Chemical synthesis of fragments of streptococcal cell wall polysaccharides

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Chapter 1

General Introduction

1. Introduction

Carbohydrates, also named saccharides or glycans, are the most abundant of the four major classes of biomolecules (DNA, proteins, lipids and carbohydrates) in all living things.[1] Due to branched structures and various types of linkages, carbohydrates are structurally the most complex. They play many different roles in all forms of living organisms, as nutrients, in the storage of energy (e.g. starch and glycogen), as structural components (e.g. cellulose and chitin) and as signaling molecules and recognition elements, for example in the immune system and blood types. Besides, they are also important components of DNA, proteins, and

lipids. For example, the monosaccharide ribose (or deoxyribose) is a key constituent of coenzymes (e.g. ATP, FAD and NAD) and the backbone of the RNA (or DNA). A large part of all proteins is glycosylated, and glycans and lipids can be combined to provide glycolipids. As a result of the multiple asymmetric carbons in monosaccharides, varying stereoselectivity and regioselectivity of glycosidic linkages, different oligo/polysaccharide chain lengths, branching and additional functionalities such as carboxylate, sulfates and acetamides, an infinite number of saccharides exist in nature.

A small structural difference in a monosaccharide or glycosydic linkages can result in a significant change of the functions and properties of a glycan. For example, glucose and galactose only differ in one chiral center, but have a very different taste (Figure 1A). Difference of one hydroxyl group between ribose and deoxyribose defines DNA or RNA. Several structurally similar and important polysaccharides show in Figure 1B present how small structural changes lead to very different properties. Chitin and cellulose only differ between the acetylamino group and hydroxyl on position C-2. The difference between cellulose and amylose, which can be regarded as “unbranched starch”, is the configuration of the glycosydic bond (1,4-α vs 1,4-β). Glycogen contains a higher density of branches compared to amylopectin, a branched starch, on the same skeleton. The function and properties of these glycans are very different. Cellulose and chitin are both structural polysaccharides, and are components of plants and arthropods, respectively. Most non-ruminant animals cannot break down these polymers because they lack the cellulase enzyme. However, starch and glycogen are nutritional polysaccharides, which are easily broken down by these organisms. The more branched the polysaccharide is, the better its solubility in water and the more quickly it is digested.

A well-known example of cell-cell recognition that is determined by carbohydrates is the ABO blood group system.[2] Only one single terminal monosaccharide on the red blood cell surface determines blood types, establishing the rules for blood and organ transfusion.[3] The terminal carbohydrate structure of the A, B and H type II antigens, shown in Figure 1C, present on glycoproteins and glycolipids determines the blood group, and antigen(s) co-exists with antibodies directed at the missing antigen(s). For example, blood of group O only contains the H antigen (which is the precursor of A and B antigen), and has antibodies against both A and B.

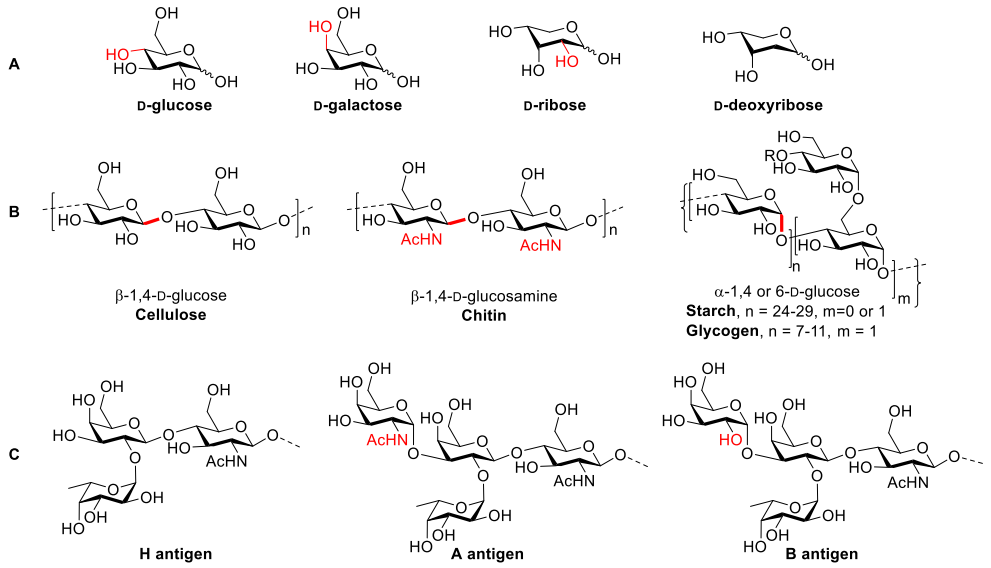


Figure 1. The structure of several mono-, oligo- and polysaccharides.

Specific recognition plays an all-important role in the immune system, which defends against infections by pathogens.[4] The cell surface of the most bacteria is surrounded by a cell wall polysaccharide (CWPS), which often is structurally unique and absent in host cells. Thus, CWPS can be regarded as pathogen signatures, and they can be used as a valuable epitopes (antigenic determinants) for the development of vaccines.[5] In general, carbohydrates are classified as T-cell-independent antigens, that can only induce specific short-lived IgM responses after being recognized by a B-cell receptor (BCR) with the aid of follicular dendritic cells (FDCs) (Fig. 2A). However, carbohydrates cannot be presented by major histocompatibility complex (MHC) proteins to activate T helper (T_h) cells, and therefore, they cannot induce immunological memory.[6] This deficiency can be made up by covalently conjugating the carbohydrate antigen with a carrier protein to provide a glycoconjugate vaccine (Fig. 2B).[7] The polysaccharide of this conjugate can react with the BCR to produce IgM response, while the peptide from the carrier protein can engage the T cell receptor (TCR) leading to the secretion of cytokines, inducing the switch from low affinity IgM to high affinity IgG antibodies and eliciting memory T (T_M) and memory B (B_M) cells for a long lasting immune response.

Some specific polysaccharides have been found that can activate the T cells, without conjugation to a protein (Fig. 2C). What these structures all have in common are negatively and positively charged groups, and therefore they are termed as zwitterionic polysaccharide (ZPS). They include a variety of structures, such as Sp1 from *Streptococcus pneumoniae*,

CP5 and CP8 from *Staphylococcus aureus*, and PS A1 from *Bacteroides fragilis* (see chapter 4).[8] ZPSs are regarded as T cell-dependent antigens, and they can be processed by antigen presenting cells (APCs) and presented by MHC-II proteins, leading to T helper cell activation.[9] Based on these unique properties, it has been suggested that ZPSs can be used to replace the carrier proteins to generate entirely carbohydrate-based vaccines.[10]

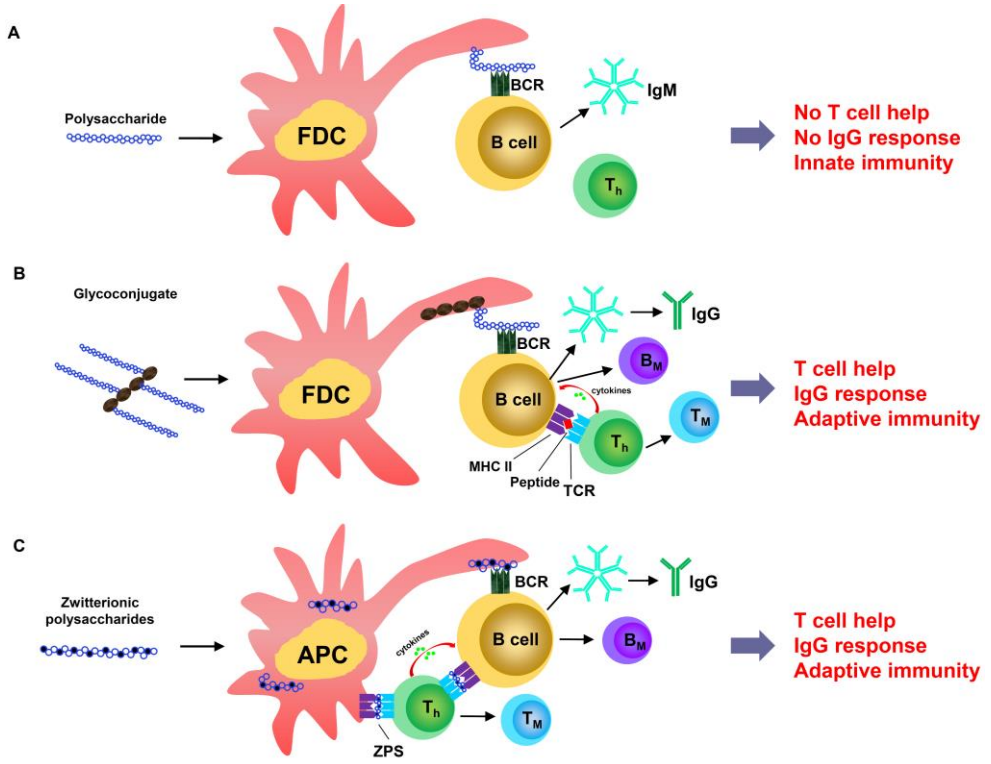


Figure 2. Interactions of polysaccharides and conjugate vaccines with the host immune system. A) Polysaccharides are recognized by a BCR leading to the production of IgM antibodies and no class switch to an IgG-responses or immunological memory. B) Glycoconjugates can not only interact with a BCR to produce the IgM, but also can recruit T cell help to provide the necessary co-stimulation to induce memory B cells and memory T cells to generate a long-lasting immune response. C) Zwitterionic polysaccharides can elicit a T helper cell response, upon presentation of APCs to T cells.

2. Carbohydrate-based Vaccines

Prevention is always better than treatment. Vaccination is a very powerful and selective weapon to combat pathogens. Compared to antibiotics or other medicine, vaccination can be

safer and cheaper. It has resulted in the eradication of smallpox, the near eradication of polio, and prevents numerous other infectious diseases. A vaccine trains the host immune system to recognize and combat pathogens. Carbohydrate-based vaccines aim to generate CWPS-specific antibodies which can provide protection against the targeted pathogen.[11] While glycoconjugate vaccines have provided a massive impact on public health, and every year billions of doses are used to vaccinate against for example *H. influenzae* type B (Hib), *Neisseria meningitidis* and pneumococcus, it is still not fully understood how the host immune system responds and processes these conjugates.

2.1 Carbohydrate-based vaccine development

CWPS often constitute the outermost layer of the cell surface, and it is the first moiety of a pathogen recognized by the host immune system. Therefore it is a natural antigen candidate.[12] The development of capsular polysaccharide (CPS)-based vaccines dates back to 1923 when the CPSs isolated from *Streptococcus pneumoniae* were shown to be immunoreactive.[9c, 13] It was shown in 1930s that carbohydrate-conjugates can induce the production of antibodies both in rabbits and humans.[14] The first CPS-based vaccine was approved by FDA in 1947 against *S. pneumoniae* using isolated pneumococcal polysaccharides. However, with the discovery of antibiotics, vaccine production was stalled in the 1950s. Due to the steady increase of antibiotic-resistant bacterial strains, CPS-based vaccines have received renewed interest. Because, CPSs elicit a poor immune response in children below two years old, vaccine development soon refocused on glycoconjugate vaccines,[14a] and the first CPS-conjugate vaccine was approved in the USA in 1987 for protection against Hib. Inspired by this success, various conjugate vaccines have been developed and licensed resulting in the remarkable reduction of Hib, *S. pneumoniae*, and *N. meningitidis* infections. Customarily, not all serogroups are covered in a vaccine. Among the 13 serogroups of *N. meningitidis*, at present, the inclusion of serotypes A, C, Y and W135 in the approved conjugate vaccines, like Menveo® (GSK), Menactra® (Sanofi Pasteur) and Nimenrix® (Pfizer) have effectively prevented most infections by this bacterium.

2.2 Novel synthetic glycoconjugate vaccine development

Although most of the above mentioned vaccines are based on isolated polysaccharides, vaccines based on synthetic oligosaccharides are being widely explored in recent years.[15] Synthetic oligosaccharide can be an attractive candidate because it guarantees minimal batch-to-batch variation, allows more controlled and precise conjugation chemistry and it can be used for detailed structure-activity relationships, for example, establishing minimal epitopes that can be used in vaccine optimization.

Taking advantage of ever more sophisticated strategies in carbohydrate synthesis, including one-pot sequential glycosylation reactions[16], (automated) solid-phase[17] and chemo-enzymatic syntheses[18], many oligosaccharides have been obtained to generate new vaccine candidates. The first licensed synthetic conjugate vaccine, Quimi-Hib® was developed in Cuba against the bacterium *Hemophilus influenza* type b (Hib). In this vaccine, the Hib ribose-ribitol phosphate oligomer is conjugated to the carrier protein tetanus toxin (TT) through a spacer attached at a pre-defined position of the oligosaccharide[19] (Fig. 3A). In 1974, Bundle and co-workers established that the CPS of the serotype A of *N. meningitidis* (MenA) as a structure composed of 1,6-linked 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate repeating units (Fig. 3B).[20] Due to the instability of the anomeric phosphodiester, 1-C-phosphono analogues of MenA CPS were developed, which were conjugated to human serum albumin (HAS) to provide a vaccine entity, featuring a CPS mimic (as opposed to a close copy) (Fig. 3B). The immune response of the mimic showed promising responses in vivo.[21] A fully synthetic conjugate vaccine against *N. meningitidis* serotype C has also been developed. Guo and co-workers generated oligo-(2,8)-sialic acids, which were linked to monophosphoryl lipid A (MPLA) to provide a vaccine entity, capable of eliciting a robust immune responses (Fig. 3C).[22] Many CPSs of various bacteria have been synthesized over the years to develop synthetic conjugate vaccines, some of which are outlined in more detail below.

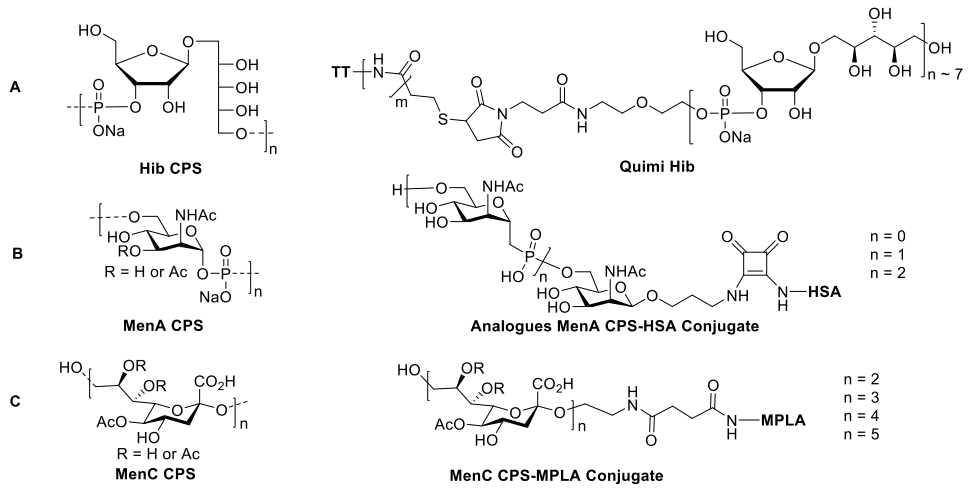


Figure 3. Structures of the repeating unit of several gram-negative bacteria CPS and their synthetic conjugate vaccines.

3. Examples of synthetic oligosaccharide vaccine for *Streptococcus*

Streptococci can cause a wide variety of fatal diseases, such as pneumonia, septicemia, meningitis, rheumatic fever and glomerulonephritis, and it tends to infect individuals with a weaker immune system, such as infants, young children, pregnant women, and the elderly.[23] Based on their hemolytic properties, they can be classified into three categories. The α-hemolytic species, such as *S. pneumoniae* and *S. viridans*i, show a greenish color on blood agar caused by the oxidization of iron in red blood cells; β-hemolytic species, such as Group A, B to V *Streptococci*, lead to a clear zone on blood agar due to complete rupture of red blood cells; γ-hemolytic species cause no hemolysis. Clinically, the most important bacteria are *S. pneumoniae*, *S. viridans*, Group A *Streptococcus* and Group B *Streptococcus*. In this chapter, examples of the CWPS syntheses of *S. pneumoniae*, GAS and GBS are provided.

3.1 Group A *Streptococcus* (GAS)

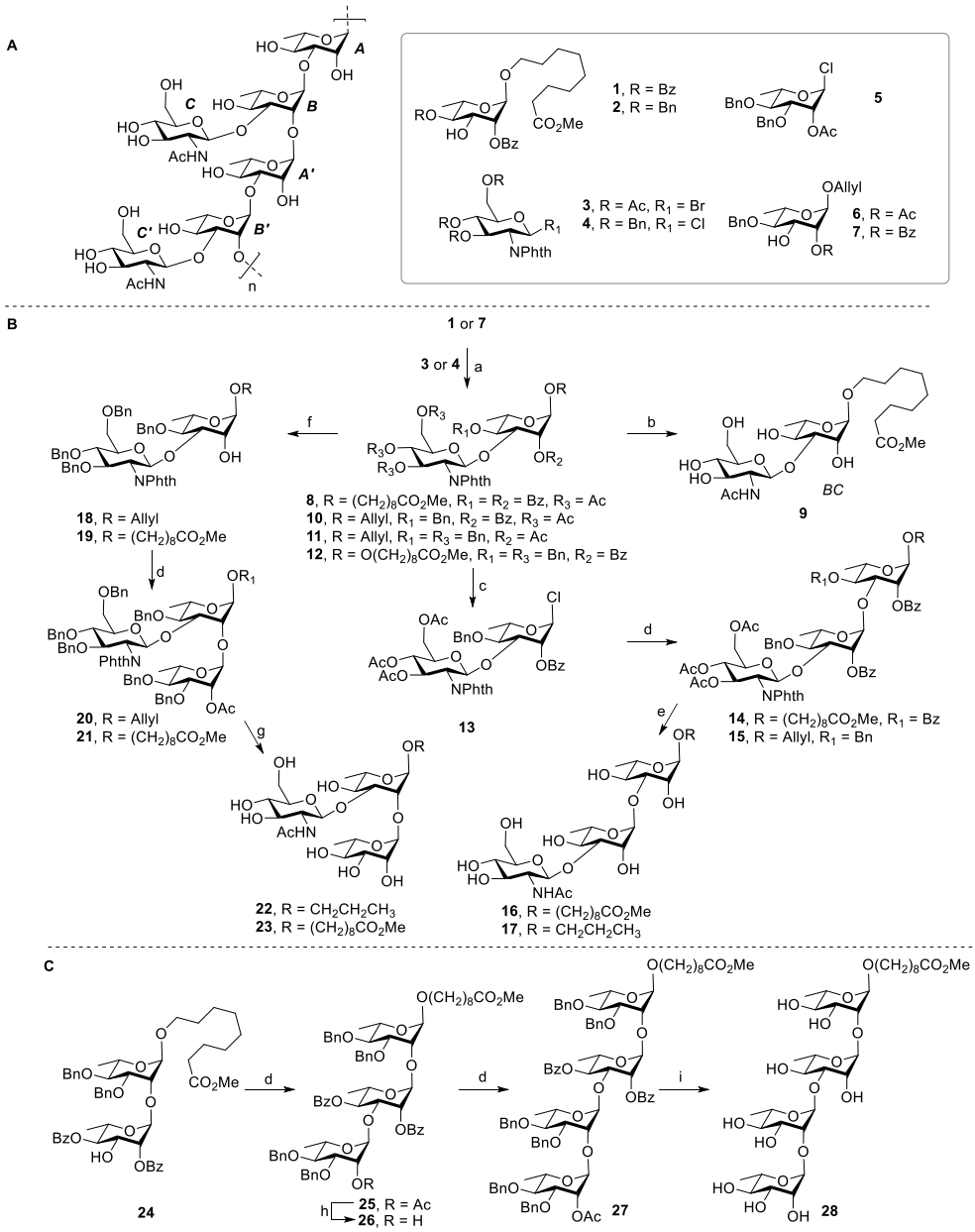
GAS also known as *Streptococcus pyogenes*, is an important human pathogen and ranks among the top ten causes of mortality and morbidity from an infectious disease. Although the GAS vaccines has been in development for almost a century, no vaccine has so far been licensed for use in humans.[24] The group A carbohydrate (GAC) was found to elicit immune responses in rabbits, mice and human,[25] and its structure, defined in 1970s, is composed of a polyrhamnose backbone containing immunodominant GlcNAc appendages[26] at the 3-position of the rhamnose residue (Scheme 1A).[27] So far, a variety of synthetic approaches including [3 + 1], [1 + 3], [3 + 2], [3 + 3], [3 + 6], [4 + 5] and [3 + 9] assembly strategies, have been developed to generate GAC fragments from di- up to dodecasaccharides.

The first synthesis of a GAC fragments was accomplished in 1981 by the Bundle group, who synthesized *BC* disaccharide **9** and tetrasaccharide *ABA′B′* **28**, representing a small rhamnose backbone (Scheme 1B and 1C).[28] In a Koenigs–Knorr reaction, rhamnose acceptor **1** and glucosyl bromide donor **3** were condensed using silver trifluoromethanesulfonate (AgOTf) and 2,4,6-trimethylpyridine (collidine) to give disaccharide **8** in 90% yield. A carboxylic ester terminated linker was used to allow for later modifications. Removal of the acetyl and benzoyl groups using NaOMe in MeOH, followed by deprotection of the phthaloyl group using hydrazine hydrate and acetylation of the released amine gave the deprotected disaccharide **9**. Meanwhile, a longer fragment of the rhamnose backbone was generated employing a Koenigs–Knorr glycosylation reaction between disaccharide acceptor **24** and rhamnose chloride donor **5** using AgOTf and tetramethylurea to provide trisaccharide **25** in 81% yield. The acetyl group was selectively removed by treatment with magnesium methoxide to generate trisaccharide acceptor **26**,

which could be glycosylated with donor **5** leading to tetrasaccharide **27** in 85% yield. The deprotected tetrasaccharide **28** was obtained after the global deprotection by subsequent basic hydrolysis and hydrogenation.

In the GAC structure, two trisaccharides can be recognized as repeating units, the linear *ABC* trimer or branched *B(C)A'* moiety. The linear *ABC* **16** and **17** were first constructed by the Pinto group via a [2 + 1] strategy (Scheme 1B).[29] Rhodium(I)-catalyzed isomerization and hydrolysis of the anomeric allyl group of disaccharide **10**, followed by a Vilsmeier-Haack reaction provided disaccharide chloride **13**. Then, glycosylation with rhamnose acceptors **1** or **7**, bearing different linkers, led to trisaccharides **14** and **15**. The deprotections were performed via methanolysis, hydrazinolysis, *N*-acetylation and hydrogenolysis to afford the trisaccharide as its 8-methoxycarbonyloctyl glycoside **16** for the preparation of conjugates and its propyl glycoside **17** for use as hapten for immunochemical studies. Besides, the branched *B(C)A'* **22** and **23** were prepared by the same group using a [1 + 2] pathway. Glycosylation of the monosaccharide donor **4** with rhamnose **6** or **2**, followed by acetyl removal gave disaccharide acceptors **18** and **19**. Treatment of these two acceptors with donor **5** under the silver triflate catalysis afforded the protected branched trisaccharides **20** and **21** in 81% and 62% yield, respectively. Deprotection of **20** and **21** was accomplished by methanolysis, hydrogenation, hydrazinolysis and *N*-acetylation to provide the trisaccharides **22** and **23**.

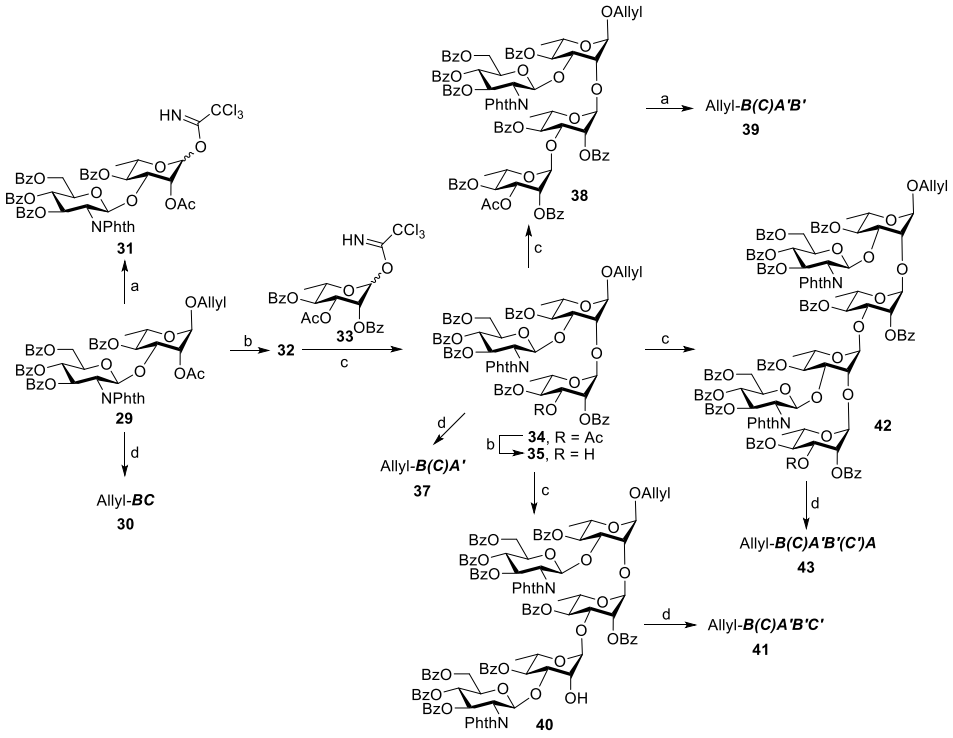
Next, pathways for the longer and modifiable GAC oligosaccharides were developed. Adopting the branched *B(C)A'* building block, several GAC fragments were generated via [1 + 3], [2 + 3] and [3 + 3] strategies (Scheme 2).[30] Trisaccharide **34** contains an allyl group on its anomeric position that could be used as a temporary protecting group for the assembly of longer oligosaccharides or as a permanent functionality that allows for conjugation of the synthetic antigens. Trisaccharide acceptor **35** could be obtained from **34** by removal of the acetyl group using HCl in MeOH. For ensuing further glycosylations, both the disaccharide **29** and trisaccharide **34** were converted to the corresponding imidate donors **31** and **36**, via the isomerization of the anomeric allyl group by Wilkinson's catalyst and hydrolysis of the so-formed enol ether, followed by the installment of trichloracetimidate moiety. Subsequently, the trisaccharide **35** was glycosylated with mono-, di- or trisaccharide imidates **31**, **33** and **36** under catalysis of TESOTf to construct tetra-, penta- and hexasaccharides **38**, **40** and **42**, respectively. Global deprotection and *N*-acetylated generated the allyl glycosides **30**, **37**, **39**, **41** and **43** in good yield.



Scheme 1. The structure of GAC and synthesis pathways of di-, tri, and tetrasaccharides

Reagents and conditions: a) AgOTf, collidine, 4Å MS, DCM, **8**, 90%; **10**, 90%; **11**, 61%; **12**, 57%. b) i, NaOMe, MeOH; ii, hydrazine hydrate, EtOH; iii, Ac_2O, MeOH, 80% (over three steps). c) i, $RhCl(PPh_3)_3$, EtOH, water, 86%; ii, HgO, $HgCl_2$, acetone, water, 93%; iii) Oxalyl chloride, *N*,*N*-dimethyl(chloromethyl-1-ene)ammonium chloride, DMF, DCM, 99%. d) AgOTf, tetramethylurea, DCM, -78 °C to rt, **14**, 53%; **15**, 76%; **20**, 81%; **21**, 62%; **25**, 81%; **27**, 85%. e) i, NaOMe, MeOH; ii, hydrazine hydrate, EtOH; iii, Ac_2O, MeOH; iv, Pd/C, H_2, AcOH, water, 75% (over

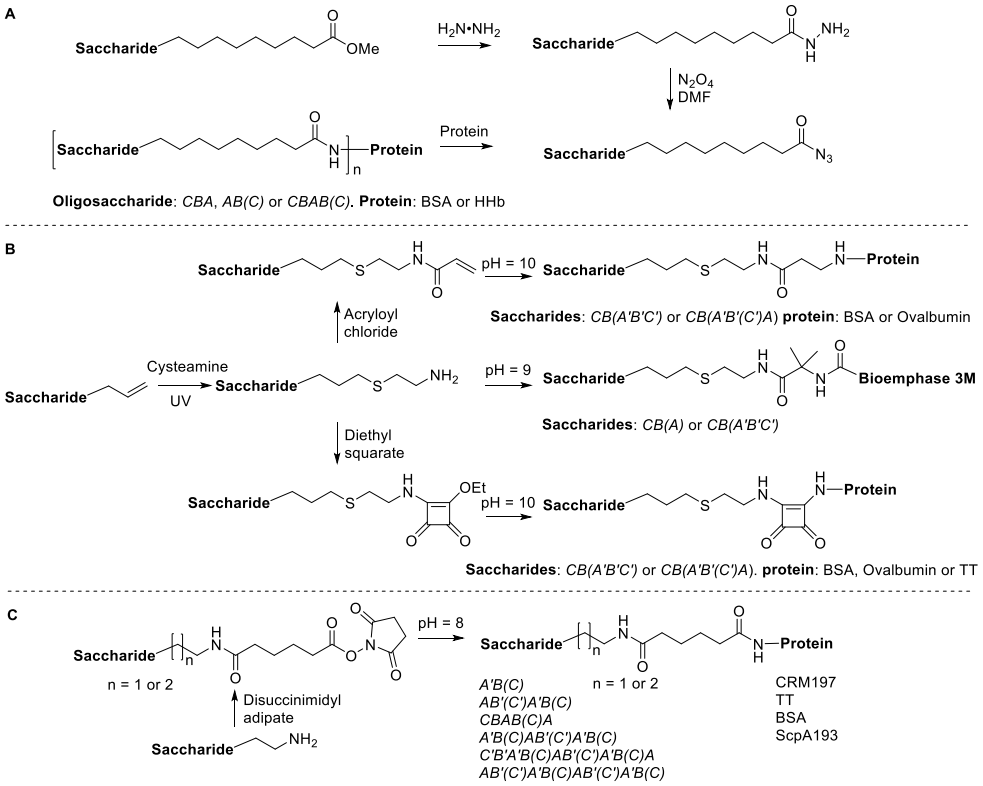
four steps). f) HCl in MeOH, **18**, 90%; NaOMe, NaOH, **19**, 58%. g) i, HCl in MeOH or NaOMe, MeOH; ii, Pd/C, H_2, AcOH, water; iii, N_2H_2•H_2O, EtOH; iv, Ac_2O, MeOH, **22**, 49%; **23**, 33% (over four steps). h) $Mg(OMe)_2$, MeOH, THF, 0 °C, 85%. i) i, Pd/C, AcOH; ii, NaOMe, MeOH, 63% (over 2 steps).



Scheme 2. The assembly of fragments of GAC using the branched *B(C)A'* as building block Reagents and conditions: a) i, $RhCl(PPh_3)_3$, 1,4-diazabicyclo[2.2.2]octane (DABCO), EtOH, water; ii, HgO, $HgCl_2$, acetone, water, 74%; iii, Cl_3CCN, K_2CO_3 or DBU, DCM, 76%. b) HCl in MeOH, **32**, 81%; **35**, 84%. c) TESOTf, DCM, -78 °C, 4Å MS, **34**, quant.; **38**, 80%, **40**, 76%; **42**, 43%. d) i, NaOMe, MeOH; ii, ethylenediamine, EtOH, reflux; iii, Ac_2O, MeOH, **30**, 68%; **37**, 70%; **39**, 88%; **41**, 86%; **43**, 84% (over four steps).

In comparing to the use of the branched *B(C)A'* trisaccharide repeating unit, the linear *CBA* trimer allows for effective neighboring group participation to control all the construction of the required 1,2-*trans* glycosidic bonds. The Pinto's group has assembled a variety of GAC fragments, such as *AB(C)A'*, *B(C)A'B'C'*, and *AB(C)A'B'C'* using the linear trisaccharide via [1 + 3],[31] [3 + 2][32] and [3 + 3][33] glycosylation strategies. So far, the largest GAC fragment was synthesized by Costantino and co-workers using the linear trisaccharide as repeating unit in a [3 + 3 + 3 + 3] glycosylation pathway to provide a dodecasaccharide.[34] Recently, Guo's group has also accomplished tri-, hexa- and nonasaccharides of GAC via a highly convergent strategy.[35]

To develop a GAS vaccine, many synthetic oligosaccharides were conjugated with different proteins. The carboxylic esters of the tri- and pentasaccharide, generated by the groups of Bundle and Pinto, were transformed via hydrazide derivatives to acyl azide intermediates[36] which were coupled to lysine residues of bovine serum albumin (BSA) and horse hemoglobin (HHb) (Scheme 3A).[37] For the allyl glycosides, different conjugation methods were developed to attach the oligosaccharides to both proteins or solid supports (Bioemphase 3M) to generate immunoaffinity columns (Scheme 3B).[38] Cysteamine hydrochloride was first added to the allyl glycosides, and then the adducts could be further functionalized via the introduced amine functionality. Different procedures were performed for conjugation of the penta- and hexasaccharides with proteins, such as BSA, ovalbumin and TT.[39] The ε-amino group of the protein lysines could be used for Michael-type addition[40] with the double bond of the *N*-acryloylated oligosaccharides, generated from the cysteamine adducts. Alternatively, the amines were linked with 3,4-diethoxy-3-cyclobutene-l,2-dione (diethyl squarate) and then coupled to the proteins. Use of the squarate adducts led to the incorporation of more glycans per protein. The immunogenicity of a hexasaccharide-TT glycoconjugate was confirmed in mice, inducing effective immunological memory. Disuccinimidyl adipate has also been used as a cross-linker to conjugate a hexa- and two dodecasaccharides with CRM197 (Scheme 3C).[34] Compared to conjugates of the bacterial polysaccharide, the oligosaccharide conjugates displayed similar immunogenicity and elicited comparable specific IgG titers in mice. Recently, branched tri-, hexa- and nonasaccharides, equipped with an amino spacer were conjugated with four different proteins, including CRM197, TT, BSA and Group A streptococcal C5a peptidase (ScpA193).[35] The latter protein was shown to be highly immunogenic and could be used to elicit specific antibodies that can inhibit streptococcal colonization (Scheme 3C). The ScpA193 was proved to be an effective carrier protein and its activity of boosting the immunogenicity was better than or at least comparable to CRM197 and TT. Because both the oligosaccharide and protein are derived from GAS, the ScpA193-conjugate could be functionalized as a promising bi-valent vaccine.



Scheme 3. Conjugation of the GAC fragments with various proteins or solid phase material

3.2 Group B *Streptococcus* (GBS)

Similar to GAS, infections of GBS, also known as *Streptococcus agalactiae*, represent a significant global public health problem, and a major cause of infections for pregnant women and newborns, but also immunocompromised people.[41] Intrapartum antibiotic prophylaxis (IAP) can substantially reduce early-onset infection of newborns, but do not present a solution for late onset infections (after 7 days of life) or later in life. So far, no vaccine is commercially available although much effort is being undertaken for this direction.[42] The capsular polysaccharides of GBS have been shown to be a major virulence factor, and it has been proposed that GBS uses its polysaccharide capsule to circumvent innate immune defenses of the host.[43] Based on the structure of the CPS (Figure 4A), currently, ten identified serotypes (Ia, Ib, and II to IX) have been described.[44] Serotype III is the most dominant serotype causing infection and colonizing 28% of mothers worldwide, with the serotypes Ia, Ib, II and V together responsible for more than 95% infections of GBS worldwide.[45]

The CPS of these serotypes represent promising vaccine candidates, as first demonstrated in 1930s by Rebecca Lancefield with CPS-specific protective rabbit sera.[46] Nowadays, a major focus is on the development of GAS glycoconjugate vaccines.[47] In 1996, the first glycoconjugate vaccine trial in humans was reported, conjugating type III CPS to a carrier protein TT (III-TT), which proved to be well-tolerated and immunogenic.[48] Afterwards, diverse monovalent and multivalent conjugate vaccines have been prepared and tested in preclinical or phase I/II clinical trials, including a CPS-CRM197 trivalent (serotypes Ia, IIb, III) and pentavalent (Ia, IIb, II, III, V) developed by Novartis/GSK.[49] These vaccines used CPSs, isolated from bacteria, but there is also significant interest in synthetic oligosaccharide conjugate vaccines. The structure of all CPS serotypes is made up of tetra- to heptasaccharide repeating units containing *N*-acetyl-D-neuraminic acid, L-rhamnose, D-galactose, D-glucose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine (Figure 4A). Besides the above CPSs, all GBS strains express the group B-specific antigen (GBC), first isolated by Jennings and co-workers (Figure 4B and Chapter 2).[50] This multiantennary structure is composed of L-rhamnose, D-galactose, D-*N*-acetylglucosamine and D-glucitol, with phosphate joints between different subunits. The synthesis of cell wall carbohydrate of GBS can be dated back to the 1980s, when several fragments of substructure III of GBC were accomplished with the assembly of a rhamnose trisaccharide and an oligosaccharide including the characteristic glucitol.[51]

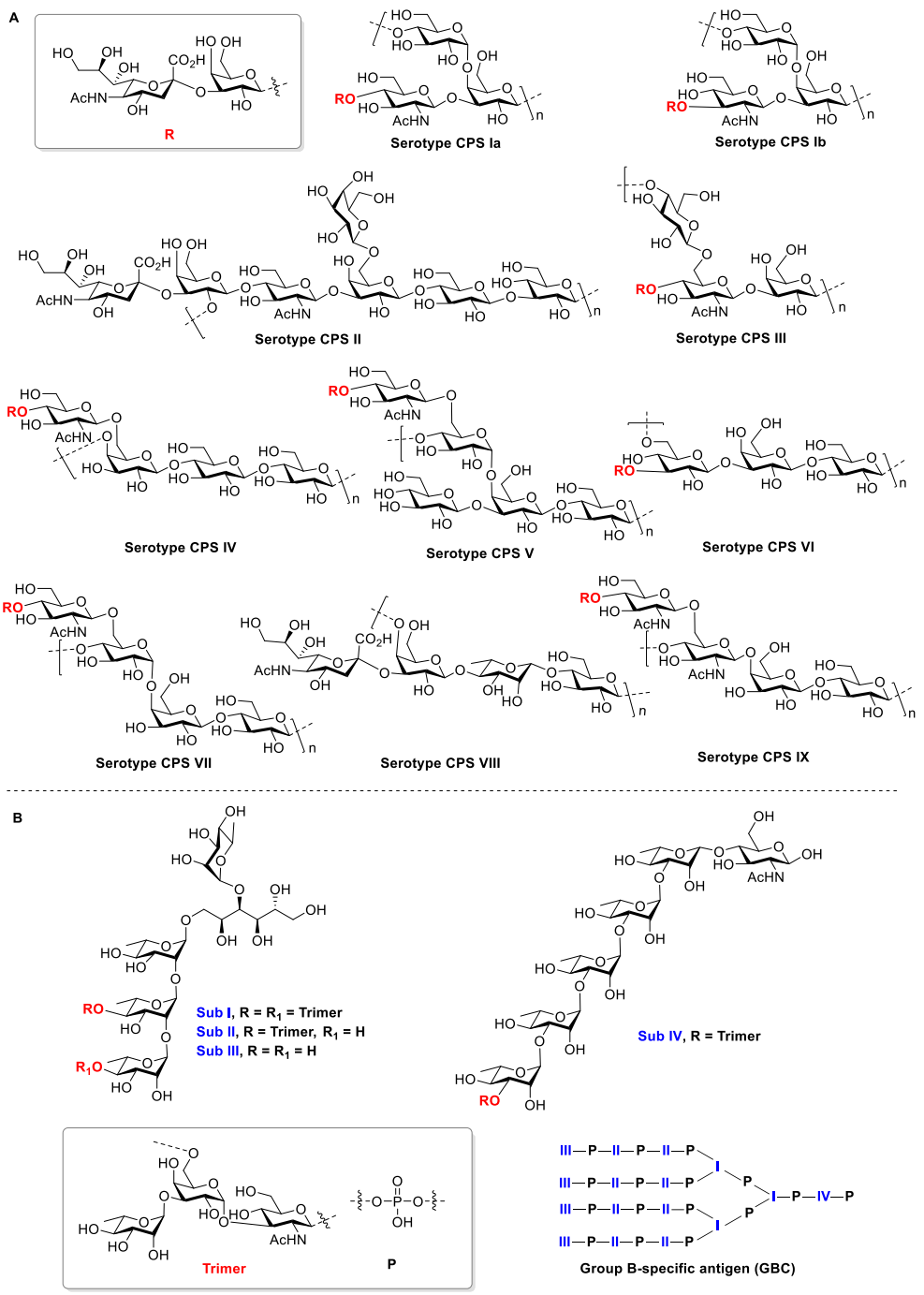
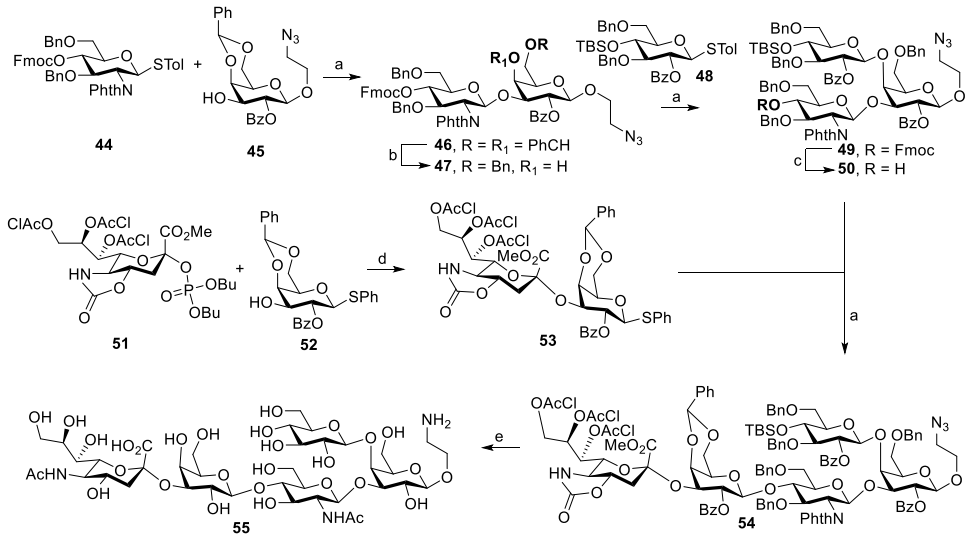


Figure 4. Structure of cell wall carbohydrate antigen of GBS

Serotype Ia and Ib

Even though the structure of the CPS serotypes were defined many years ago, and a chemoenzymatic synthesis strategy for the assembly of a type Ia CPS hexasaccharide was reported in 1996,[52] the first chemical synthesis of the type Ia repeating unit was not accomplished until 2015 when Guo's group assembled the pentasaccharide[53]. After probing various glycosylation strategies, the pentasaccharide repeating unit of serotype Ia CPS was obtained employing a convergent [2 + 3] pathway with a sialogalactoside disaccharide as the donor and a branched trisaccharide as the acceptor (Scheme 4). It is worth mentioning that the monosaccharide at the C-4-*O*-position of galactose had a big impact on the glycosylation of the 3-hydroxyl group, while the monosaccharide at the C-3 hydroxyl had little influence on the glycosylation of the C-4 alcohol. The synthesis commenced with the preparation of branched trisaccharide **50**. Glycosylation of **44** with **45** under the promotion of NIS/AgOTf provided **46** in 75% yield. Regioselective opening of the benzylidene ring, followed by glycosylation with **48** generated trisaccharide **49**. The branched acceptor **50** was generated by Fmoc-removal with triethylamine. The key [2 + 3] glycosylation was performed in the presence of NIS and AgOTf to yield **54** in 55% yield using acceptor **50** and sialogalactose disaccharide donor **53**, which was synthesized from **51** and **52**. Global deprotection of **54** was accomplished by desilylation, basic hydrolysis, acetylation and hydrogenation to provide the desired pentasaccharide **55**, which contained a free amino group at the reducing end for the further conjugation.



Scheme 4. Synthesis of the repeating unit of serotype **Ia** GBS CPS

Reagents and conditions: a) NIS, AgOTf, 4Å MS, DCM, **46**, 75%; **49**, 72%; **54**, 55%. b) $NaBH_3CN$, HCl, 4Å MS,

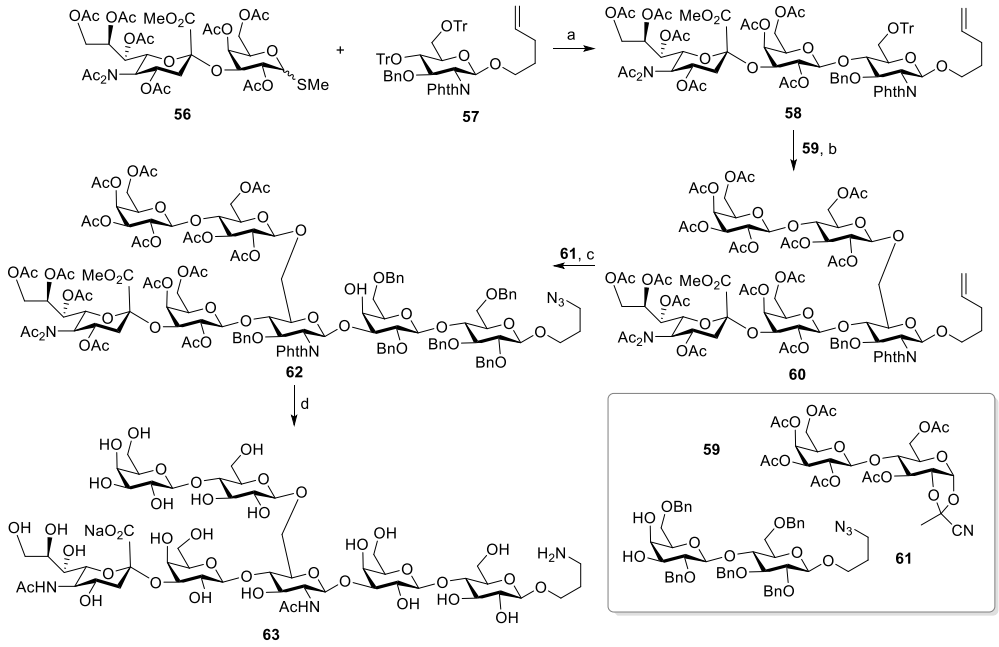
DCM, 95%. c) Et_3N, DCM, 100%. d) TMSOTf, DCM/MeCN, 4Å MS, 64%. e) i, Et_3N•3HF, THF; ii, LiOH, MeOH, H_2O, NH_2NH_2•H_2O; iii, Ac_2O, Pyridine; iv, NaOMe, MeOH; v, Pd/C, H_2, MeOH, 67% (over five steps).

Afterwards, more fragments of CPS type Ia were assembled including a decasaccharide composed of two repeating units,[54] a linear pentasaccharide repeating unit and a non-sialylated tetrasaccharide[55]. The synthesis of the decasaccharide was accomplished employing a convergent [2 * 2 + 6] dual glycosylation strategy using a double glycosylation with two copies of the sialogalactoside disaccharide donor and the branched hexasaccharide acceptor, which was generated using a [1 + 2 + 3] one-pot two-step synthesis pathway. Based on the successful [2 + 3] approach described above, the linear pentasaccharide repeating unit and the non-sialylated fragment were constructed by regioselective glycosylations using a trisaccharide diol containing a free Glc C3-OH and a free Gal C4-OH, exploiting the higher reactivity of the former alcohol over the latter one. The minimal structural difference between CPS Ia and Ib, (*i.e.* the 1-4 or 1-3 linkage of the sialogalactoside to the GlcNAc moiety), the synthesis of the repeating unit of CPS Ib, a branched and a linear pentasaccharide, containing a free amino group for future conjugation with carrier proteins, were accomplished employing similar strategies.[55]

Serotype III

On account of being the most prevalent and virulent, serotype III CPS has been the most studied CPS of the GBS polysaccharides. As early as 1990, desialylated tri- and tetrasaccharide fragments of the CPS were chemically synthesized by the group of Jennings.[56] Based on this success, one year later, the first complete repeating unit, a pentasaccharide, was achieved employing a combined chemical and enzymatic synthesis strategy, using a specific rat liver sialyltransferase to install the α-NeuNAc-moiety.[57] Taking advantage of this chemoenzymatic approach, two decasaccharides, representing two repeating units, were generated via enzymatic sialylation of the two terminal galactose residues of an octasaccharide.[58] A complete chemical synthesis of a heptasaccharide carrying an artificial spacer was accomplished in Boons group through a highly convergent strategy using a sialogalactosyl thioglycosyl donor and a *n*-pentenyl glucosamine building block (Scheme 5).[59] A chemoselective glycosylation of the thioglycoside donor **56** with di-*O*-tritylated acceptor **57** in the presence of MeOTf provided trisaccharide **58** with absolute regio- and stereoselectivity in excellent yield. The reactivity of C-4 hydroxyl in **57** is increased by tritylation due to the steric requirements of the C-4-*O*-trityl ether, resulting in elongation and polarization of the C-O bond of the secondary trityl ether. Next, the primary trityl ether was glycosylated with disaccharide donor **59** to assemble pentasaccharide **60** in

82% yield. Synthesis of the fully protected heptasaccharide **62** was accomplished employing the regioselective glycosylation of the *n*-pentenyl pentasaccharide donor **60** with disaccharide acceptor **61** under the promotion of NIS/TMSOTf. In the end, the target heptasaccharide **63** was obtained in 31% yield after a five-step deprotection, including removal of the esters, one sialyl *N*-acetyl group and the phthalimido group, *N*-acetylation of the glucosamine residue and hydrogenation of the benzyl ethers and azide. To detect antibodies in serum of pregnant women against the CPS type III by ELISA assay, the heptasaccharide was coupled to a poly(*N*-acryloyloxy)-succinimide polymer using the aminopropyl spacer.



Scheme 5. Synthesis of the repeating unit of serotype III GBS CPS

Reagents and conditions: a) MeOTf, 3Å MS, DCM, 96%. b) $TrClO_4$, DCM, 82%. c) NIS, TMSOTf, 3Å MS, 62%. d) i, LiI, pyridine; ii, ethylenediamine, *n*-butanol, H_2O; iii, Ac_2O, MeOH; iv, Pd/C, H_2, EtOH; v, 1N aq NaOH, 31%.

To explore the minimal structural requirements for antibody binding, various fragments of the type III CPS and mimics thereof were assembled, including a sialotrisaccharide, different pentasaccharides and a desialylated hexasaccharide.[60] Interestingly, the oligosaccharides of type III CPS could also be transformed to *S. pneumoniae* type 14 glycans using an enzymatic approach.[61] Recognition of the glycoconjugates with polyclonal CPS III specific serum indicated that the Glc β-(1→6) branch to GlcNAc is an important motif for antibody binding.[60b, 62]

3.3 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a gram-positive bacterium causing fatal pneumonia, septicemia, otitis media and meningitis.[63] Pneumococcal infections lead to a very high morbidity and mortality rate worldwide, especially occurring in individuals with weaker immune systems, such as infants, young children and the elderly. The highly variable CPSs of *S. pneumoniae* have been identified to be one of the most important virulence determinants of pneumococci. The first-generation carbohydrate-based vaccine PPV23 which contains the 23 most prevalent serotypes, was developed by Merck and approved in the United States and in Europe in 1983.[64] However, PPSV23 cannot elicit a protective immune response in children, younger than 2 years old. To improve the immunogenicity, the second-generation pneumococcal conjugate vaccines (PCV7, PCV10 and PCV13, approved in 2010) were developed for use in children younger than 2 years of age. The vaccines PPSV23 (containing 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) and PCV13 (containing 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) cover the most important, but not all of the 90 serotypes.[65]

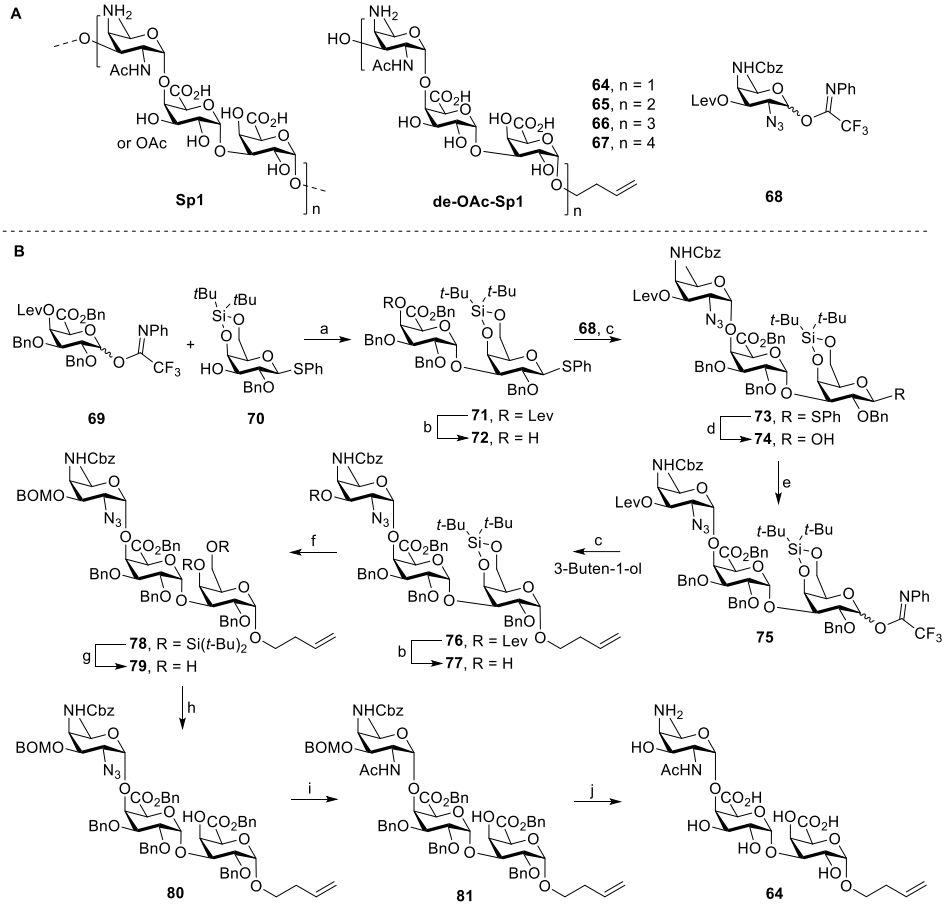
As described above most CPSs are T-cell-independent antigens which cannot lead to immunological memory without conjugating to a protein.[8a, 66] However, the serotype 1 CPS of *S. pneumoniae* (Sp1) is a zwitterionic polysaccharide (ZPS), which can provoke a T-cell dependent immune response as mentioned above (Figure 2). Sp1 is a linear polysaccharide consisting of a trisaccharide repeating unit, which contains two galacturonic acids and a rare 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (D-AAT) monosaccharide (Scheme 6A).[67] The structure presents three major challenges to a synthetic pathway, including stereoselective construction of the 1,2-*cis* glycosidic bonds, the introduction of the two uronic acids moieties and the rare AAT monosaccharide. Bundle and co-workers reported the first synthesis of Sp1-fragments with the assembly of oligosaccharides one and two repeating units.[68] The α-stereoselectivity of all glycosylations was achieved using remote participation effects. The uronic acids were introduced by the simultaneous oxidation of the two and four primary alcohols of trisaccharide and hexasaccharide, respectively, using a post glycosylation−oxidation strategy. Christina *et al.* reported the synthesis of all three possible frame-shifted Sp1 trisaccharides employing galacturonic acid-[3,6]-lactone as building blocks.[69] Longer oligomers could not be obtained using this approach because of poor selectivity in the crucial glycosylation reaction.

Recently, the assembly of larger fragments of Sp1, trisaccharide **64** up to dodecasaccharide **67**, were accomplished by Zhang *et al.* using a combination of a pre glycosylation−oxidation

with a post glycosylation−oxidation strategy to introduce the carboxylate groups (Scheme 6).[70] Crucial in the approach was the use of a trisaccharide featuring a silylidene protected galactose donor moiety to construct the 1,2-*cis* glycosydic linkages between the trimer repeating units. To generate the trimer repeating unit, first a glycosylation between galacturonic acid **69** and galactose acceptor **70** were performed to provide disaccharide **71** in 77% yield with 13:1 α/β stereoselectivity. Delevulinoylation and glycosylation with D-AAT donor **68** obtained the desired trisaccharide **73** in 85% yield with the same stereoselectivity. Next, this intermediate was transformed into trisaccharide imidate donor **75**. The spacer was stereoselectively glycosylated with donor **75** to furnish **76** as a single anomer. Removal of the levulinoyl group provided the trisaccharide acceptor **77**, which was used to generate the trisaccharide target, and for further elongated to acquire the longer oligosaccharides. To prevent the formation of the cyclic C-3-*O*-C-4-*N*-carbamate[69,71] during further modifications, a benzyloxymethyl group was installed on the free alcohol of **77**. Next, removal of the silylidene group, followed by regioselective oxidation of **79** using the TEMPO/BAIB reagent combination and benzylation gave trisaccharide **80** in good yield. The azide group of **80** was transformed into an acetamide using thioacetic acid. Finally, the global deprotection was accomplished by basic hydrolysis and Birch reduction to finish the trisaccharide **64**.

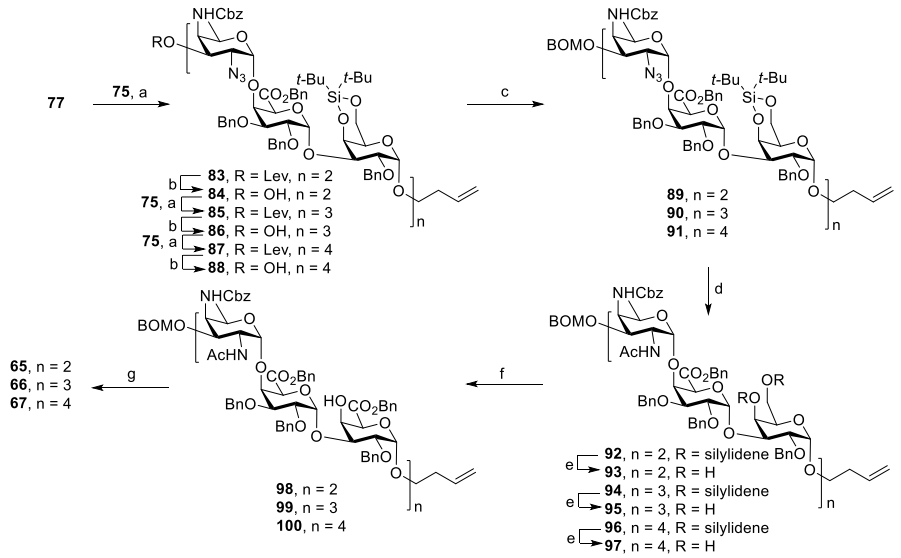
According to the successful synthesis of **64**, a next round of glycosylation with trisaccharide donor **75** and deprotection reactions provided the longer oligosaccharide hexasaccharide **83**, nonasaccharide **85** and dodecasaccharide **87** with good yield and excellent stereoselectivity (Scheme 7). However, to transform the generated oligomers into the target hexa-, nona- and dodecasaccharides, important improvements to the oxidation protocol had to be implemented as the TEMPO/BAIB oxidation conditions were not suitable to oxidize the multiple alcohols of the longer oligosaccharides. After a series of optimizations, the oxidation of the longer oligosaccharides was accomplished using a TEMPO/BAIB mediated oxidation under basic conditions, followed by benzylation using phenyldiazomethane, which provided a better yield than the use of benzyl bromide and K_2CO_3. Deprotection of the target oligosaccharides led to hexasaccharide **65** in 39% yield, nonasaccharide **66** in 55% yield and dodecasaccharide **67** in 47% yield. Structural studies and antibody recognition experiments with these oligosaccharides were conducted using NMR experiments, molecular dynamics (MD) calculations and ELISA experiments. It was revealed that the nona- and dodecasaccharides completed a full helical turn and showed better binding with both human and murine antibodies than the shorter oligosaccharides. The nonasaccharide may be an attractive candidate for the generation of an anti-Sp1 vaccine, representing the minimal epitope containing all structural features of the polysaccharide. To

this end, the alkene spacer can be used for conjugation purposes using a thiol-ene reaction.



Scheme 6. Synthesis of the monomeric repeating unit of Sp1 **64**

Reagents and conditions: a) TfOH, DCM, - 78 °C, 77%, α:β = 13:1. b) N_2H_4•H_2O, pyridine, AcOH, **72**, 89%; **77**, 98%. c) TBSOTf, DCM, 0 °C, **73**, 85%, α:β = 13:1; **76**, 82%, α only. d) NIS, TFA, DCM, 96%. e) *N*-phenyltrifluoroacetimidoyl chloride, K_2CO_3, acetone, 89%. f) BOMCl, DIPEA, TBAI, DCM, 89%. g) HF•Py, pyridine, THF, 94%. h) i. TEMPO, BAIB, DCM/tBuOH/H_2O, 4 °C; ii. Cs_2CO_3, BnBr, DMF, 84%. i) AcSH, pyridine, rt, 20 h, 66%. j) i, 1 M NaOH, THF, MeOH; ii, Na, NH_3 (liq.), THF, *t*-BuOH, allylcarbinol, 95%.



Scheme 7. Synthesis of the mono-, di-, tri- and tetrameric repeating unit of Sp1 **65**, **66**, **67**

Reagents and conditions: a) TBSOTf, DCM, **83**, 83%; **85**, 80%; **87**, 72%. b) N_2H_4•H_2O, pyridine, AcOH, **84**, 97%; **86**, 89%; **88**, 91%. c) BOMCl, DIPEA, TBAI, DCM, **89**, 81%; **90**, 89%; **91**, 84%. d) i, PPh_3, pyridine, H_2O, THF; ii. Ac_2O, pyridine, **92**, 93%; **94**, 88%; **96**, 99%. e) HF•Py, pyridine, THF, **93**, 91%; **95**, 88%; **97**, 91%. f) i. TEMPO, BAIB, $NaHCO_3$, EtOAc/*t*-BuOH/H_2O, 4 °C; ii. Cs_2CO_3, BnBr, DMF, or $PhCHN_2$, DCM, Et_2O, **98**, 45%; **99**, 51%; **100**, 49% (over two steps). g) i, 1 M NaOH, THF, MeOH; ii, Na, NH_3 (liq.), THF, *t*-BuOH, allylcarbinol, **65**, 39%; **66**, 55%; **67**, 47% (over two steps).

4. Aim and outline of this thesis

The aim of this thesis is to develop synthetic approaches to generate streptococcal oligosaccharides, which can be used to unravel structure-activity relationships and eventually to generate well-defined synthetic glycoconjugate vaccines. **Chapter 1** introduces the use of isolated and synthetic carbohydrates in vaccine development. Several syntheses of streptococcal CPSs are summarized to show the state-of-the-art in oligosaccharide synthesis and illustrate the use of synthetic oligosaccharides in the generation of anti-streptococcal conjugate vaccines. **Chapter 2** describes a chemical synthesis strategy to generate the terminal fragments of the Group B-specific antigen (GBC) of Group B *Streptococcus*. Highly convergent methods were employed to assemble GBC structures including a pentasaccharide, an octasaccharide through a [3 + 5] glycosylation strategy and a tridecasaccharide using a [5 + 8] phosphoramidite coupling. All structures were equipped with a spacer terminating in a free amine for the further conjugation with protein or other functional molecules. **Chapter 3** presents the first synthesis of fragments of the recently discovered glycerol phosphate (GroP)

modified group A carbohydrate (GAC), termed GroP GAC. Employing a linear trisaccharide as key repeating unit building block, tri-, hexa-, and nonasaccharides, with and without the GroP appendage, all bearing a free amine spacer at the reducing end were synthesized. **Chapter 4** describes the first assembly of the fragments of *O*-acetylated type 1 capsular polysaccharide of *Streptococcus pneumoniae* (Sp1), ranging from tri- to nonasaccharides. All fragments contain a diol terminated spacer that can be selectively oxidized in a Malaprade reaction to give an aldehyde for further modification. **Chapter 5** summarizes all the research presented in this thesis and provides some future prospect, including results of initial conjugation reactions and new synthesis pathways of fragments of another zwitterionic polysaccharide, PS A1, found in *Bacteroides fragilis*.

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