glycans. In **Chapter 2** the synthesis of a set of CP8-oligosaccharides, varying in length from a trisaccharide to a dodecasaccharide, is presented (**1-4** in Figure 1A). The oligosaccharides were synthesized in a [3+3n] matter employing a key trisaccharide intermediate, which was transformed into the required acceptor and donor synthons. The synthetic plan relied on introducing the acid functionality and the *O*-acetylation in the ManN₃A building prior to assembly of the oligosaccharides, to minimize the post-glycosylation modification steps, in

contrast to the majority of reported synthetic methods.^{4,5,7} Deprotection could be achieved in only two steps to yield the pure target compounds. The synthetic fragments were conjugated to CRM₁₉₇ and evaluated for their ability to act as an antigen in interaction studies with both monoclonal antibodies (mAb) and polyclonal antibodies (pAb). Western Blot and ELISA experiments showed that the trisaccharide was poorly recognized and did not bind to either the mAb or pAb, while a clear concentration-dependent competition for hexasaccharide **2**, nonasaccharide **3** and dodecasaccharide **4** was detected. Binding to **3** and **4** was comparable and significantly stronger than binding to **2**, indicating that **3** holds the minimal binding epitope for the mAb/pAb-CP8. Structural studies revealed that the CP8 oligosaccharides adopt a linear structure with the acetamides of the repeating units oriented in the same direction with respect to the oligosaccharide backbone and forming extended hydrophobic anchor points for binding. The spatial orientation of the *O*-acetyl and acetamide groups provided an energy barrier for the rotation of the FucNAc-ManNAcA linkage, which locked the saccharide in a linear conformation. STD-NMR revealed the binding epitope to span over two repeating units (RUs), in which the ManNAcA and L-FucNAc residues provided key interactions. Immunization studies confirmed the length-dependent recognition, and the long synthetic oligosaccharides, having a minimum of 3 RUs, mimicked the antigenicity of the natural polysaccharide well.

In **Chapter 3**, a set of CP5-oligosaccharide ranging in length from a trisaccharide to a nonasaccharide is presented (**7-9** in Figure 1B). Also for this CP, previously published synthetic approaches have only delivered a trisaccharide unit,⁸⁻¹² where the work of Adamo and co-workers⁸ highlighted the need for longer saccharides to induce a useful antibody response. The focus in this Chapter therefore relied on producing longer fragments and for this, the same synthetic principals as implemented in Chapter 2 were applied, with the minor difference that now the *O*-acetyl was installed on larger fragments to ensure the selectivity in the glycosylations. Instead, a temporary 2-methylnaphthyl (Nap) ether was installed, leading to the need for two trisaccharide building blocks to keep the orthogonality – one for elongation and one for the terminal end. Deprotection was found to be more difficult than anticipated, as complete reduction of the TCA groups was difficult to obtain. A two-step purification after the reduction using both silica-gel column chromatography and HPLC were utilized to provide pure oligosaccharides, unfortunately leading to loss of material. The synthetic oligosaccharides were conjugated to CRM₁₉₇ and Western Blots and SPR experiments showed trisaccharide **7** to be too short to bind to antibodies, in line with the findings of Adamo and co-workers. The longer hexasaccharide **8** and nonasaccharide **9** showed equal

antibody binding and similar IC₅₀ values. Conformational analysis revealed a linear conformation with the acetyls within a RU pointing in the same direction and the RUs being flipped $\sim 180^\circ$ with respect to the flanking RUs.

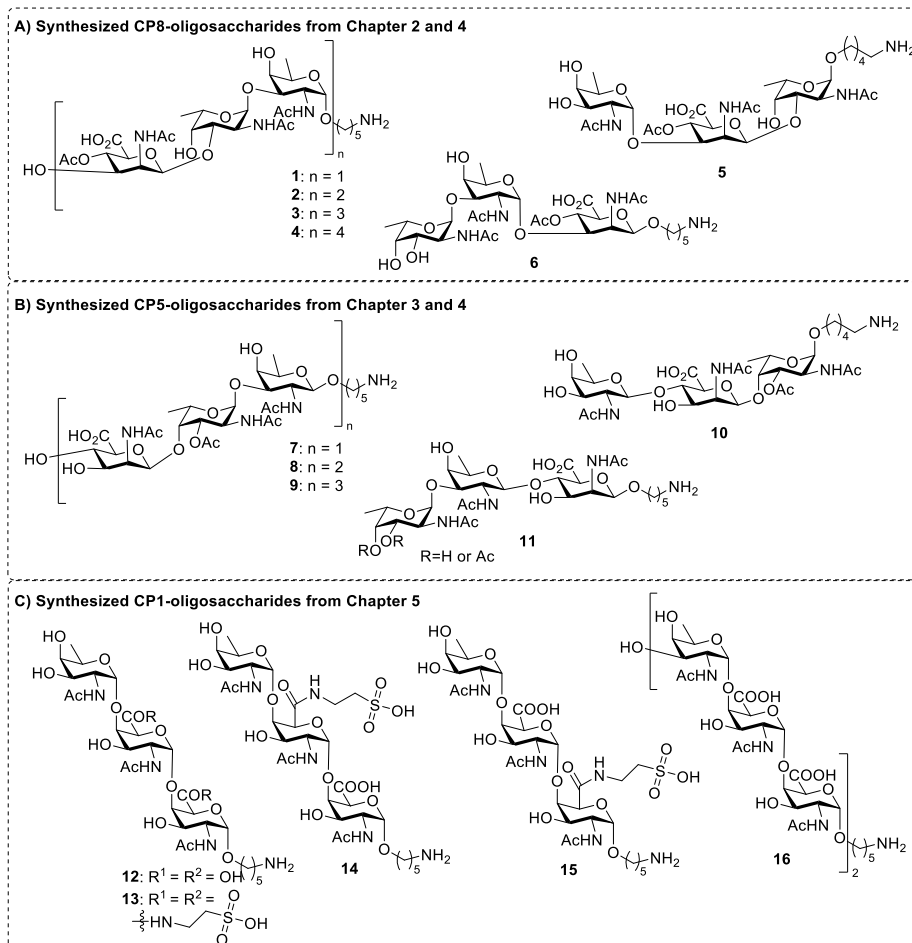


Figure 1: The synthesized oligosaccharides in this Thesis. A) A set of CP8-oligosaccharides ranging from a trisaccharide to a dodecasaccharide and two frameshifted trisaccharides synthesized in Chapter 2 and 4. B) A set of CP5-oligosaccharides ranging from a trisaccharide to a nonasaccharide and two frameshifted trisaccharides synthesized in Chapter 3 and 4. C) A set of CP1-trisaccharides with varying taurine pattern and a hexasaccharide synthesized in Chapter 5.

Chapter 2 and 3 only report on the synthesis and evaluation of a single trisaccharide, where three different frameshift trisaccharides can be defined, and therefore the question remains whether the length of the fragments or the exact frameshift is important for antibody recognition. In **Chapter 4** the other frameshifted CP5 and CP8 trisaccharides were investigated by synthesizing the

remaining frameshifted trisaccharides **5**, **6**, **10** and **11** (Figure 1A and B). The synthetic principals from Chapter 2 and 3 were implemented with the difference that the linker was installed prior to elongation to spare valuable trisaccharide material. In general, the yields were lower and the stereoselectivity in the used glycosylation reactions was worse than those reported in Chapter 2 and 3. Nonetheless, the set of four frameshifted trisaccharides was obtained. For **11**, *O*-acetyl migration from C-3 in the L-FucNAc to the C-4 in the L-FucNAc was observed due to the *cis*-configuration of the hydroxy groups, making this trisaccharide unfit for binding studies. The CP8 frameshift trisaccharides **5** and **6**, were found not to bind to the anti-CP8 antibodies, in either ELISA experiments or STD-NMR. This is in line with the finding in Chapter 2, where the minimal binding epitope was determined to span at least 3 RU. On the other hand, CP5 trisaccharide **10** was found to bind to anti-CP5 antibodies, and binding was comparable to binding of hexasaccharide **8** in the SPR experiments. This indicated that the binding epitope likely consists of the D-FucNAc–D-ManNAcA–L-FucNAc trisaccharide, which is present in both **10** and **8**.

Besides CP5 and CP8, several other *S. aureus* CP types have been found, including CP1. In the past, two synthetic approaches towards a CP1 trisaccharide unit without the characteristic taurine substitution have been published,^{13,14} and **Chapter 5** was therefore focused on the synthesis of the four possible CP1 trisaccharides (*i.e.* none, one or two taurines per repeating unit) with a different taurine substitution pattern and a non-taurinated hexasaccharide (**12–16** in Figure 1C). In contrast to the work in Chapter 2–4, now major modifications were performed on the larger saccharides, because of the following reasons. Firstly, to obtain α -selectivity in the glycosylation reactions, a di-*tert*-butylsilylidene protected galactosyl donor was used because previous work found galacturonic acid donors to give poor selectivity.¹³ Secondly, this allowed to steer the position of the taurine substitution pattern by using orthogonal C-6-OH protecting groups. A four-step modification/deprotection sequence gave the non-taurinated trisaccharide **12** and a five-step sequence gave double-taurinated trisaccharide **13**, while a seven-step modification/deprotection sequence gave taurinated trisaccharide **14** and **15**. For the assembly of hexasaccharide **16** a [3+3] strategy was implemented yielding the hexasaccharide in fine yield. Again, a four-step modification and deprotection sequence gave non-taurinated hexasaccharide **16**.

Potential antibody interaction with the CP5 oligosaccharides

To investigate the interaction between synthetic oligosaccharides and monoclonal antibodies, saturation transfer difference nuclear magnetic resonance (^1H STD-NMR) experiments have been conducted where the STD-NMR results revealed the structural elements that defined the binding epitopes. In Chapter 2, clear binding epitopes were identified for oligosaccharides **2** and **3**, with the epitope for **2** originating from the terminal end and that for **3** from the middle of the fragments. In contrast, no binding was observed for trisaccharides **1**, **5**, and **6**. Initial STD-NMR studies for the CP5 oligosaccharides showed different results as illustrated in Figure 2. For trisaccharide **7**, which was found to give very low binding in the SPR experiment, a binding epitope was found by STD-NMR. The STD signals were found to arise from the central L-FucNAc unit and the terminal D-ManNAcA unit with the strongest STD signals arising from the *O*-acetyl moiety and the *N*-acetyl groups of the L-FucNAc and D-ManNAcA units. The *N*-acetyl of the D-FucNAc unit provided lower STD signals. On the other end, STD-NMR analysis of hexasaccharide **8** and nonasaccharide **9** provided no STD signals. The absence of signals can be caused by either too weak binding or very strong binding. Given the clear STD binding epitope identified for trisaccharide **7** and the strong binding observed for both hexasaccharide **8** and nonasaccharide **9** in the SPR experiments, it is most likely that the lack of signals originates from too strong binding with the antibody. To further investigate whether these results originate from weak or strong binding, competitive STD-NMR experiments can be conducted by including trisaccharide **7**, which shows binding. The appearance of STD NMR signals will indicate weak binding of the longer saccharides, while absence of STD-NMR signals will be an indication for binding of the antibody with the longer fragments and not to the competing trisaccharide.

STD-NMR analysis using trisaccharide **10** also showed no STD, as illustrated in Figure 2B, consistent with the findings for hexasaccharides **8** and nonasaccharide **9**. Again, the absence of signals may indicate very weak or very strong binding. Considering the similar IC_{50} values for **10** and **8** and **9**, along with the binding epitope identified for trisaccharide **7**, it is likely that the lack of STD signals reflects strong binding. Again, conducting competition NMR experiments with trisaccharide **7** could further validate these results.

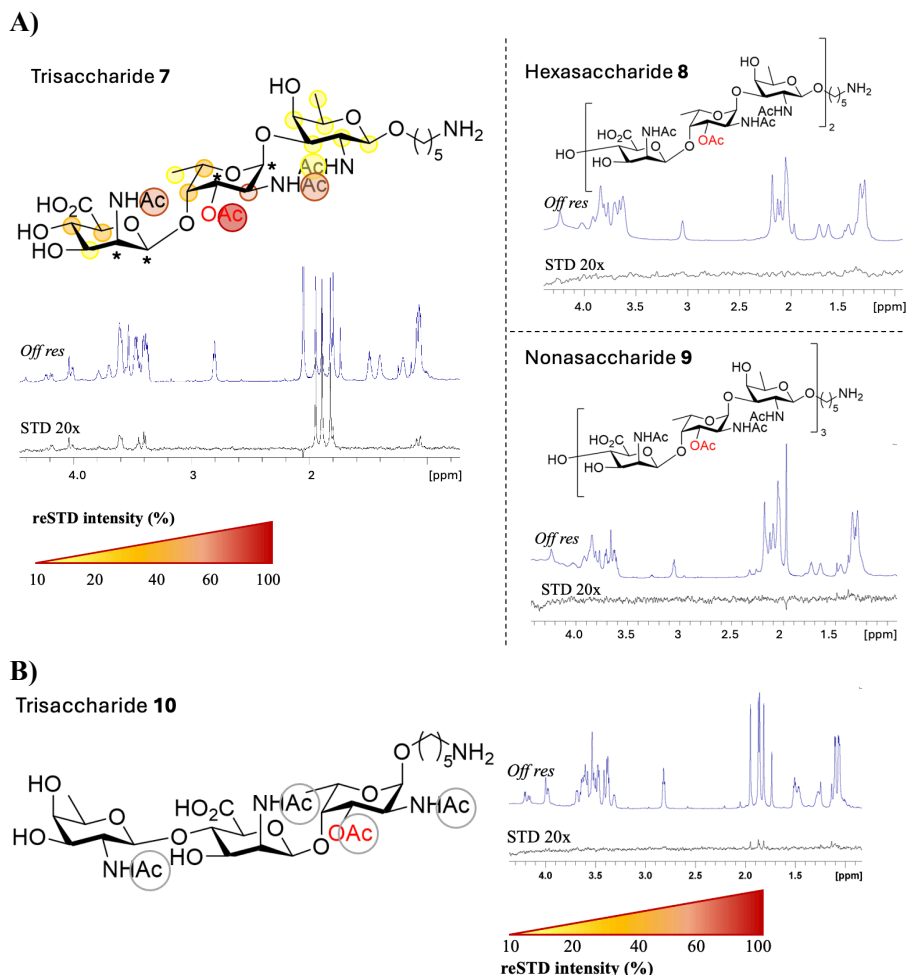


Figure 2: A) ^1H STD-NMR spectra performed for the complexes of mAb-CP5 and the trisaccharide **7**, the hexasaccharide **8**, and the nonasaccharide **9**. B) ^1H STD-NMR spectra performed for the complexes of mAb-CP5 and **10**. Off-resonance spectra (in blue) and corresponding STD-NMR spectra at 310 K (in black). The representation of the epitope map disclosed by the analysis of the relative STD-NMR signal intensities for each oligosaccharide is reported as color legend associated with the STD% value. *Cannot be estimated due to water suppression.

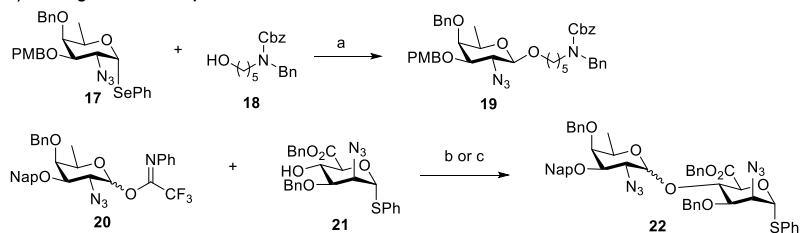
Potential new amine protecting group for the CP5 synthesis

In all Chapters of this Thesis, an azide moiety was implemented in the building blocks as a precursor for the acetamides, if the neighboring glycosylic linkage was of a 1,2-*cis* nature. The azides could easily be reduced to the corresponding amines and acetylated in a one-pot fashion to obtain the acetamides. For the CP5

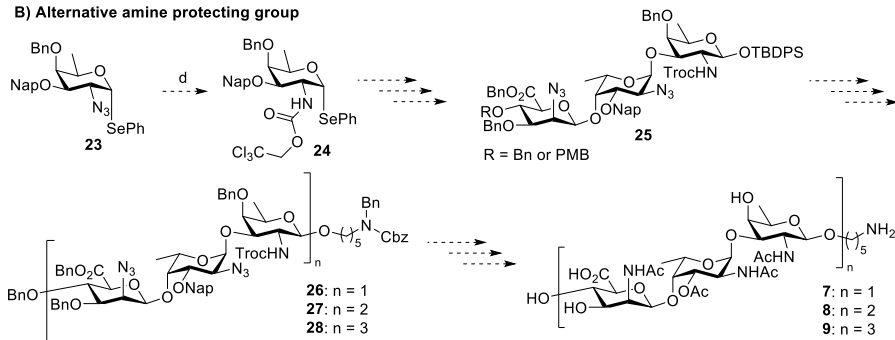
fragments, described in Chapter 3 and 4, a D-FucNAc 1,2-*trans* linkage had to be installed and to obtain the β -selective glycosylations, a protecting group enabling neighboring group participation was implemented. The trichloroacetyl (TCA) group was chosen as it can enable neighboring group participation and is stable under various conditions. In addition, the TCA group can be transformed into the corresponding acetamide under the same conditions used for the transformation of the azides, reducing the amount of deprotection steps on the larger saccharides. Unfortunately, partial reduction of the TCA group(s) led to product mixtures, that were difficult to purify, which led to low yields in the reduction reaction and the generation of only small amounts of pure material.

To overcome these problems, an alternative will have to be found to replace the TCA groups. By only using azides as precursors for the acetamides, the reduction may be achieved without problems. However, as a D-FucNAc 1,2-*trans* linkages is needed, alternative glycosylation conditions will have to be developed to install this linkage stereoselectively. In Chapter 2, linker **18** was installed on the D-FucN₃ donor **17** in a highly β -selective manner, which can be explained to arise from the high reactivity from the primary alcohol, being capable of directly displacing the anomeric α -triflate (Scheme 1A). However, when connecting the trisaccharides in a [3+3n] manner, a bond between the D-FucN₃ and the ManN₃A is required, and the glycosylation between monosaccharide D-FucN₃ donor **20** and monosaccharide ManN₃A acceptor **21** provided mainly α -disaccharide **22**. Unfortunately, also the use of participating nitrile solvents proved unsuccessful in promoting the formation of the β -linked product (Scheme 1A). Alternatively, a different acetamide protecting group, capable of neighboring group participation can be studied. To this end the trichloroethyl carbamate (Troc) group can be explored, which has been found to ensure 1,2-*trans* selectivity in glycosylation reactions.¹⁵ The Troc group can be installed on the FucN building block, in a similar fashion as for the TCA, by reduction of the azide in **23** with zinc and acetic acid followed by treatment with Troc-Cl and NaHsCO₃ (Scheme 1B). Just like TCA, the Troc group is stable under hydrolytic, strongly acidic, nucleophilic, and mild reductive conditions, and it can be reduced using zinc and acetic acid, after which acetylated in a one-pot reaction can provide the required acetamides. Substituting the TCA for a Troc should thus allow the same synthetic strategy to obtain the CP5-fragments.

A) Investigation of azide protected amine



B) Alternative amine protecting group



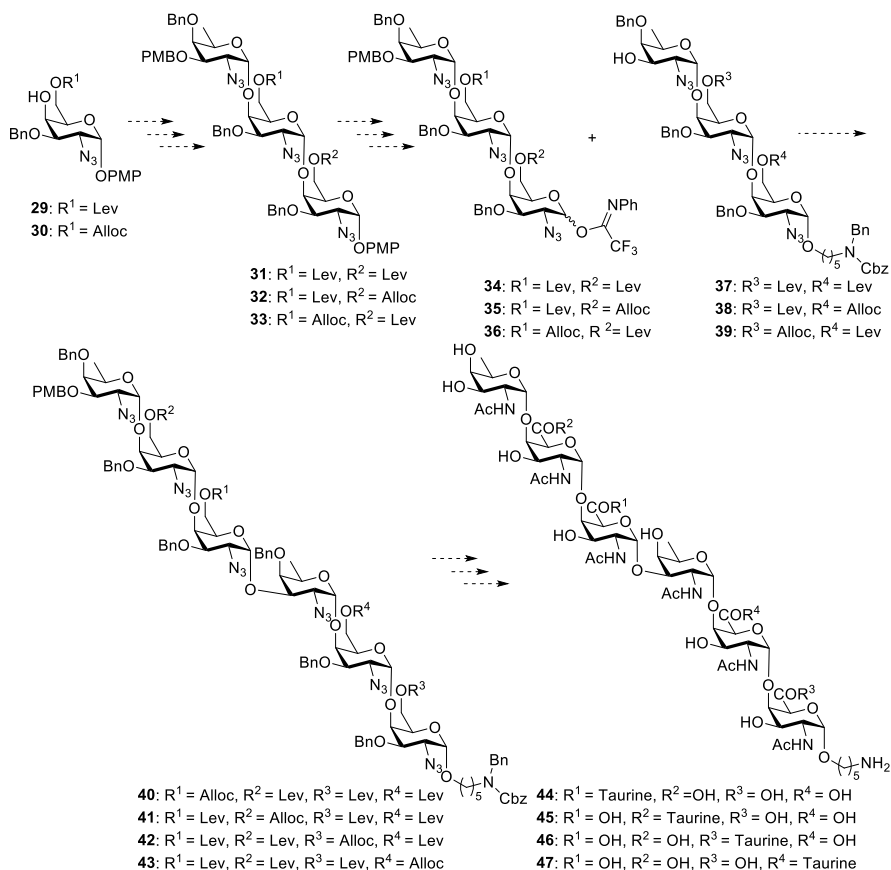
Scheme 1: Alternative amine protecting group for the D-Fuc-N in CP5. A) The investigation of using only azides a) TMSOTf, NIS, DCM/Et₂O 1:1, 78%, $\alpha/\beta=10:90$, b) TBSOTf, DCM/MeCN 2:1, -78 °C, 98% $\alpha/\beta=78:22$, c) TBSOTf, MeCN, -40 °C, 98% $\alpha/\beta=75:25$. B) The proposed route using Troc protection of the amine d) i) zinc, AcOH, THF, ii) Troc-Cl, NaHCO₃, THF.

No immunization studies have yet been performed with the generated CP5 oligomers. It would thus be of interest to test the glycoconjugates generated with the synthetic oligosaccharides and especially trisaccharide **10** is of interest, as it represents a relatively small saccharide, but was shown to be capable of binding anti-CP5 antibodies, raised against the native polysaccharide, in contrast to the trisaccharide frame shift reported by Adamo and co-workers.⁸

Potential synthesis of CP1 strain M hexasaccharides with a various taurine pattern

In Chapter 5, CP1 trisaccharides with different taurine pattern were synthesized. To investigate the synthesis of longer oligomers, a synthetic method for a non-taurinated hexasaccharide was developed. The glycosylation reactions to generate the trisaccharide donor went in moderate yield (56-65%), which may be improved by replacing the anomeric phenylselenenyl group in the acceptor building blocks, as this moiety may react with electrophilic species, generated during the glycosylation reactions. For example, a *p*-methoxyphenyl (PMP) protecting group

may be used, as it can selectively be removed under mild conditions, in the presence of both levulinoyl (Lev), allyloxycarbonyl (Alloc), *p*-methoxybenzyl (PMB) and silyl groups, with Ag(II)(hydrogen dipicolinate)₂ and sodium acetate.¹⁶



Scheme 2: Global overview of the proposed synthesis of CP1 hexasaccharides bearing taurine residues.

With the [3+3n] methodology confirmed to give the α -linked product, the next step would be to investigate the synthesis of the hexasaccharides bearing taurine amides. Three trisaccharides, bearing orthogonal protecting groups on the reducing and non-reducing end, as well as on the GalN₃ C-6-hydroxy groups, can be synthesized and used to provide different taurine patterns. The linker would be installed on the trisaccharides and through [3+3n] couplings longer oligomers may be attained. Scheme 2 shows a global overview of the proposed synthetic pathway.

Potential multivalent conjugate vaccines using synthetic material

The anti-*S. aureus* CP8 model vaccine candidates described in this Thesis were shown to be capable of inducing an effective antibody response, comparable to the response induced by a conjugate vaccine carrying the natural polysaccharide (Chapter 2). Previously, vaccine candidates using isolated material have been shown to induce an immune response in healthy adults, but these failed in late-stage clinical trials when moving to immunocompromised subjects (see Chapter 1),¹⁷ for yet unknown reasons. The complexity of the isolated polysaccharides and heterogeneity of the conjugates can be prevented by using synthetic material.

The model vaccines described in this Thesis incorporated a single type of antigen, either CP5 or CP8. Previously, vaccine candidates have reported incorporating of isolated material of both CP5/8 as well as other antigens and these have been shown to induce an immunogenic response in healthy subjects. Instead of conjugating the carrier protein with one type of synthetic antigen, different synthetic antigens can be introduced to obtain a more effective vaccine candidate, which can target several *S. aureus* strains. Different types of synthetic CPs can be used, which in this case would be CP1, CP5 and CP8. A different approach would be to include other types of antigens, such as well-defined synthetic wall teichoic acid (WTA) fragments. This approach is not unprecedented; various classes of cell wall glycopolymers have been utilized previously in vaccine candidates. For instance, the pentavalent vaccine PentaStaph, which incorporates two detoxified toxin components alongside conjugates of isolated CP5, CP8, as well as β -1,3-GlcNAc-modified WTA, was patented in 2005 by Nabi Biopharmaceuticals¹⁸ and later acquired by GlaxoSmithKline in 2009. This vaccine has demonstrated promising results and has undergone phase I/II clinical development.^{19,20} The advantages of using synthetic material over isolated fragments, include improved definition and homogeneity of the glycoconjugates, and better control of the relative composition of the different incorporated antigens, which may enhance their efficacy. When working with multiple antigens of varying size and carrying different (labile) functionalities, controlling and quantifying the sugar content in glycoconjugate vaccines can become increasingly complex and the availability of well-defined fragments will aid in the generation of better defined and controlled conjugates.

Acknowledgement

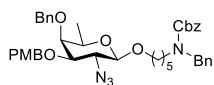
LucaUnione from CIC BioGune is acknowledged for his contribution to the STD-NMR.

Experimental

General experimental procedures

All reagents were of commercial grade and used as received unless otherwise noted. All moisture sensitive reactions were performed under an argon or nitrogen (N_2) atmosphere. Dried solvents (DCM, DMF, THF, toluene, Et_2O) were stored over flame-dried 3 or 4 Å molecular sieves. Reactions were monitored by thin layer chromatography (TLC) analysis conducted with Merck aluminum sheets with 0.20 mm of silica gel 60. The plates were detected by UV (254 nm) and were applicable by spraying with 20% sulfuric acid in EtOH or with a solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g/L) in 10% sulfuric acid (aq.) followed by charring at $\sim 150^\circ C$. Flash column chromatography was performed with silica gel (40–63 μm). Size-exclusion chromatography was carried out using SephadexTM (LH-20, GE Healthcare Life Sciences) by isocratic elution with DCM/MeOH (1:1, v:v). High-resolution mass spectra were recorded on a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature $275^\circ C$) with resolution $R=60,000$ at $m/z=400$ (mass range 150–4000). 1H and ^{13}C spectra were recorded on a Bruker AV-400 (400 and 101 MHz respectively), Bruker AV-500 (500 and 126 MHz respectively), Bruker AV-600 (600 and 151 MHz respectively), Bruker AV-850 (800 and 214 MHz respectively) or a Bruker AV-1200 (1200 and 302 MHz respectively). Chemical shifts (δ) are given in ppm relative to the residual signal of the deuterated solvent (1H -NMR: 7.26 ppm for $CDCl_3$, 3.31 ppm for MeOD, 1.94 for $CNCD_3$ or 4.79 for D_2O . ^{13}C -NMR: 77.16 ppm for $CDCl_3$, 49.00 ppm for MeOD, 1.32 for $CNCD_3$). Coupling constants (J) are given in Hz. All ^{13}C spectra are proton decoupled. NMR peak assignments were made using COSY and HSQC experiments, where applicable, HMBC and GATED experiments were used to further elucidate the structure. The anomeric product ratios were analyzed through integration of proton NMR signals.

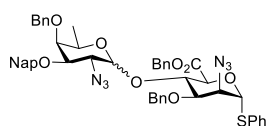
5-(Benzyl(benzyloxycarbonyl)amino)pentyl 2-azide-4-O-benzyl-2-deoxy-3-O-*p*-methoxybenzyl- β -D-fucopyranoside (**18**)



Donor **17** (82 mg, 0.151 mmol, 1 equiv.) and acceptor **16** (99 mg, 0.308 mmol, 2 equiv.) and were co-evaporated with toluene (x3) before being dissolved in dry DCM/ Et_2O (1:1, 1.5 mL, 0.1 M). Activated 3 Å molecular sieves were added and the solution was stirred for 30 min under argon at rt. The reaction was cooled to $-40^\circ C$ followed by addition of NIS (44 mg, 0.198 mmol, 1.3 equiv.) and TMSOTf (3 μL , 0.0303 mmol, 0.2 equiv.). The reaction was allowed to warm to $-20^\circ C$ and stirred for 1 h under argon until TLC (pentane/ $EtOAc$, 8:2) showed full conversion. The reaction was quenched with Et_3N and diluted with $EtOAc$. The organic phase was washed $Na_2S_2O_3$ (sat. aq.; x1), $NaHCO_3$ (sat. aq.; x1) and brine (x1), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. Column chromatography (pentane/ $EtOAc$, 95:5 \rightarrow 80:20) yielded **18** in 78% yield (83 mg, 0.117 mmol) in a $\alpha/\beta = 1:9$. NMR reported for the β -anomer. 1H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.08 (m, 17H, Ar-*H*), 6.93 – 6.87 (m, 2H, Ar-*H*), 5.17 (d, $J = 11.8$ Hz, 2H, CH_2 -Linker), 4.92 (d, $J = 11.6$ Hz, 1H, CH_2 -Ar), 4.65 (d, $J = 11.5$ Hz, 3H, CH_2 -Ar), 4.49 (d, $J = 7.2$ Hz, 2H, CH_2 -Linker), 4.12 (t, $J = 8.8$ Hz, 1H, H-1), 3.81 (s, 3H, CH_3 -PMB),

3.79 – 3.71 (m, 1H, H-2), 3.49 (dd, $J = 2.9, 1.0$ Hz, 1H, H-4), 3.45 – 3.30 (m, 2H, H-5, CH₂-Linker), 3.28 – 3.24 (m, 2H, H-3, CH₂-Linker), 3.19 (t, $J = 7.4$ Hz, 1H, CH₂-Linker), 1.65 – 1.44 (m, 4H, CH₂-Linker), 1.39 – 1.22 (m, 3H, CH₂-Linker), 1.17 (d, $J = 6.4$ Hz, 3H, H-6). ¹³C NMR (101 MHz, CDCl₃) δ 159.50 (C=O), 138.38 (C_q-Ar), 138.07 (C_q-Ar), 129.91 (C_q-Ar), 129.60 (C-Ar), 128.62 (C-Ar), 128.60 (C-Ar), 128.47 (C-Ar), 128.31 (C-Ar), 127.99 (C-Ar), 127.93 (C-Ar), 127.79 (C-Ar), 127.39 (C-Ar), 127.33 (C-Ar), 114.00 (C-Ar), 102.34 (C-1), 80.66 (C-3), 75.01 (C-4), 74.72 (CH₂-Ar), 72.39 (CH₂-Ar), 70.61 (C-5), 67.22 (CH₂-Linker), 63.20 (C-2), 55.40 (CH₃-PMB), 50.60 (CH₂-Linker), 50.29 (CH₂-Linker), 47.16 (CH₂-Linker), 46.30 (CH₂-Linker), 29.29 (CH₂-Linker), 27.99 (CH₂-Linker), 27.68 (CH₂-Linker), 23.31 (CH₂-Linker), 17.00 (C-6).

Phenyl 2-azide-4-*O*-benzyl-2-deoxy-3-*O*-(2-naphthylmethyl)- β / α -D-fucopyranosyl (1 \rightarrow 4)-(Benzyl (2-azido-3-*O*-benzyl-2-deoxy-1-thio- α -D-mannopyranosiduronate)) (21)



Acceptor **20** (66 mg, 0.134 mmol, 1 equiv.) and donor **19** (118 mg, 0.200 mmol, 1.5 equiv.) were co-evaporated with toluene (x3) before being dissolved in dry DCM/MeCN (2:1, 1.5 mL, 0.1 M). Activated 3 Å molecular sieves were added and the solution was stirred for 30 min under argon at rt. The reaction was cooled to -78 °C followed by addition of TBSOTf (6 μ L, 0.0267 mmol, 0.2 equiv.). The reaction was stirred at -78 °C and stirred for 1 h under argon until TLC (pentane/EtOAc, 8:2) showed full conversion. The reaction was quenched with Et₃N and diluted in EtOAc. The organic phase was washed Na₂S₂O₃ (sat. aq.; x1), NaHCO₃ (sat. aq.; x1) and brine (x1), dried over Na₂SO₄, filtered and concentrated *in vacuo*. Column chromatography (pentane/EtOAc, 95:5 \rightarrow 75:25) yielded **21** in 98% yield (117 mg, 0.131 mmol) in a $\alpha/\beta = 78:22$. NMR reported for the major α -anomer. ¹H NMR (400 MHz, CDCl₃) δ 7.95 – 7.84 (m, 6H), 7.67 – 7.47 (m, 7H), 7.40 – 7.25 (m, 18H), 7.24 – 7.10 (m, 5H), 5.71 (d, $J = 9.6$ Hz, 1H), 5.06 (d, $J = 3.6$ Hz, 1H), 5.02 – 4.91 (m, 3H), 4.91 – 4.76 (m, 4H), 4.72 (d, $J = 2.7$ Hz, 1H), 4.66 (d, $J = 11.5$ Hz, 1H), 4.56 (q, $J = 11.2$ Hz, 2H), 4.44 (dd, $J = 4.5, 2.6$ Hz, 1H), 4.04 (dd, $J = 4.5, 2.8$ Hz, 1H), 3.90 – 3.78 (m, 3H), 3.73 – 3.65 (m, 2H), 3.58 – 3.44 (m, 3H), 1.17 (d, $J = 6.4$ Hz, 5H). ¹³C NMR (101 MHz, CDCl₃) δ 168.49, 138.11, 136.58, 134.98, 133.69, 133.41, 131.20, 128.79, 128.65, 128.59, 128.52, 128.49, 128.44, 128.38, 128.33, 128.31, 128.26, 128.12, 128.08, 128.02, 127.97, 127.85, 127.78, 126.52, 126.39, 126.21, 125.87, 125.62, 100.08, 77.73, 76.53, 75.75, 75.13, 75.04, 74.93, 73.25, 72.52, 67.72, 67.30, 63.38, 59.58, 57.58, 31.86, 29.80, 19.40, 16.82.

Ligand-antibody interaction studies

¹H-STD NMR experiments & methods. For the acquisition of the ¹H-STD-NMR experiments the mAb-CP8 antibody was buffer exchanged to deuterated PBS 1X pD 7.8 using centrifuge filters (Sartorius Vivaspin 6 50000 MWCO) up to an antibody concentrated of 2 μ M. 100 equivalents of ligands (**1-3**) were added, which resulted into a solution of 2 μ M of mAb and 200 μ M of the ligand.

The STD experiments were recorded using Bruker AVANCE II 800 MHz NMR spectrometer equipped with cryo-probe (Bruker Inc.; Billerica, MA, US) at different temperatures that ranged

between 288 and 310 K. The used ^1H -STD pulse sequence includes T2 filter, for protein NMR signal suppression, and excitation sculpting, for residual water NMR signal suppression. The STD NMR spectra were acquired with 2880 scans and 5 s of relaxation delay. Different conditions were screened for STD experiments. All the STD experiments were performed at both on-resonances, at the aliphatic (0.8 ppm) and aromatic (7.0 ppm) regions. The resulting STD spectra provided similar results. The on- and off-resonance spectra were registered in the interleaved mode with the same number of scans. The on-resonance protein saturation was obtained using a Gaussian shape pulse of 50 ms with a total saturation time of 2 s at a frequency of δ 0.8 ppm (aliphatic region). The off-resonance frequency was always set at δ 100 ppm.

The analysis was carried out using the ^1H NMR signals of the STD spectrum and from their comparison with the off-resonance spectrum, the STD-AF (Average Factor) was obtained. The strongest STD intensity was used as reference (100% of STD effect). On this basis, the relative STD intensities for the other protons were estimated from the comparison of the corresponding integrals. These relative STD intensities (STD%) were used to map the ligand-binding epitope.

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