Chemical synthesis of oligosaccharide antigens as vaccine candidates for *Streptococcus suis* serotypes 2 and 14

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by
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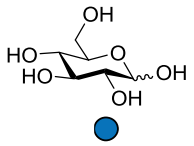
ABBREVIATIONS

Ac	Acetyl
Bn	Benzyl
BSA	Bovine serum albumin
Bz	Benzoyl
Cbz	Benzyloxycarbonyl
CPS	Capsular polysaccharide
CRM	Cross-reacting material
DCM	Dichloromethane
DIC	*N*,*N*′-Diisopropylcarbodiimide
DMF	Dimethylformamide
ESI-MS	Electrospray ionization-mass spectrometry
Fmoc	9-Fluorenylmethoxycarbonyl
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
Lev	Levulinoyl (4-Oxopentanoyl)
MALDI-MS	Matrix-assisted laser desorption ionization-mass spectrometry
Nap	2-Naphtylmethyl
NBS	*N*-bromosuccinimide
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
Pico	Pyridine-2-carboxyl
r.t.	Room temperature
SD	Standard deviation
TBA	Tetrabutylammonium
TBS	*tert*-butyldimethylsilyl
TCA	Trichloroacetyl
TCCA	Trichloroisocyanuric acid
TEA	Triethylamine
TfOH	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Tol	p-Tolyl
Tris	*tris*-(Hydroxymethyl)aminomethane

SYMBOLS



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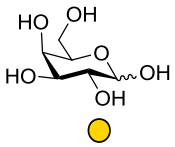

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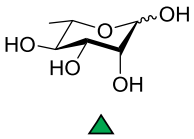

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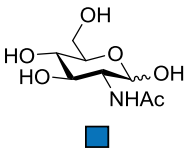

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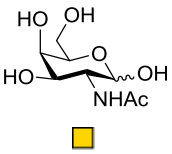

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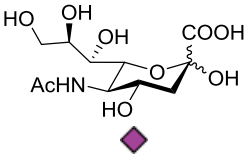


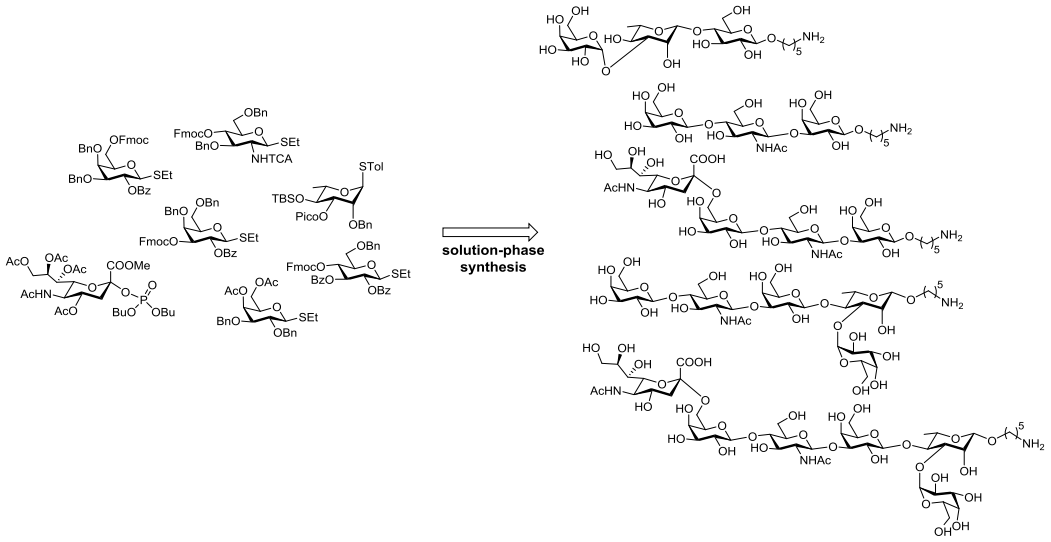
***N*-Acetyl D-neuraminic acid (Neu5Ac)**

ZUSAMMENFASSUNG

Bakterielle Polysaccharide, vor allem die, die am Aufbau der Bakterien-Kapsel beteiligt sind, werden seit einiger Zeit zur Impfstoffentwicklung gegen Pathogene eingesetzt werden. Üblicherweise wurden Impfstoffe aus Polysacchariden, die aus natürlichen Quellen isoliert wurden, hergestellt. Dazu stellen Impfstoffe, die auf synthetischen Oligosacchariden basieren, eine Alternative dar, die die Möglichkeit bietet, Impfstoffe mittels rationalem Design zu entwickeln oder schon bestehende zu verbessern. Zugang zu ausreichenden Mengen hochreinen und genau charakterisierten Oligosacchariden ist die Grundvoraussetzung für die Durchführung von Studien, die das Ziel haben, die Struktur des minimalen Zuckerepitops mit immunogenem Potential aufzuklären.

Das Hauptziel dieser Arbeit ist die Entwicklung neuer Synthesewege, um Oligosaccharide, die die Sequenzen kapsulärer Polysaccharide (KPS) pathogener Bakterien repräsentieren, zu erhalten und um minimale Epitope von Antikörpern aufzuklären. Das Ziel wurde durch Kombination von verschiedenen synthetischen Methoden, darunter Flüssigphasensynthese und automatisierte Festphasensynthese, erreicht. Das finale Ziel dieser Arbeit war es, synthetische Polysaccharid-Antigene, die für die Entwicklung neuartiger semisynthetischer Glykokonjugat-Impfstoffe für die Anwendung am Menschen oder in Tieren genutzt werden können, zu synthetisieren.

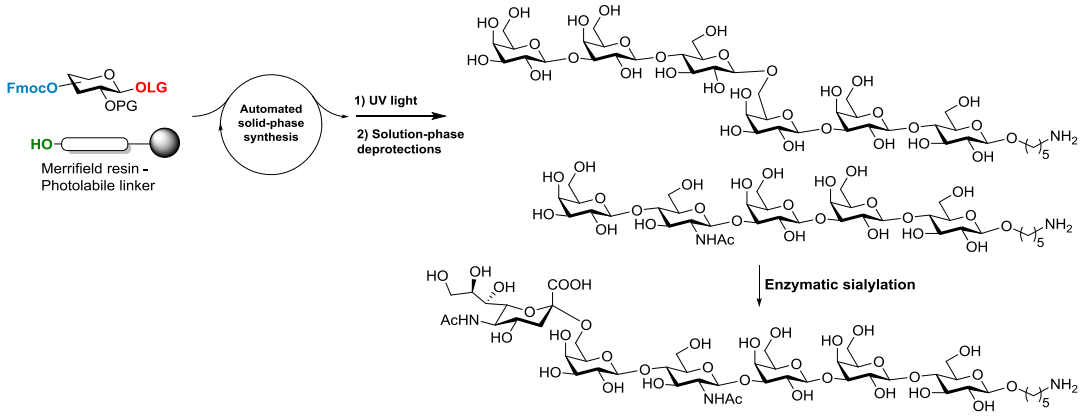
Im ersten Teil der vorgelegten Dissertation wird die Synthese von fünf Fragmenten, die den KPS des porzinen Pathogens *Streptococcus suis* Serotyp 2 zugeordnet werden können, mittels Flüssigphasensynthese beschrieben. Als Startpunkt wurden sieben Monosaccharid-Bausteine, die geeignete Schutzgruppen tragen, genutzt, um durch eine Reihe von chemischen Glykosylierungen und nachfolgender Manipulation der Schutzgruppen die Zielmoleküle zu erhalten. Die so erhaltene Bibliothek wurde genutzt, um Glykan-Arrays durchzuführen, die die Bindungsspezifität von Antikörpern, die in Seren von mit *Streptococcus suis* Typ 2 infizierten Schweinen vorkommen, evaluieren.



Schema I: Synthese der *Streptococcus suis* Typ 2 Glykan-Bibliothek.

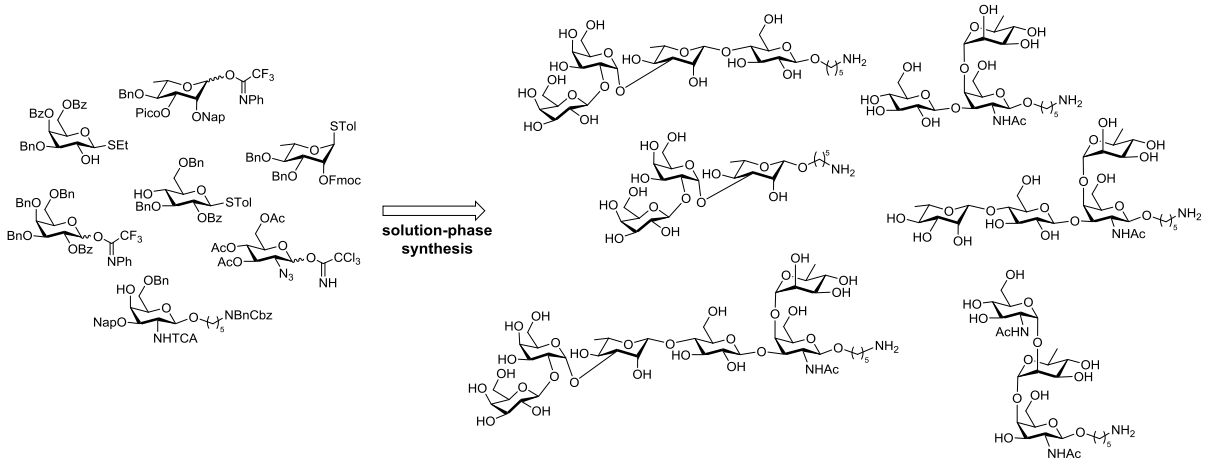
Der zweite Teil dieser Arbeit beschreibt die Kombination von automatisierter Festphasensynthese mit enzymatischen Glykosylierungen, um drei Fragmente, die den KPS von *Streptococcus suis* Serotyp

14 zugeordnet werden können, zu erhalten. Diese Glykane sollen analog zu den oben beschriebenen Experimenten ebenfalls durch Glykan-Mikroarrays evaluiert werden.



Schema II: Synthese der *Steptococcus suis* type 14 Glykan-Bibliothek.

Der letzte Abschnitt beschreibt die Synthese von Oligosacchariden, die den CPS von *Streptococcus pneumoniae* 7F zugeordnet werden können. Sechs Oligosaccharide wurden mittels Flüssigphasensynthese synthetisiert und mithilfe von Glykan-Arrays untersucht. Die Mikroarrays wurden mit humanem Anti-Pneumokokken-Serum getestet, um spezifische Sequenzen, die für die Antikörper-Bindung wichtig sind, zu identifizieren.



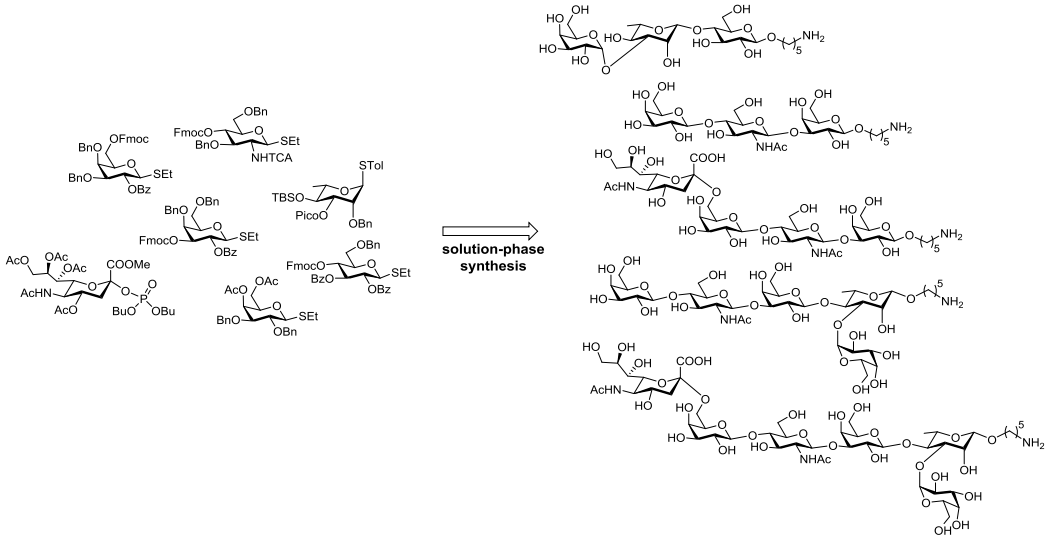
Schema III: Synthese der *Steptococcus pneumoniae* 7F Glykan-Bibliothek.

SUMMARY

Carbohydrates from bacteria, in particular polysaccharides constituting bacterial capsules, have been used to develop vaccines against pathogens. Traditionally, these vaccines are made from polysaccharides isolated from natural sources. As an alternative, vaccines based on synthetic oligosaccharides offer the possibility of rationally designing new vaccines or improve the existing ones. Having access to amounts of highly-pure and well-characterized oligosaccharides is fundamental for performing studies aimed at understanding the structures of minimal sugar epitopes with immunogenic potential.

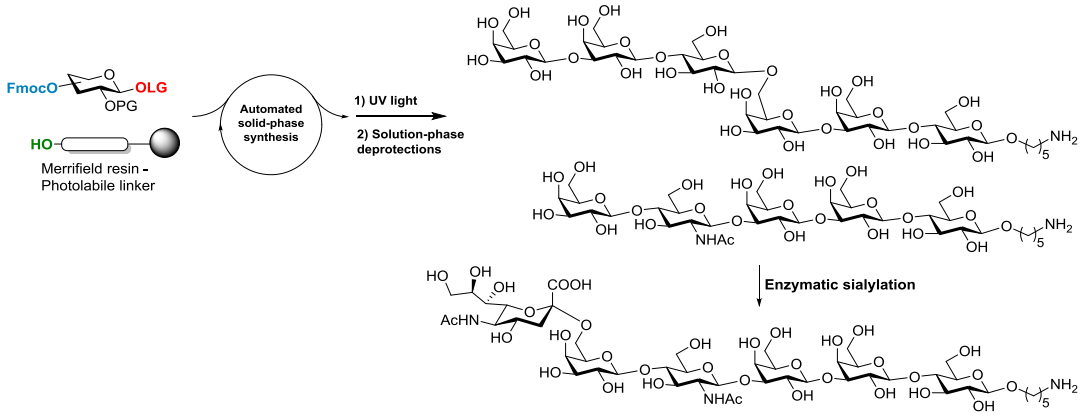
The main objective of this work is the development of synthetic routes to obtain oligosaccharides representing sequences of capsular polysaccharides (CPSs) from pathogenic bacteria to elucidate minimal epitopes of antibodies. The aim was achieved via a combination of synthetic chemical methods and employed both solution-phase and automated solid-phase techniques. The ultimate goal was to design synthetic carbohydrate antigens useful for developing new semi-synthetic glycoconjugate vaccines for human or animal use.

The first part of this dissertation describes the synthesis of five fragments related to the CPS of the pig pathogen *Streptococcus suis* serotype 2 using solution phase chemistry. Starting from the synthesis of seven monosaccharide building blocks bearing appropriate protecting groups, a series of chemical glycosylations and successive protecting group manipulations gave access to the target compounds. The obtained library was used to create glycan microarrays to evaluate binding specificities of antibodies contained in samples of sera from pigs infected with *Streptococcus suis* type 2.



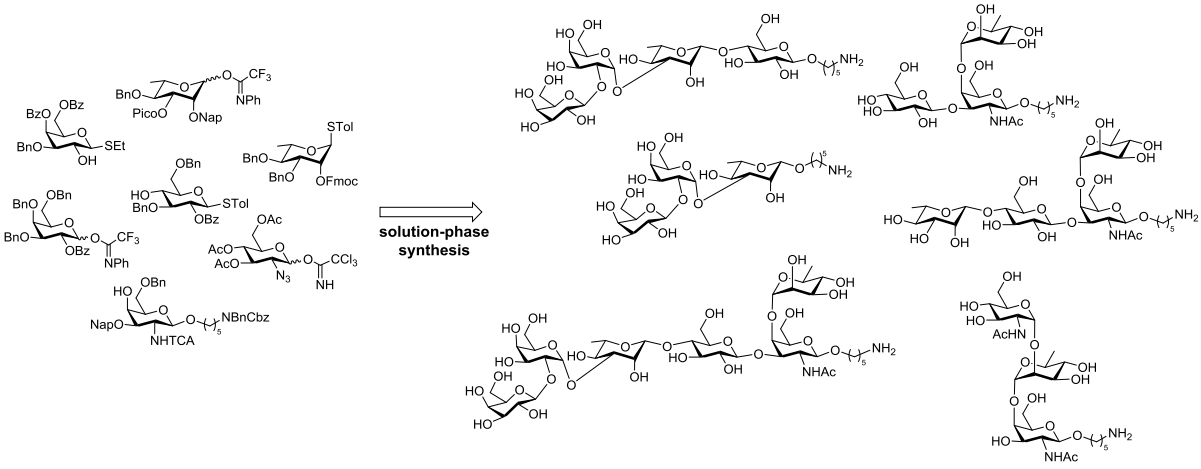
Scheme IV: Synthesis of a *Streptococcus suis* type 2 glycan library.

The second part describes the use of a combination of automated solid-phase synthesis and enzymatic glycosylations to synthesize three fragments related to the CPS of *Streptococcus suis* serotype 14. These glycans will be evaluated in glycan microarrays experiments as described above.



Scheme V: Synthesis of a *Streptococcus suis* type 14 glycan library.

The Supplementary section describes the synthesis of oligosaccharides related to the CPS from the human pathogen *Streptococcus pneumoniae* 7F. Six oligosaccharides were synthesized through solution phase chemistry and used to create glycan arrays. Microarrays were screened with human anti-pneumococcal sera, identifying specific sequences involved in antibody binding.



Scheme VI: Synthesis of a *Streptococcus pneumoniae* 7F glycan library.

CHAPTER 1

INTRODUCTION

1.1. *STREPTOCOCCUS SUIS*

Streptococcus suis is an important pathogen of swine, widely found in farm pigs all over the world and is one of the main causes of bacterial infections[1], therefore posing animal health and economic concerns for today´s industrialized pig farming. *S. suis* was first isolated and described as the causative agent of bacteremia, arthritis and meningitis occurring in animals during first weeks of life[2-4] as part of infection outbreaks in the Netherlands and the UK in the 1950s and 1960s. At the same time the first reports of infection and death in humans surfaced[5]. During the following decades the widespread distribution of this bacterium became more obvious as cases of infection in both pigs and humans were reported in other European countries[6,7], Asia[8-10], Australia[11] and in North America[12-14]. *S. suis* is a commensal bacterium that commonly inhabits the upper respiratory, digestive and reproductive systems of pigs, localized mostly in the animal's tonsils and saliva[15,16]. Most animals are healthy carriers of nonvirulent strains but some acquire virulent strains that can infect the bloodstream eventually resulting in septic shock and most frequently meningitis. Mortality rates during infection outbreaks have reached peak levels of 20%[17]. While *S. suis* is largely responsible for infections in pigs, the bacterium's natural host, it can also cause disease in humans, leading mostly to septicemia and meningitis. The majority of human infections occur in the southeastern region of Asia[18], particularly Vietnam and Thailand, where it has been recognized as one of the main causes of bacterial meningitis in adults[9,19]. In Europe, infections are rare and are considered to be occupationally related, affecting mostly farmers, butchers or veterinarians.

Streptococcus suis is a gram-positive coccus belonging to the group of encapsulated bacteria. The bacterial cell wall is surrounded by a layer of polysaccharides forming the bacterial capsule. Based on the chemical composition of the capsules 35 different serotypes of *S. suis*, named with progressive numbers from serotype 1 to 34 plus serotype 1/2, have been identified so far, although six serotypes have been suggested to belong to different species[20,21]. However, only a small number of the known serotypes are considered virulent. According to recent reports[20] serotype 2, 3, 7, 9 and 1/2 are the most frequently isolated from infected animals and their distribution follows some geographical trends, with serotype 2 being prevalent worldwide but especially frequent in Europe and Asia, while serotypes 3 and 9 were mostly reported in North America.

1.2. CAPSULAR POLYSACCHARIDES

Encapsulated bacteria are surrounded by a capsule, an extracellular shell of variable thickness linked to the cell surface, detectable under a light microscope after application of appropriate staining reagents or upon "swelling" with antibodies[22]. Bacterial capsules are cellular components that play a

fundamental role in the pathogen`s survival. Chemically they consist of a number of monosaccharides, often negatively charged at physiological pH, organized in repeating units and forming a high molecular weight hydrophilic polymer. This highly hydrated external layer coats the cell preventing cellular dehydration, helping adhesion to surfaces and facilitating the formation of biofilms[23]. Bacterial capsules are also important virulence factors: during an infection the capsule interferes with the immune system of the host, protecting the bacterium from mechanisms aimed at killing the invading pathogen, thus increasing its virulence. When the host lacks specific antibodies, CPSs might confer resistance to the host innate immune system. For instance, as demonstrated in the cases of *Staphylococcus aureus*[24,25] and *Streptococcus pneumoniae*[26], the capsule acts as a cover for several components of the bacterial membrane, lipopolysaccharides or teichoic acids, which would promptly activate the classical and alternative pathways of the complement system ultimately leading to cell lysis. The capsule also confers resistance to phagocytosis, possibly impairing contacts with phagocytic cells by displaying a negative charge at the surface[27]. Finally, CPSs from some pathogens have been suggested to counteract the activity of antimicrobial peptides[28].

Today it is widely established that CPSs, as the outermost antigen coming in contact with the host immune system, are able to trigger an adaptive immune response resulting in the production of specific antibodies. Therefore, these polysaccharides are attractive targets for the development of antibacterial vaccines[29].

1.3. CARBOHYDRATE VACCINES

Vaccines against bacterial pathogens can be broadly classified into different classes according to the antigen used for their preparation: live vaccines made using live attenuated cells, inactivated vaccines using inactivated killed pathogens, or subunit vaccines made using just a specific bacterial component such as detoxified toxic proteins (toxoids), polysaccharides or glycoconjugates. In addition, recombinant vaccines use genetic engineering to express defined bacterial antigens.

A large array of carbohydrates is displayed on the surface of many pathogenic bacteria, either as part of cell envelopes, cell membranes or as extracellular materials such as capsules or slime layers. They are key mediators of virulence mechanisms and cell surface antigens that can initiate immune responses, therefore representing targets for the design of new bacterial vaccines.

1.4. CAPSULAR POLYSACCHARIDE-BASED VACCINES

It was not until the 1920s that carbohydrates, in addition to proteins, were considered antigens. The earliest characterizations of capsular saccharides[30,31] and immunizations of animals and humans[32-34] were conducted using pneumococcal polysaccharides. These early experiments later evolved into deeper evaluations of CPSs from *Streptococcus pneumoniae*[35], *Salmonella typhi*[36] and *Haemophilus influenzae* type b[37] as vaccine candidates, all eventually resulting in licensed and marketed vaccines for human immunization.

All polysaccharide-based vaccines induce moderate to high levels of protection in adults. However, they also suffer a significant limitation: polysaccharides alone are not able to induce a strong immune response and immunological memory in young children, older adults and immunocompromised individuals, the parts of the population that are most prone to bacterial infections[38]. This is the effect of the T cell-independent nature of the immune response generated by plain polysaccharides, discussed more in detail in Section 1.6.

Since the early years of research on polysaccharide-based vaccines, it has been noted that enhanced immunogenicity and antibody response directed against the saccharide antigen could be achieved upon conjugation of these saccharides to a protein[39]. The mechanism underlying this effect was at that time unknown but it is now well established that these constructs can trigger a T cell-dependent response, resulting in strong antibody production and immunological memory, when plain polysaccharide vaccines fail. Despite these early findings, the strategy of saccharide-protein conjugates (glycoconjugates) as vaccines was not thoroughly investigated until the 1970s. The last 40 years have seen extensive research in this field and several glycoconjugate vaccines are available to protect against some of the most important human pathogens and more are being developed[40].

1.5. GLYCOCONJUGATES AS NEW VETERINARY VACCINES

Animal vaccination represents an effective strategy for limiting disease and reducing the spread of pathogens between animals and between animals and humans. Vaccination is currently common in veterinary practice and animal husbandry. In particular, livestock vaccination is a powerful tool to limit highly infectious diseases in farm animals and it can ultimately bring concrete socio-economic benefit through overall improved production efficiency, reduced antibiotic consumption[41] and a limited occurrence of antimicrobial resistance[42]. As an example, a recent study has shown that vaccination of a large group of pigs in Denmark against the common pathogen *Lawsonia intracellularis* resulted in a decreased use of antibiotics to treat related diseases by 79% without affecting production parameters[43]. All antibacterial vaccines available for animals are still made from live attenuated or inactivated bacterial cells[44], and therefore suffer shortcomings in terms of safety, stability and in some cases limited immunogenicity[42,44,45].

Significant advances in understanding the immune system, the pathogenicity of bacteria and viruses and new technologies for vaccine production seen in the last decades, have translated into several new generations of vaccines for human use. On the other hand, a similar development has not occurred for veterinary vaccines. Progress in biomedical research has resulted in a shift in interest toward new strategies in veterinary vaccination as well, such as the development of subunit vaccines[42,46,47]. In this regard, glycoconjugates represent a largely unexplored opportunity[48]. So far, only very few studies have been reported describing the immunological evaluation of polysaccharide-protein conjugates against porcine pathogens *Actinobacillus pleuropneumoniae*[49,50] and *Streptococcus suis*[51] and the ruminant pathogen *Mannheimia haemolytica*[52].

Widespread success was achieved through glycoconjugate vaccination in humans. Accordingly, new research efforts could be oriented towards the development of glycoconjugate veterinary vaccines.

1.6. IMMUNE RESPONSE TO CARBOHYDRATE VACCINES

Mammals possess an elaborate immune system composed of cells and macromolecules that helps them to fight and eliminate dangerous pathogens. Even though differences between animals belonging to the same class (such as humans and pigs) exist, the global structure and mechanisms are largely identical[53].

The immune system can be subdivided into the innate and adaptive branches. The adaptive branch is a highly specific mechanism that, after exposure to a particular foreign (non-self) antigen displayed by a pathogen, triggers mechanisms aimed at eliminating the infective agent and also results in immunological memory, allowing for an immediate reaction of the immune system in case of a subsequent exposure to the same microorganism. The main components of the adaptive system are antibodies, B cells and T cells.

Vaccines induce protection against a specific pathogen by artificially stimulating the development of adaptive immunity. The goal is the induction of antibodies of the IgG subclass and IgG-secreting memory B cells[54]. IgG antibodies are high-affinity antibodies, secreted by plasma B cells, that can mediate neutralization. As a result of their higher affinity they can effectively opsonize (coat) and “tag” the bacteria to ultimately facilitate phagocytosis. The IgM subclass typically displays lower binding affinities and lower protective potential and therefore is less desirable.

Bacterial polysaccharides, like other large polymeric antigens, are commonly classified as T cell-independent antigens, meaning that they activate B cells directly without the cooperation of T cells. In a simplified depiction of the mechanism[55,56] they are recognized on the extracellular side of B cells by saccharide-specific receptors (Fig. 1-1). Due to their polymeric multivalent character they bind several receptors simultaneously causing cross-linking of the receptors and activation of intracellular pathways that stimulate the B cells to maturate into plasma B cells, white blood cells capable of secreting large amounts of antibodies, mostly of the IgM subclass. Moreover this mechanism does not allow for B cells to maturate into memory cells, therefore no immunological memory is obtained. In young children, possibly due to the inherent immaturity of parts of their immune system[57,58], these mechanisms fail.

In contrast, in the case of glycoconjugates (Fig. 1-1), B cells can act as antigen-presenting cells (APCs) and a T cell-dependent pathway is activated[55,56,59]. The glycoconjugate can be recognized by receptors of polysaccharide-specific B cells but in this case the antigen is internalized through phagocytosis into endosomes. Here proteases and reactive radical species process and degrade the peptide and carbohydrate moieties[60]. Whether the products of degradation are just peptides, glycopeptides, or both, is still debated and subject of ongoing research[59,61]. These smaller antigens are then loaded onto major histocompatibility complex (MHC) class II, a protein that is subsequently transferred to the surface of the B cell. The MHC-antigen complex can now bind specific receptors on T cells (also named T-helper cells). This interaction activates the T cell, provoking the secretion of stimulatory cytokines which in turn activate the B cells and induce them into a complex process of proliferation, differentiation and DNA mutations resulting in maturation into specific plasma cells able to secrete polysaccharide-specific IgG antibodies. These cells have a relatively short lifespan,

however, some B cells also become memory cells, long-lasting and ready to quickly initiate a new response upon future exposure to the same specific antigen, providing lifelong immunity.

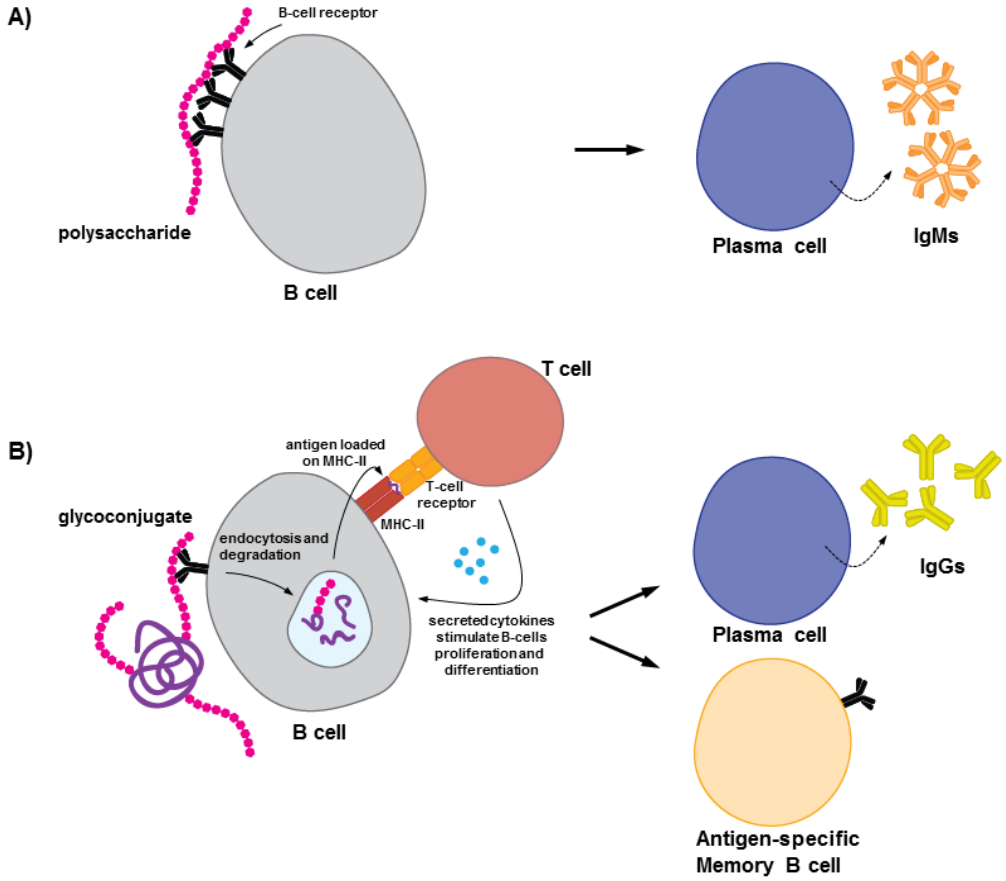


Figure 1-1: Simplified mechanisms of immune activation from carbohydrate-based vaccines. A) Direct activation of the B cell from plain polysaccharides leads to differentiation into IgM-secreting and short-lived plasma cells. B) Polysaccharides as part of a glycoconjugate are recognized by the same receptor but in this case the antigen is internalized. Fragments of the polysaccharide–protein conjugate are presented on B cells as epitopes of T cells, activating a mechanism that leads to differentiation into Memory B cells specific for that antigen and plasma cells producing antibodies of higher affinity.
Adapted from: *Nature Reviews Immunology,* 9, 213–220 (2009).

1.7. GLYCOCONJUGATE VACCINE DEVELOPMENT

Glycoconjugate vaccines are composed of a protein carrier to which a poly- or oligosaccharide chain is covalently linked, and an adjuvant to increase immunogenicity[56]. Carriers are proteins of bacterial origin that retain peptide sequences representing T cell epitopes which are presented on MHC class II and involve T cells, thus enforcing a T cell dependent mechanism and an effective adaptive immunity. After years of clinical experimentation[62], five carrier proteins are available and licensed for use in conjugate vaccines[63]: CRM_{197} and DT (Diphtheria Toxoid) from *Corynebacterium diphteriae*, TT (tetanus toxoid) from *Clostridium tetani*, OMPC (Outer Membrane Protein Complex) from *Neisseria meningitidis* and HiD from *Haemophilus influenzae*. Some of them, DT and TT, represent active ingredients in widely used diphtheria and tetanus vaccines.

CRM_{197} is the most well studied and well defined carrier used in recently developed glycoconjugate vaccines. It consists of a modified version of DT in which a single amino acid mutation avoids its toxic effect[64]. Usually carbohydrates are covalently attached to the protein by exploiting the nucleophilicity of ε-amino groups of lysine residues exposed on the protein surface. Due to steric impediments and their position within the secondary structure around one half of the total 39 lysine residues present in the polypeptide chain are available for covalent bond formation with carbohydrates[65] but commonly, depending on the conjugation method employed, a maximum of around 10 saccharide chains are attached per protein[66].

Almost all glycoconjugate vaccines are made from polysaccharides obtained from bacteria. Long polysaccharide chains are normally depolymerized to obtain lower molecular weight polymers that are more easily conjugated to proteins[67]. A number of techniques can be employed to achieve this scope such as chemical depolymerization (acidic or basic hydrolysis), physical methods (ultrasonication) or, in a few cases, enzymatic degradation. Unfortunately, these treatments are often not specific, cutting molecules at different glycosidic linkages and often resulting in the removal of functional groups such as acetyl or pyruvate groups. The obtained saccharides are later fractionated to a narrower range of molecular weights by using size-exclusion chromatography[68].

Following depolymerization, a step of chemical activation introduces electrophilic or nucleophilic functional groups, often non-selectively at random positions along the chain[67,69], to perform the subsequent chemical conjugation steps. The most commonly employed conjugation method is reductive amination[69] to obtain a secondary amine from the lysine ε-amino groups through the aldehyde form of reducing-end aldose monosaccharides or via aldehydes produced through periodate cleavage of diols on the sugar rings, or ozonolysis. This procedure presents the considerable disadvantage of directly modifying the chemical structure of the carbohydrate, with possible introduction of artificial epitopes. Cyanylation involves the reaction of the sugar with a mild cyanylating agent to form cyanide esters that can react with amines to form stable *O*-alkyl isourea functionalities[70]. Where present, carboxyl groups along the saccharide chain can be activated with coupling agents to generate amide bonds. All these methods involve direct attachment of sugars to the protein.

Alternatively, linkers can be introduced at the reducing end of the sugar, allowing reaction of a functionality on the spacer with lysines or acidic amino acids. Examples of such functionalities include

N-hydroxysuccinimide esters, squarate esters and adipic acid dihydrazides[69]. Additionally, linkers can also be introduced on the protein side. This allows for the use of other types of chemical strategies such as cycloaddition and click-chemistry reactions[71].

Recently, studies have suggested that a more controlled attachment of sugars to precise amino acids on the protein could have a positive impact on the response to the vaccine[72]. To achieve this, other amino acids such as cysteine or tyrosine were functionalized[73,74]. These residues are not abundant but allow for more chemoselective modifications. In the last ten years a new methodology has emerged, based on a process named Protein Glycan Coupling Technology, or bioconjugation[75,76]. The glycoconjugate is produced *in vivo* by engineered *Escherichia coli* cells expressing both the polysaccharide and the carrier protein; enzymes called oligosaccharyltransferases transfer the saccharide chain with very high specificity onto a residue of the acceptor protein. The glycoconjugate can be then purified from the cell lysate.

1.8. SEMISYNTHETIC GLYCOCONJUGATE VACCINES

As an alternative to the processes described in the previous section, glycoconjugate vaccines can be manufactured from synthetic carbohydrates resembling portions of the native bacterial polysaccharides. The latter methodology is still considered challenging and is not widely employed, but it is generally believed that it has the potential to overcome some limitation of the former process and provides several advantages[29,72,77,78].

Costly large scale fermentations of bacteria[79] and problematic purifications[80,81] from mixtures containing various biomolecules are avoided using this technique. As a result, the obtained products are totally free from any possible biological contamination. Synthetic sugars can be easily conjugated to proteins; introducing reducing-end spacers enables diverse conjugation chemistries to ensure higher loadings or site-selective attachments[82]. Moreover, in the conjugation process the original structure of the carbohydrate remains intact and some functional groups often lost during isolation of polysaccharides[83,84] are preserved. Due to higher control over the conjugation chemistry[72], physico-chemical properties of the glycoproteins are maintained and reproduced between different batches.

The resulting glycoproteins are homogeneous molecules[82], rather than cross-linked constructs as in the case of polysaccharides[78]. They contain sugar moieties with precise lengths and molecular weights as opposed to more heterogeneous polydisperse polysaccharides[84]. The number of saccharide chains attached to each protein can be more easily determined resulting in better characterized glycoproteins.

Semi-synthetic glycoconjugates are also suitable to evaluate how some characteristics affect the immunogenicity of the resulting vaccine[72], such as the type of protein carrier employed, the identity and length of the spacer and the saccharide-to-protein ratio. Most importantly, a precise correlation between the chemical structure of the saccharide antigen and its effect on the protective response induced by the vaccine can be derived.

A key step for the creation of an effective semi-synthetic glycoconjugate vaccine is represented by the elucidation of minimal glycan epitopes, or glycotopes, the precise sequence of sugars that are involved in binding interactions to immunoglobulins[77].

Vaccines based on polysaccharides contain high-molecular-weight antigens, chemically and conformationally equivalent to the natural antigen displayed on the bacterium and therefore contain sequences representing B-cell epitopes eventually inducing antibody responses. On the other hand, synthetic oligosaccharide antigens are shorter and less complex and must be designed to exactly mimic these epitopes. According to an old hypothesis[85] minimal glycan epitopes are represented by a minimum of one to a maximum of 6-7 monosaccharide units, based on the size of the antigen binding pockets of antibodies. This found partial confirmation in recent studies[86-88] where short linear sugar sequences were identified as minimal epitopes.

Recently, some biochemical techniques directed towards detailed analyses of sugar-proteins interactions have greatly evolved and nowadays represent powerful tools also in the field of glycoconjugate vaccine development[89]. One of the most important technologies exploiting this interaction is carbohydrate microarrays.

1.8. CARBOHYDRATE ARRAYS

Glycan microarrays are a technique that offers the possibility of studying interactions between carbohydrates and carbohydrate-binding proteins in a high-throughput fashion[90]. Since the creation of the first glycan arrays[91,92], many studies have focused on studying interactions between mammalian carbohydrates and proteins[93]. However, recently several bacterial carbohydrate arrays have been created and have helped provide insight into binding specificities of bacterial carbohydrate-binding proteins[94,95].

Glycan arrays are practically created by immobilizing sugars on the surface of microscope-size slides through covalent bonds or non-covalent interactions[90]. Several types of commercially available slides offer the possibility to exploit different immobilization strategies. Among these, glass slides modified with a hydrophilic polymer coating functionalized with reactive groups such as *N*-hydroxysuccinimide esters (NHS esters) are often used. This permits reactions with amino groups on sugars modified with suitable linkers to form robust covalent bonds. Polysaccharides on the other hand can be adsorbed on these surfaces through a combination of hydrophilic and non-hydrophilic interactions[91].

Under typical experimental conditions the layer obtained after immobilization displays carbohydrates with a high surface density, maintaining at the same time a degree of flexible orientation[96]. Sugar-binding proteins possess relatively low binding affinities, often in the micromolar range for a monovalent binding, and therefore the high density of glycans at the surface ensures the achievement of stronger bindings through multivalent interactions.

Immobilization is commonly performed using robotic equipment allowing for a miniaturized and highly reproducible printing process consuming only very small amounts, nanomoles or even less, of the carbohydrate sample and producing spots around 100 μm in diameter[96].

A solution containing antibodies, commonly serum from humans or animals previously infected or vaccinated, is applied after printing on the surface of the slide and with a subsequent washing step the unbound proteins are washed away. Later the array is probed with a solution of a class-specific secondary antibody conjugated to a fluorescent dye. After another washing, fluorescence is measured in a scanner, giving an indirect detection of bound primary antibodies. After elaboration of the data, the measured mean fluorescence intensities can be used to derive qualitative and in some cases quantitative information on protein binding events[96].

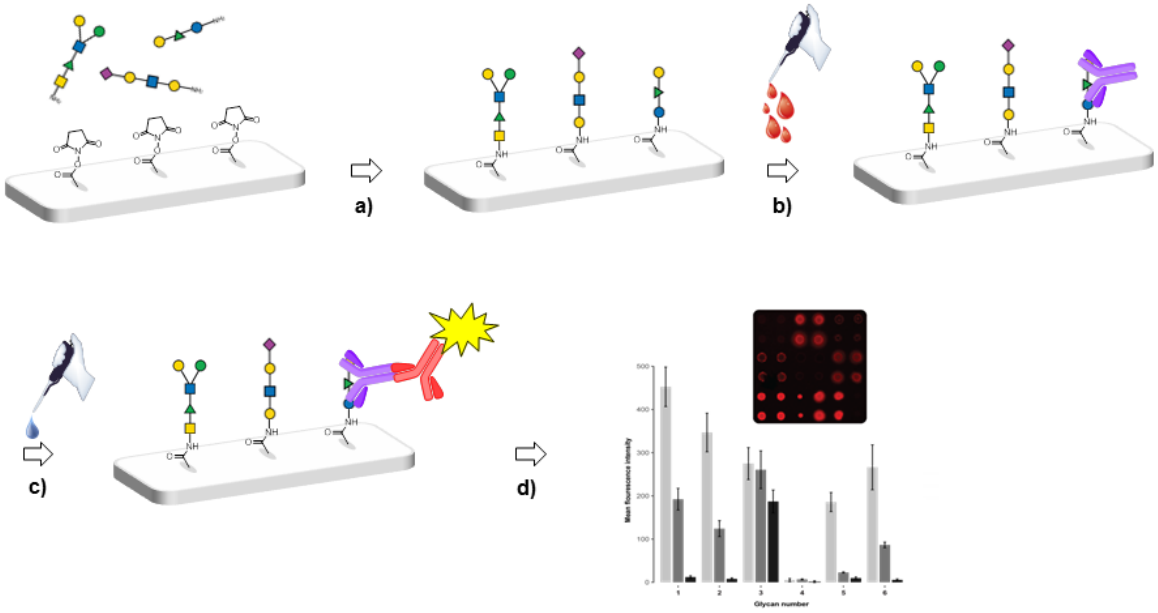


Figure 1-2: Typical glycan array workflow. a) Immobilization on the solid surface; b) incubation with an antibody-containing sample followed by washing; c) incubation with a fluorescence-tagged secondary antibody followed by washing; d) readout and data elaboration (image and graph were adapted from the Supplementary section).

During glycoconjugate vaccine development, glycan arrays serve a double function: they can be used to identify minimal glycan epitopes of antibodies from infected humans or animals, assisting in the design of synthetic antigens, or they can be used after vaccination to detect the presence of specific antibodies and monitor the magnitude of the immune response[77].

The major limitation for glycan arrays analyses of complex bacterial sugars is still obtaining libraries of carbohydrates that contain an appropriate number of compounds with sufficient structural diversity, in order to maximize conclusions drawn from the experiment. To this end, chemical synthesis can provide access to the necessary amounts of highly pure, well-characterized and structurally homogeneous oligosaccharides.

1.9. METHODS OF OLIGOSACCHARIDE SYNTHESIS

Large collections of glycans for biochemical studies, some containing hundreds of compounds, contain mostly sugars isolated and purified from natural sources[93,97-99]. Importantly, small-sized but more focused chemical libraries can be created by chemical synthesis. The scope of the experiment

determines which approach is more suitable to obtain the desired library[95,97]. As already introduced in Section 1.8, due to the high complexity of the bacterial glycome[100], bacterial oligosaccharide analogues obtained by chemical synthesis are extremely useful tools for studying a variety of processes in which these glycans are involved, among others recognition by antibodies to help diagnostic applications or vaccine development.

The field of carbohydrate chemistry has seen major advances in the last decades[101-103]. However, despite significant improvements, more research efforts are needed to improve efficacy of synthetic procedures and technologies in order to advance knowledge in the field of glycobiology[104]. Three main approaches for obtaining oligosaccharides can be employed: solution-phase synthesis, automated solid-phase synthesis and chemoenzymatic synthesis. Often these approaches tend to be applied separately depending on the target of the research project or the expertise of the research group. Starting from an overview of principles of carbohydrates synthesis, the three approaches will be discussed in the following chapters.

1.9.1. CHEMICAL GLYCOSYLATION REACTIONS

The most important chemical transformation in oligosaccharides synthesis is the reaction that forms the glycosidic bond[105]. In virtually all cases it is achieved by nucleophilic attack of a non-anomeric hydroxyl group from a sugar residue (the glycosyl acceptor) on the electrophilic anomeric carbon of another residue (the glycosyl donor) formed upon departure of a leaving group. An acetal is then formed with two possible stereochemical configurations, *alfa* or *beta* configuration (Fig 1-3). The product with axial configuration is generally viewed as lower in energy due to the Edward-Lemieux effect, or anomeric effect[106]. Different models have been proposed to explain this effect but the true reasons behind it are still a matter of debate[107].

Glycosylations display intricate mechanisms[108,109], the same transformation can proceed through several discrete pathways and more than one mechanism might occur at once. According to current understanding, the proposed mechanisms range through a whole spectrum from S_N1-like to S_N2-like reactions, going through contact ion pairs, solvent-separated ion pairs and covalent adducts[108,110-112]. Multiple factors are involved in determining the mechanism: type of leaving group, activator, solvent, temperature and additives[113]. Consequently, it is practically difficult to control which mechanism is operating under given conditions. A simplified mechanism is generally depicted as follows: the leaving group is first activated by interaction with the activator, then following its departure a cationic species is formed, which is attacked by the nucleophilic hydroxyl. An irreversible deprotonation leads then to the formation of the glycosidic bond. Under most employed conditions the final products are not interconverted through equilibrium and therefore no thermodynamic control can be established[110], however, exceptions exist[114,115].

The cationic intermediate can be viewed as a secondary carbocation (glycosyl cation) stabilized by delocalization from the adjacent oxygen, producing a resonance form of an oxonium ion. The true nature of this intermediate and even its existence has been subject of debate and research for over 30 years[116-121].

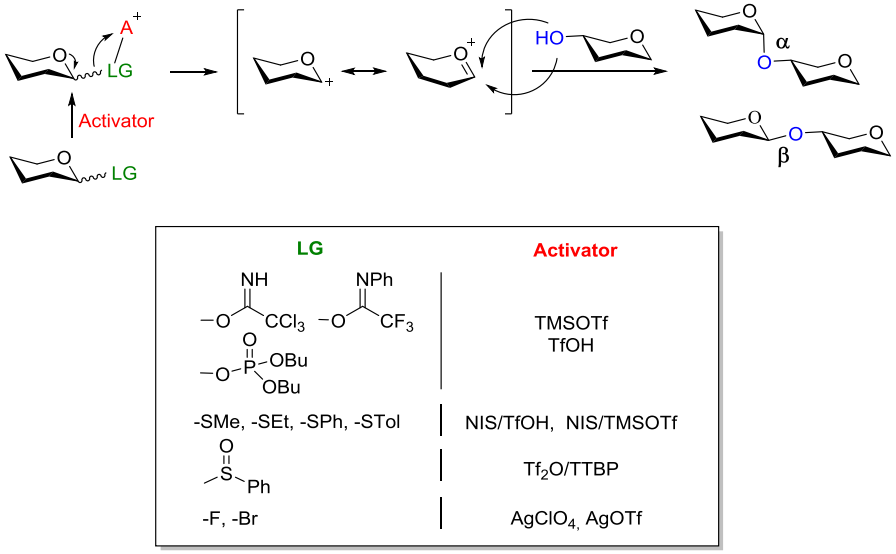


Figure 1-3: Generic mechanism of a chemical glycosylation and most common combinations of leaving groups (LG) and activators (A) presently employed.

Several classes of functional groups are employed today as appropriate leaving groups[105]. The most commonly employed groups are imidates, thioethers, phosphates and halides (Fig 1-3). These leaving groups necessitate an activator that facilitates breakage of the bond with the anomeric carbon. The type of promoter used is determined by the chemical nature of the leaving group and is often a Lewis or Brønsted acid. The choice of leaving group affects the synthetic route. Some are introduced at an early stage as stable functional groups and are maintained throughout several steps, such as in the case of thioethers. Others – imidates or phosphates – are more reactive and must be introduced with an additional step just before the glycosylation.

Regioselectivity in oligosaccharide synthesis is a challenge that arises from the chemical nature of this class of compounds. Carbohydrates are polyhydroxyaldehydes or ketones and in solutions form pyranose or furanose rings through their cyclic hemiacetal form, displaying several hydroxyl groups with almost identical nucleophilicity. In practice, differences in nucleophilicity exist but are rarely differentiated directly in glycosylations[98]. Hydroxyl groups or other nucleophilic functionalities are usually masked with protecting groups[105] through a complex stepwise sequence in which differences of nucleophilicity and steric hindrance are exploited to achieve regioselective installation of different groups at distinct positions of the sugar ring. A careful balance of orthogonalities between different protecting groups ensures that one single hydroxyl group will be exposed and used in the formation of the glycosydic bond. Later, upon selective removal of another group a hydroxyl at another position on the same or another sugar unit will become a nucleophile for a subsequent glycosylation. The choice is greatly complicated by the fact that protecting groups can either positively or negatively affect the reactivity of the reaction partners through electronic effects[122-124]. Stereoselectivity is also affected by other protecting-group related effects: steric or conformational constraints [125,126] or anchimeric assistance[105].

Careful design of protected monosaccharide building blocks is crucial[105]. As a general strategy, hydroxyls which will be used as nucleophiles in glycosylations or will be involved in later-stage modifications are protected with “temporary” protecting groups such as esters, carbonates, acetals or

silyl ethers. Hydroxyls neighboring anomeric centers are protected with ester groups to exploit anchimeric assistance in case a 1,2-*trans* configuration is required, otherwise as ethers. All other hydroxyls are converted into “permanent” protecting groups, commonly chemically inert benzyl ethers, and removed simultaneously in a late or terminal stage of the synthesis.
The introduction of protecting groups greatly reduces hydrophilicity of protected carbohydrates. For this reason, together with the fact that nucleophiles interfere with glycosylation, reactions need to be performed under an inert atmosphere in apolar aprotic solvents, commonly dichloromethane or toluene, with use of molecular sieves. Mixtures of solvents including ethers or acetonitrile are employed in order to exploit solvent participation in some specific transformations.

Stereoselectivity is of superior importance in glycosylation reactions[105]. Two distinct situations can be identified: glycosidic bonds can be in a *trans* relationship with the neighboring group in position 2 or conversely in a *cis* relation. In the fomer case very high, often complete stereochemical control can be obtained in their formation through anchimeric assistance (neighboring group participation) offered by ester or amide functionalities at the neighboring positon on the donors. This approach is commonly employed to install β glycosidic bonds on sugars of the D-*gluco* and D-*galacto* configurations and α glycosidic bonds on D-*manno* and L-*rhamno* derivatives.
Oppositely, the introduction of 1,2-*cis* glycosidic bonds represents a much larger synthetic challenge. Several strategies to improve stereochemical control have been developed by carbohydrate chemists over the years[127], although none of them of a level high enough for general application. Solvent effects, remote anchimeric assistance, chiral auxiliaries, conformational strain or variations in the electronic properties of the acceptor are just some examples of such methods (Fig. 1-4).

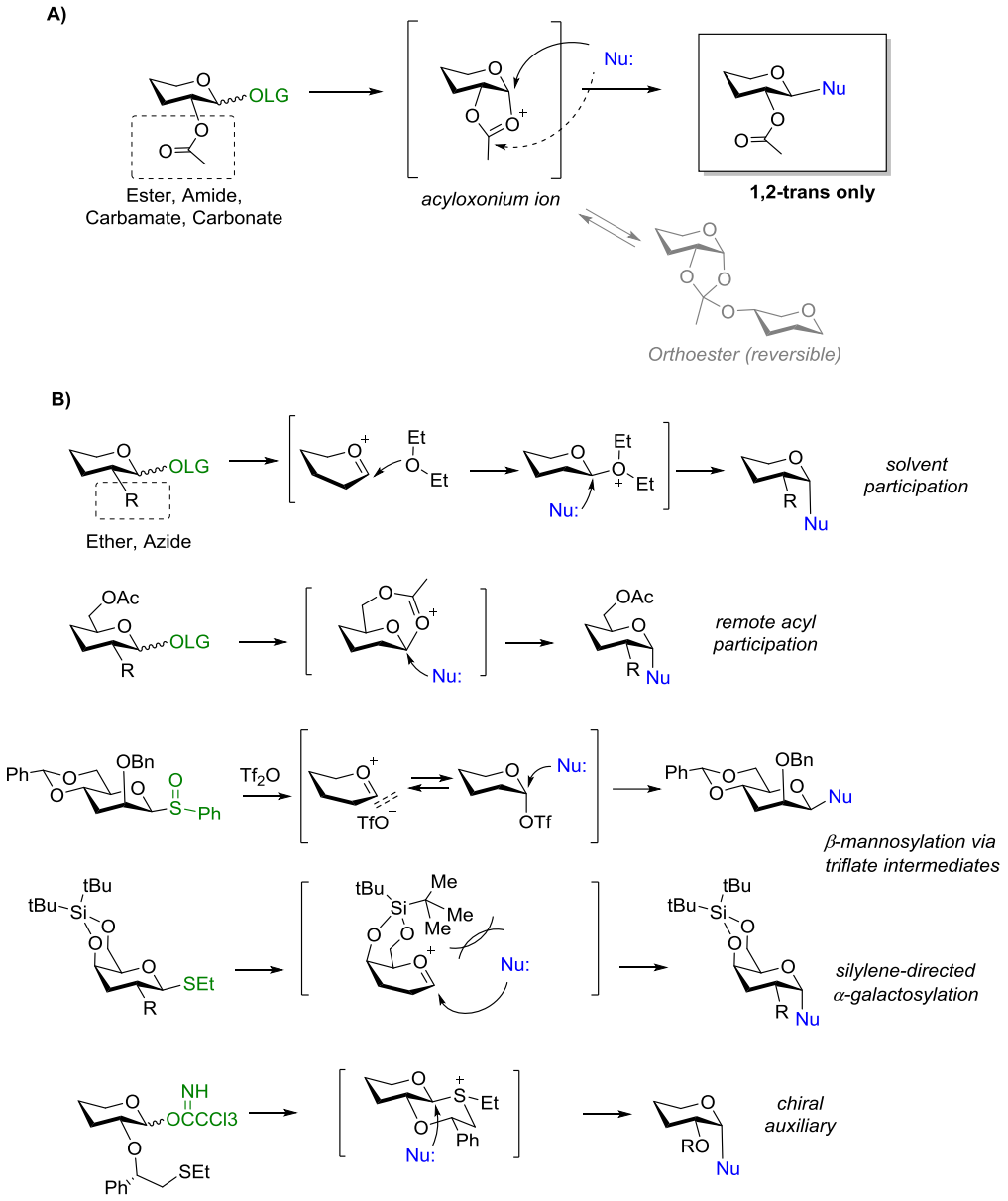


Figure 1-4: Selected examples of strategies to achieve stereoselective control over formation of A) 1,2-*trans* and B) 1,2-*cis* glycosidic bonds.

When planning an oligosaccharide synthesis, retrosynthetic analyses are facilitated since disconnections at glycosidic bonds are obvious. They must follow a "building-block oriented" approach[128] since sugars are readily available "chiral pools" and most synthetic efforts are directed towards their functionalization with protecting groups. As a result of all these considerations, oligosaccharide syntheses often involve a number of chemical steps comparable to the most complex syntheses of natural products[129], although the variety of chemical transformations is typically more limited. Finally, in most of the described synthetic routes amounts of oligosaccharides obtained are in the range of milligrams, enough for basic research or pre-clinical studies, but larger amounts needed for successive developments can be obtained through scaling-up[130].

1.9.2. AUTOMATED SOLID-PHASE SYNTHESIS

Similar to peptides and oligonucleotides, oligosaccharides are chemical compounds constituted by multiple repetitive units and can be assembled blockwise with a linear synthetic approach[99]. In the case of the former compounds, methods for solid-phase synthesis executed by automated systems are well established and widely employed[131,132]. On the contrary, automated solid-phase of oligosaccharides is a fairly recent technique. The first automated systems for carbohydrate synthesis were developed in the earliest years of this century[133], despite the earliest studies of solid phase synthesis of carbohydrates date back to the 1970s[134].

In solid-phase oligosaccharide synthesis, carbohydrate chains are synthesized on the surface of a solid support consisting in resin beads (Fig 1-5). The resin is typically functionalized with appropriate linkers offering nucleophilic sites for creating chemical bonds with monosaccharides through glycosylation. Similarly to peptide synthesis, the carbohydrate is protected on one hydroxyl group with a temporary protecting group such as Fmoc. After a step of selective deprotection by using a mild base, nucleophilic hydroxyls on resin-bound acceptors are exposed and subsequent glycosylations can attach another monosaccharide unit. In case branching needs to be introduced along the chain, an orthogonal protecting group such as a levulinyol ester is placed on a specific hydroxyl group of the sugar residue and can be cleaved to give a site for a second glycosylation. The sequence is repeated cyclically until the desired composition of the growing chain is reached. Afterward, the resin is separated from the reaction mixture and subjected to a chemical treatment that results in cleavage of the bond between the compound and the resin, releasing protected or semi-protected oligosaccharides to the solution phase. If needed, further purification can be performed with chromatographic techniques. Deprotection steps such as deacylations and hydrogenolysis are usually performed in solution phase.

The main advantage commonly attributed to solid-phase synthesis is that high overall yields can be obtained for long synthetic sequences. This is achieved mainly by using a large excess of donor in each glycosylation. Reactions can theoretically be driven towards completion reducing the formation of side-products, namely incomplete sequences arising from unsuccessful glycosylations. Several chemical steps (glycosylations, capping, deprotections) are performed sequentially as a single process and intermediate purifications are avoided since the excess of reactants is simply removed by washing steps. The final result is a more time-effective synthesis.

Automated synthesizers[99] can execute the entire assembly of the oligosaccharide from building blocks, limiting the operator's intervention. Bench work operations involve therefore synthesis of the protected donors and, after the automated synthesis, deprotections and purifications.

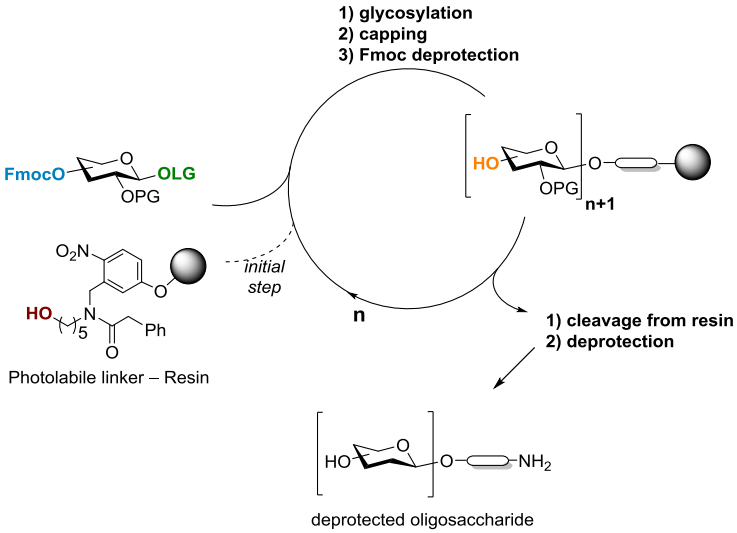


Figure 1-5: Schematic representation of a solid-phase synthesis of oligosaccharides.

The resin employed for automated glycan assembly (AGA)[99] is typically Merrifield resin (polystyrene-divinylbenzene cross-linked polymer), insoluble, inert in all reaction conditions and able to swell in the most common organic solvents.

Several classes of linkers have been employed: the most practically useful for AGA are photocleavable[135], methatesis-labile[136] and base-labile linkers[137]. The photocleavable linker has found most application (Fig 1-5). It is an *ortho*-nitrobenzyl-type linker cleavable by near-UV light irradiation in a photochemical reaction that gives optimal results when performed in a continuous-flow reactor[138]. These conditions are compatible with the survival of all protecting groups on the synthesized product. Moreover, after cleavage it leads to an aminopentyl spacer attached to the reducing-end monosaccharide, allowing for further use of the oligosaccharide in applications such as protein modifications, glycan microarrays or nanoparticle synthesis. Complementary to this, a photolabile linker that furnishes an unfunctionalized reducing-end sugar was recently developed[139]. Thereby, compounds can be obtained for applications where unnatural spacers are not needed and for further use of the products as donors in successive synthetic sequences.

Considerations regarding stereoselectivity issues in chemical glycosylations parallel those encountered in automated solid-phase synthesis. No general solution for stereoselective 1,2-*cis* glycosylations can be applied. However, strategies for some cases have been identified[140] and mostly rely on remote anchimeric assistance of protecting groups.

Optimized protocols for glycosylations using rare sugars often found in bacterial glycans are yet to be explored, together with the possibility of introducing functional groups by performing oxidations, reductions or nucleophilic displacements. The main bottleneck is represented by the long multistep syntheses required to access appropriate amounts of building blocks. Developments in this sense can be expected if in the future more semi-protected carbohydrates will increasingly become commercially available. For these reasons, syntheses of complex bacterial carbohydrates are still better performed using traditional solution-phase chemistry, where also more complex convergent syntheses can be performed[129]. However, provided the availability of automated synthesizers, solid-phase synthesis can

be considered the fastest choice for obtaining libraries of bacterial oligosaccharides of low and medium complexity.

1.9.3. ENYZMATIC SYNTHESIS

In nature, glycosidic bonds are formed with complete regio- and stereoselectivity by enzyme catalysts[141]. Two classes of enzymes have potential for use in oligosaccharides synthesis[104]: glycosidases (or glycosyl hydrolases) and glycosyltransferases. Glycosidases catalyze hydrolysis of glycosidic bonds through stereochemical inversion or retention with respect to the original stereochemistry of the donor. In the case of retaining glycosidases, they can perform transglycosylation by accepting an alcohol as acceptor instead of a molecule of water. These enzymes have not been employed extensively for synthetic purposes[104]. However, synthetically useful mutated variants of glycosidases have been recently created. Glycosynthases are retaining hydrolases obtained by site-directed mutagenesis[142]; substitution of one single nucleophilic residue in the active site allows irreversible glycosylations using glycosyl fluorides as donors.

Glycosyltransferases are more frequently employed in chemoenzymatic synthesis[104,143]. Most enzymes of this class catalyze the transfer of one monosaccharide unit, activated as sugar nucleotide, to an acceptor which consists often in a glycoside, to create oligo- and polysaccharides, or an aminoacid residue, to start sequences of *N*- or *O*-glycans on glycoproteins[141]. Similarly to hydrolases, they are known to give either inverting or retaining stereochemical outcomes but detailed mechanisms for some of these enzymes are less clearly understood[143]. Glycosyltransferases guarantee higher yields and complete stereoselectivity at the expense of a higher specificity for the substrates they can accept[143], therefore limiting their synthetic scope to structures resembling products they generate *in vivo*. Structural variation on the acceptor can be tolerated to a certain degree, especially by bacterial glycosyltransferases, while the use of unnatural sugar donors might result in a loss of activity[144]. Glycosyltransferases have been employed in several syntheses of human glycans[104,145] and glycopeptides[146,147]. Recently, automated systems able to perform fully enzymatic oligosaccharide syntheses are being developed[148].

Arguments in favor of chemoenzymatic approaches can be briefly summarized as follows: enzymatic reactions are completely regio- and stereoselective due to the high specificity of the catalyst; they are performed in water solutions in very mild conditions; syntheses are more straightforward as they are not based on protection-deprotection sequences. On the other hand, limited availability and cost of both enzymes and sugar nucleotide donors are major drawbacks that still limit the widespread use of this methodology.

Due to the previously mentioned factors, especially the limited substrate scope, complete chemoenzymatic approaches for total synthesis of bacterial oligosaccharides seem impractical. The use of enzymes can be considered a complementary solution to solve stereoselectivity issues encountered in specific glycosylations within complex synthetic routes. The most representative example is sialylated oligosaccharides. A general solution to obtain full stereoselectivity in the formation of sialosides by chemical synthesis has not been developed. The use of sialyltransferases,

where applicable, represents a powerful method for obtaining this class of compounds[149]. Examples of syntheses using combinations of solution-phase[149-151] or automated solid-phase synthesis[152] and enzymatic sialylations with sialyltransferases have been described. Such approach was pursued also in this work as described in Chapter 3.

1.10. AIMS OF THE THESIS

The overall goal of this work was to contribute to the rational design of new glycoconjugate vaccines with the use of synthetic organic chemistry tools. Small focused libraries of oligosaccharides related to capsular polysaccharides of pathogenic bacteria were synthesized and can be employed to elucidate minimal epitopes of anti-carbohydrate antibodies in glycan microarray experiments. Synthetic oligosaccharides emerging as hits from these experiments can be chemically conjugated to carrier proteins and the immunogenic properties of the resulting glycoconjugates evaluated in animal models.
In particular, research was carried out on two main serotypes of *Steptococcus suis*, an important pig pathogen for which an effective vaccine is not currently available. Synthesis routes designed to obtain five oligosaccharides related to the CPS of *S. suis* serotype 2 are described in Chapter 2. The synthesis of three oligosaccharides related to the CPS of *S. suis* serotype 14 is described in Chapter 3 and was accomplished with different chemical approaches. Finally, the Supplementary section describes the synthesis of sub-structures related to the CPS from the human pathogen *Streptococcus pneumoniae* 7F. The library of compounds was screened in a glycan array experiment and used to identify structural elements of a minimal glycan epitope of human antibodies.

CHAPTER 2

SYNTHESIS OF OLIGOSACCHARIDES RELATED TO *STREPTOCOCCUS SUIS* SEROTYPE 2 CAPSULAR POLYSACCHARIDE

2.1. STREPTOCOCCUS SUIS SEROTYPES

To date, 35 different serotypes of *Streptococcus suis* have been identified. The subdivision of the species into serotypes is based on the diverse chemical compositions of bacterial capsules. As mentioned in Section 1.1, serotype distribution follows geographical trends, but according to the most recent report[20], serotype 2 is the most often found in diseased pigs worldwide, followed by serotypes 9 and 3. Moreover, serotype 2 is the most common serotype causing human infections globally[20].

In recent years, *S. suis* has gained recognition as an important animal pathogen and a number of studies aimed at determining structures of CPSs from important serotypes have been carried out[141,153-155]. By using a combination of NMR experiments, CPS structures of the major serotypes have been elucidated and are shown in Fig. 2-1.

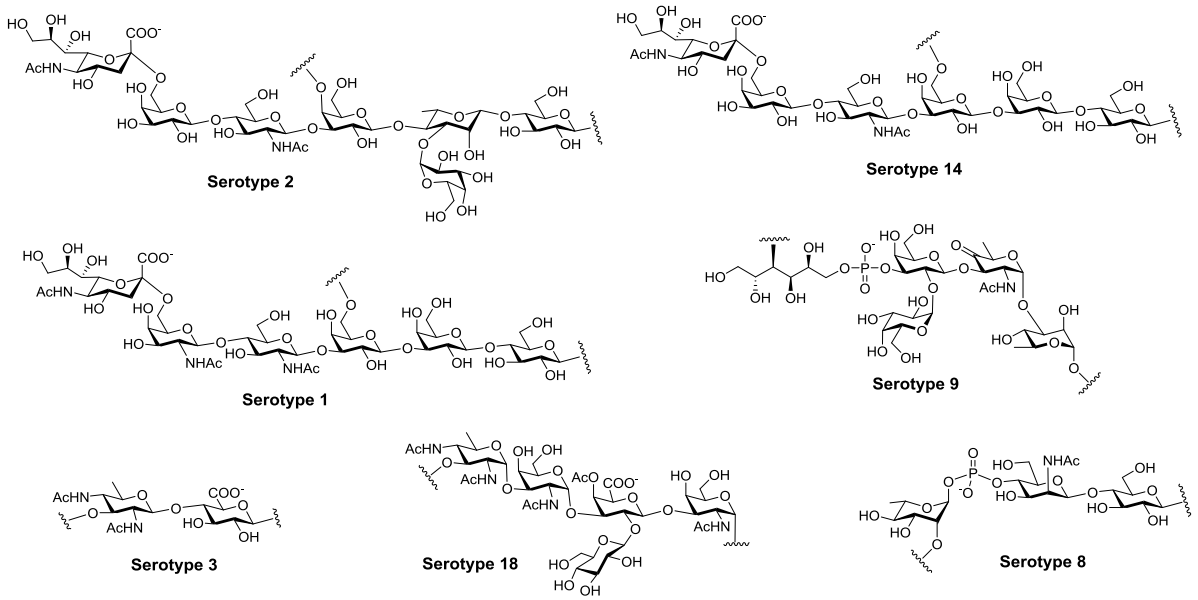


Figure 2-1: Chemical structures of CPSs from common *S. suis* serotypes.

As commonly observed with other encapsulated bacteria, the CPSs of *S. suis* are polysaccharides of high complexity. They normally include rare sugars, a variety of glycosidic linkages, often anionic charges and sometimes peculiar functional groups such as acetyls or phosphodiester bridges. Interestingly, a high structural similarity was found between the structures of serotypes 1, 2, 1/2 and 14.

2.2. SEROTYPE 2 CAPSULAR POLYSACCHARIDE

The structure of the CPS from serotype 2 was determined in 2010[155] and consists of branched heptasaccharide repeating units of sequence [→4)[α-Neu5Ac(2→6)-β-D-Gal(1→4)-β-D-GlcNAc(1→3)]-β-D-Gal(1→4)-[α-D-Gal(1→4)]-β-L-Rha(1→4)-β-D-Glc(1→] (Fig. 2-2). Four sugars constitute the backbone of this polysaccharide and a β1→4 glycosidic linkage between glucose and galactose within this sequence connects the repeating units forming the polysaccharide. A trisaccharide side-chain is connected to the backbone and consists of a lactosamine (galactose linked 1→4 to glucosamine) terminating with an *N*-acetyl neuraminic acid (Neu5Ac) linked α2→6 to galactose.

This polysaccharide presents structural and composition similarities to CPSs from Group B *Streptococcus* (GBS), for example in the backbone sequence β-Gal(1→4)-β-L-Rha(1→4)-β-D-Glc, identical to GBS type VIII, and the peculiar sialylated side-chain present also in several GBS serotypes. However, *S. suis* serotype 2 displays Neu5Ac linked to galactose with an α2→6 connectivity rather than α2→3.

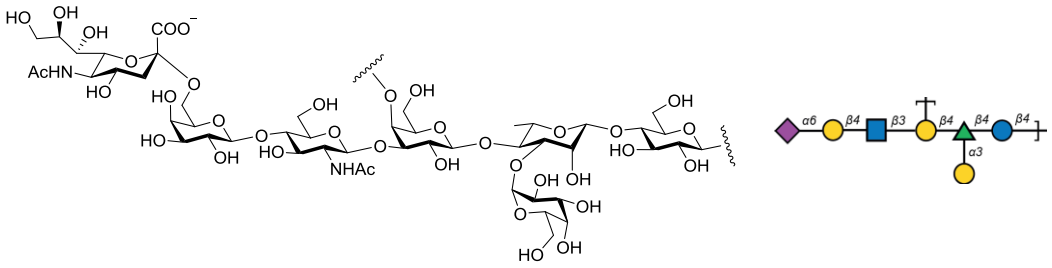


Figure 2-2: Characterized structure of serotype 2 CPS repeating unit.

Sialic acids are a family of nine-carbon sugars widely present as terminal residues in glycoconjugates on the surfaces of mammalian cells, but a small number of pathogenic bacteria also possess them in their surface[156]. The reason behind the presence of glycans resembling mammalian antigens on bacteria is not fully understood but hypotheses have been advanced. The presence of sialic acids could be useful for the survival of the pathogens, since the mimicking of host self antigens retards activation of the immune system and hides underlying antigens from recognition[156]. It has been demonstrated that the CPS from serotype 2 is fundamental for its virulence as it can prevent phagocytosis when the bacterium infiltrates the bloodstream[157].

2.3. CAPSULAR POLYSACCHARIDE AS VACCINE TARGET

Over the last three decades vaccine candidates against *S. suis* serotype 2 have been proposed[158]. The first serotype-specific vaccines to be evaluated were killed whole-cell formulations and these vaccines showed unsatisfactory results. Very low or undetectable levels of antibodies were produced and no significant protection from infection was observed in pigs[158]. Several subunit vaccines made from proteins were proposed and tested for their immunogenic properties with different adjuvant systems[158] but few were thoroughly investigated for protection *in vivo*[159]. These vaccines have

potential for cross-protection, as some proteins are expressed in several serotypes, but stronger evidence has yet to be given.

Between the antigen candidates, the CPS is considered the most promising[158,160]. Despite being poorly immunogenic, low levels of anti-CPS antibodies were seen in pigs after infection[161] or immunization[160], it can induce protective antibodies[162,163] for the most part of the IgM subclass. These observations led to a study in which for the first time a glycoconjugate as vaccine against *S. suis* serotype 2 was evaluated[51]. Capsular polysaccharides isolated from bacterial fermentations were depolymerized and linked via reductive amination to tetanus toxoid (TT). The resulting glycoconjugate was evaluated in immunization experiments in mice and pig models. When the immunogenicity was tested, it was found that significantly higher antibody titers were induced in both models by vaccinating with the conjugate vaccine compared to the plain CPS. Importantly, production of high-affinity IgG antibodies was observed. The induced antibodies were useful to achieve protection: their protective capacity was demonstrated with an opsonophagocytic killing assay (OPA), a technique that assesses the ability of antibodies to induce bacterial killing *in vitro*, which can correlate with protection *in vivo*. As final proof, in a challenge study pigs that received immunization with the glycoconjugate vaccine showed good levels of protection against a systemic infection. The results in terms of survival levels were, however, not significantly different from those conferred by an inactivated whole-cell vaccine.

This important study represented the first proof-of-principle study evidencing that the poorly-immunogenic nature of the native CPS can be overcome and that protection against *S. suis* can be achieved by active immunization with a glycoconjugate vaccine. Even though the achieved results were not optimal, the study left room for improvement since several parameters can be reconsidered. Some variables that are likely to influence the outcome of vaccination are the type of protein carrier, a different conjugation strategy, and different sugar-to-protein ratios. Moreover, the structure of carbohydrate epitopes responsible for the production of protective antibodies is still unknown. The elucidation of such structures serves as the basis for evaluating structure-immunogenicity relationships and designing optimized carbohydrate antigens. Few studies were conducted in this regard and relied on analyses performed using the native CPS, and produced inconclusive results. It was first found that after removal of sialic acid residues from the native CPS by hydrolysis, either a monoclonal antibody or polyclonal mouse serum maintained their ability to bind the CPS. This suggested a non-prominent role of the sialic acid in the epitope of such antibodies[164]. In a subsequent study[153] on cross-reactions between structurally related CPSs from serotypes 1, 2, 1/2 and 14 (Fig. 2-3) it was noted that a polyclonal rabbit sera against serotype 2 was able to recognize to a lower extent only the CPS from serotype 1/2. The latter is almost identical to serotype 2 except for an *N*-acetyl galactosamine residue on the side-chain replacing the galactose. In addition, this serum recognized only weakly the desialylated CPS. No cross-reaction was observed with serotype 14, containing a different backbone and an identical side-chain. These results suggested that the side-chain, including the sialic acid, is possibly an important sequence forming epitopes of the predominant antibody population and that anti-backbone antibodies were rare. However, the lack of cross-reactivity with serotype 14 was seen as a sign of conformational differences affecting the binding.

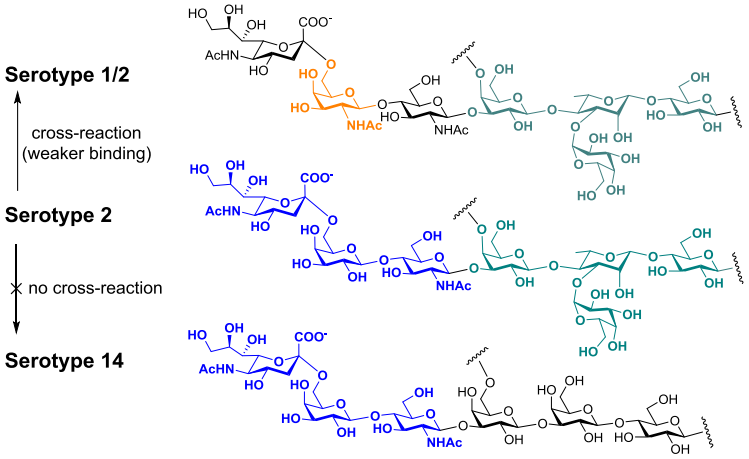


Figure 2-3: Cross-reactivity of anti-serotye 2 polyclonal rabbit serum as observed in Ref. 153.

Later, the same approach was followed to study epitopes of monoclonal antibodies generated after immunization of mice with the above described serotype 2 glycoconjugate[165]. After examining cross-reactions between serotypes 1, 2, 1/2 and 14, an unclear specificity pattern emerged as three IgM antibodies showed different cross-reactivities and one IgG showed no cross-reactions. In this case, all antibodies did not recognize the native CPS if the sialic acid residues were hydrolyzed and the authors concluded that the sialylated side-chain represents a dominant sequence of antibody epitopes. In addition, monoclonal antibodies were evaluated for their protective potential with passive immunization in mice and significant levels of protection were noted only with two antibodies at high doses.

The empirical evidence obtained from these studies can be confirmed and brought to a more detailed level by using synthetic oligosaccharides related to the CPS, which can help a more exact determination of antibody epitopes.

2.4. RESULTS AND DISCUSSION

Five fragments of the repeating unit of serotype 2 CPS were designed in order to obtain detailed structural information of antigenic epitopes of antibodies from *S. suis*-infected pigs. To determine whether antibody binding involves specific parts of a repeating unit to different extents, three shorter fragments were included. They represent two distinct portions obtained by ideally cutting the heptasaccharide shown in Fig. 2-4 unit along the **D**-**B** (βGal→βRha) linkage. Trisaccharide **2-1** represents the backbone sequence up to the α galactose residue, while compounds **2-2** and **2-3** represent the side-chain. To evaluate if the sequence to which antibodies bind is more extended and structurally more complex, pentasaccharide **2-4** and hexasaccharide **2-5** were also included in the library. These oligosaccharides cover almost entirely the length of one repeating unit and represent branched sequences. Compounds **2-2** and **2-4** differ from **2-3** and **2-5** respectively only in the presence of a terminal *N*-acetyl neuraminic acid. This difference could be used to determine if this sugar is directly playing a role in the binding.

All the compounds were synthesized carrying an aminopentyl spacer at the reducing end sugar, a linker commonly used in carbohydrate chemistry to obtain compounds ready for creating microarrays or for protein conjugations.

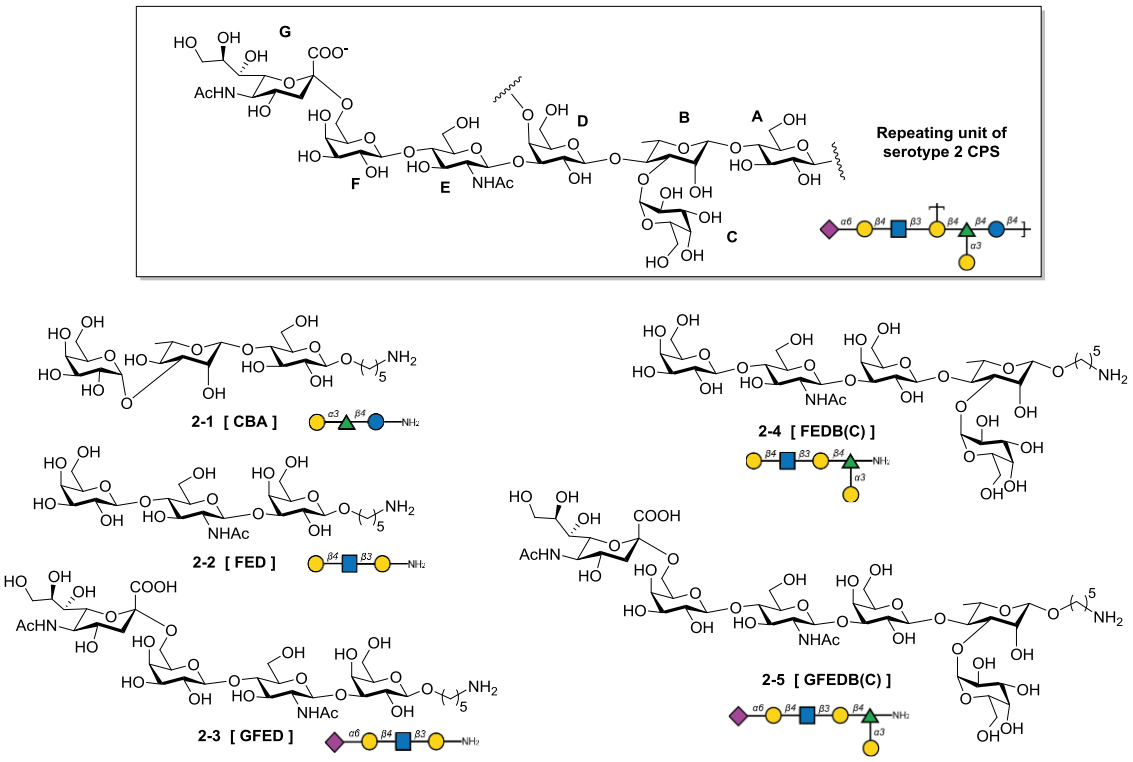


Figure 2-4: CPS repeating unit and synthesized sub-structures.

Since the repeating unit of this CPS is composed by five monosaccharides (D-Glc, D-Gal, L-Rha, D-GlcNAc, D-Neu5Ac), all connected through different glycosidic linkages, a minimum number of seven orthogonally protected monosaccharide building blocks, shown in Fig. 2-5, were identified as targets. Two 1,2-*cis* glycosidic bonds, a branching point on L-rhamnose, together with the α sialyl linkage, constituted considerable synthetic challenges. Some of the building blocks from this initial set were later substituted or adapted as described in the following Sections. Monosaccharide donors **2-6**, **2-9**, **2-10** and **2-11** were commercially available while other building blocks were prepared by chemical synthesis.

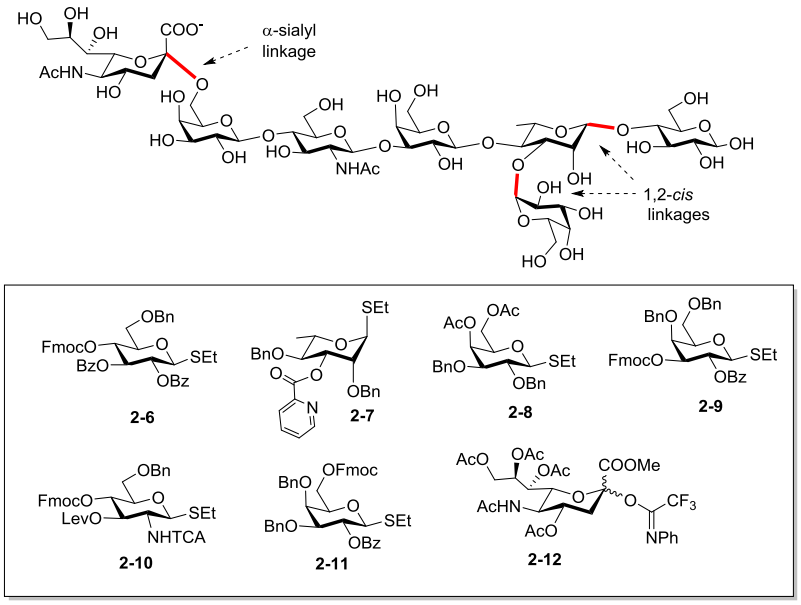


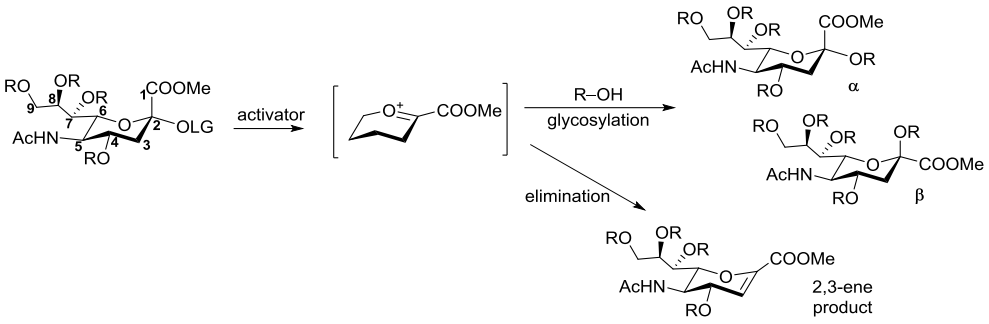
Figure 2-5: Initial set of building blocks.

The β rhamnosidic and the α sialyl linkages represent two particularly difficult bonds to be formed through chemical synthesis. β Rhamnosidic linkages are fairly uncommon in natural carbohydrates and a limited number of syntheses of rhamnose-containing bacterial oligosaccharides have been reported. Several strategies to provide stereocontrol in chemical rhamnosylation have been explored, including solvent effects[166], promoter effects[167], unusual protecting group patterns on donors[167,168], and special acceptors giving unconventional mechanisms[169]. However, most studies report glycosylations with structurally simple acceptors and none of them have resulted in a universal methodology applicable to the synthesis of more complex oligosaccharides.

Recently, another strategy for the formation of 1,2-*cis* glycosidic bonds was introduced and relies on the effect of protecting groups in the form of esters or ethers of pyridine derivatives[170]. According to the proposed mechanism, the nitrogen atom on these groups acts as a hydrogen bond-acceptor. In apolar solvents donor and acceptor can reversibly form pairs connected through a hydrogen bond between the hydroxyl group of the acceptor and the pyridine nitrogen on the donor. The directionality of the hydrogen bond consequently orients the approach of the nucleophile from one of the two faces of the oxocarbenium ion formed upon donor activation, resulting in the formation of a glycosidic bond *cis* with respect to the orientation of the "directing" group. This strategy was therefore named "H-bond mediated aglycone delivery" and was explored to stereoselectively form 1,2-*cis* glycosidic bonds for both glucose and mannose. Recently, syntheses of oligosaccharides containing β rhamnose have employed this methodology and showed high stereoselectivities[171,172], demonstrating the relevance of this method for forming this glycosidic bond.

Similarly, a method for completely stereoselective formation of α sialyl glycosidic bonds is lacking. Sialylation represents perhaps the most complicated chemical glycosylation[173] for a combination of reasons: sialic acid donors do not have a hydroxyl neighboring the anomeric center so no anchimeric assistance can be exploited; upon donor activation the oxocarbenium ion is destabilized by the neighboring electron-withdrawing ester and the attack of the nucleophile must occur on a sterically

hindered tertiary carbon; finally, the α configuration (the exclusive configuration found in nature) corresponds to an equatorial glycosidic bond, higher in energy when compared to the axial bond, as mentioned in Section 1.9.



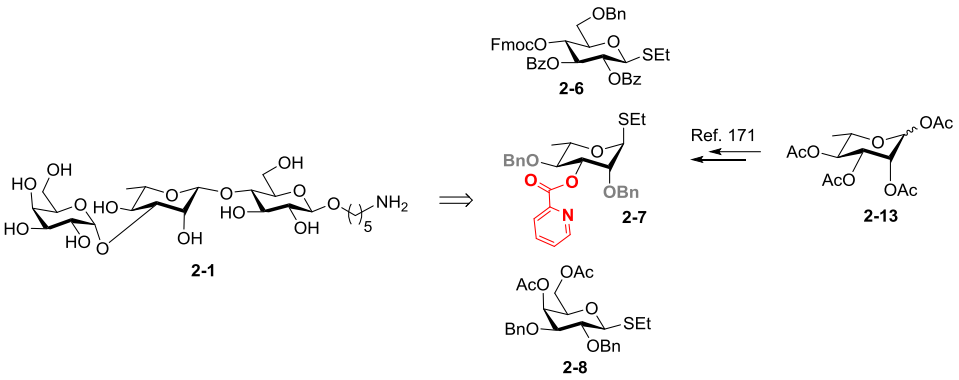
Scheme 2-1: General glycosylation with a sialyl donor.

Important parameters that can be tuned to improve stereoselectivity of sialylations are the type of leaving group and the nature of the protecting groups. It was found that higher levels of α product were formed when sialic acid donors were protected with electron-withdrawing groups. An α-directing effect was observed when donors were protected with fused oxazolidinone rings[174]. This is believed to be a consequence of favorable dipole alignments which lower the potential energy of the α diastereoisomer[175]. Lower temperatures will increase the formation of the kinetically favored α product, therefore good leaving groups such as phosphates or imidates guarantee optimal results[149,173,176] but high levels of stereocontrol are not always achieved. The obtained diastereoisomers often show very similar chromatographic behavior, resulting in complex purifications that can affect yields. To complicate things further, an acid-catalyzed elimination giving a conjugate ester competes with glycosylation and leads to an often observed side-product (Scheme 2-1).

2.4.1. SYNTHESIS OF LINEAR OLIGOSACCHARIDES

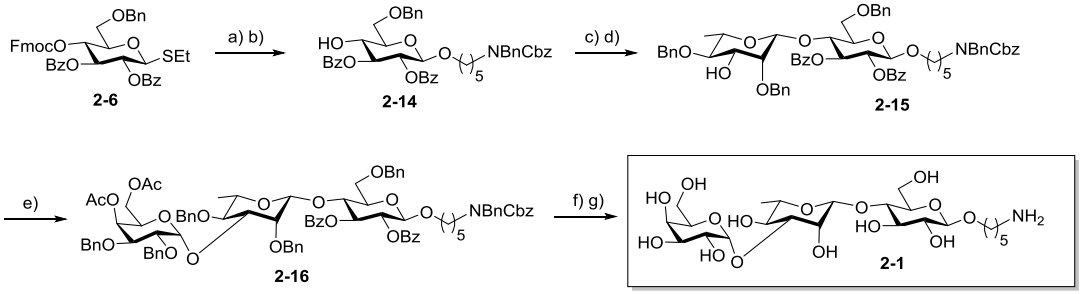
Preparation of **2-1** required a linear synthesis using three monosaccharide building blocks (Scheme 2-2). Rhamnose building block **2-7** was synthesized according to reported protocols[171]. To perform a glycosylation that can introduce the following α galactose unit, the C-3 on this donor needs to display a group that can be selectively deprotected over the other two positions. A 2-pyridinecarbonyl ester (picoloyl ester – Pico) was chosen, also to exploit the H-bond stereodirecting effect discussed above. The other two positions were protected with permanent benzyl ether groups.

The non-reducing end galactose had to be introduced with α configuration. Therefore, known donor **2-8**[140], equipped with acetyl esters at C-4 and C-6 and benzyl ethers at C-2 and C-3, was used. This protecting group pattern was used to ensure high levels of α stereoselectivity in both solution phase[172] and solid phase syntheses[140], possibly through a combination of remote participation and solvent effects.



Scheme 2-2: Retrosynthesis of 2-1.

Next, the assembly of **2-1** (Scheme 2-3) started with the introduction of the spacer at the reducing end monosaccharide with a glycosylation between *N*-protected aminopentanol and donor **2-6**. Without further chromatographic purification, the Fmoc group was removed to obtain **2-14**. The monosaccharide acceptor was then glycosylated with donor **2-7** by employing high dilution conditions (10 mM). These conditions should decrease the probability of acceptors attacking non-hydrogen bound donors, resulting in a loss of the stereodirecting effect of the picoloyl ester. β-Linked disaccharide **2-15** was obtained in two steps after glycosylation and picoloyl ester hydrolysis. Presumably traces of α-linked disaccharide were formed, but only on an undetectable level. Acceptor **2-15** was glycosylated with donor **2-8** in a DCM/Et_2O mixture to increase α selectivity. Only the α-linked product was detected on TLC and isolated. Finally, protected trisaccharide **2-16** was fully deprotected by ester hydrolysis with NaOMe in MeOH followed by catalytic hydrogenation, obtaining trisaccharide **2-1**.

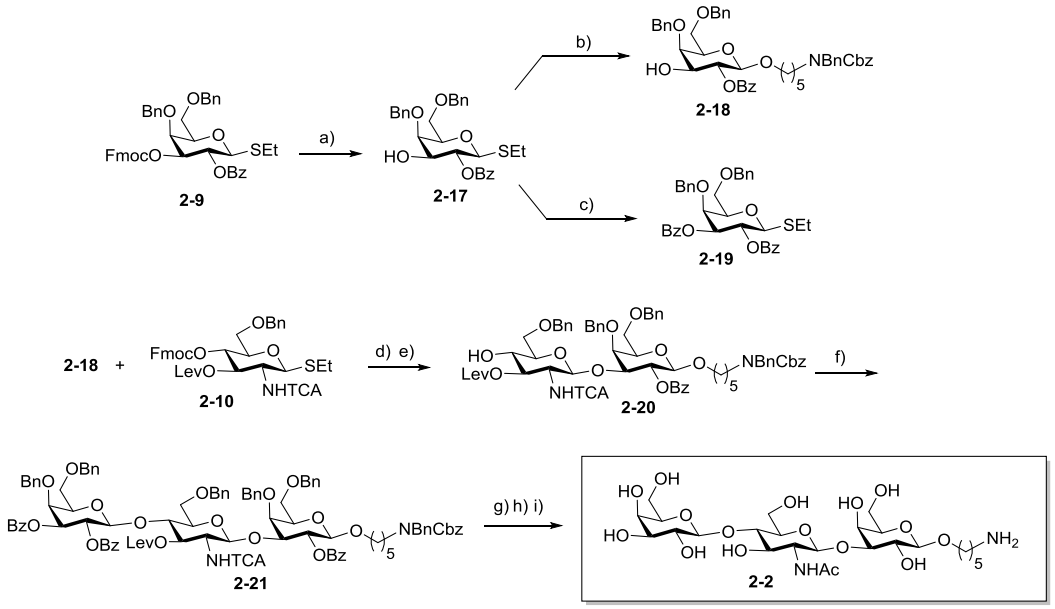


Scheme 2-3: Synthesis of 2-1. Reagents and conditions: a) $HO(CH_2)_5NBnCbz$, NIS, TfOH, DCM, –15 °C; b) TEA, DCM, 52% over two steps; c) **2-7**, NIS, TfOH, DCM, –30 °C; d) $Cu(OAc)_2{\cdot}H_2O$, DCM/MeOH 2:1, 60% over two steps; e) **2-8**, NIS, TfOH, DCM/Et_2O 1:1, –15 °C, 62%; f) NaOMe, MeOH/THF 1:1; g) H_2, Pd/C, EtOAc/tBuOH/H_2O/AcOH 2:1:1:0.1, 40% over two steps.

The synthesis of trisaccharide **2-2** also proceeded linearly using three easily accessible building blocks (Scheme 2-4). First, commercially available galactose building block **2-9** was the starting point for obtaining both **2-18** with an attached spacer and also thioglycoside **2-19** which was used as donor to attach at the terminal position.

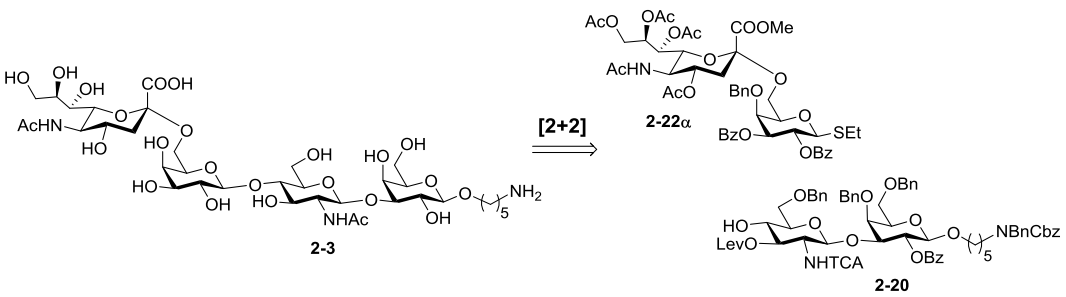
Galactose **2-17** was obtained by simple removal of the Fmoc group on **2-9** and then directly employed in a glycosylation with *N*-protected aminopentanol and by exploiting the higher nucleophilicity of the primary alcohol at low temperature, **2-18** was obtained and could be employed directly in the following

transformation. Then, galactose **2-18** was glycosylated with commercially available glucosamine **2-10**, and following deprotection of the Fmoc group, disaccharide **2-20** was obtained. It should be noted that the levulinoyl ester on **2-10** was not intended as a protecting group to introduce a branching but instead this monosaccharide was chosen as the most accessible glucosamine donor at the time of the synthesis. The disaccharide was finally glycosylated with galactose **2-19** to obtain a fully protected trisaccharide. Compound **2-21** was then deacylated with sodium methoxide in methanol at 35 °C. During this step, mass spectrometry monitoring of the reaction revealed partial hydrolysis of the trichloroacetamide, likely caused by the large excess of base employed, therefore an intermediate step of *N*-acetylation had to be performed. Finally, catalytic hydrogenation was removed all ethers and afforded deprotected trisaccharide **2-2**.



Scheme 2-4: Synthesis of 2-2. Reagents and conditions: a) TEA, DCM, 85%; b) $HO(CH_2)_5NBnCbz$, NIS, TfOH, DCM, –50 °C, 83%; c) Bz_2O, TEA, DCM, 90%; d) NIS, TfOH, DCM, –30 °C; e) TEA, DCM, 84% over two steps; f) **2-19**, NIS, TfOH, –15 °C, 66%; g) NaOMe, MeOH/THF 4:1, 35 °C; h) Ac_2O; i) H_2, Pd/C, iPrOH/H_2O/AcOH 3:1:0.1; 74% over two steps.

A convergent strategy based on a [2+2] glycosylation was followed to assemble tetrasaccharide **2-3**. Previously synthesized compound **2-20** was selected as the disaccharide acceptor. As donor, disaccharide **2-22α**, containing a preinstalled α sialyl glycosydic bond was designed.

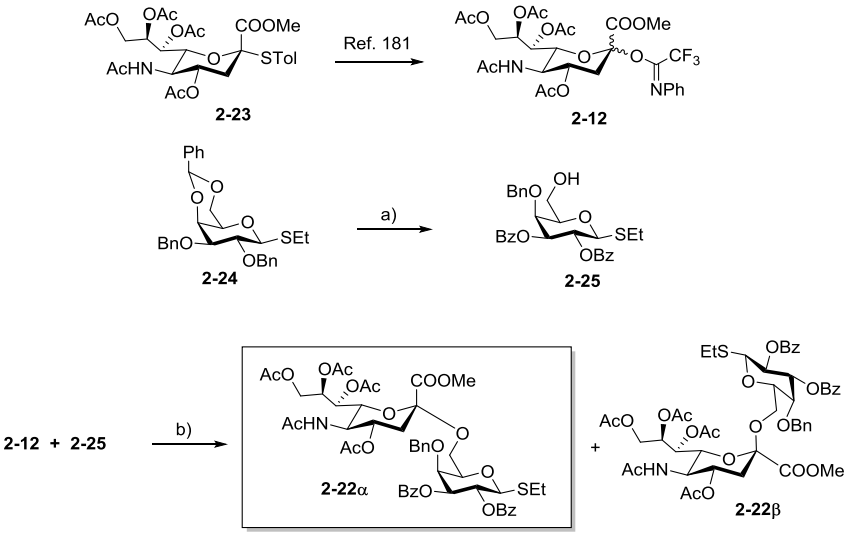


Scheme 2-5: Retrosynthesis of 2-3.

To obtain disaccharide **2-22α**, the first step involved selection of an appropriate sialic acid donor for glycosylation with a galactose acceptor. Phosphate donors bearing oxazolidinone protecting group are

considered the most efficient to achieve higher diastereoselectivities. It was considered that good results are also reported for simpler protecting group patterns[177-179] and that these donors require few additional synthetic steps to introduce the oxazolidinone ring. A peracetylated sialic acid methyl ester was therefore preferred.

Considering that ideally the donor should be activated at very low temperature, phosphates or imidates have to be employed as leaving groups. Traditionally, phosphates are preferred due to their higher stability. However, the necessity of using stoichiometric amounts of strong Lewis acids for activation, together with the fact that considerable differences in reactivity between the α and β phosphate anomers are reported[176], led to the choice of an *N*-phenyl trifluoroacetimidate as leaving group. Few reports describe the use of imidate sialic acid donors, showing high levels of α steroselectivity[177,180]. Due to the availability of thioglycoside **2-23** at the time of the synthesis(†), known donor **2-12**[180] was prepared using reported procedures. A mixture of anomers was obtained and used as such in glycosylations, as differences in reactivity were not reported in the literature. Galactose acceptor **2-25** was readily obtained from known **2-24** after regioselective opening of the benzylidene acetal.



Scheme 2-6: Synthesis of disaccharide 2-22. Reagents and conditions: a) BH_3-THF, TMSOTf, 80%; b) TMSOTf, DCM/CH_3CN 1:1, −60 °C, 62% α, 10% β.

Acetimidate groups can be chemoselectively activated in the presence of thioethers, therefore thioglycoside **2-25** was used as acceptor and glycosylated with sialyl donor **2-12**. TMSOTf was used as activator in a 1:1 DCM/acetonitrile solvent mixture. Nitrilic solvents have a beneficial effect as they can participate in the mechanism through an intermediate "axial" ion pair and favor the formation of equatorial bonds. To maximize the effect, sialylations can be performed in pure acetonitrile, but in this case the use of DCM was necessary to solubilize the reactants. Surprisingly, donor **2-12** could not be activated at −78 °C, temperature at which many imidate donors are reactive. No formation of products was observed by TLC, even after prolonged reaction times, for temperatures up to −60 °C. Likely, the electron-withdrawing effect of the esters results in deactivation of the donor and opposes the increase

† Compound 2-23 was synthesized by Dr. Chian-Hui Lai

in reactivity guaranteed by the leaving group. The reaction was therefore performed at −60 °C and proceeded slowly, reaching completion only after 2 h. Isolation of the pure α diastereoisomer from a complex crude mixture proved challenging and a careful purification by chromatography on silica eventually gave pure sialylated galactosides **2-22α** and **2-22β** in a 6:1 ratio.

The configuration of glycosidic bonds is commonly deducted from values of $J_{H\text{-}1,H\text{-}2}$ or $J_{C\text{-}1,H\text{-}1}$ in NMR measurements. This method is not applicable to sialic acids since their anomeric center is a quaternary carbon. Instead, an indicator of the configuration is the chemical shift of the equatorial H-3. It was empirically observed that its signal, which appears as *dd*, it is often found at δ 2.6-2.5 ppm in α-sialyl configurations, while in β isomers the signal is shifted to higher fields, commonly around δ 2.1-2.0 ppm[181]. Axial H-3 appears as a triplet at δ 1.9-1.8 ppm, often overlapped with singlets given by acetyl groups. A more accurate determination relies on measuring three bonds C,H-couplings, which follow the Karplus relationship. Sialic acid derivatives are predominantly found in a $^2C_5$ chair conformation, therefore coupling between C-1 and the axial H-3 in an α configuration would result in a $J_{C\text{-}1,H\text{-}3ax}$ of 6-7 Hz while a β configuration would give values <1 Hz [182]. As an additional indicator, $J_{C\text{-}2,H\text{-}3ax}$ is commonly in the range of 7-8 Hz for α linkages compared to smaller values of about 3-4 Hz for β linkages[183].

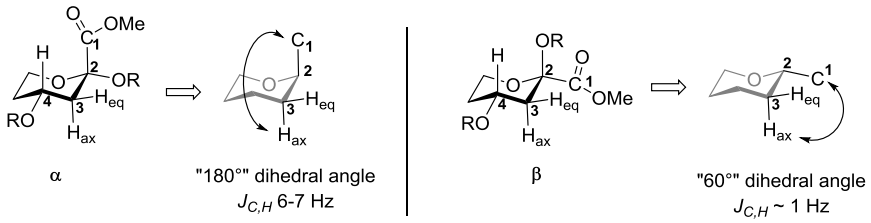


Figure 2-6: Correlations between configuration and heteronuclear coupling in sialic acid derivatives.

Configuration of the disaccharides was unequivocally determined by measuring the long-range $J_{C\text{-}1,H\text{-}3ax}$ using EXSIDE[184]. This bidimensional technique creates cross peaks, appearing in the same position as in an HMBC spectrum, split along the carbon dimension by a value that is correlated to the coupling constant. For practical reasons a scaling factor is introduced in the experiment, acting as a multiplier to increase splitting and allowing easier interpretation. The relation becomes therefore: *splitting* (Hz) = *scaling factor* * $J_{C,H}$ (Hz).

As shown in Fig. 2-7, an HMBC spectrum revealed chemical shifts of C-1 and C-2 of the sialic acid and the long-range correlation between C-1 and the axial H-3. Both signals of C-1 (δ 168.0 ppm) and axial H-3 (δ 1.96 ppm) were clearly separated from adjacent peaks in the respective spectra, simplifying the analysis. The major product was found to have a $J_{C\text{-}1,H\text{-}3}$ of 6.3 Hz and a $J_{C\text{-}2,H\text{-}3}$ of 7.8 Hz and was defined as the α-linked disaccharide. A similar NMR analysis was not performed on the minor product, which gave a very similar ^{1}H-NMR spectrum with an equatorial H-3 as a *dd* at δ 2.26 ppm, and an *m/z* value identical to the major product and was therefore identified as the β-linked disaccharide.

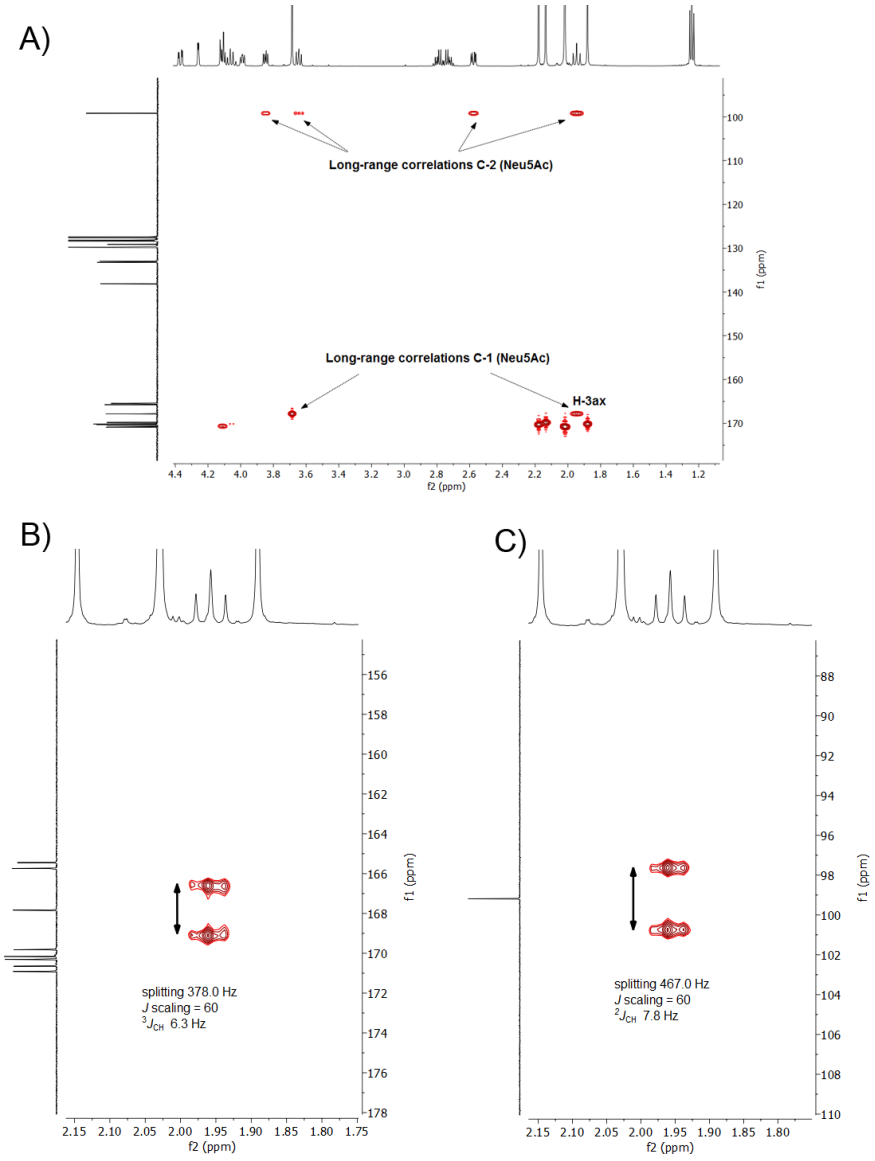
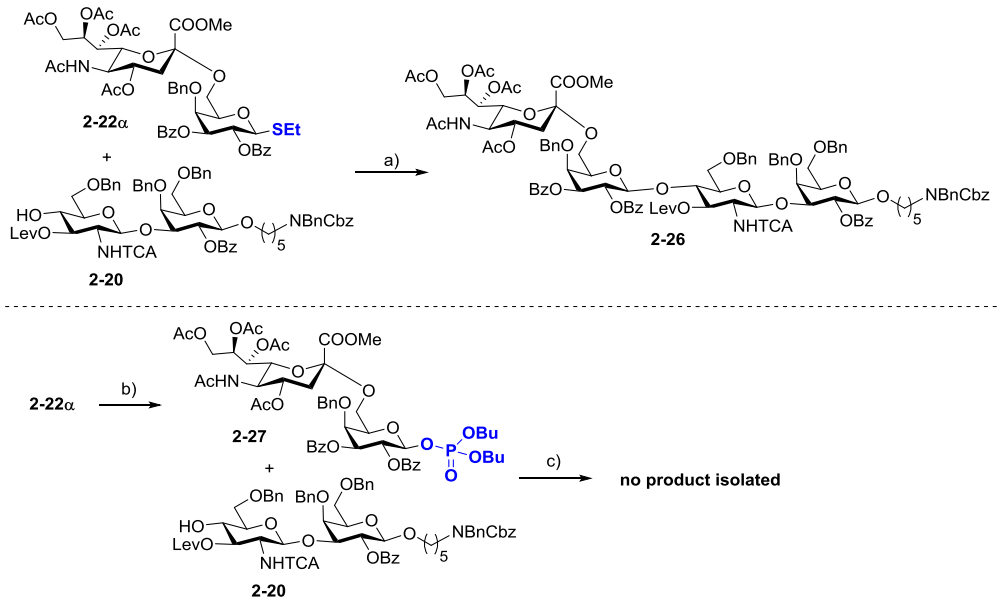


Figure 2-7: A) HMBC spectrum expansion of 2-22α. B) EXSIDE expansion showing C-1,H-3$_{ax}$ and C) C-2,H-3$_{ax}$ cross-peaks.

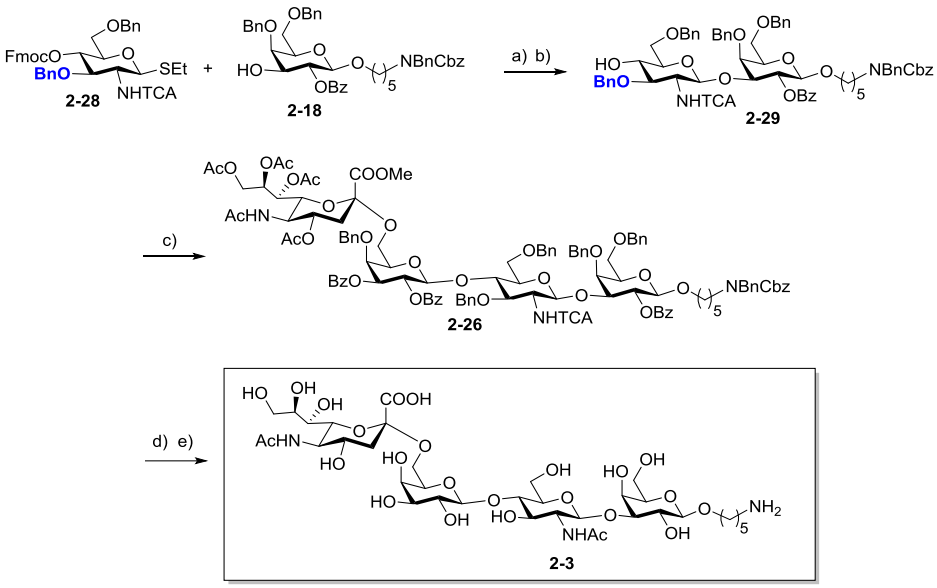
Disaccharide **2-22α** was employed as donor in a [2+2] glycosylation with acceptor **2-20**, using NIS and triflic acid (TfOH) as promoters. These conditions were found to be ineffective for the formation of the tetrasaccharide. As judged from TLC analysis, the reaction partners seem to be unreactive, even when reactions were conducted at room temperature. Reaction times of several hours resulted in progressive degradation of the donor, without substantial increase in product formation. The best result was a 15% isolated yield for tetrasaccharide **2-26**.

Poor reactivity of the thioglycoside donor due to the linked sialic acid was supposed as a first explanation for these results. Therefore, the thioglycoside donor was directly converted into a more reactive phosphate with a one-step procedure. When the new donor **2-27** was tested in a glycosylation with acceptor **2-20**, however, a similarly sluggish reaction was observed and no product could be isolated from a complex reaction mixture.



Scheme 2-7: First attempts at synthesizing tetrasaccharide 2-26. Reagents and conditions: a) NIS, TfOH, DCM; b) Dibutyl phosphate, NIS, TfOH, 0 °C, 95%; c) TMSOTf, DCM.

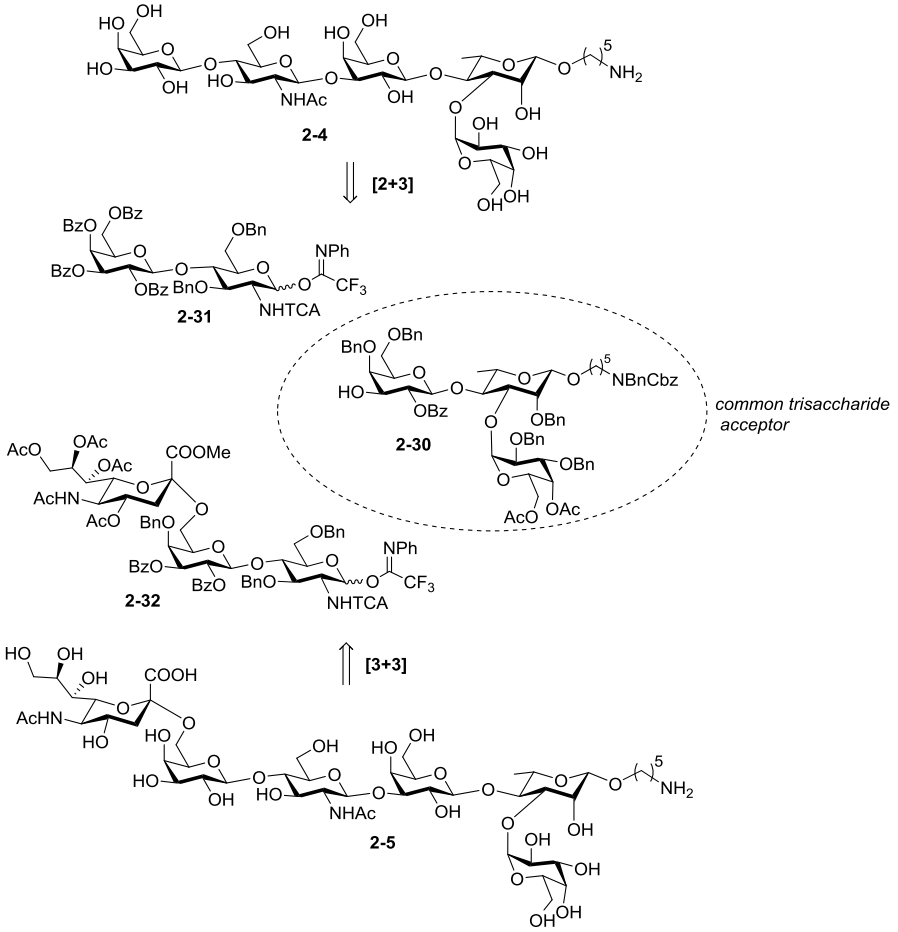
In both cases, donors could not be recovered in substantial amounts after the reaction (hydrolysis and degradation were observed) suggesting that activation was indeed taking place. Oppositely, relatively high amounts of acceptor could be recovered. It was then concluded that results could be explained by an insufficient nucleophilicity of the acceptor. It is known that 4-OH glucosamine acceptors protected with esters are poor nucleophiles[185] and that variations in the protecting group pattern can lead to improved results. Known glucosamine donor **2-28** and galactose **2-18** were used to synthesize disaccharide **2-29**, containing an ether group instead of an ester at C-3 of the glucosamine. When donor **2-27** was used to glycosylate the new acceptor, the tetrasaccharide was obtained in a satisfying 67% isolated yield. Removal of all protecting groups was performed by ester hydrolysis in basic conditions followed by catalytic hydrogenation, and gave deprotected tetrasaccharide **2-3** after HPLC purification.



Scheme 2-8: Synthesis of 2-3. Reagents and conditions: a) NIS, TfOH, DCM, −30 °C; b) TEA, DCM, 86% over two steps; c) **2-27,** TMSOTf, DCM, 0 °C, 67%; d) LiOH·H_2O, MeOH/THF, 50 °C; e) H_2, Pd/C, THF/MeOH/H_2O 1:1:1, 23% over two steps.

2.4.2. SYNTHESIS OF BRANCHED OLIGOSACCHARIDES

Retrosynthetic analysis of target compounds **2-4** and **2-5** revealed that both can be obtained from a common trisaccharide acceptor glycosylated either with a disaccharide donor, to afford pentasaccharide **2-4**, or alternatively with a sialylated trisaccharide donor to obtain **2-5**, as shown in Scheme 2-9.

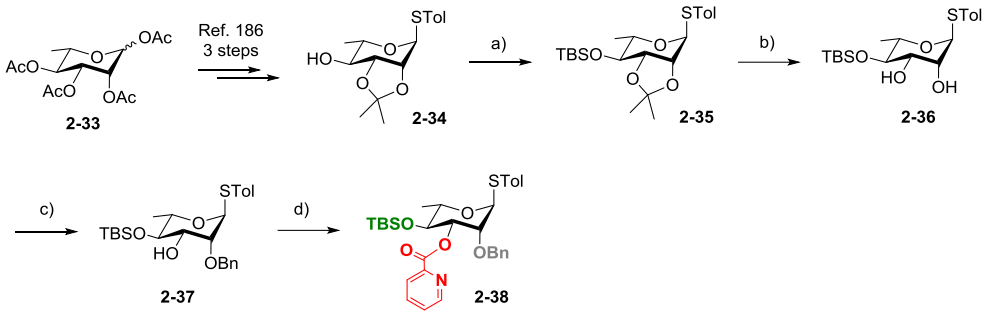


Scheme 2-9: Retrosynthesis of 2-4 and 2-5.

Trisaccharide **2-30** contains the same challenging 1,2-*cis* glycosidic bonds displayed in the previously synthesized fragments. However, acceptor **2-30** contains at the reducing end residue a double substitution on the rhamnose residue. Previously employed building block **2-7** carries a benzyl ether at C-4 and was not suitable for this synthesis. A different rhamnose building block had to be prepared. The new donor needed to display four orthogonal protecting groups. The picoloyl ester was maintained to ensure stereoselectivity in β rhamnosylation. A *tert*-butyldimethylsilyl (TBS) ether was chosen as protecting group for C-4 due to its stability in a range of acidic and basic conditions and a limited tendency to migrate.

Starting from peracetylated rhamnose **2-33**, intermediate **2-34**[186] was synthesized in three steps. Then the silyl ether was introduced, followed by hydrolysis of the isopropylidene acetal, affording diol **2-36**. To achieve selective protection of the hydroxyl group at position 2, a reported protocol was chosen[187]: it was found that slightly different acidities between axial and equatorial hydroxyls on rhamnoses could

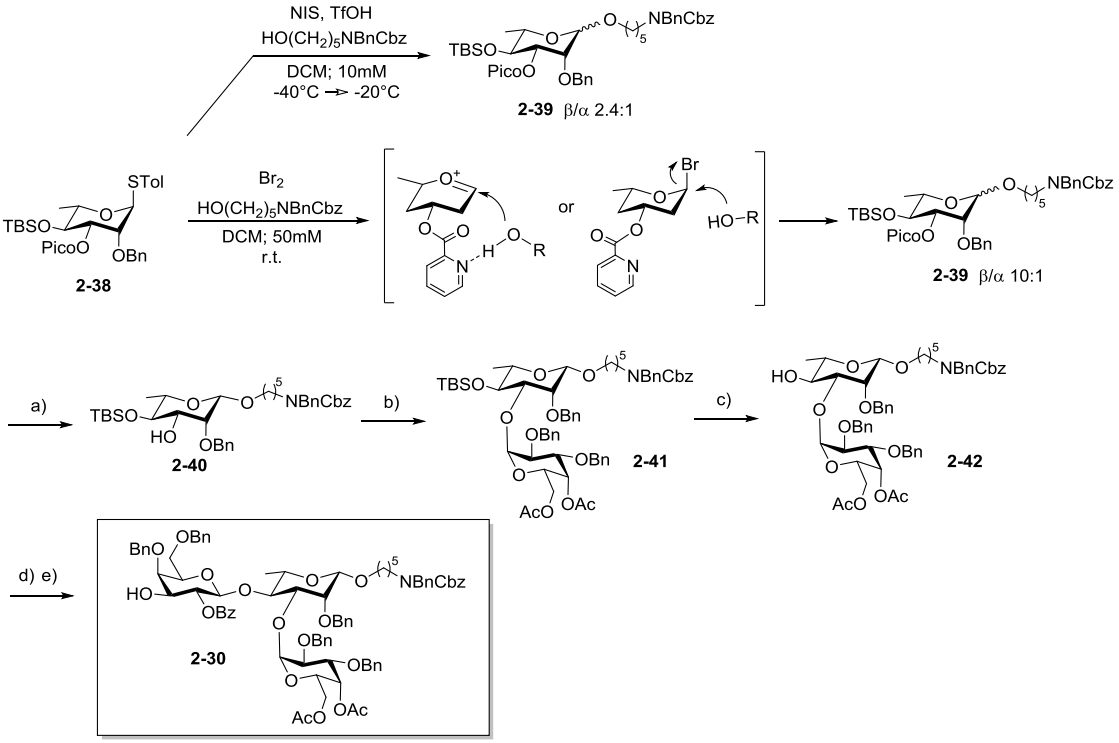
be exploited to perform regioselective etherification under phase transfer conditions. Previously, a 4-*O* benzylated rhamnose diol was treated in these conditions to form a 2-*O*-naphtylmethyl ether, which was obtained in 85% yield[172]. Unfortunately, when the same procedure was applied on diol **2-36**, the reaction showed poor regioselectivity. The appearance of comparable amounts of both 2-*O* and 3-*O* benzylated products was observed already at short reaction times and resulted in a low 26% isolated yield. The obtained amount was considered sufficient to continue the synthetic route and to access target donor **2-38**, obtained after a single esterification step with picolinic acid.



Scheme 2-10: Synthesis of rhamnose donor 2-38. Reagents and conditions: a) TBSCl, imidazole, DMF, 95%; b) Trifluoroacetic acid, DCM/H_2O 60:1; 93%; c) Benzyl bromide, TBABr, NaOH 10%(aq), DCM, 26%; d) 2-Picolinic acid, DIC, DCM, 93%.

A glycosylation involving donor **2-38** and the aminopentanol linker was attempted, using previously employed conditions for β-bond formation. In this case low stereoselectivity was observed as the two anomers were obtained in a 2.4:1 β/α ratio as judged by NMR. It was presumed that the high nucleophilicity of this primary alcohol was responsible for a competing fast glycosylation occurring on non-hydrogen bound acceptors. To improve the result, a recently reported alternative for activation of thioglycosides was considered[188]. Bromine can act as promoter and help the detachment of the thiol. Subsequently, an intermediate glycosyl bromide is formed and is the "active" donor on which the nucleophilic attack takes place. This activator gives slower reaction rates and can activate only donors that are not substituted with electron-withdrawing groups. Slower activation could yield more product deriving from the H-bond mediated mechanism, although it is possible that glycosylation through an S_N2-like mechanism will happen on intermediate glycosyl bromides (Scheme 2-11).

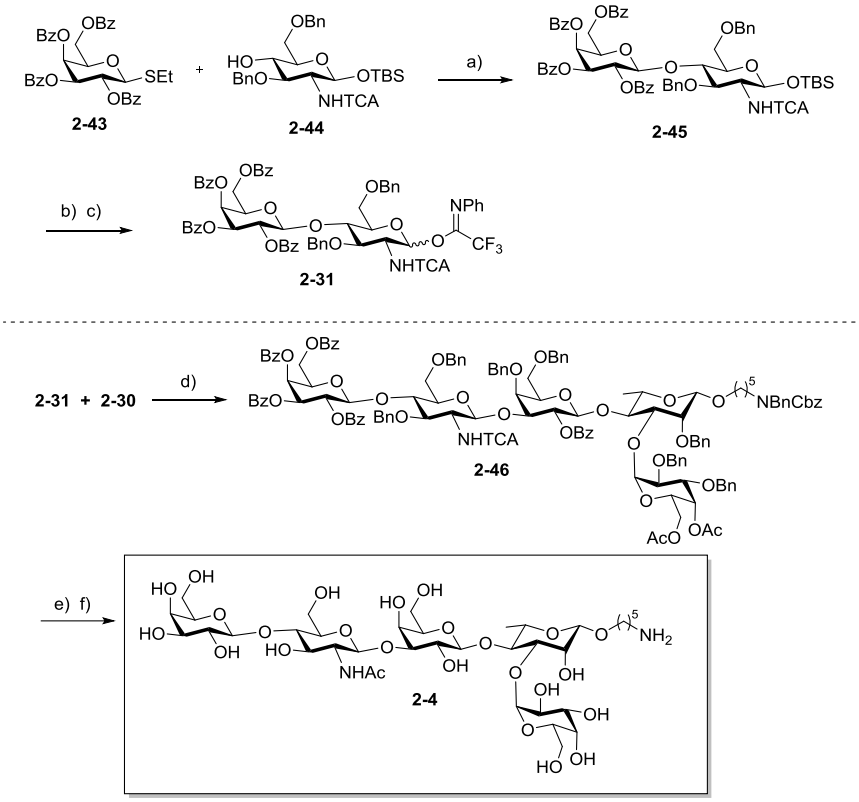
Experimentally, it was found that bromine activation resulted in a slow reaction, but when glycosylation was allowed to proceed overnight to reach full donor conversion, spacer-linked rhamnose anomers were obtained in a more satisfactory β/α ratio of approximately 10:1 as judged by NMR. The isomers were separated by chromatography after removal of the picoloyl ester. Rhamnose **2-40** was then glycosylated with galactose donor **2-8** in a DCM/Et_2O mixture. Similarly to the synthesis of trisaccharide **2-1**, no appreciable amounts of β-linked galactose were isolated. To perform a second glycosylation and install a second galactose residue, the TBS ether was deprotected with TBAF to afford disaccharide acceptor **2-42**, which was then glycosylated with donor **2-9**. Directly after glycosylation, the Fmoc group was removed to obtain desired trisaccharide **2-30**. This result proved that 4-OH on the rhamnose ring acted as a good nucleophile, despite proximity of the α galactose unit which could impose steric hindrance.



Scheme 2-11: Synthesis of trisaccharide 2-30. Reagents and conditions: a) $Cu(OAc)_2 \cdot H_2O$, DCM/MeOH 2:1, 61% over two steps; b) **2-8**, NIS, TfOH, DCM/Et_2O 1:1, −10 °C; c) TBAF, AcOH, THF, 64% over two steps; d) **2-9**, NIS, TfOH, DCM, −10 °C; e) TEA, DCM, 87% over two steps.

With trisaccharide acceptor **2-30** in hand, the assembly of **2-4** continued with the preparation of the disaccharide donor. To guarantee optimal reactivity, an imidate disaccharide was designed (Scheme 2-12). First, two known monosaccharides **2-43**[189] and **2-44**[190](‡) were glycosylated to obtain disaccharide **2-45** which, after deprotection of the anomeric silyl ether, was easily converted into an *N*-phenyl trifluoroacetimidate. Donor **2-31** and acceptor **2-30** were coupled to obtain the protected pentasaccharide in good yield. Next, the pentasaccharide was deprotected and pure **2-4** was obtained after HPLC purification.

‡ Compound 2-43 was synthesized by Dr. Benjamin Schumann. Compound 2-44 was synthesized by Dr. Lenz Kröck



Scheme 2-12: Synthesis of disaccharide donor 2-31 and synthesis of 2-4. Reagents and conditions: a) NIS, TfOH, DCM, −20 °C, 93%; b) TBAF, AcOH, THF; c) ClC=N(Ph)CF_3, Cs_2CO_3, DCM, 88% over two steps; d) TMSOTf, DCM, −30 °C, 67%; e) NaOMe, MeOH; f) H_2, Pd/C, MeOH/H_2O 4:1, 14% over two steps.

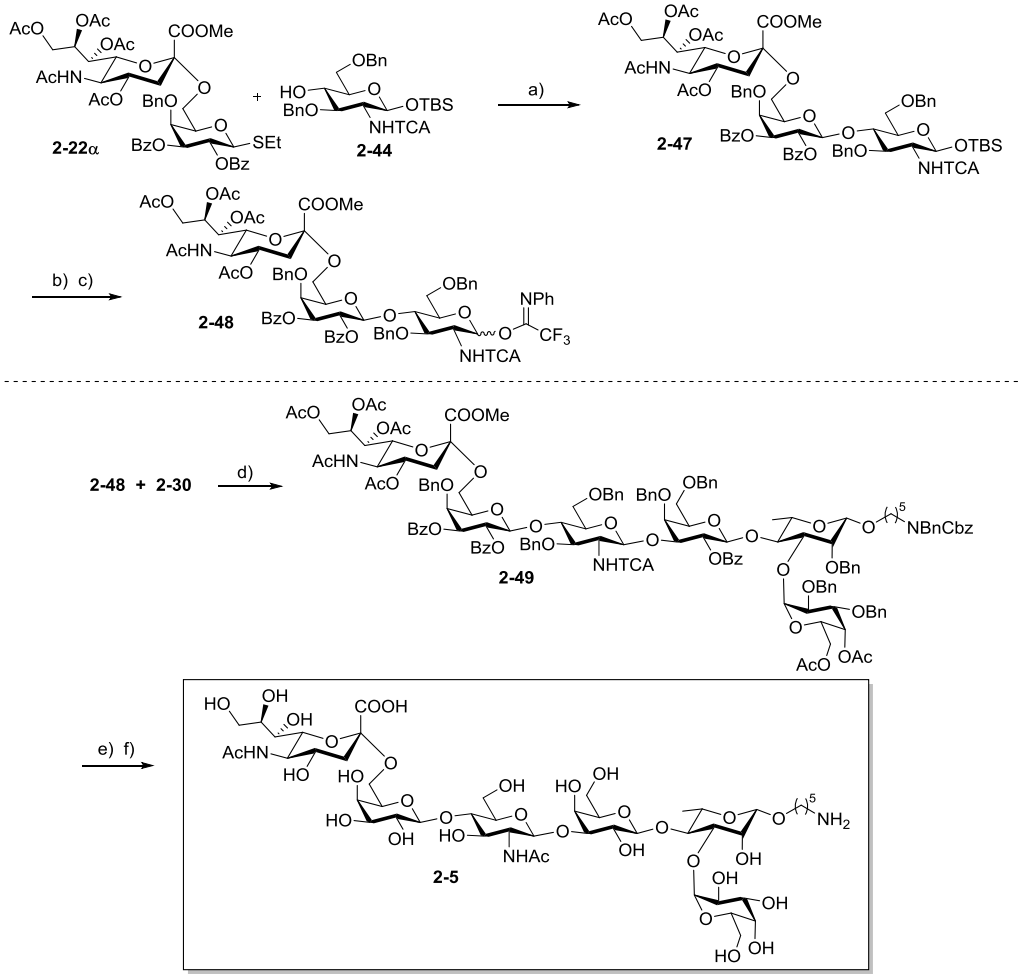
To assemble sialylated hexasaccharide **2-5** (Scheme 2-13), a trisaccharide donor was synthesized. Previously synthesized sialyl-galactose **2-22α** showed sufficient reactivity to glycosylate TBS-protected acceptor **2-44**. The silyl ether was removed from the glucosamine unit of the trisaccharide and the hemiacetal was then converted into an *N*-phenyl trifluoroacetimidate.

When **2-31** was employed in a [3+3] glycosylation with trisaccharide acceptor **2-30**, little product was detected by TLC. Glycosylation was not observed at temperatures lower than −20 °C and degradation of the donor was common. Substantial amounts of both donor and acceptor were recovered after purification, suggesting an insufficient reactivity of the reactants. Other experimental parameters were considered. An increase in the amount of promoter (TMSOTf) from 0.1 equiv to 0.3 equiv gave a similar result; a larger amount (0.5 equiv) resulted in fast donor degradation. A slight improvement was seen when the solution was warmed to 0 °C after addition of the promoter at −20 °C. The increase in temperature activated the reaction, since product formation was immediately observed. However, the reaction progression seemed to stop after a few minutes. The addition of an equal amount of acid slightly increased the amount of product but the composition of the mixture did not significantly change. Therefore, the reaction was quenched and the crude mixture was highly complex and necessitated HPLC to recover pure hexasaccharide **2-49** in low 11% yield.

Since an identical acceptor was successfully employed to synthesize fragment **2-4**, without low reactivity or unexpected side-products, it was deduced that donor **2-46** was the reason for the challenging synthesis. An explanation to this observation was not obvious. Absence of donor

activation and lower nucleophilicity of the acceptor could be ruled out, since traces of product were always formed. Steric hindrance around the anomeric carbon due to a particular conformation of the trisaccharide chain could be used as an argument but would not fully explain the observations, and would be difficult to prove. Contamination of the reactants with impurities undetectable by NMR which could interfere with activation and/or glycosylation could also not be excluded.

Although the amount of protected hexasaccharide was low, it was decided to continue with the deprotection steps. Fortunately, esters removal and catalytic hydrogenation proceeded smoothly and deprotected hexasaccharide **2-5** was obtained after HPLC purification.



Scheme 2-13: Synthesis of trisaccharide donor 2-48 and synthesis of hexasaccharide 2-5. Reagents and conditions: a) NIS, TfOH, DCM, −15 °C, 60%; b) TBAF, AcOH, THF; c) ClC=N(Ph)CF_3, Cs_2CO_3, DCM, 89% over two steps; d) TMSOTf, DCM, −20 °C→0 °C, 11%; e) LiOH·H_2O, MeOH/THF, 50 °C; e) H_2, Pd/C, MeOH/H_2O 3:1, 34% over two steps.

2.4.3. GLYCAN MICROARRAYS

Synthesized oligosaccharides were printed on NHS-activated microarray slides, together with unrelated synthetic glycans and bacterial polysaccharides as controls, as shown in detail in Section 2.5.1. The slides were probed with four samples of sera from pigs experimentally infected with *S. suis* serotype 2 (§) and bound antibodies were revealed using anti swine-IgG secondary antibodies. Considering the low number of samples, sera were not pooled but instead tested individually. Results are summarized in Fig. 2-8 as mean fluorescence intensities (MFI) from duplicate measurements. Serum samples are named with numbers 4515, 4641, 7007 and 7013.

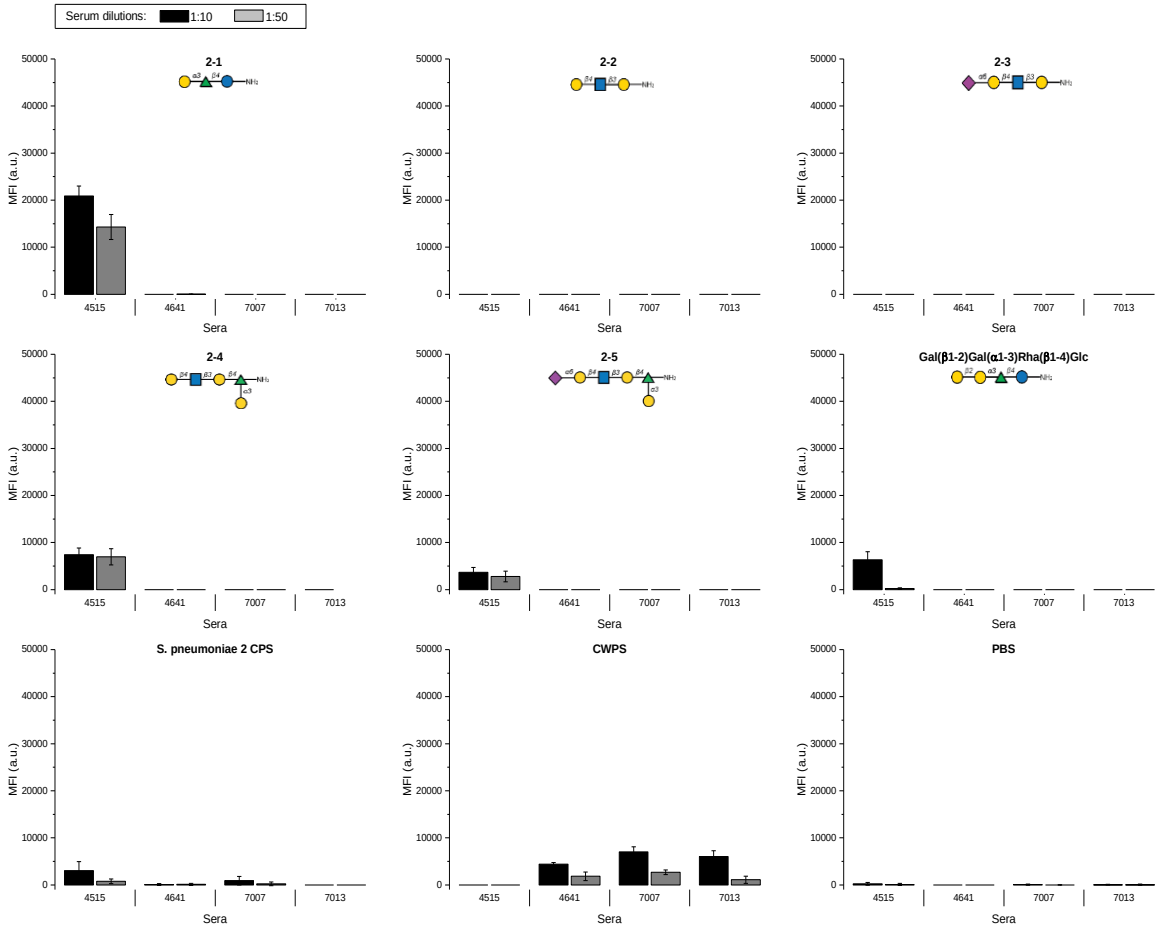


Figure 2-8: Glycan array analysis of four pig serum samples. Data shown are MFI measured for synthetic oligosaccharides and unrelated polysaccharides as controls. Each sample was measured in two dilutions to observe concentration dependence. CWPS are polysaccharides from the bacterial membrane of *Streptococcus pneumoniae.* PBS is phosphate buffer pH 8.5 (printing buffer).

The experiment revealed absence of binding to synthetic oligosaccharides from three samples – sera 4641, 7007 and 7013 – all of which showed binding to the unrelated cell-wall polysaccharide (Fig. 2-8). Such binding may indicate that similar structures are expressed on the cell wall of different types of streptococci. One sample – serum 4515 – showed strong binding to synthetic trisaccharide **2-1** (the backbone sequence) and, to a lower extent, to the longer sequences **2-4** and **2-5**. The observation

§ Serum samples were provided by Prof. Peter Valentin-Weigand (University of Veterinary Medicine Hannover)

that this serum also recognized another synthetic tetrasaccharide containing the exact sequence displayed by **2-1**, suggested an involvement of the backbone sugars in binding to these antibodies. Oppositely, the side-chain in its sialylated form or not, was never recognized. Weaker recognition of longer oligosaccharides can be hardly rationalized. A direct comparison with **2-1** cannot be made, particularly in light of the fact that **2-4** and **2-5** do not display the β-Rha-(1→4)-β-Glc sequence.

Altogether, considering the limited number of analyzed samples and that only one of them showed bindings to synthetic glycans, the experiment gave inconclusive results. More accurate conclusions could be drawn from an assay including a larger number of samples (ten or more). A positive control such as the native *S. suis* serotype 2 CPS, with which inhibition assays can be performed, could also give clear proof for the specificity of the detected antibodies. Despite this, the experiment proved that even though levels of induced IgG antibodies against the CPS are reported to be very low or even undetectable[160,161], the high sensitivity of the glycan arrays technique can guarantee their detection.

2.5. CONCLUSIONS AND OUTLOOK

A collection of five substructures related to the capsular polysaccharide of *Streptococcus suis* serotype 2 was assembled using solution-phase chemistry. The synthetic strategies applied recent carbohydrate chemistry protocols which proved useful in introducing challenging glycosidic bonds with high stereoselectivities and may be employed in future syntheses of *S. suis* oligosaccharides of higher complexity, in view of possible optimization of the synthetic antigens.

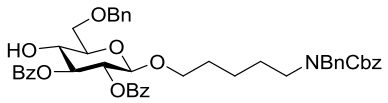
The synthesized compounds were printed on microarray slides and a preliminary screening conducted with a limited number of sera from experimentally infected pigs demonstrated that IgG antibodies are easily detectable. Future glycan arrays experiments performed with a larger number of serum samples will give more accurate information on epitopes of anti-CPS antibodies and will allow the selection of synthetic oligosaccharides which, upon conjugation to a carrier protein such as CRM_{197}, could result in the first semisynthetic glycoconjugate vaccine candidate against *S. suis* serotype 2.

2.6. EXPERIMENTAL SECTION

Commercial grade solvents and reagents were used without further purification. Anhydrous solvents were obtained from a solvent drying system (JCMeyer) or dried according to reported procedures. Analytical TLC was performed on Kieselgel 60 F254 glass (Macherey-Nagel). Spots were visualized with UV light, Sulphuric acid stain [1 mL of 3-methoxyphenol in 1 L of EtOH and 30 mL H_2SO_4] or Ceric ammonium molybdate stain [0.5 g $Ce(NH_4)_4(SO_4)_4 \cdot 2H_2O$, 12 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 15 mL H_2SO_4 in 235 mL H_2O]. Flash chromatography was performed on Kieselgel 60 230-400 mesh (Sigma-Aldrich). Preparative HPLC purifications were performed with an Agilent 1200 Series or Agilent 1260 Infinity II. NMR spectra were recorded on a Varian 400 MHz spectrometer (Agilent), Ascend 400 MHz (cryoprobe, Bruker) or Varian 600 MHz (Agilent) at 25 °C unless indicated otherwise. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CHCl_3$: δ 7.26 in ^1H and 77.16 in ^{13}C; HDO δ 4.79 in ^1H). Bidimensional and non-decoupled experiments were performed to assign identities of peaks showing relevant structural features. Configurations of sialic acid derivatives were determined by bidimensional HMBC and EXSIDE. The following abbreviations are used to indicate peak multiplicities: *s* (singlet), *d* (doublet) *dd* (doublet of doublets), *t* (triplet), *dt* (doublet of triplets), *td* (triplet of doublets), *q* (quartet), *p* (pentet), *m* (multiplet). Additional descriptors *b* (broad signal) and *app* (apparent first-order multiplet) are also employed when required. Coupling constants (*J*) are reported in Hertz (Hz). NMR spectra were processed using MestreNova 11.0 (MestreLab Research). Specific rotations were measured with a UniPol L1000 polarimeter (Schmidt & Haensch) at λ = 589 nm. Concentration (*c*) is expressed in g/100 mL in the solvent noted in parentheses. IR spectra were measured with a Perkin Elmer 100 FTIR spectrometer. High-resolution mass spectra (ESI-HRMS) were recorded with a Xevo G2-XS Q-Tof (Waters).

2.6.1. SYNTHETIC PROTOCOLS AND SPECTRAL DATA

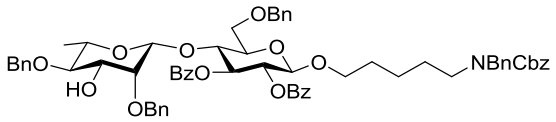
***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranoside (2-14)**



Commercially available compound **2-6** (1.565 g; 2.101 mmol) and *N*-(Benzyl)benzyloxycarbonylaminopentanol (0.989 g; 3.021 mmol) were twice coevaporated with toluene and left under vacuum overnight. They were then dissolved in DCM (20 mL) under Ar atmosphere, 4Å molecular sieves were added, the solution was stirred for 30 min and then cooled to −15 °C. NIS (566 mg; 2.521 mmol) and TfOH (19 µL; 0.210 mmol) were added. After 30 min the reaction was quenched with a large excess of triethylamine (4.0 mL) and gradually warmed to r.t. After 2 h it was diluted with DCM, filtered and washed with 10% aqueous $Na_2S_2O_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The residue was purified by chromatography on silica (Hexane/EtOAc 9:1 to 7:3) to obtain **2-14** (885 mg; 1.123 mmol; 52% over 2 steps).

$[\alpha]_D^{25}$ = 26.4° (c = 2.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3428, 3066, 3034, 2943, 2868, 1729, 1698, 1603, 1586, 1497, 1475, 1453, 1424, 1366, 1315, 1266, 1178, 1095, 1069, 1028, 988, 914, 854, 803, 736, 710, 699; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.86 (m, 4H), 7.54 – 7.47 (m, 1H), 7.45 – 7.06 (m, 20H), 5.49 – 5.34 (m, 2H, H-2/H-3), 5.18 – 5.08 (m, 2H,CH_2Ph), 4.69 – 4.55 (m, 3H, H-1/ CH_2Ph), 4.43 – 4.30 (m, 2H, CH_2Ph), 4.01 – 3.77 (m, 4H, H-4/H-6a/H-6b/-OCHH-), 3.73 – 3.63 (m, 1H, H-5), 3.50 – 3.34 (m, 1H, -OCHH-), 3.11 – 2.89 (m, 2H, -CH_2N-), 1.56 – 1.28 (m, 4H, 2xCH_2(Linker)), 1.21 – 1.00 (m, 2H, CH_2(Linker)); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 165.3, 156.8, 156.2, 138.0, 138.0, 137.7, 137.0, 136.8, 133.5, 133.3, 130.1, 129.8, 129.5, 129.2, 128.6, 128.5, 128.4, 128.1, 128.0, 127.92, 127.89, 127.8, 127.4, 127.3, 127.2, 101.2 (C-1), 76.6, 74.6, 73.9, 71.6, 71.3, 70.2, 70.0, 69.9, 67.2, 50.6, 50.3, 47.1, 46.1, 29.2, 27.8, 27.4, 23.1; HRMS (ESI+) calculated for $C_{47}H_{49}NO_{10}Na$ [M+Na]: 810.3248, found: 810.3282.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2,4-di-*O*-benzyl-β-L-rhamnopyranosyl-(1→4)- 2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranoside (2-15)**

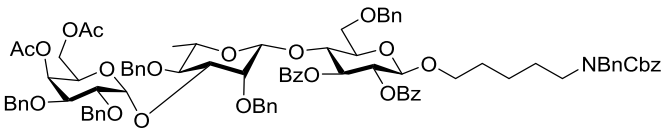


Rhamnose donor **2-7**[171] (136 mg; 0.276 mmol) and glucose acceptor **2-14** (168 mg; 0.213 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM (20 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to −30 °C. NIS (72 mg; 0.318 mmol) and TfOH (2 µL; 0.021 mmol) were added. After 30 min the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and water. The combined aqueous phases were extracted once with DCM. The combined organic phases were dried over Na_2SO_4, filtered and concentrated. Crude material was purified by column chromatography (Hexane/EtOAc 8:2 to 1:1) to obtain an impure mixture containing β-linked disaccharide, probably together with traces of α-linked product (not isolated) and hydrolyzed donor. Without further purification the mixture was redissolved in DCM/MeOH 2:1 (6.0 mL) and $Cu(OAc)_2{\cdot}H_2O$ (54 mg; 0.270 mmol) was added. After 1 h the solution was diluted with DCM and washed twice with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. Purification by chromatography on silica (Hexane/EtOAc 7:3 to 1:1) afforded pure β-**2-15** (141 mg; 0.127 mmol; 60% over 2 steps).

$[\alpha]_D^{25}$ = 46.5° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3543, 3066, 3033, 2931, 2867, 1730, 1698, 1603, 1586, 1497, 1453, 1423, 1367, 1316, 1273, 1179, 1094, 1069, 1028, 1001, 913, 854, 795, 736, 711, 698; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.86 (m, 4H), 7.56 – 7.49 (m, 1H), 7.47 – 7.06 (m, 30H), 5.73 (t, *J* = 9.6 Hz, 1H, H-3), 5.35 (dd, *J* = 10.0, 7.1 Hz, 1H, H-2), 5.19 – 5.01 (m, 2H, CH_2Ph), 4.95 (d, *J* = 11.8 Hz, 1H, -CHHPh), 4.82 (d, *J* = 10.9 Hz, 1H, -CHHPh), 4.71 – 4.29 (m, 8H, H-1/H-1'/CH_2Ph/2xCHHPh/-OCH_2-), 4.09 (d, *J* = 10.0 Hz, 1H), 3.98 (t, *J* = 9.1 Hz, 1H, H-4), 3.94 – 3.71 (m, 2H), 3.54 – 3.35 (m, 2H), 3.19 – 2.89 (m, 5H), 2.14 (d, *J* = 9.3 Hz, 1H), 1.57 – 1.27 (m, 7H, -CH_3/2xCH_2(Linker), 1.23 – 1.04 (m, 2H, CH_2(Linker)); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.9, 165.3, 156.8, 156.2, 138.6, 138.4, 138.3, 138.1, 137.0, 136.9, 133.8, 133.3, 129.8, 129.6, 129.5, 129.0,

128.9, 128.7, 128.61, 128.57, 128.51, 128.45, 128.44, 128.39, 128.3, 128.2, 128.12, 128.06, 127.94, 127.91, 127.85, 127.6, 127.4, 127.3, 127.2, 102.3 (C-1'), 101.0 (C-1), 81.8, 78.4, 76.3, 75.5, 75.4, 75.2, 75.0, 73.8, 73.6, 71.9, 71.6, 70.0, 69.9, 69.5, 67.2, 50.6, 50.3, 47.2, 46.2, 29.2, 27.8, 27.4, 23.2, 18.0 (-CH_3); HRMS (ESI+) calculated for $C_{67}H_{71}NO_{14}Na$ [M+Na]: 1136.4767, found: 1136.4780.

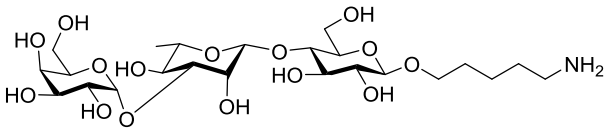
***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 4,6-di-*O*-acetyl-2,3-di-*O*-benzyl-α-D-galactopyranosyl-(1→3)-2,4-di-*O*-benzyl-β-L-rhamnopyranosyl-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranoside (2-16)**



Disaccharide acceptor **2-15** (60 mg; 0.054 mmol) and monosaccharide donor **2-8**[140] (34mg; 0.070 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM/Et_2O 1:1 (2 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to −15 °C. NIS (21 mg; 0.093 mmol) and TfOH (0.1 M in Et_2O; 54 µL; 5.4 µmol) were added. After 30 min the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phases were dried over Na_2SO_4, filtered and concentrated. Crude material was purified by chromatography on silica (Hexane/EtOAc 7:3) to obtain pure trisaccharide **2-16** (52 mg; 0.034 mmol; 62%).

$[\alpha]_D^{25}$ = 56.8° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3066, 3034, 2928, 2859, 2309, 1733, 1699, 1603, 1586, 1498, 1454, 1427, 1370, 1315, 1273, 1250, 1230, 1178, 1095, 1069, 1028, 947, 913, 853, 798, 737, 711, 698, 665; ^{1}H NMR (600 MHz, $CDCl_3$, 10 °C) δ 7.95 – 7.87 (m, 4H), 7.49 – 7.08 (m, 41H), 5.65 (t, *J* = 9.6 Hz, 1H, H-3), 5.33 (t, *J* = 8.7 Hz, 1H, H-2), 5.20 – 5.07 (m, 3H), 4.71 – 4.45 (m, 11H, H-1/H-1'/H-1''/3xCH_2Ph/-OCH_2-), 4.44 – 4.30 (m, 4H), 4.05 (t, *J* = 6.7 Hz, 1H), 4.03 – 3.97 (m, 2H), 3.92 – 3.81 (m, 2H), 3.81 – 3.71 (m, 2H), 3.69 – 3.62 (m, 3H), 3.57 (dd, *J* = 11.1, 6.6 Hz, 1H), 3.53 – 3.36 (m, 2H), 3.29 – 3.25 (m, 1H), 3.17 – 3.09 (m, 1H), 3.07 – 2.89 (m, 2H, -CH_2N-), 2.07 (s, 3H, $COCH_3$), 1.83 (s, 3H, $COCH_3$), 1.60 – 1.25 (m, 7H, -CH_3/2xCH_2(Linker)), 1.16 – 1.01 (m, 2H, CH_2(Linker)); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.4, 170.3, 165.9, 165.3, 139.3, 138.7, 138.3, 138.2, 138.1, 133.7, 133.2, 129.8, 129.6, 129.2, 128.9, 128.62, 128.59, 128.5, 128.44, 128.42, 128.37, 128.07, 128.06, 128.04, 128.01, 127.96, 127.92, 127.89, 127.8, 127.7, 127.63, 127.60, 127.58, 127.3, 127.2, 101.8 (C-1'), 101.0 (C-1), 94.2 (C-1''), 79.1, 75.9, 75.8, 75.63, 75.58, 75.3, 75.1, 75.0, 74.8, 73.54, 73.51, 72.4, 72.1, 71.8, 69.9, 69.5, 67.5, 67.2, 66.6, 62.0, 50.6, 50.3, 47.2, 46.2, 29.2, 27.9, 27.5, 23.2, 21.0 ($CO\underline{C}H_3$), 20.8 ($CO\underline{C}H_3$), 18.0 (-CH_3); HRMS (ESI+) calculated for $C_{91}H_{97}NO_{21}Na$ [M+Na]: 1562.6445, found: 1562.6466.

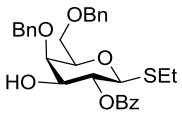
5-Aminopentyl α-D-galactopyranosyl-(1→3)-β-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2-1)



Compound **2-16** (26 mg; 16.9 µmol) was dissolved in Methanol/THF 1:1 (2.0 mL). Sodium methoxide 0.5 M in MeOH was added (0.85 mL; 0.43 mmol). The reaction was stirred for 16 h, then neutralized with the addition of Amberlite IR120 H^+, filtered and concentrated. The crude residue was purified by column chromatography (DCM/MeOH 95:5). The obtained product was dissolved in EtOAc/tBuOH/H_2O/AcOH 2:1:1:0.1 (2.0 mL). Pd/C was added, the solution was purged with argon and hydrogen and left stirring under H_2 atmosphere with a balloon for 24 h at 35 °C. The mixture was filtered through a PTFE filter (0.45 µm pore size) and concentrated. The crude trisaccharide was purified by RP-HPLC (Hypercarb column, 150x10 mm, H_2O (0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (10 min)) and lyophilized to obtain **2-1** as a formic acid salt (4.3 mg; 6.8 µmol; 40% over 2 steps).

^{1}H NMR (600 MHz, D_2O) δ 8.46 (s, 1H, $HCOO^-$), 5.16 (d, *J* = 3.9 Hz, 1H, H-1''), 4.89 (br s, 1H, H-1'), 4.47 (d, *J* = 8.0 Hz, 1H, H-1), 4.32 (d, *J* = 3.0 Hz, 1H), 4.23 – 4.19 (m, 1H), 4.03 (dd, *J* = 3.5, 1.1 Hz, 1H), 3.98 – 3.92 (m, 3H), 3.89 – 3.81 (m, 2H), 3.77 – 3.67 (m, 4H), 3.67 – 3.62 (m, 2H), 3.55 – 3.42 (m, 3H), 3.33 – 3.24 (m, 1H), 3.02 (app t, *J* = 7.5 Hz, 2H), 1.70 (m, 4H), 1.47 (m, 2H), 1.35 (d, *J* = 5.9 Hz, 3H, $-CH_3$); ^{13}C NMR (151 MHz, D_2O) δ 170.9 ($HCOO^-$), 102.1 (C-1), 100.5 (C-1'), 95.3 (C-1''), 77.3, 76.6, 75.6, 74.6, 73.1, 72.1, 70.7, 70.2, 70.0, 69.3, 69.1, 68.2, 67.0, 60.74, 60.71, 39.3, 28.1, 26.3, 22.0, 16.7; HRMS (ESI+) calculated for $C_{23}H_{44}NO_{15}$ [M+H]: 574.2705, found: 574.2708.

Ethyl 2-*O*-benzoyl-4,6-di-*O*-benzyl-1-thio-β-D-galactopyranoside (2-17)

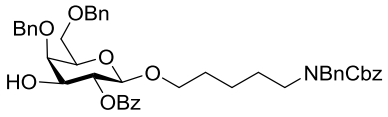


Commercially available compound **2-9** (500 mg; 0.684 mmol) was dissolved in DCM (5 mL) and Triethylamine (1.5 mL). After 2 h the reaction was cooled to 0 °C and neutralized with acetic acid. It was then diluted with DCM and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified by chromatography on silica (Hexane/EtOAc 9:1 to 7:3) to obtain **2-17** (297 mg; 0.584 mmol; 85%).

$[\alpha]_D^{25}$ = -1.9° (c = 0.9, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3458, 3065, 3033, 2927, 2871, 1723, 1603, 1586, 1497, 1453, 1401, 1352, 1316, 1265, 1210, 1178, 1091, 1071, 1053, 1028, 994, 881, 804, 734, 710, 699, 677; ^{1}H NMR (400 MHz, CDCl3) δ 8.11 – 8.04 (m, 2H), 7.62 – 7.55 (m, 1H), 7.51 – 7.30 (m, 12H), 5.33 (t, *J* = 9.7 Hz, 1H, H-2), 4.83 – 4.69 (m, 2H, CH_2Ph), 4.61 – 4.49 (m, 3H, H-1/CH_2Ph), 4.03 (dd, *J* = 3.5, 0.9 Hz, 1H, H-4), 3.85 – 3.70 (m, 4H, H-3/H-5/H-6a/H-6b), 2.84 – 2.66 (m, 2H, $SC\underline{H_2}CH_3$), 1.27 (t, *J* = 7.4 Hz, 3H, $SCH_2C\underline{H_3}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 138.2, 137.7, 133.3, 130.0, 129.9, 128.7, 128.6, 128.5, 128.08, 128.06, 128.0, 83.4 (C-1), 76.8, 75.5, 74.1, 73.7,

72.4, 68.2, 24.0 ($S\underline{C}H_2CH_3$), 15.1 ($SCH_2\underline{C}H_3$); HRMS (ESI+) calculated for $C_{29}H_{32}O_6SNa$ [M+Na]: 531.1812, found: 531.1819.

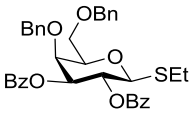
_N_-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2-_O_-benzoyl-4,6-di-_O_-benzyl-β-D-galactopyranoside (2-18)



Compound **2-17** (0.122 g; 0.240 mmol) and *N*-(Benzyl)benzyloxycarbonylaminopentanol (0.216 g; 0.660 mmol) were twice coevaporated with toluene and left under vacuum overnight. They were then dissolved in DCM (10 mL) under Ar atmosphere, 4Å molecular sieves were added, the solution was stirred for 30 min and then cooled to –50 °C. NIS (80 mg; 0.356 mmol) and TfOH (0.45 M in Et_2O; 50 µL; 0.023 mmol) were added. After 30 min the reaction was diluted with DCM, quenched with triethylamine and gradually warmed to r.t. The solution was filtered, washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. The residue was purified by chromatography on silica (Hexane/EtOAc 7:3) to obtain **2-18** (174 mg; 0.199 mmol; 83%).

$[\alpha]_D^{25}$ = -9.8° (c = 2.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3453, 3065, 3033, 2937, 2867, 1727, 1697, 1604, 1587, 1497, 1475, 1454, 1423, 1367, 1315, 1270, 1178, 1114, 1071, 1028, 999, 910, 843, 804, 769, 735, 712, 699; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 7.6 Hz, 2H), 7.53 – 7.44 (m, 1H), 7.41 – 7.22 (m, 20H), 7.21 – 7.15 (m, 1H), 7.13 – 7.07 (m, 1H), 5.22 (dd, *J* = 10.0, 7.9 Hz, 1H, H-2), 5.17 – 5.10 (br s, 2H, CH_2Ph), 4.78 – 4.69 (m, 2H, CH_2Ph), 4.58 – 4.32 (m, 5H, H-1/CH_2Ph/-OCH_2-), 3.95 (br d, *J* = 3.5 Hz, 1H, H-4), 3.90 – 3.65 (m, 5H, H-3/ H-5/H-6a/CH_2Ph), 3.48 – 3.30 (m, 1H, H-6b), 3.15 – 2.89 (m, 2H, -CH_2N-), 2.49 – 2.38 (m, 1H), 1.57 – 1.30 (m, 4H, 2xCH_2(Linker)), 1.24 – 1.04 (m, 2H, CH_2(Linker)); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.8, 156.8, 156.2, 138.1, 138.0, 138.0, 137.8, 137.0, 136.9, 133.3, 129.92, 129.88, 128.7, 128.62, 128.60, 128.55, 128.4, 128.3, 128.2, 128.14, 128.08, 128.05, 128.03, 128.00, 127.92, 127.86, 127.4, 127.31, 127.25, 101.2 (C-1), 76.6, 75.6, 74.3, 73.7, 73.6, 73.3, 69.8, 69.7, 68.3, 67.2, 50.6, 50.3, 47.2, 46.2, 29.2, 27.8, 27.4, 23.2; HRMS (ESI+) calculated for $C_{47}H_{51}NO_9Na$ [M+Na]: 796.3456, found: 796.3469.

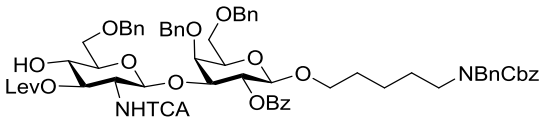
Ethyl 2,3-di-_O_-benzoyl-4,6-di-_O_-benzyl-1-thio-β-D-galactopyranoside (2-19)



Compound **2-17** (150 mg; 0.235 mmol) was dissolved in DCM (5.0 mL). Triethylamine (0.13 mL; 0.940 mmol), benzoic anhydride (106 mg; 0,470 mmol) and a catalytic amount of DMAP were added, the reaction was stirred for 16 h at room temperature then diluted with EtOAc and extracted with 1 M HCl, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified by chromatography on silica (Hexane/EtOAc 8:2) to obtain **2-19** (130 mg; 0.212 mmol; 90%);

$[\alpha]_D^{25}$ = 56.1° (c = 0.9, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3066, 3034, 2930, 2871, 1726, 1603, 1586, 1497, 1454, 1354, 1316, 1276, 1213, 1179, 1152, 1096, 1071, 1028, 1001, 885, 803, 735, 709; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.92 (m, 4H), 7.53 – 7.47 (m, 2H), 7.41 – 7.16 (m, 14H), 5.89 (t, *J* = 10.0 Hz, 1H, H-2), 5.40 (dd, *J* = 10.0, 3.1 Hz, 1H, H-3), 4.73 (d, *J* = 11.6 Hz, 1H), 4.69 (d, *J* = 9.9 Hz, 1H, H-1), 4.52 (app d, *J* = 11.8 Hz, 2H), 4.46 (d, *J* = 11.8 Hz, 1H), 4.29 – 4.25 (m, 1H, H-4), 3.95 – 3.90 (m, 1H, H-5), 3.73 – 3.64 (m, 2H, H-6a/H-6b), 2.84 – 2.70 (m, 2H, $SC\underline{H}_2CH_3$), 1.26 (t, *J* = 7.4 Hz, 3H, $SCH_2C\underline{H}_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.0, 165.6, 138.0, 137.9, 133.5, 133.2, 130.0, 129.9, 129.7, 129.2, 128.6, 128.42, 128.36, 128.1, 127.98, 127.95, 127.8, 83.9 (C-1), 77.5, 75.9, 75.1, 74.4, 73.7, 68.7, 68.3, 24.0 ($S\underline{C}H_2CH_3$), 15.0 ($SCH_2\underline{C}H_3$); HRMS (ESI+) calculated for $C_{36}H_{36}O_7SNa$ [M+Na]: 635.2074, found: 635.2082.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 6-*O*-benzyl-3-*O*-levulinoyl-2-trichloroacetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranoside (2-20)**

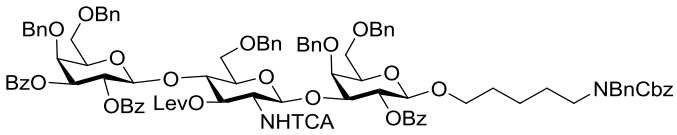


Galactose acceptor **2-18** (151 mg; 0.195 mmol) and glucosamine donor **2-10** (186 mg; 0.239 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM (2.5 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to −30 °C. NIS (59 mg; 0.263 mmol) and TfOH (0.1 M in dioxane; 195 µL; 0.020 µmol) were added. After 1 h the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phases were dried over Na_2SO_4, filtered and concentrated. The obtained crude product was redissolved in DCM (10 mL) and triethylamine (2.5 mL). After 2 h the solution was cooled to 0 °C and carefully neutralized with acetic acid. It was then diluted with DCM and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified by chromatography on silica (Hexane/EtOAc 1:1 to 4:6) to obtain disaccharide **2-20** (209 mg; 0.165 mmol; 84% over 2 steps).

$[\alpha]_D^{25}$ = -22.8° (c = 1.5, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3341, 3066, 3033, 2928, 2868, 1718, 1604, 1587, 1524, 1498, 1475, 1454, 1423, 1365, 1314, 1269, 1161, 1098, 1069, 1028, 914, 839, 821, 737, 699; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 – 7.87 (m, 2H), 7.53 – 7.45 (m, 1H), 7.37 – 7.23 (m, 25H), 7.17 (d, *J* = 7.4 Hz, 1H), 7.10 (d, *J* = 7.3 Hz, 1H), 6.51 (d, *J* = 8.3 Hz, 1H, NH), 5.51 (dd, *J* = 10.0, 7.8 Hz, 1H, H-2), 5.12 (s, 2H, CH_2Ph), 4.97 (d, *J* = 11.6 Hz, 1H, C$\underline{H}$HPh), 4.88 (dd, *J* = 10.9, 8.8 Hz, 1H, H-3'), 4.67 (d, *J* = 8.2 Hz, 1H, H-1'), 4.63 – 4.55 (m, 3H, 3xC$\underline{H}$HPh), 4.49 – 4.26 (m, 5H, 2xCH$\underline{H}$Ph/-OCH_2-(Linker)/H-1), 4.13 – 4.01 (m, 2H, H-3/H-4), 4.01 – 3.90 (m, 1H, H-2), 3.89 – 3.46 (m, 8H), 3.43 – 3.19 (m, 2H), 2.82 – 2.65 (m, 2H, CH_2(Lev)), 2.58 – 2.46 (m, 1H, C$\underline{H}$H(Lev)), 2.46 – 2.36 (m, 1H, CH$\underline{H}$(Lev)), 2.14 (s, 3H, -CH_3 (Lev)), 1.46 – 1.23 (m, 4H, 2xCH_2 (Linker)), 1.12 – 0.93 (m, 2H, CH_2 (Linker)); ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.1 ($CH_3\underline{C}O$- (Lev)), 173.2, 165.2, 162.3, 156.7, 156.2, 138.7, 138.0, 137.8, 133.6, 129.80, 129.79, 128.83, 128.75, 128.64, 128.59, 128.5, 128.2, 128.02, 127.96, 127.9, 127.83, 127.77, 127.6, 127.4, 127.3, 127.2, 101.6 (C-1), 100.7 (C-1'), 92.1 ($CO\underline{C}Cl_3$),

78.8, 75.9, 75.3, 75.0, 73.9, 73.8, 73.7, 73.0, 70.1, 69.8, 69.6, 69.2, 68.9, 67.2, 55.6, 50.5, 50.3, 38.5, 29.94, 29.91, 29.1, 28.3, 27.8, 27.4, 23.2; HRMS (ESI+) calculated for $C_{67}H_{73}Cl_3N_2O_{16}Na$ [M+Na]: 1289.3918, found: 1289.3965.

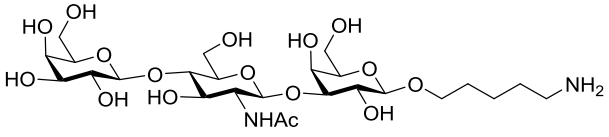
_N_-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2,3-di-_O_-benzoyl-4,6-di-O-benzyl-_6_-_O_-benzyl-β-D-galactopyranosyl-(1→4)-3-_O_-levulinoyl-2-trichloroacetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2-_O_-benzoyl-4,6-di-_O_-benzyl-β-D-galactopyranoside (2-21)



Disaccharide acceptor **2-20** (89 mg; 0.070 mmol) and galactose donor **2-19** (74 mg; 0.121 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM (3.0 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to −15 °C. NIS (27 mg; 0.121 mmol) and TfOH (0.1 M in Dioxane; 70 µL; 7 µmol) were added. After 1 h the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and brine. Crude material was purified using a RevelerisX2 Flash Chromatography System (Hexane/EtOAc 85:15 to 45:55) and size-exclusion chromatography (Sephadex LH-20, $CHCl_3$/MeOH 1:1) to obtain trisaccharide **2-21** (84 mg; 0.046 mmol; 66%).

$[\alpha]_D^{25}$ = 0.8° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3333, 3066, 3034, 2927, 2868, 1725, 1603, 1586, 1528, 1497, 1454, 1422, 1363, 1315, 1274, 1211, 1160, 1098, 1071, 1029, 843, 820, 736, 713, 700; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 – 7.80 (m, 5H), 7.56 – 7.07 (m, 45H), 6.45 (d, *J* = 9.2 Hz, 1H), 5.61 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.47 (dd, *J* = 9.8, 7.9 Hz, 1H), 5.22 (dd, *J* = 10.5, 3.2 Hz, 1H), 5.15 – 5.09 (m, 2H), 5.00 (d, *J* = 11.7 Hz, 1H), 4.96 – 4.91 (m, 1H), 4.68 – 4.61 (m, 2H), 4.60 – 4.51 (m, 4H), 4.49 – 4.28 (m, 8H), 4.17 (d, *J* = 3.2 Hz, 1H), 4.01 – 3.95 (m, 4H), 3.80 – 3.71 (m, 2H), 3.67 – 3.49 (m, 7H), 3.32 – 3.21 (m, 2H), 3.03 – 2.95 (m, 0.5 H), 2.91 – 2.83 (m, 1.5H), 2.60 – 2.38 (m, 4H), 1.96 (s, 3H), 1.43 – 1.21 (m, 4H), 1.10 – 0.89 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 206.3, 172.6, 165.9, 164.9, 162.2, 138.7, 138.1, 137.9, 137.84, 137.75, 133.49, 133.45, 133.3, 130.0, 129.9, 129.76, 129.75, 129.5, 129.1, 128.9, 128.8, 128.7, 128.64, 128.58, 128.56, 128.54, 128.52, 128.40, 128.38, 128.3, 128.2, 128.10, 128.05, 128.03, 128.01, 128.00, 127.99, 127.98, 127.92, 127.90, 127.87, 127.8, 127.6, 101.7, 101.1, 100.6, 92.1, 79.0, 75.9, 75.2, 74.94, 74.91, 74.8, 74.6, 74.5, 74.4, 74.0, 73.9, 73.7, 73.62, 73.60, 73.56, 73.4, 72.7, 72.3, 70.6, 69.4, 69.3, 69.0, 67.62, 67.57, 67.2, 56.0, 50.6, 50.3, 47.2, 46.2, 37.9, 29.8, 29.1, 28.1, 27.8, 27.4, 23.2; HRMS (ESI+) calculated for $C_{101}H_{103}Cl_3N_2O_{23}Na$ [M+Na]: 1839.5909, found: 1839.5867.

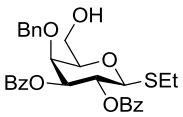
5-Aminopentyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)- β-D-galactopyranoside (2-2)



Compound **2-21** (84 mg; 46 µmol) was dissolved in Methanol/THF 4:1 (2.5 mL). Sodium methoxide 0.5 M in MeOH was added (0.30 mL; 150 µmol). The reaction was warmed to 35 °C and stirred for 72 h. MS analysis showed formation of both the desired product and a derivative resulting from cleavage of the amidic bond of the trichloroacetamide. The solution was cooled to 0 °C, Ac_2O (0.25 mL; 2.63 mmol) was added and the reaction was warmed to r.t.. After 30 min MS analysis showed appearance of a new peak corresponding to the *N*-acetylated trisaccharide. The solvents were evaporated and the crude residue was purified by column chromatography (DCM/MeOH 95:5). Without further characterization the obtained mixture was dissolved in iPrOH/H_2O/AcOH 3:1:0.1 (2.0 mL). Pd/C was added, the solution was purged with Argon and Hydrogen and left stirring under H_2 atmosphere with a balloon for 60 h at 30 °C. The mixture was filtrered through a PTFE filter (0.45 µm pore size) and concentrated. The crude material was purified by size-exclusion chromatography (Sephadex LH-20, H_2O/MeOH 9:1). Lyophilization afforded **2-2** (22 mg, 34 µmol, 74%).

^{1}H NMR (600 MHz, D_2O) δ 4.73 (d, *J* = 8.3 Hz, 1H), 4.49 (d, *J* = 7.8 Hz, 1H), 4.40 (d, *J* = 7.9 Hz, 1H), 4.16 (d, *J* = 3.4 Hz, 1H), 4.00 – 3.91 (m, 3H), 3.86 (m, 1H), 3.83 – 3.66 (m, 12H), 3.63 – 3.53 (m, 3H), 3.04 – 2.99 (m, 2H), 2.05 (s, 3H), 1.74 – 1.65 (m, 4H), 1.47 (m, 2H); ^{13}C NMR (151 MHz, D_2O) δ 174.9, 102.8, 102.7, 102.5, 82.4, 78.1, 75.3, 74.6, 74.5, 72.4, 72.1, 70.9, 69.9, 69.7, 68.5, 68.2, 61.0, 60.8, 59.8, 55.2, 39.3, 28.1, 26.4, 22.2, 22.0; HRMS (ESI+) calculated for $C_{25}H_{47}N_2O_{16}$ [M+H]: 631.2920, found: 631.2930.

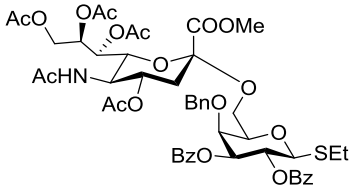
Ethyl 2,3-di-*O*-benzoyl-4-*O*-benzyl-1-thio-β-D-galactopyranoside (2-25)



Compound **2-24**[191] (700 mg; 1.345 mmol) was dissolved in DCM (13 mL). BH_3 1 M in THF (6 mL; 6 mmol) was added followed by TMSOTf (36 µL; 0.202 mmol). The reaction was left stirring for 16 h at r.t. then cooled to 0 °C and neutralized with triethylamine and MeOH. The solvent was evaporated and the residue was purified using a RevelerisX2 Flash Chromatography System (Hexane/EtOAc 70:30 to 30:70) to obtain pure **2-25** (565 mg; 1.081 mmol; 80%).

$[\alpha]_D^{25}$ = 83.0° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3516, 3068, 3034, 2932, 2873, 1724, 1603, 1585, 1495, 1453, 1355, 1316, 1276, 1179, 1133, 1092, 1071, 1028, 1002, 871, 803, 735, 709, 675; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.93 (m, 4H), 7.55 – 7.47 (m, 2H), 7.42 – 7.33 (m, 4H), 7.31 – 7.21 (m, 5H), 5.90 (t, *J* = 10.0 Hz, 1H), 5.39 (dd, *J* = 10.0, 3.0 Hz, 1H), 4.78 (d, *J* = 11.7 Hz, 1H), 4.69 (d, *J* = 9.9 Hz, 1H, H-1), 4.50 (d, *J* = 11.7 Hz, 1H), 4.18 (d, *J* = 2.9 Hz, 1H), 3.88 (dd, *J* = 11.2, 7.0 Hz, 1H), 3.81 – 3.73 (m, 1H), 3.58 (dd, *J* = 11.3, 5.2 Hz, 1H), 2.87 – 2.68 (m, 2H, $SC\underline{H_2}CH_3$), 1.25 (t, *J* = 7.4 Hz, 3H, $SCH_2C\underline{H_3}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 165.6, 137.4, 133.6, 133.3, 129.94, 129.86, 129.5, 129.0, 128.64, 128.58, 128.5, 128.4, 128.2, 83.9 (C-1), 79.1, 76.0, 74.8, 73.6, 68.5, 61.9, 24.1, 15.0; HRMS (ESI+) calculated for $C_{29}H_{30}O_7SNa$ [M+Na]: 545.1604, found: 545.1606.

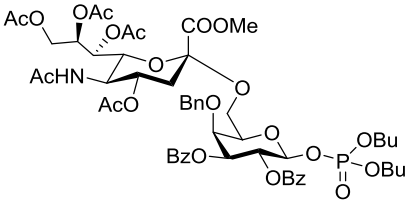
Ethyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-1-thio-β-D-galactopyranoside (2-22α)



Acceptor **2-20** (360 mg; 0.688 mmol) and donor **2-12**[180] (380 mg; 0.573 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM/CH_3CN 1:1 (10 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min, then cooled to −60 °C. TMSOTf (0.5 M in DCM; 230 µL; 0.115 mmol) was added. After 2 h the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM, filtered and concentrated. The crude product was purified by chromatography on silica (Toluene/Acetone 8:2 to 6:4) to obtain pure α sialyl disaccharide **2-22α** (354 mg; 0.355 mmol; 62%) and the corresponding β disaccharide (59 mg; 0.059 mmol).

$[\alpha]_D^{25}$ = 30.0° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3364, 2960, 1729, 1667, 1603, 1586, 1538, 1498, 1453, 1369, 1215, 1179, 1156, 1124, 1070, 1029, 1002, 947, 904, 859, 824, 752, 709, 667; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99 – 7.95 (m, 2H), 7.93 – 7.87 (m, 2H), 7.51 – 7.45 (m, 2H), 7.38 – 7.28 (m, 6H), 7.24 – 7.19 (m, 2H), 7.19 – 7.13 (m, 1H), 5.85 (t, *J* = 10.0 Hz, 1H, H-2), 5.45 – 5.37 (m, 2H, H-3/H-8'), 5.32 (dd, *J* = 8.3, 2.0 Hz, 1H, H-7'), 5.21 (d, *J* = 9.8 Hz, 1H, NH), 4.90 – 4.83 (m, 1H, H-4'), 4.79 (d, *J* = 9.9 Hz, 1H, H-1), 4.68 (d, *J* = 11.5 Hz, 1H, C<u>H</u>HPh), 4.63 (d, *J* = 11.5 Hz, 1H, CH<u>H</u>Ph), 4.38 (dd, *J* = 12.5, 2.7 Hz, 1H, H-9'a), 4.27 (d, *J* = 3.1, 1.0 Hz, 1H, H-4), 4.16 – 4.03 (m, 3H, H-9'b/H-5'/H-6'), 4.02 – 3.97 (m, 1H, H-5), 3.86 (dd, *J* = 10.0, 5.8 Hz, 1H, H-6a), 3.70 (s, 3H, OCH_3), 3.66 (dd, *J* = 10.0, 8.5 Hz, 1H, H-6b), 2.85 – 2.70 (m, 2H, S<u>CH_2</u>CH_3), 2.59 (dd, *J* = 12.9, 4.6 Hz, 1H, H-3'$_{eq}$), 2.19 (s, 3H, $COCH_3$), 2.15 (s, 3H, $COCH_3$), 2.05 – 2.00 (m, 6H, 2x$COCH_3$), 1.96 (t, *J* = 12.6 Hz, 1H, H-3'$_{ax}$), 1.89 (s, 3H, $COCH_3$), 1.25 (t, *J* = 7.5 Hz, 3H, SCH_2C<u>H_3</u>); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.1, 170.8, 170.5, 170.3, 170.0, 168.0 (C-1', $^3J_{C,H}$ = 6.3 Hz), 166.0, 165.6, 138.3, 133.4, 133.1, 129.93, 129.90, 129.80, 129.3, 128.5, 128.4, 128.3, 127.8, 127.6, 99.3 (C-2'), 83.7 (C-1), 76.6, 75.8, 74.9, 74.1, 72.9, 69.0, 68.78, 68.76, 67.5, 63.0, 62.7, 53.0 (OCH_3), 49.6, 38.2 (C-3'), 24.1, 23.3, 21.2, 20.97, 20.96, 20.9, 15.0; HRMS (ESI+) calculated for $C_{49}H_{57}NO_{19}SNa$ [M+Na]: 1018.3138, found: 1018.3136.

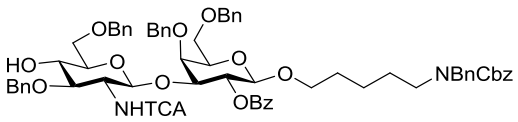
Dibutyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-β-D-galactopyranosyl phosphate (2-27)



Disaccharide **2-22α** (80 mg; 0.080 mmol) was coevaporated three times with toluene, left under vacuum overnight, then it was in dissolved in DCM (2 mL). Dibutyl phosphate (48 µL; 0.241 mmol) was dissolved in DCM (1 mL) and 4Å molecular sieves were added. After stirring for 30 min the solution of disaccharide was added to the solution of dibutyl phosphate and cooled to 0 °C. NIS (27 mg; 0.120 mmol) and TfOH (0.1 M in dioxane; 160 µL; 0.016 mmol) were added and the reaction was gradually warmed to r.t. After 2 h it was neutralized with triethylamine, diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phases were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by chromatography on silica (Toluene/Acetone 6:4) to obtain pure β phosphate **2-27** (87 mg; 0.076 mmol; 95%).

$[\alpha]_D^{25}$ = 21.9° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3292, 2963, 2878, 1742, 1687, 1603, 1546, 1453, 1370, 1262, 1221, 1180, 1098, 1035, 955, 870, 803, 758, 713; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 – 7.97 (m, 2H), 7.94 – 7.89 (m, 2H), 7.54 – 7.44 (m, 2H), 7.39 – 7.28 (m, 6H), 7.24 – 7.13 (m, 3H), 5.90 (dd, *J* = 10.5, 8.0 Hz, 1H, H-2), 5.54 (t, *J* = 7.7 Hz, 1H, H-1), 5.44 – 5.38 (m, 1H, H-7'), 5.37 – 5.30 (m, 2H, H-8'/H-3), 5.17 (d, *J* = 9.6 Hz, 1H, NH), 4.94 – 4.84 (m, 1H, H-4'), 4.65 (br s, 2H, CH_2Ph), 4.30 – 4.22 (m, 2H), 4.15 – 3.97 (m, 7H), 3.82 – 3.60 (m, 6H), 2.59 (dd, *J* = 12.8, 4.6 Hz, 1H, H-3'$_{eq}$), 2.18 (s, 3H, $COCH_3$), 2.13 (s, 3H, $COCH_3$), 2.03 (m, 6H, 2x$COCH_3$), 1.98 – 1.88 (m, 4H, H-3'$_{ax}$/$COCH_3$), 1.68 – 1.59 (m, 2H), 1.43 – 1.33 (m, 2H), 1.32 – 1.22 (m, 2H) 1.08 – 0.97 (m, 2H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.68 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 170.9, 170.4, 169.6, 168.0, 165.9, 165.4, 138.0, 133.6, 133.4, 130.01, 129.95, 129.3, 129.0, 128.6, 128.5, 128.4, 127.9, 127.7, 98.8 (C-2'), 96.88 (d, $^2J_{C,P}$ = 4.6 Hz, H-1), 75.1, 74.3, 73.8, 73.7, 72.5, 70.2, 70.10, 69.0, 68.2, 68.1, 68.0, 67.94, 67.91, 67.1, 62.4, 62.3, 53.1 (OCH_3), 49.6, 38.1 (C-3'), 32.2, 32.1, 31.9, 31.9, 23.4, 21.2, 21.03, 21.01, 20.9, 18.7, 18.4, 13.8, 13.5; ^{31}P NMR (162 MHz, $CDCl_3$) δ -2.78; HRMS (ESI+) calculated for $C_{55}H_{70}NO_{23}PNa$ [M+Na]: 1166.3968, found: 1166.3976.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranoside (2-29)**

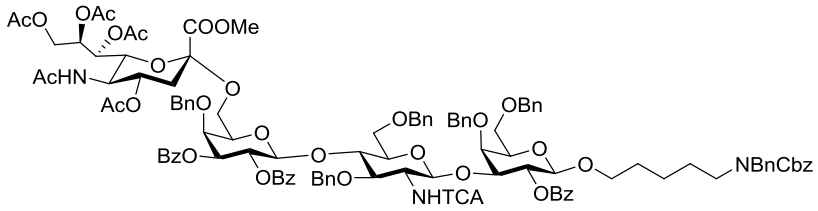


Galactose acceptor **2-18** (237 mg; 0.306 mmol) and glucose donor **2-28** (286 mg; 0.371 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM (6 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to −30 °C. NIS (96 mg; 0.428 mmol) and TMSOTf (0.1 M in dioxane; 300 µL; 0.030 µmol) were added. The reaction was gradually warmed to −10 °C and quenched after 1 h with the addition of triethylamine. It was then diluted with DCM and filtered. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phases were dried over Na_2SO_4, filtered and concentrated. The obtained crude product was redissolved in DCM (5 mL) and triethylamine (1.5 mL). After 2 h the solution was cooled to 0 °C and carefully neutralized with acetic acid. It was then diluted with DCM and extracted three times with water. The aqueous phases were extracted once with DCM and the combined organic phases were dried over Na_2SO_4, filtered and concentrated. Crude material

was purified by chromatography on silica (Toluene/EtOAc 9:1 to 7:3) and size-exclusion chromatography (Sephadex LH-20, $CHCl_3$/MeOH 1:1) to obtain disaccharide **2-29** (336 mg; 0.266 mmol; 86% over 2 steps).

$[\alpha]_D^{25}$ = -19.2° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3417, 3066, 3034, 2927, 2865, 1701, 1604, 1586, 1521, 1498, 1455, 1424, 1365, 1269, 1070, 1028, 913, 821, 736, 698; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.89 (m, 2H), 7.49 – 7.42 (m, 1H), 7.37 – 7.07 (m, 32H), 6.62 (d, *J* = 8.0 Hz, 1H, NH), 5.50 (t, *J* = 8.7 Hz, 1H, H-2), 5.11 (br s, 2H, CH_2Ph), 4.92 (d, *J* = 11.8 Hz, 1H), 4.86 (br d, *J* = 8.1 Hz, 1H, H-1'), 4.69 – 4.61 (m, 2H), 4.61 – 4.49 (m, 3H), 4.46 – 4.25 (m, 5H, CH_2Ph/-OCH_2-/H-1), 4.08 – 3.98 (m, 2H), 3.83 – 3.51 (m, 9H), 3.49 – 3.42 (m, 1H), 3.33 – 3.21 (m, 1H), 3.02 – 2.96 (m, 0.5H), 2.91 – 2.81 (m, 1.5H, -CH_2N-), 1.43 – 1.26 (m, 4H, 2xCH_2 (Linker)), 1.09 – 0.91 (m, 2H, CH_2 (Linker)); ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.3, 162.0, 156.8, 156.2, 138.8, 138.1, 138.0, 137.7, 137.0, 133.4, 130.1, 129.9, 128.7, 128.60, 128.57, 128.56, 128.53, 128.50, 128.4, 128.3, 128.10, 128.07, 128.03, 128.00, 127.98, 127.92, 127.89, 127.85, 127.8, 127.6, 127.3, 101.7 (C-1'), 100.0 (C-1), 92.3 (CO<u>C</u>Cl_3), 80.2, 78.7, 76.2, 75.0, 74.6, 74.1, 73.91, 73.89, 73.7, 73.0, 58.1, 50.6, 50.3, 47.2, 46.2, 29.8, 29.2, 27.8, 27.4, 23.2; HRMS (ESI+) calculated for $C_{69}H_{73}Cl_3N_2O_{14}Na$ [M+Na]: 1281.4020, found: 1281.4014.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-trichloroacetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranoside (2-26)**

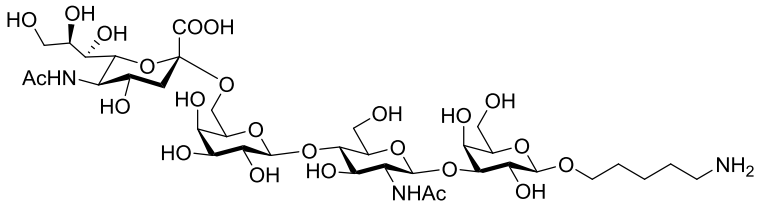


Disaccharide acceptor **2-29** (40 mg; 0.032 mmol) and disaccharide donor **2-27** (55 mg; 0.048 mmol) were coevaporated three times with toluene and left under vacuum overnight. They were then dissolved in DCM (1.5 mL) under Ar atmosphere. 4Å molecular sieves were added, the solution was stirred for 30 min then cooled to 0 °C. TMSOTf (0.5 M in DCM; 95 µL; 0.045 mmol) was added. After 1 h the reaction was quenched with triethylamine, warmed to r.t., diluted with DCM, filtered and concentrated. The crude product was purified by chromatography on silica (Toluene/Acetone 7:3) and HPLC (YMC-diol-300NP column, 150 x 20 mm, 30% EtOAc in Hex (5 min), linear gradient to 75% AcOEt (30 min), linear gradient to 100% AcOEt (5 min)) to obtain pure tetrasaccharide **2-26** (47 mg; 0.0214 mmol; 67%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 – 7.87 (m, 6H), 7.54 – 7.48 (m, 2H), 7.45 – 7.13 (m, 41H), 7.11 – 7.07 (m, 1H), 6.74 (d, *J* = 7.6 Hz, 1H), 5.73 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.49 (dd, *J* = 10.2, 7.8 Hz, 1H), 5.33 – 5.26 (m, 3H), 5.15 – 5.07 (m, 3H), 4.95 (d, *J* = 11.8 Hz, 1H), 4.91 – 4.79 (m, 3H), 4.74 (d, *J* = 6.6 Hz, 1H), 4.65 (d, *J* = 11.4 Hz, 1H), 4.62 – 4.53 (m, 3H), 4.44 (d, *J* = 12.0 Hz, 1H), 4.40 (d, *J* = 11.9 Hz, 1H), 4.38 – 4.26 (m, 6H), 4.26 – 4.22 (m, 1H), 4.16 (d, *J* = 3.1 Hz, 1H), 4.08 – 3.95 (m, 6H), 3.87

(dd, *J* = 10.3, 2.8 Hz, 1H), 3.83 – 3.66 (m, 6H), 3.64 – 3.52 (m, 9H), 3.48 – 3.39 (m, 2H), 3.24 (d, *J* = 32.2 Hz, 1H), 3.03 – 2.93 (m, 0.5H), 2.91 – 2.81 (m, 1.5H), 2.53 (dd, *J* = 12.9, 4.6 Hz, 1H, H-3'''$_{eq}$), 2.10 (s, 3H, $COCH_3$), 2.06 (s, 3H, $COCH_3$), 2.02 (s, 3H, $COCH_3$), 1.93 – 1.90 (m, 4H, $COCH_3$/ H-3'''$_{ax}$), 1.88 (s, 3H, $COCH_3$), 1.40 – 1.28 (m, 4H), 1.10 – 0.93 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.89, 170.7, 170.2, 170.1, 169.9, 167.6, 165.7, 165.17, 164.9, 161.7, 138.7, 138.2, 138.1, 138.02, 138.0, 133.3, 133.2, 133.00, 130.02, 129.8, 129.73, 129.68, 129.5, 129.0, 128.50, 128.46, 128.42, 128.38, 128.3, 128.2, 128.1, 128.0, 127.82, 127.76, 127.6, 127.5, 127.4, 127.3, 127.1, 101.6, 100.5, 100.4, 99.2, 92.1, 79.1, 78.3, 75.9, 75.1, 74.8, 74.6, 74.1, 73.8, 73.7, 73.5, 73.2, 72.8, 72.7, 72.2, 70.8, 69.0, 68.8, 68.7, 68.6, 67.2, 67.0, 62.2, 57.2, 52.9, 50.4, 50.1, 49.5, 47.0, 46.0, 37.7, 31.9, 29.7, 27.7, 27.2, 23.2, 21.0, 20.8, 20.73, 20.68; HRMS (ESI+) calculated for $C_{116}H_{124}Cl_3N_3O_{33}Na_2$ [M+2Na]: 1118.8484, found: 1118.8470.

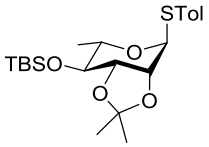
5-Aminopentyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranoside (2-3)



Compound **2-26** (54 mg; 0.026 mmol) was dissolved in THF/MeOH 1:1 (2.5 mL), $LiOH{\cdot}H_2O$ (24 mg; 1.002 mmol) was added and the solution was warmed to 50 °C. After 3 h it was cooled to r.t. and neutralized with the addition of Amberlite IR120 H^+, filtered and concentrated. The crude residue was purified by size-exclusion chromatography (Sephadex LH-20, $CHCl_3$/MeOH 1:1). The obtained product was dissolved in THF/MeOH/H_2O 1:1:1 (2.0 mL), Pd/C was added, the solution was purged with argon and left stirring under H_2 atmosphere (10 bar) for 72 h. The mixture was filtered through a PTFE filter (0.45 µm pore size) and concentrated. The crude material was purified by HPLC (Hypercarb column, 150x10 mm, H_2O (0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (10 min)) and lyophilized to obtain **2-3** (5.5 mg; 6.0 µmol; 23% over 2 steps).
^{1}H NMR (600 MHz, D_2O) δ 4.77 – 4.75 (m, 1H)**, 4.47 (d, *J* = 7.9 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.17 (d, *J* = 3.4 Hz, 1H), 4.04 – 3.61 (m, 22H), 3.60 – 3.52 (m, 4H), 3.05 – 3.00 (m, 2H), 2.69 (dd, *J* = 12.4, 4.7 Hz, 1H), 2.10 – 2.01 (m, 6H), 1.77 – 1.65 (m, 5H), 1.53 – 1.45 (m, 2H); ^{13}C NMR (151 MHz, D_2O) δ 174.86, 174.85, 173.5 (C-1''', $^3J_{C,H}$ = 5.2 Hz), 103.4, 102.7, 102.4, 100.1, 82.3, 80.4, 74.6, 74.2, 73.6, 72.5, 72.4, 72.2, 71.6, 70.7, 69.9, 69.7, 68.4, 68.3, 68.2, 68.1, 63.3, 62.6, 60.8, 60.1, 55.0, 51.8, 40.0, 39.3, 28.1, 26.3, 22.3, 22.02, 21.96; HRMS (ESI+) calculated for $C_{36}H_{64}N_3O_{24}$ [M+H]: 922.3874, found: 922.3894.

****Note:** the appearance of this signal as multiplet is presumably an artifact since it can be attributed to an anomeric proton.

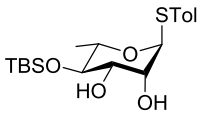
***p*-Tolyl 2,3-*O*-isopropylidene-4-*O*-*tert*-butyl dimethylsilyl-1-thio-α-L-rhamnopyranoside (2-35)**



Compound **2-34**[186] (3.366 g; 10.844 mmol) was dissolved in DMF (20 mL) and the solution was cooled to 0 °C. *tert*-Butyldimethylsilyl chloride (2.499 g; 16.580 mmol) and imidazole (2.300 g; 33.784 mmol) were added portionwise. After the addition it was warmed to r.t. and left stirring overnight. The solution was then concentrated, diluted with EtOAc and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified using a RevelerisX2 Flash Chromatography System (Silica Cartridge - Hexane/EtOAc 90:10 to 70:30) to obtain compound **2-35** (4.382 g; 10.318 mmol; 95%).

$[\alpha]_D^{25}$ = -179.3° (c = 2.2, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3379, 2957, 2933, 2898, 2859, 1494, 1473, 1463, 1381, 1362, 1311, 1279, 1245, 1220, 1164, 1106, 1092, 1073, 1056, 1019, 1002, 939, 923, 869, 838, 809, 792, 778, 749, 713, 667; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (d, *J* = 8.2 Hz, 2H), 7.12 (d, *J* = 8.4 Hz, 2H), 5.66 (d, *J* = 0.8 Hz, 1H, H-1), 4.32 (dd, *J* = 5.6, 0.8 Hz, 1H, H-2), 4.08 – 3.99 (m, 2H, H-3/H-5), 3.40 (dd, *J* = 9.7, 7.2 Hz, 1H, H-4), 2.33 (s, 3H, -PhCH_3), 1.52 (s, 3H, -CH_3), 1.35 (s, 3H, -CH_3), 1.18 (d, *J* = 6.2 Hz, 3H, -CH_3), 0.91 (s, 9H, tBu-Si), 0.15 (s, 3H, Me-Si), 0.09 (s, 3H, Me-Si); ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.9, 132.6, 129.94, 129.91, 109.3, 84.4, 79.0, 76.8, 76.3, 67.6, 28.3, 26.7, 26.0 (3C), 21.3, 18.3, 17.8, -3.8, -4.7; HRMS (ESI+) calculated for $C_{22}H_{36}O_4SSiNa$ [M+Na]: 447.1996, found: 447.2018.

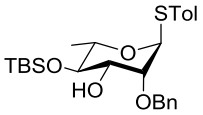
***p*-Tolyl 4-*O*-*tert*-butyl dimethylsilyl-1-thio-α-L-rhamnopyranoside (2-36)**



Compound **2-35** (4.382 g; 10.318 mmol) was dissolved in DCM (60 mL). Water (1 mL) and Trifluoroacetic acid (81 µL; 1.06 mmol) were added. The solution was left stirring overnight, then it was neutralized with triethylamine, diluted with DCM and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified using a RevelerisX2 Flash Chromatography System (Silica Cartridge - Hexane/EtOAc 90:10 to 50:50) to obtain compound **2-36** (3.682 g; 9.573 mmol; 93%).

$[\alpha]_D^{25}$ = -116.6° (c = 2.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3374, 2956, 2931, 2859, 1494, 1473, 1464, 1381, 1363, 1257, 1105, 1062, 1020, 985, 927, 835, 811, 778, 728, 672; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.32 (m, 2H), 7.15 – 7.10 (m, 2H), 5.26 (d, *J* = 1.7 Hz, 1H, H-1), 4.20 – 4.06 (m, 2H), 3.71 (dd, *J* = 9.2, 3.4 Hz, 1H), 3.46 (t, *J* = 9.3 Hz, 1H), 2.33 (s, 3H, -PhCH_3), 2.25 (br s, 2H), 1.31 (d, *J* = 6.2 Hz, 3H, -CH_3), 0.90 (s, 9H, tBu-Si), 0.10 (s, 3H, Me-Si), 0.07 (s, 3H, Me-Si); ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.9, 132.7, 130.3, 130.0, 89.2, 74.2, 73.4, 72.5, 69.2, 25.8, 21.3, 18.1, 17.6, -4.5, -4.7; HRMS (ESI+) calculated for $C_{19}H_{32}O_4SSiNa$ [M+Na]: 407.1683, found: 407.1686.

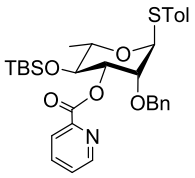
***p*-Tolyl 2-*O*-benzyl-4-*O*-*tert*-butyl dimethylsilyl-1-thio-α-L-rhamnopyranoside (2-37)**



Compound **2-36** (3.682 g; 9.573 mmol) was dissolved in DCM (33 mL). Aqueous NaOH (10% w/v; 7 mL) was added. Under vigorous stirring, tetrabutylammonium bromide (720 mg; 2.233 mmol) and benzyl bromide (1.25 mL; 10.508 mmol) were added and the solution was left stirring at r.t. After 2 h the reaction was diluted with DCM, layers were separated and the organic layer was extracted twice with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified using a RevelerisX2 Flash Chromatography System (Silica Cartridge - Hexane/EtOAc 95:5 to 75:25) to obtain desired compound **2-37** (1.161 g; 2.446 mmol; 26%).

$[\alpha]_D^{25}$ = -90.2° (c = 1.1, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3560, 2957, 2931, 2896, 2859, 1494, 1473, 1456, 1400, 1361, 1303, 1251, 1211, 1104, 1089, 1068, 1019, 1007, 891, 838, 809, 778, 737, 699, 670; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.28 (m, 7H), 7.15 – 7.09 (m, 2H), 5.47 (d, *J* = 1.3 Hz, 1H, H-1), 4.72 (d, *J* = 11.8 Hz, 1H, -CHHPh), 4.50 (d, *J* = 11.8 Hz, 1H, -CHHPh), 4.10 – 4.01 (m, 1H, H-5), 3.96 (dd, *J* = 3.8, 1.4 Hz, 1H, H-2), 3.69 (dd, *J* = 9.0, 3.7 Hz, 1H, H-3), 3.47 (t, *J* = 9.0 Hz, 1H, H-4), 2.34 (s, 3H, -PhCH_3), 1.28 (d, *J* = 6.2 Hz, 3H, -CH_3), 0.91 (s, 9H, tBu-Si), 0.15 (s, 3H, Me-Si), 0.09 (s, 3H, Me-Si); ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.8, 137.5, 132.4, 130.7, 130.0, 129.0, 128.3, 128.1, 85.5, 79.9, 75.9, 72.4, 72.2, 69.9, 26.1, 21.3, 18.2, -3.6, -4.4; HRMS (ESI+) calculated for $C_{26}H_{38}O_4SSiNa$ [M+Na]: 497.2152, found: 497.2158.

***p*-Tolyl 2-*O*-benzyl-3-*O*-picoloyl-4-*O*-*tert*-butyl dimethylsilyl-1-thio-α-L-rhamnopyranoside (2-38)**

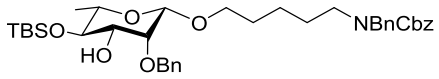


Compound **2-37** (1.141 g; 2.403 mmol) was dissolved in DCM (25 mL). 2-Picolinic acid (0.385 g; 3.127 mmol), DIC (550 µL; 3.605 mmol) and two crystals of DMAP were added sequentially. After 2 h the reaction was filtered through Celite, diluted with DCM and washed once with saturated aqueous $NaHCO_3$ and once with brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified using a RevelerisX2 Flash Chromatography System (Hexane/EtOAc 90:10 to 60:40) to obtain compound **2-38** (1.300 g; 2.242 mmol; 93%).

$[\alpha]_D^{25}$ = -32.9° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 2931, 2860, 1723, 1587, 1494, 1473, 1353, 1307, 1292, 1248, 1130, 1104, 1089, 1044, 994, 838, 809, 779, 745, 700, 668; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (br d, *J* = 4.6 Hz, 1H), 8.06 (d, *J* = 7.8 Hz, 1H), 7.83 (t, *J* = 7.7 Hz, 1H), 7.50 (m, 1H), 7.37 (d, *J* = 7.8 Hz, 2H), 7.24 – 7.18 (m, 2H), 7.11 (m, 5H), 5.45 – 5.34 (m, 2H, H-1/H-2), 4.65 (d, *J* = 12.3 Hz, 1H, CHHPh), 4.50 (d, *J* = 12.3 Hz, 1H, CHHPh), 4.25 – 4.10 (m, 2H, H-5/H-2), 4.04 (t, *J* = 9.0 Hz, 1H, H-4)), 2.34 (s, 3H, -PhCH_3), 1.35 (d, *J* = 6.2 Hz, 3H, -CH_3), 0.78 (s, 9H, tBu-Si), 0.12 (s, 3H, Me-Si), -0.05 (s, 3H, Me-Si); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.2, 150.0, 147.9, 137.8, 137.6, 137.0, 132.4, 130.6, 130.0, 128.3, 128.2, 127.8, 127.0, 125.4, 85.9 (C-1), 77.0, 75.6, 72.4, 72.0, 70.7, 25.9,

21.3, 18.5, 18.1, -3.8, -4.2; HRMS (ESI+) calculated for $C_{32}H_{42}NO_5SSiNa$ [M+Na]: 580.2547, found: 580.2553.

_N_-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2-_O_-benzyl-4-_O_-_tert_-butyl dimethylsilyl-β-L-rhamnopyranoside (2-40)

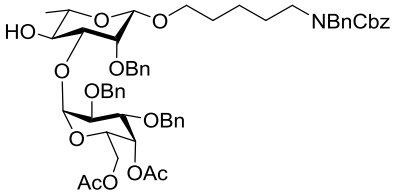


Compound **2-38** (0.203 g; 0.350 mmol) and *N*-(Benzyl)benzyloxycarbonylaminopentanol (210 mg; 0.641 mmol) were coevaporated twice with toluene and left under vacuum for 3 h. They were then dissolved in DCM (6 mL), 4Å Molecular sieves were added and the solution was stirred for 30 min. Bromine (20 µL; 0.778 mmol) was added and the reaction was left stirring at r.t. After 16 h it was filtered, diluted with EtOAc and washed with 10% aqueous $Na_2S_2O_3$ and brine. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified by chromatography on silica (Hexane/EtOAc 8:2) to obtain an impure glycosylated monosaccharide (presumably mixed with traces of α-linked isomer or hydrolyzed donor). Without further characterization, the residue was dissolved in DCM/Methanol 2:1 (6 mL), $Cu(OAc)_2{\cdot}H_2O$ (0.110 g; 0.551 mmol) was added and the solution was left stirring overnight. It was then diluted with DCM and extracted with water; the organic phase was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified using a RevelerisX2 Flash Chromatography System (Silica Cartridge - Hexane/EtOAc 95:5 to 60:40) to obtain pure β-**2-40** (0.146 g; 0.215 mmol; 61% over 2 steps).

$[\alpha]_D^{25}$ = 45.4° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3516, 3068, 3034, 2932, 2873, 1724, 1602, 1585, 1495, 1453, 1355, 1316, 1276, 1179, 1133, 1092, 1071, 1028, 1002, 871, 803, 735, 709, 675; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.13 (m, 15H), 5.16 (app d, 2H, CH_2Ph), 5.02 (m, 1H, C<u>H</u>HPh), 4.64 – 4.56 (m, 1H, CH<u>H</u>Ph), 4.49 (m, 3H, -OCH_2(linker)/H-1), 3.96 – 3.83 (m, 1H, C<u>H</u>HPh), 3.75 (m, 1H, H-2), 3.46 – 3.11 (m, 6H, -NCH_2-(Linker)/H-3/H-4/H-5/ CH<u>H</u>Ph), 2.19 (s, 1H), 1.70 – 1.48 (m, 4H, 2xCH_2(Linker)), 1.30 (d, *J* = 6.2 Hz, 5H, -CH_2-(Linker)/-CH_3), 0.87 (s, 9H, tBu-Si), 0.10 (s, 3H, Me-Si), 0.06 (s, 3H, Me-Si); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.9, 156.3, 138.5, 138.0, 137.0, 136.9, 128.7, 128.59, 128.56, 128.4, 128.1, 128.01, 127.95, 127.4, 127.3, 101.8 (C-1), 78.0, 75.8, 75.1, 73.9, 72.9, 69.8, 69.7, 67.3, 50.6, 50.3, 47.2, 46.3, 29.9, 29.5, 28.0, 27.6, 26.1, 23.5, 18.4, 18.3, -3.7, -4.5; HRMS (ESI+) calculated for $C_{39}H_{55}NO_7SiNa$ [M+Na]: 700.3640, found: 700.3647.

Note: the unusual splittings and multiplicities, together with broad proton signals, were indicative of a possible conformational equilibrium in solution.

_N_-(Benzyl)benzyloxycarbonyl-5-aminopentyl 4,6-di-_O_-acetyl-2,3-di-_O_-benzyl-α-D-galactopyranosyl-(1→3)-2-_O_-benzyl-β-L-rhamnopyranoside (2-42)

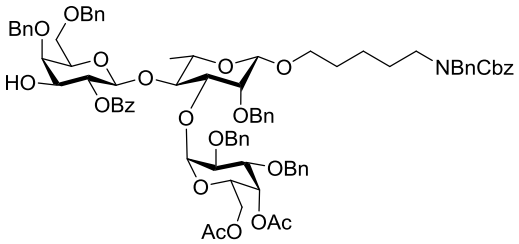


Donor **2-8**[140] (198 mg; 0.406 mmol) and acceptor **2-38** (220 mg; 0.325 mmol) were coevaporated three times with Toluene and left under high vacuum overnight. Under argon atmosphere they were dissolved in DCM/Et_2O 1:1 (6 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −10 °C. NIS (137 mg; 0.609 mmol) and TfOH (0.5M in dioxane; 66 µL; 0.033 mmol) were added. After 30 min the reaction was quenched with triethylamine, warmed to r.t., filtered and diluted with EtOAc. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified by chromatography on silica (Hexane/EtOAc 8:2 to 3:1) to obtain the desired disaccharide.

Without complete characterization, the obtained product was dissolved in THF (6 mL), acetic acid (82 µL; 1.44 mmol) and TBAF (1M in THF; 1.40 mL; 1.40 mmol) were added and the reaction was left stirring at r.t. for 16 h. It was then diluted with EtOAc and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude was purified by chromatography on silica (Hexane/EtOAc 6:4 to 4:6) to obtain pure disaccharide **2-42** (207 mg; 0.209 mmol; 64% over 2 steps).

$[\alpha]_D^{25}$ = 64.0° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3487, 3034, 2927, 2857, 1746, 1698, 1607, 1498, 1455, 1423, 1371, 1310, 1227, 1157, 1125, 1091, 1071, 1028, 946, 910, 822, 794, 736, 698; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38 – 7.14 (m, 25H), 5.44 (br s, 1H), 5.17 (br d, *J* = 19.2 Hz, 2H), 5.00 (br s, 1H, H-1 or H-1'), 4.87 – 4.80 (m, 2H), 4.69 – 4.62 (m, 2H), 4.58 (d, *J* = 12.0 Hz, 1H), 4.53 – 4.46 (m, 2H), 4.42 (d, *J* = 11.5 Hz, 1H), 4.33 – 4.23 (m, 2H), 4.12 (dd, *J* = 11.5, 3.7 Hz, 1H), 4.05 – 3.99 (m, 1H), 3.91 – 3.78 (m, 4H), 3.59 (t, *J* = 9.1 Hz, 1H), 3.38 – 3.15 (m, 5H), 2.12 (s, 3H), 2.07 (s, 3H), 1.64 – 1.46 (m, 4H), 1.37 (d, *J* = 6.1 Hz, 3H), 1.32 – 1.20 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.6, 170.3, 156.8, 156.3, 139.23, 138.6, 138.0, 137.9, 137.0, 136.9, 128.7, 128.6, 128.50, 128.48, 128.03, 127.98, 127.94, 127.93, 127.91, 127.90, 127.87, 127.86, 127.83, 127.80, 127.43, 127.38, 127.3, 127.2, 100.9 (C-1 or C-1'), 99.3 (C-1 or C-1'), 86.1, 77.6, 75.7, 75.5, 74.9, 73.8, 72.1, 72.1, 72.1, 69.6, 69.6, 68.2, 67.8, 67.3, 63.2, 50.7, 50.3, 47.3, 46.3, 29.8, 29.4, 28.1, 27.6, 23.4, 20.9, 20.7, 18.0; HRMS (ESI+) calculated for $C_{57}H_{67}NO_{14}Na$ [M+Na]: 1012.4453, found: 1012.4449.

_N_-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2-_O_-benzoyl-4,6-di-_O_-benzyl-β-D-galactopyranosyl-(1→4)-[4,6-di-_O_-acetyl-2,3-di-_O_-benzyl-α-D-galactopyranosyl-(1→3)]-2-_O_-benzyl-β-L-rhamnopyranoside (2-30)



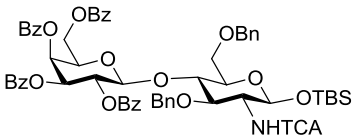
Commercially available donor **2-9** (199 mg; 0.251 mmol) and acceptor **2-42** (207 mg; 0.209 mmol) were coevaporated three times with toluene and left under high vacuum overnight. Under argon atmosphere they were dissolved in DCM (5 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −10 °C. NIS (70 mg; 0.311 mmol) and TfOH (0.5M in dioxane; 42

µL; 0.021 mmol) were added. After 1 h the reaction was quenched carefully with triethylamine, warmed to r.t., filtered and diluted with EtOAc. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated.

The crude product was redissolved in DCM (8 mL) and TEA (2 mL) was added. After 2 h it was neutralized with acetic acid, diluted with DCM and washed three times with water. The organic solution was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by chromatography on silica (Hexane/EtOAc 7:3 to 6:4) to obtain trisaccharide **2-30** (261 mg; 0.181 mmol; 87% over 2 steps).

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10 – 8.04 (m, 2H), 7.58 (m, 1H), 7.46 (m, 2H), 7.38 – 7.14 (m, 33H), 7.03 (m, 2H), 5.29 (d, *J* = 3.3 Hz, 1H), 5.21 – 5.11 (m, 4H), 4.90 (m, 2H), 4.74 (d, *J* = 11.8 Hz, 1H), 4.68 – 4.61 (m, 2H), 4.55 (d, *J* = 11.7 Hz, 4H), 4.43 – 4.36 (m, 3H), 4.00 (d, *J* = 11.1 Hz, 1H), 3.87 (m, 5H), 3.80 – 3.70 (m, 4H), 3.67 – 3.57 (m, 3H), 3.53 (dd, *J* = 9.9, 3.4 Hz, 1H), 3.47 (d, *J* = 6.3 Hz, 1H), 3.40 (dd, *J* = 9.6, 3.1 Hz, 1H), 3.31 – 3.15 (m, 3H), 3.10 (m, 1H), 2.17 (s, 3H), 2.03 (s, 3H), 1.53 (m, 4H), 1.37 – 1.23 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.4, 170.3, 166.5, 138.29, 138.27, 138.2, 137.9, 133.4, 130.3, 129.9, 128.74, 128.72, 128.66, 128.62, 128.60, 128.56, 128.5, 128.3, 128.08, 128.06, 128.04, 128.00, 127.9, 127.73, 127.70, 127.66, 127.6, 127.3, 101.4, 99.7, 95.4, 79.2, 76.9, 76.39, 76.37, 75.4, 75.2, 74.7, 74.5, 73.7, 73.2, 73.0, 72.5, 71.5, 71.4, 69.7, 69.6, 68.00, 67.95, 67.8, 67.3, 63.0, 50.7, 50.4, 47.3, 46.3, 29.5, 28.1, 27.7, 23.5, 21.1, 21.0, 18.0; HRMS (ESI+) calculated for $C_{84}H_{93}NO_{20}Na$ [M+Na]: 1458.6183, found: 1458.6185.

tert-Butyl dimethylsilyl 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (2-45)

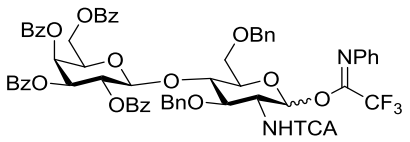


Donor **2-43**[189] (136 mg; 0.212 mmol) and acceptor **2-44**[190] (102 mg; 0.165 mmol) were coevaporated three times with toluene and left under high vacuum overnight. Under Argon atmosphere they were dissolved in DCM (4 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −20 °C. NIS (55 mg; 0.244 mmol) and TfOH (0.5M in dioxane; 33 µL; 0.017 mmol) were added. After 30 min the reaction was quenched with triethylamine, warmed to r.t., filtered and diluted with EtOAc. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by chromatography on silica (Toluene/EtOAc 30:1 to 9:1) to obtain disaccharide **2-45** (178 mg; 0.151 mmol; 93%).

$[\alpha]_D^{25}$ = 18.5° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3345, 3067, 3034, 2932, 2860, 1731, 1603, 1586, 1530, 1497, 1453, 1362, 1316, 1264, 1177, 1094, 1069, 1028, 938, 911, 840, 821, 785, 738, 709, 687; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 – 7.98 (m, 2H), 7.91 (m, 4H), 7.80 – 7.73 (m, 2H), 7.60 – 7.51 (m, 3H), 7.49 – 7.29 (m, 13H), 7.28 – 7.16 (m, 6H), 6.89 (m, 1H), 5.90 (dd, *J* = 3.5, 1.0 Hz, 1H), 5.75 (dd, *J* = 10.4, 8.0 Hz, 1H), 5.45 (dd, *J* = 10.4, 3.5 Hz, 1H), 5.17 (d, *J* = 10.8 Hz, 1H), 5.03 (d, *J* = 7.7 Hz, 1H), 4.97 (d, *J* = 8.1 Hz, 1H), 4.75 (m, 2H), 4.48 – 4.37 (m, 2H), 4.32 – 4.18 (m, 2H), 4.14 –

4.05 (m, 2H), 3.72 (dd, *J* = 11.1, 3.1 Hz, 1H), 3.57 – 3.46 (m, 2H), 3.34 (m, 1H), 0.86 (s, 9H), 0.08 (s, 3H), 0.04 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 165.6, 165.5, 165.0, 161.7, 138.5, 138.0, 133.63, 133.56, 133.4, 129.92, 129.86, 129.8, 129.5, 129.10, 129.06, 128.84, 128.82, 128.71, 128.65, 128.6, 128.41, 128.38, 128.37, 128.2, 127.8, 127.7, 100.4, 94.6, 92.6, 77.2, 76.6, 74.7, 74.5, 73.8, 71.8, 71.3, 70.3, 68.0, 67.8, 61.7, 60.3, 25.7, 18.0, -4.1, -5.1; HRMS (ESI+) calculated for $C_{62}H_{64}Cl_3NO_5SiNa$ [M+Na]: 1218.3003, found: 1218.3008.

2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-α/β-D-glucopyranosyl *N*-phenyltrifluoroacetimidate (2-31)



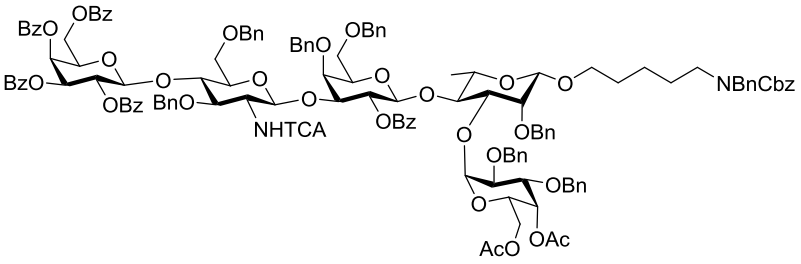
Disaccharide **2-45** (178 mg; 0.151 mmol) was dissolved in THF (3 mL). Acetic acid (26 µL; 0.45 mmol) and TBAF (1M in THF; 450 µL; 0.450 mmol) were added and the reaction was stirred at r.t. for 30 min. It was then diluted with EtOAc and extracted three times with water. The organic phase was dried over Na_2SO_4, filtered and concentrated.

The obtained residue was dissolved in DCM (3 mL); *N*-phenyltrifluoroacetimidoyl chloride (75 µL; 0.46 mmol) and Cs_2CO_3 (158 mg; 0.485 mmol) were added and the solution was stirred at r.t. for 30 min. The solution was then filtered and concentrated. The crude residue was purified by chromatography on silica (Hexane/EtOAc 3:1) to obtain imidate **2-31** as a mixture of diastereoisomers (167 mg; 0.133 mmol; 88%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 – 8.02 (m, 2H), 7.99 – 7.96 (m, 2.4H), 7.95 – 7.91 (m, 4.4H), 7.85 – 7.79 (m, 4.4H), 7.74 – 7.71 (m, 4.4H), 7.59 – 7.55 (m, 1H), 7.54 – 7.12 (m, 55H), 7.01 – 6.96 (m, 1H), 6.66 (d, *J* = 7.7 Hz, 2.2H), 6.50 – 6.30 (m, 2H), 6.22 (d, *J* = 7.0 Hz, 1H), 5.89 (dd, *J* = 3.5, 1.1 Hz, 1H), 5.83 (d, *J* = 3.4 Hz, 1.2H), 5.74 (dd, *J* = 10.4, 8.0 Hz, 1.2H), 5.66 (dd, *J* = 10.5, 8.0 Hz, 1H), 5.36 – 5.31 (m, 2.2H), 5.13 (d, *J* = 11.4 Hz, 1.2H), 4.85 – 4.77 (m, 2.4H), 4.73 (d, *J* = 12.0 Hz, 1.2H), 4.67 (d, *J* = 12.1 Hz, 1H), 4.63 – 4.53 (m, 3.2H), 4.50 (dd, *J* = 11.4, 6.3 Hz, 1H), 4.40 – 4.33 (m, 5.4H), 4.29 – 4.20 (m, 3.4H), 4.16 – 4.06 (m, 3.4H), 4.01 – 3.97 (m, 1.2H), 3.84 – 3.77 (m, 1.2H), 3.71 – 3.64 (m, 1.2H), 3.60 – 3.51 (m, 2H), 3.48 – 3.36 (m, 3.2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.13, 166.06, 165.7, 165.6, 165.5, 165.4, 165.1, 164.6, 162.8, 162.0, 143.0, 138.0, 137.8, 137.7, 137.6, 133.8, 133.70, 133.67, 133.51, 133.47, 133.44, 133.41, 130.2, 129.91, 129.88, 129.84, 129.83, 129.77, 129.76, 129.5, 129.30, 129.27, 129.2, 129.10, 129.06, 129.02, 128.97, 128.84, 128.79, 128.78, 128.75, 128.73, 128.71, 128.68, 128.63, 128.61, 128.57, 128.5, 128.43, 128.41, 128.37, 128.08, 128.05, 128.0, 127.8, 126.5, 124.7, 120.6, 119.4, 104.1, 102.4, 100.5, 92.1, 86.3, 76.5, 75.9, 75.2, 74.7, 73.9, 73.4, 73.2, 71.9, 71.8, 71.53, 71.52, 71.3, 70.7, 70.2, 69.6, 68.4, 68.1, 68.0, 66.8, 65.9, 62.4, 61.6, 53.9; HRMS (ESI+) calculated for $C_{64}H_{54}Cl_3F_3N_2O_{15}Na$ [M+Na]: 1275.2434, found: 1275.2397.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-**

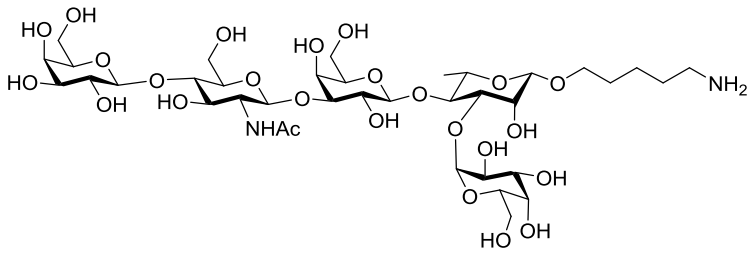
4,6-Di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-[4,6-di-*O*-acetyl-2,3-di-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-2-*O*-benzyl-β-L-rhamnopyranoside (2-46)



Donor **2-31** (131 mg; 0.105 mmol) and acceptor **2-30** (112 mg; 0.078 mmol) were coevaporated three times with Toluene and left under high vacuum overnight. Under Argon atmosphere they were dissolved in DCM (3 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −30 °C. TMSOTf (0.1 M in DCM; 78 µL; 0.0078 mmol) was added. After 30 min, an additional 0.1 eq of TMSOTf was added. After 1 h the reaction was quenched carefully with triethylamine, warmed to r.t., filtered and concentrated. The crude residue was purified by chromatography on silica (Toluene/EtOAc 30:1 to 9:1) and size-exclusion chromatography (Sephadex LH-20, $CHCl_3$/MeOH 1:1) to obtain pentasaccharide **2-46** (130 mg; 0.052 mmol; 67%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 8.11 (d, *J* = 7.7 Hz, 2H), 8.01 (d, *J* = 7.8 Hz, 2H), 7.88 (m, 4H), 7.79 (s, 2H), 7.61 – 7.12 (m, 58H), 6.99 (d, *J* = 7.1 Hz, 2H), 6.60 (d, *J* = 7.6 Hz, 1H), 5.87 (d, *J* = 3.5 Hz, 1H), 5.71 (dd, *J* = 10.3, 8.1 Hz, 1H), 5.48 (dd, *J* = 10.1, 7.8 Hz, 1H), 5.40 (dd, *J* = 10.4, 3.5 Hz, 1H), 5.24 (br s, 1H), 5.22 – 5.16 (m, 3H), 5.02 (m 2H), 4.92 (m, 2H), 4.87 (d, *J* = 7.8 Hz, 1H), 4.79 (t, *J* = 12.2 Hz, 1H), 4.72 (d, *J* = 11.9 Hz, 1H), 4.61 (m, 2H), 4.57 – 4.49 (m, 3H), 4.43 (d, *J* = 11.6 Hz, 1H), 4.38 (dd, *J* = 11.2, 6.3 Hz, 1H), 4.36 – 4.28 (m, 3H), 4.27 – 4.21 (m, 3H), 4.18 (dd, *J* = 11.2, 7.5 Hz, 1H), 4.12 – 4.06 (m, 2H), 3.99 (t, *J* = 7.0 Hz, 1H), 3.92 (m, 2H), 3.88 – 3.83 (m, 2H), 3.80 – 3.51 (m, 10H), 3.44 (m, 2H), 3.36 (d, *J* = 9.7 Hz, 1H), 3.33 – 3.16 (m, 4H), 3.11 (m, 1H), 3.00 (m, 1H), 2.20 (s, 3H), 2.00 (s, 3H), 1.53 (m, 4H), 1.33 – 1.21 (m, 5H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 170.4, 170.3, 166.0, 165.6, 165.5, 165.0, 164.8, 161.6, 156.8, 156.3, 139.73, 139.67, 139.2, 138.4, 138.1, 138.03, 137.98, 137.9, 137.8, 136.9, 136.8, 133.64, 133.57, 133.44, 133.42, 130.3, 130.0, 129.89, 129.85, 129.83, 129.78, 129.4, 129.00, 128.98, 128.91, 128.87, 128.85, 128.82, 128.79, 128.75, 128.71, 128.66, 128.64, 128.59, 128.57, 128.52, 128.48, 128.40, 128.36, 128.34, 128.29, 128.24, 128.21, 128.12, 128.06, 128.01, 127.95, 127.94, 127.91, 127.87, 127.82, 127.76, 127.73, 127.66, 127.6, 127.44, 127.36, 127.33, 127.25, 127.09, 127.07, 101.0, 100.3, 100.2, 100.0, 96.4, 92.1, 80.0, 79.5, 78.4, 76.7, 76.7, 76.64, 76.56, 76.3, 74.94, 74.91, 74.89, 74.58, 74.55, 74.2, 73.7, 73.6, 73.3, 73.0, 72.6, 71.7, 71.49, 71.47, 71.2, 71.1, 70.2, 69.6, 69.5, 68.5, 68.0, 67.8, 67.71, 67.69, 67.6, 67.3, 67.2, 62.8, 61.3, 58.9, 50.6, 50.2, 47.2, 46.3, 29.51, 29.46, 28.1, 27.7, 23.5, 23.4, 21.2, 21.0, 18.0; HRMS (ESI+) calculated for $C_{140}H_{145}Cl_3N_3O_{34}$ [M+NH_4]: 2516.8770, found: 2516.8711.

5-Aminopentyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-D-galactopyranosyl-(1→4)-[α-D-galactopyranosyl-(1→3)]-β-L-rhamnopyranoside (2-4)

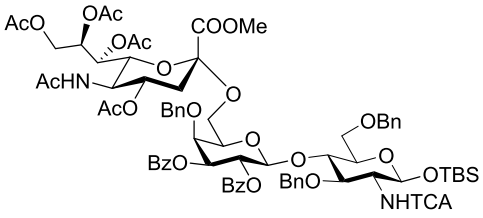


Compound **2-46** (38 mg; 15.2 µmol) was dissolved in MeOH (1.5 mL). Sodium methoxide 1 M in MeOH (0.20 mL; 0.20 mmol) was added. The reaction was stirred for 24 h, then neutralized with the addition of Amberlite IR120 H^+, filtered and concentrated. The crude residue was purified by chromatography on silica (DCM/MeOH 25:1). The obtained product was dissolved in MeOH/H_2O 4:1 (2.0 mL). Pd/C was added, the solution was purged with argon and hydrogen and left stirring under H_2 atmosphere (7 bar) for 5 days at room temperature. The mixture was then filtered through Celite and concentrated. The crude product was purified by RP-HPLC (Synergi column, 250x10 mm, H_2O (0.1% formic acid) isocratic (5 min), linear gradient to 25% ACN (30 min), linear gradient to 100% ACN (5 min)) and a C18-SPE Cartridge (H_2O/ACN 1:0 to 0:1), to obtain pentasaccharide **2-4** (2.0 mg; 2.1 µmol; 14%).

^{1}H NMR (700 MHz, D_2O) δ 5.26 (d, *J* = 3.9 Hz, 1H), 4.70 – 4.64 (m, 2H), 4.49 (d, *J* = 7.9 Hz, 1H), 4.30 (br s, 1H), 4.24 (m, 1H), 4.17 (br s, 1H), 4.01 – 3.92 (m, 6H), 3.91 – 3.84 (m, 3H), 3.82 – 3.65 (m, 14H), 3.60 (br s, 1H), 3.57 – 3.50 (m, 3H), 2.98 (t, *J* = 7.6 Hz, 2H), 2.05 (s, 3H), 1.67 (m, 4H), 1.45 (m, 2H), 1.37 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (176 MHz, D_2O) δ 174.9, 102.9, 102.7, 102.5, 99.5, 92.7, 82.9, 78.2, 75.3, 75.2, 74.9, 74.6, 74.5, 72.5, 72.3, 71.2, 71.02, 70.95, 70.0, 69.6, 69.5, 69.4, 68.5, 68.3, 68.2, 65.4, 61.3, 61.0, 60.9, 59.9, 55.3, 39.4, 28.1, 26.8, 22.3, 22.1, 17.2; HRMS (ESI+) calculated for $C_{37}H_{67}N_2O_{25}$ [M+H]: 939.4027, found: 939.4021.

Note: an unreported anomeric proton signal overlaps with the residual solvent peak.

***tert*-Butyldimethylsilyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (2-47)**

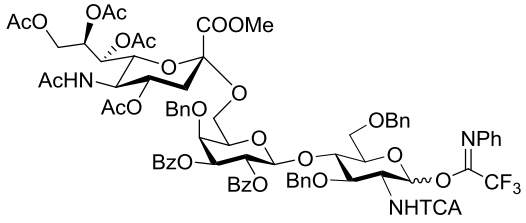


Disaccharide donor **2-22α** (255 mg; 0.256 mmol) and acceptor **2-44**[190] (122 mg; 0.197 mmol) were coevaporated three times with toluene and left under high vacuum overnight. Under argon atmosphere they were dissolved in DCM (5 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −15 °C. NIS (67 mg; 0.30 mmol) and TfOH (0.5 M in dioxane; 52 µL; 0.026

mmol) were added. After 1 h the reaction was quenched with triethylamine, warmed to r.t., filtered and diluted with EtOAc. The organic solution was washed with 10% aqueous $Na_2S_2O_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by chromatography on silica (Toluene/EtOAc 9:1 to 7:3) to obtain trisaccharide **2-47** (185 mg; 0.151 mmol; 60%).

$[\alpha]_D^{25}$ = 9.7° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max}: 3356, 2934, 2860, 1748, 1603, 1530, 1454, 1370, 1261, 1220, 1095, 1070, 840, 785, 737, 711; ^1H NMR (600 MHz, $CDCl_3$) δ 7.86 – 7.78 (m, 3H), 7.41– 7.35 (m, 2H), 7.29 – 7.12 (m, 17H), 7.10 – 7.05 (m, 3H), 7.01 – 6.97 (m, 1H), 5.69 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.30 – 5.23 (m, 2H), 5.21 (dd, *J* = 10.5, 3.1 Hz, 1H), 5.17 – 5.14 (m, 1H), 4.90 – 4.85 (m, 2H), 4.82 (d, *J* = 7.3 Hz, 1H), 4.80 – 4.74 (m, 1H), 4.61 – 4.55 (m, 2H), 4.50 (d, *J* = 11.3 Hz, 1H), 4.45 (d, *J* = 12.2 Hz, 1H), 4.28 (d, *J* = 12.2 Hz, 1H), 4.23 (dd, *J* = 12.4, 2.8 Hz, 1H), 4.10 (d, *J* = 3.1 Hz, 1H), 4.04 – 3.96 (m, 4H), 3.92 (dd, *J* = 9.1, 7.9 Hz, 1H), 3.73 – 3.67 (m, 2H), 3.62 – 3.57 (m, 1H), 3.56 – 3.50 (m, 6H), 3.37 – 3.33 (m, 1H), 2.48 (dd, *J* = 12.9, 4.6 Hz, 1H), 2.04 – 1.99 (m, 6H), 1.94 (s, 3H), 1.90 (s, 3H), 1.86 (t, *J* = 12.5 Hz, 1H), 1.80 (s, 3H), 0.74 (s, 9H), -0.04 (s, 3H), -0.07 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.1, 170.9, 170.4, 170.3, 170.1, 167.8, 165.9, 165.3, 161.7, 138.6, 138.3, 138.2, 133.4, 133.3, 129.9, 129.8, 129.6, 129.2, 128.54, 128.52, 128.51, 128.33, 128.31, 128.28, 128.2, 127.8, 127.7, 127.63, 127.57, 127.5, 100.4, 99.3, 94.9, 92.8, 77.8, 76.3, 75.0, 74.9, 74.5, 74.0, 73.9, 73.4, 72.96, 72.95, 70.8, 69.1, 68.9, 68.8, 67.5, 62.5, 59.2, 53.0, 49.6, 37.9, 25.7, 23.3, 21.2, 21.0, 20.92, 20.90, 17.9, -4.2, -5.2; HRMS (ESI+) calculated for $C_{75}H_{89}Cl_3N_2O_{25}SiNa$ [M+Na]: 1573.4481, found: 1573.4510.

(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-α/β-D-glucopyranosyl *N*-Phenyltrifluoroacetimidate (2-48)

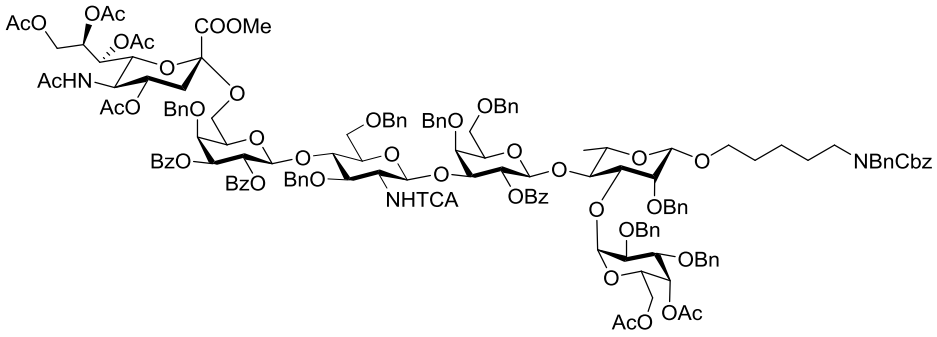


Trisaccharide **2-47** (160 mg; 0.103 mmol) was dissolved in THF (10 mL). Acetic acid (17 µL; 0.30 mmol) and TBAF (1M in THF; 300 µL; 0.30 mmol) were added and the reaction was stirred at r.t. for 2 h. It was then diluted with EtOAc and extracted twice with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by chromatography on silica (Hexane/EtOAc 2:8 to 0:1) to obtain the trisaccharide hemiacetal.

Without complete characterization, the product was dissolved in DCM (10 mL); *N*-phenyl trifluoroacetimidoyl chloride (45 µL; 0.28 mmol) and Cs_2CO_3 (90 mg; 0.28 mmol) were added and the solution was stirred at r.t. for 30 min. The solution was then filtered and concentrated. The crude residue was purified by chromatography on silica (Hexane/EtOAc 3:7 to 0:1) to obtain imidate **2-48** as a mixture of diastereoisomers (148 mg; 0.092 mmol; 89% over 2 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.75 (m, 3.8H), 7.45 – 7.05 (m, 24H), 6.97 – 6.90 (m, 1H), 6.73 (d, *J* = 7.8 Hz, 0.7H), 6.63 – 6.54 (m, 0.7H), 6.44 – 6.22 (m, 0.5H), 6.20 – 6.12 (m, 0.2H), 5.78 – 5.63 (m, 1H), 5.34 – 5.01 (m, 4H), 4.94 (d, *J* = 11.2 Hz, 0.8H), 4.88 – 4.47 (m, 5.8H), 4.41 – 3.80 (m, 9.6H), 3.75 – 3.31 (m, 8.6H), 2.51 – 2.42 (m, 1H), 2.10 – 2.00 (m, 5.4H), 1.98 – 1.93 (m, 3.6H), 1.91 (s, 2.4H), 1.89 – 1.77 (m, 4.6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.1, 138.32, 138.26, 138.2, 138.10, 138.08, 138.0, 133.5, 133.4, 133.2, 130.00, 129.96, 129.8, 129.7, 129.6, 129.52, 129.47, 129.1, 128.8, 128.73, 128.68, 128.58, 128.55, 128.5, 128.4, 128.34, 128.31, 128.26, 128.2, 128.11, 128.06, 128.02, 127.92, 127.88, 127.8, 127.7, 127.59, 127.58, 126.5, 124.6, 120.6, 119.4, 104.0, 102.7, 100.5, 99.3, 99.1, 92.2, 76.5, 75.6, 75.2, 75.0, 74.9, 74.5, 74.4, 74.2, 74.0, 73.8, 73.5, 73.3, 73.2, 73.1, 73.0, 72.8, 72.6, 72.1, 70.8, 70.6, 70.0, 69.1, 69.0, 68.91, 68.87, 67.7, 67.4, 67.1, 66.0, 63.1, 62.4, 62.2, 60.6, 53.8, 53.1, 53.0, 49.6, 49.5, 38.2, 37.9, 23.4, 21.22, 21.19, 21.1, 21.01, 20.99, 20.91, 20.89, 20.86; HRMS (ESI+) calculated for $C_{77}H_{79}Cl_3F_3N_3O_{25}Na$ [M+Na]: 1630.3913, found: 1630.3925.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-2,3-di-*O*-benzoyl-4-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-[4,6-di-*O*-acetyl-2,3-di-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-2-*O*-benzyl-β-L-rhamnopyranoside (2-49)**

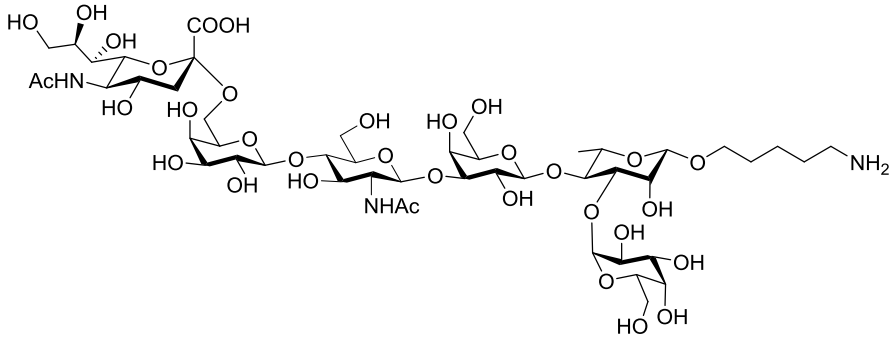


Donor **2-48** (63 mg; 39.1 µmol) and acceptor **2-30** (45 mg; 31.3 µmol) were coevaporated three times with toluene and left under high vacuum overnight. Under argon atmosphere they were dissolved in DCM (2 mL), 4Å Molecular sieves were added, the solution was stirred for 30 min and then cooled to −20 °C. TMSOTf (0.1 M in DCM; 31 µL; 31 µmol) was added. After 30 min additional 0.1 eq of TMSOTf were added and the reaction was warmed to 0 °C. After 2 h the reaction was quenched carefully with triethylamine, warmed to r.t., filtered and concentrated. The crude residue was purified by chromatography on silica (Toluene/Acetone 1:0 to 7:3) and HPLC (YMC-Diol-300 column, 150x20 mm, Hex/EtOAc 80:20 (5 min), linear gradient to 50% EtOAc (35 min), linear gradient to 100% EtOAc (5 min)) to obtain hexasaccharide **2-49** (10 mg; 3.5 µmol; 11%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10 – 8.07 (m, 2H), 7.92 – 7.85 (m, 4H), 7.54 – 7.43 (m, 5H), 7.37 – 7.12 (m, 52H), 6.98 – 6.95 (m, 2H), 6.54 (d, *J* = 8.4 Hz, 1H), 5.73 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.45 (dd, *J* = 10.2, 7.8 Hz, 1H), 5.33 – 5.28 (m, 2H), 5.24 (dd, *J* = 10.5, 3.2 Hz, 1H), 5.19 – 5.13 (m, 3H), 5.11 – 5.07 (m, 1H), 4.97 (d, *J* = 11.9 Hz, 1H), 4.92 (d, *J* = 10.8 Hz, 1H), 4.90 – 4.87 (m, 2H), 4.85 – 4.80 (m, 3H), 4.78 – 4.73 (m, 1H), 4.63 (d, *J* = 11.4 Hz, 1H), 4.58 (d, *J* = 11.4 Hz, 1H), 4.55 – 4.46 (m, 6H),

4.40 (d, *J* = 11.8 Hz, 1H), 4.34 (d, *J* = 11.7 Hz, 1H), 4.28 – 4.19 (m, 5H), 4.15 (d, *J* = 3.2 Hz, 1H), 4.12 – 3.97 (m, 5H), 3.93 – 3.79 (m, 5H), 3.76 – 3.51 (m, 17H), 3.43 – 3.38 (m, 2H), 3.29 – 3.05 (m, 5H), 3.01 – 2.94 (m, 1H), 2.50 (dd, *J* = 12.8, 4.6 Hz, 1H), 2.16 (s, 3H), 2.10 (s, 3H), 2.05 – 2.01 (m, 6H), 1.92 – 1.86 (m, 10H), 1.56 – 1.42 (m, 4H), 1.30 – 1.20 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.1, 170.8, 170.4, 170.33, 170.28, 170.2, 169.9, 167.8, 165.9, 165.3, 164.7, 161.6, 139.3, 138.5, 138.3, 138.21, 138.19, 138.17, 133.5, 133.39, 133.35, 130.3, 130.2, 130.0, 129.8, 129.6, 129.2, 128.8, 128.7, 128.6, 128.5, 128.42, 128.40, 128.32, 128.26, 128.24, 128.21, 128.08, 128.06, 127.99, 127.96, 127.9, 127.78, 127.75, 127.72, 127.69, 127.58, 127.55, 127.43, 127.38, 127.36, 127.1, 101.1, 101.0, 100.7, 100.2, 99.3, 96.5, 92.2, 80.0, 79.3, 78.1, 76.8, 76.73, 76.65, 75.03, 74.99, 74.96, 74.9, 74.6, 74.4, 74.1, 73.9, 73.64, 73.59, 73.4, 73.1, 72.9, 72.8, 71.6, 71.4, 70.9, 69.1, 68.8, 68.7, 68.4, 68.3, 67.9, 67.4, 67.3, 62.9, 62.3, 62.2, 57.7, 53.0, 49.6, 37.7, 29.5, 23.5, 23.4, 21.2, 21.0, 20.9, 20.9, 20.8, 18.1; HRMS (ESI+) calculated for $C_{153}H_{166}Cl_3N_3O_{44}Na$ [M+Na]: 2876.9802, found: 2876.9995.

5-Aminopentyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-D-galactopyranosyl-(1→3)]-β-L-rhamnopyranoside (2-5)



Compound **2-49** (10 mg; 3.5 µmol) was dissolved in THF/MeOH 1:1 (2 mL), $LiOH{\cdot}H_2O$ (7 mg; 167 µmol) was added and the solution was warmed to 40 °C. After 16 h it was cooled to r.t. and neutralized with the addition of Amberlite IR120 H^+, filtered and concentrated. The obtained crude product was dissolved in MeOH/H_2O 3:1 (2.0 mL), Pd/C was added, the solution was purged with argon, and then left stirring under H_2 atmosphere (3 bars) for 24 h. The mixture was filtered through Celite and concentrated. The crude material was purified using a C18-SPE Cartridge (H_2O/ACN 1:0 to 1:1) and size-exclusion chromatography (Sephadex LH-20, H_2O/MeOH 1:1). Lyophilization afforded pure **2-5** (1.4 mg; 1.2 µmol; 34 % over 2 steps).

^{1}H NMR (700 MHz, D_2O) δ 5.26 (d, *J* = 3.8 Hz, 1H), 4.68 (m, 2H), 4.47 (d, *J* = 7.9 Hz, 1H), 4.30 (br s, 1H), 4.25 (m, 1H), 4.17 (d, *J* = 3.3 Hz, 1H), 4.03 – 3.50 (m, 34H), 3.01 (t, *J* = 7.6 Hz, 2H), 2.68 (dd, *J* = 12.5, 4.7 Hz, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 1.69 (m, 5H), 1.45 (m, 2H), 1.37 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (176 MHz, D_2O) δ 175.0, 174.9, 173.5, 103.5, 102.7, 102.3, 100.2, 99.6, 92.7, 82.9, 80.5, 75.3, 74.9, 74.5, 74.3, 73.7, 72.6, 72.44, 72.38, 71.7, 71.2, 71.1, 70.7, 70.0, 69.6, 69.5, 68.43, 68.38, 68.3, 68.2, 65.4, 63.4, 62.7, 61.4, 61.0, 60.2, 55.1, 51.9, 40.1, 39.4, 28.1, 26.5, 22.5, 22.1, 22.0, 17.2; HRMS (ESI+) calculated for $C_{48}H_{84}N_3O_{33}$ [M+H]: 1230.4981, found: 1230.4973.

Note: an unreported anomeric proton signal overlaps with the residual solvent peak.

2.5.1. GLYCAN ARRAYS PREPARATION AND SCREENING

Glycan microarray slides were prepared by robotically spotting solutions on NHS activated glass slides. Synthetic glycans were dissolved in printing buffer (50 mM sodium phosphate, pH 8.5) to obtain 0.2 mM solutions. Similarly, polysaccharides were dissolved in printing buffer to obtain 0.2 mg/mL solutions. The solutions were transferred to a 384 well V bottom plate (Genetix) and robotically printed onto NHS activated glass slides (CodeLink slides) using an S3 non-contact microarray spotter (Scienion) equipped with a Type 4 coated nozzle (PDC80). Humidity in the printing chamber was maintained at 45% during the entire print run. Following printing, the slides were left overnight at room temperature in a humidity-saturated chamber. To quench residual reactive groups the slides were incubated in quenching solution (50 mM sodium phosphate, 100 mM ethanolamine, pH 9) at room temperature for one hour. The slides were then washed twice with water, dried by centrifugation at 300 x g for three minutes (Eppendorf CombiSlide system) and stored dry at 4 °C until use.

To avoid nonspecific bindings between antibodies and the surface, directly before the assay the slides were blocked with a solution of 3% (w/v) BSA in PBS (BSA-PBS) for 60 min at room temperature, washed 3 × 2 min with PBS and dried by centrifugation. A 64-well incubation gasket (FlexWell 64 grid, Grace BioLabs) was attached to the slide. Pig serum was diluted in 3% (w/v) BSA-PBS-0.1% Tween, and added in duplicates to the glycan arrays. After incubation for 1 h at r.t., slides were washed 3 × 2 min with PBS containing 0.1% (v/v) Tween-20 (PBST) by adding 50 µL to each well. The secondary antibody (goat anti-swine IgG AlexaFluor 488 1:400, Dianova) diluted in 3% (w/v) BSA-PBS-0.1% Tween was directly added with 25 µL to the wells of the gasket and incubated for 1 h at room temperature in the dark. After incubation the slides were washed twice with PBS-T, twice with PBS, rinsed with deionized water and dried by centrifugation (300 x g, 3 min) prior to scanning with a GenePix 4300A microarray scanner (Molecular Devices). Intensities were evaluated as mean fluorescence intensity of circles of identical diameter for all glycans with local background subtraction using GenePix 7 (Molecular Devices).

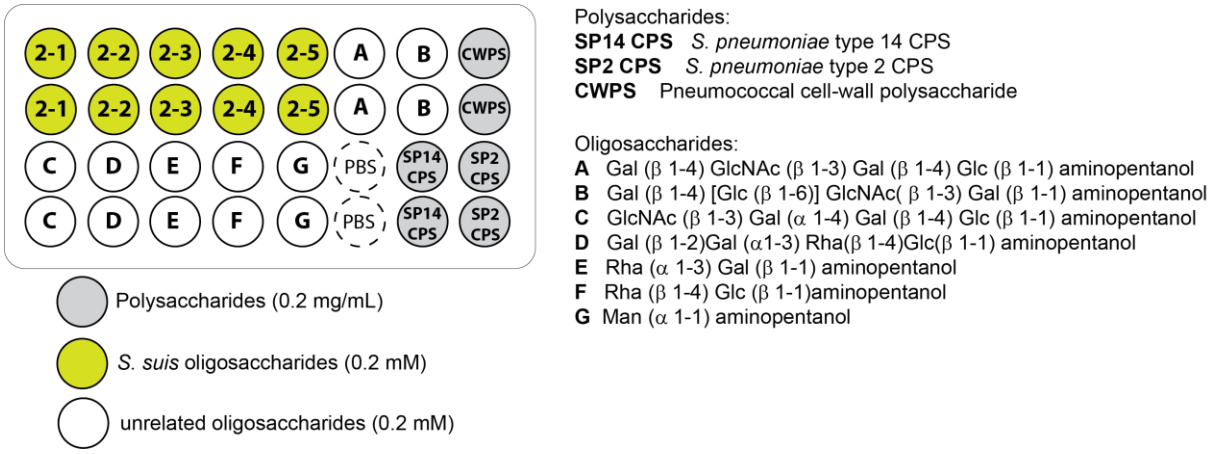


Figure 2-9: Schematic representation of printing pattern on microarray slides with employed printing concentrations and compound descriptions. Synthetic oligosaccharides (yellow), native polysaccharides (grey) and unrelated synthetic oligosaccharides (white) were printed according to the above described procedure.

CHAPTER 3

SYNTHESIS OF OLIGOSACCHARIDES RELATED TO *STREPTOCOCCUS SUIS* SEROTYPE 14 CAPSULAR POLYSACCHARIDE

3.1. *STREPTOCOCCUS SUIS* SEROTYPE 14 CAPSULAR POLYSACCHARIDE

Serotype 14 is an important *S. suis* serotype, responsible for pig infections and for disease in humans, the majority of cases being reported in Vietnam and Thailand[20,192]. Nevertheless, this serotype has been less studied than the most prevalent serotype 2. It was found that expression of the CPS is fundamental to inhibit phagocytosis *in vitro* and that mutated non-encapsulated bacteria are significantly less virulent in mice models[193]. These results suggest a prominent role of the CPS in virulence, similarly to what observed for serotype 2, and more in general with encapsulated bacteria and could be anticipated since the two serotypes show structural similarities in their CPSs. The structure of serotype 14 CPS was elucidated in 2012[154] and consists of hexasaccharide repeating unit of sequence: [→6)[α-Neu5Ac(2→6)-β-D-Gal(1→4)-β-D-GlcNAc(1→3)]-β-D-Gal(1→3)-β-D-Gal(1→4)-β-D-Glc(1→]. Similarly to serotype 2, repeating units are formed by a backbone, in this case a three-sugars sequence, and a sialylated lactosamine side chain. Structural differences with serotype 2 consist in the absence of the β rhamnose in the backbone and the linkage between the units: a β1→6 linkage, instead of β1→4, connects glucose and galactose.

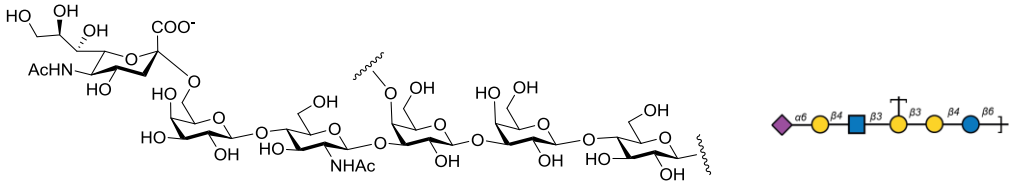


Figure 3-1: Characterized repeating unit of serotype 14 capsular polysaccharide.

In studies aimed at identifying cross-reactivities between structurally similar serotypes 1, 2, 1/2 and 14[153], already mentioned in Section 2.3, it was found that a polyclonal rabbit serum against serotype 14 was able to strongly recognize also the CPS from serotype 1. CPSs from serotype 14 and 1 share an identical backbone sequence and have slightly different side-chain (Fig. 3-2), containing respectively a galactose and a galactosamine. Since no cross-reactivity was observed with serotype 2, which displays an identical side-chain, it was proposed that the backbone residues represent the major part of epitopes of antibodies present in these sera.

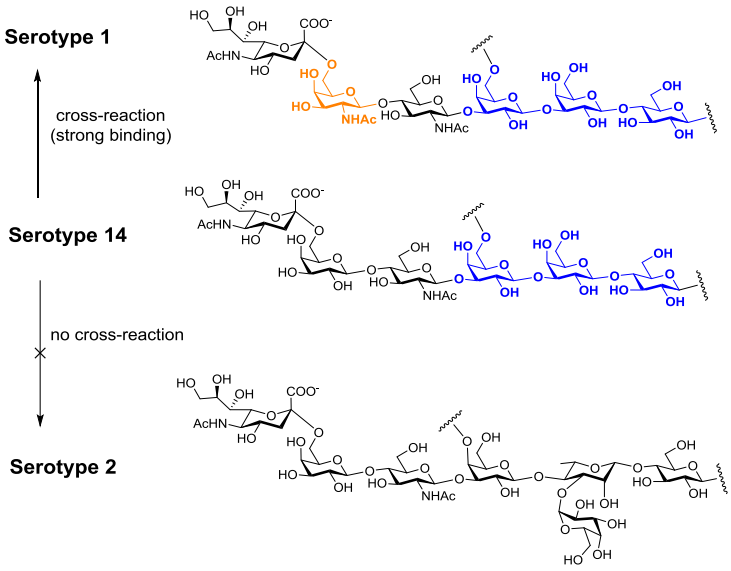


Figure 3-2: Cross-reactivity of anti-serotype 14 polyclonal rabbit serum as observed in Ref. 153.

Results from the above described experiment represent a starting point for the elucidation of glycotopes responsible for the production of protective antibodies against *S. suis* serotype 14, but more detailed information on the structure of carbohydrate epitopes is needed. Moreover, the antigenic properties of the CPS, either alone or as part of a glycoconjugate, have not been evaluated. Synthetic oligosaccharides as substructures of the CPS can help a more exact determination of antibody epitopes, to design new synthetic antigens for developing glycoconjugate vaccines against *S. suis* serotype 14.

3.2. RESULTS AND DISCUSSION

A library of substructures related to the repeating unit of serotype 14 CPS was designed, and included three oligosaccharides carrying an aminopentyl spacer at the reducing end. To identify whether antibody binding involves mostly the backbone residues, as suggested in the aforementioned study, hexasaccharide **3-1** was synthesized. Due to the non-complex nature of the backbone sequence, containing two types of sugars and only 1,2-*trans* glycosidic linkages, it was decided to synthesize a backbone fragment that spans two units. Longer oligosaccharides can result in higher binding affinities and facilitate detection on glycan arrays.

To evaluate if antibody epitopes include the whole repeating unit, pentasaccharide **3-2** and hexasaccharide **3-3** were also synthesized. Compounds **3-2** and **3-3** constitute respectively a non-sialylated and a sialylated repeating unit. Presence (or absence) of sialic acid on the oligosaccharides can directly indicate if this sugar is important for binding. In this sense, hypotheses on the role of sialylation in serotype 14 CPS have not yet been advanced.

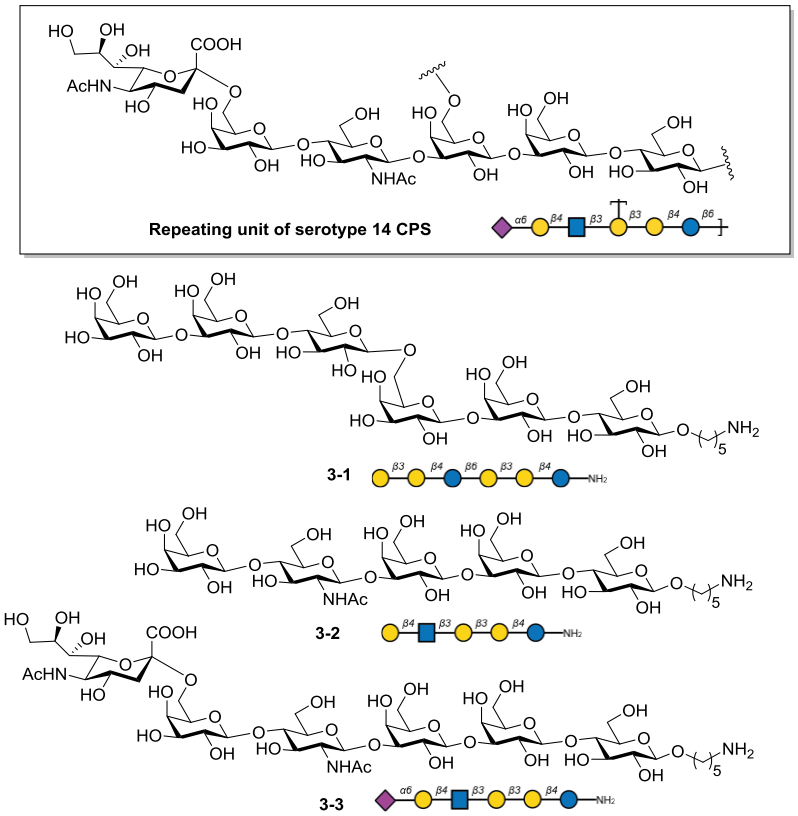
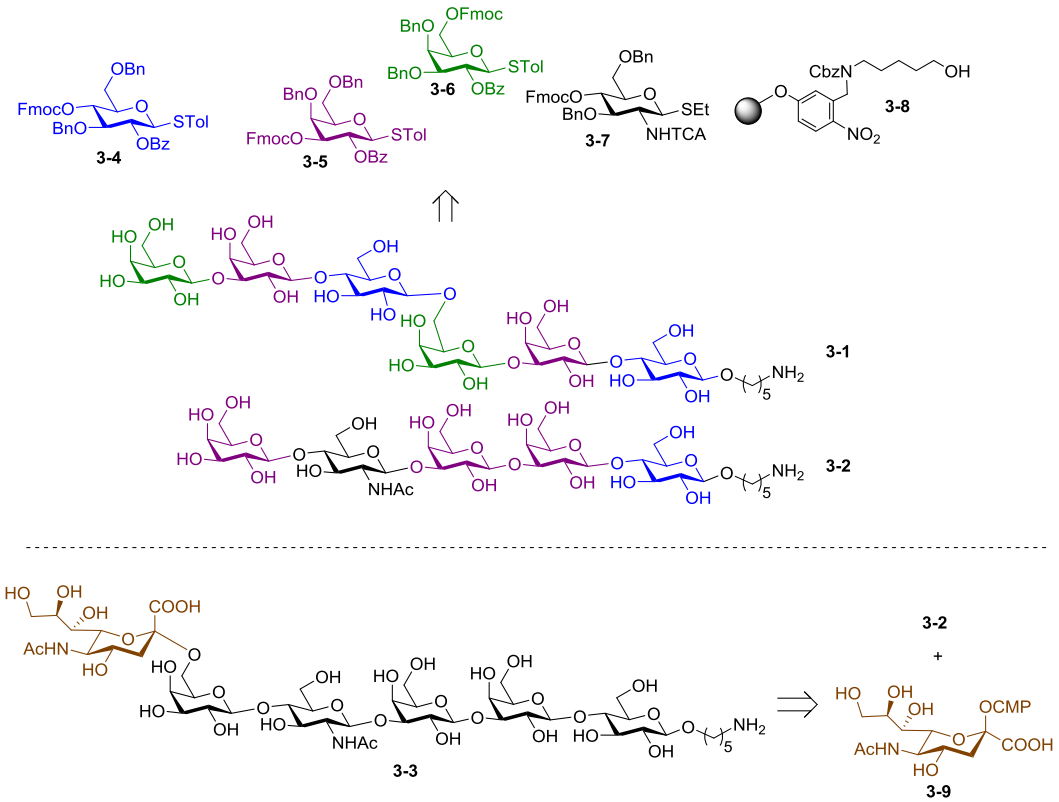


Figure 3-3: CPS repeating unit and synthetic sub-structures.

Considering that the planned structures were linear, included exclusively 1,2-*trans* glycosidic bonds and did not contain rare sugars, automated solid-phase synthesis represented the fastest method to obtain the desired compounds. As shown in Fig. 3-4, fragments **3-1** and **3-2** can be assembled from four building blocks and Merrifield resin functionalized with a photolabile linker, described in Section 1.9.2. These thioglycoside building blocks were common donors previously employed in automated oligosaccharide syntheses. Considering the challenges encountered in the formation of sialyl bonds on serotype 2 structures, to obtain hexasaccharide **3-3** a chemoenzymatic approach was preferred, as it could furnish in one single step the desired hexasaccharide from compound **3-2** and an activated sialyl nucleotide, using a sialyltransferase. Donors **3-4**, **3-5**, **3-6**, **3-7**, **3-9** were commercially available and resin **3-8** was prepared according to reported procedures[135].

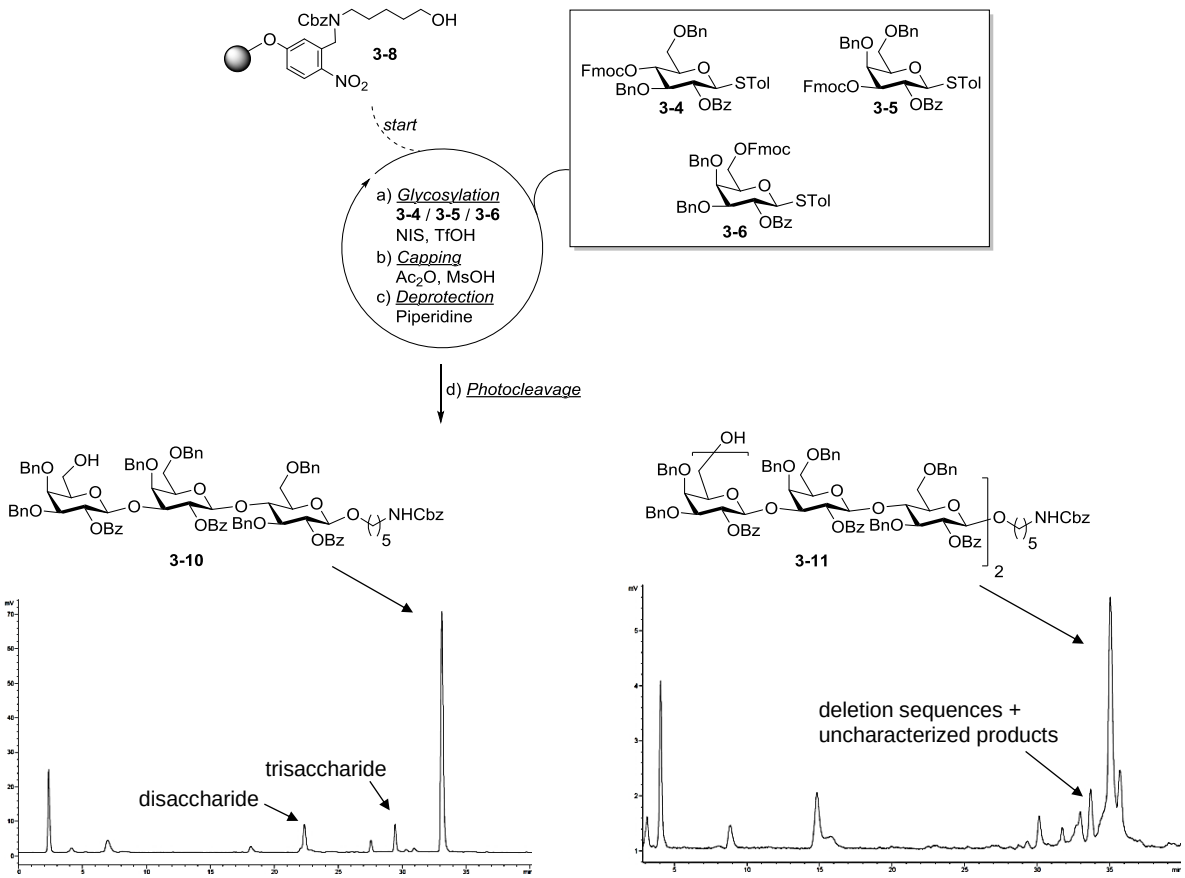


Scheme 3-1: Retrosynthesis of 3-1, 3-2 and 3-3. (CMP=cytidine monophosphate).

Hexasaccharide **3-1** consists of a repetition of a three-sugars sequence. To verify if building blocks **3-4**, **3-5** and **3-6** were suitable for the assembly of this sequence, the synthesis of trisaccharide **3-10** (Scheme 3-2) was fist attempted. Using a home-built automated synthesizer, the linker-functionalized Merrifield resin was coupled with building block **3-4**, followed by **3-5** and **3-6**, through cyclices of glycosylation, followed by capping (esterification) to protect potentially unreacted hydroxyl groups, and base-assisted removal of Fmoc groups.

The employed conditions were typical conditions developed to activate standard thioglycoside donors, such as those employed in this synthesis, in recent automated glycan assembly protocols. The activator consisted of a solution containing NIS and triflic acid; a donor excess of 6.5 equivalents was employed, based on the initial resin loading; temperature of the reaction was first set at an incubation temperature of −20 °C for five minutes followed by warming up to a reaction temperature of 0 °C, maintained for forty minutes; acid-catalyzed capping was performed with a solution of acetic anhydride and methanesulfonic acid; Fmoc deprotections were carried out with piperidine.

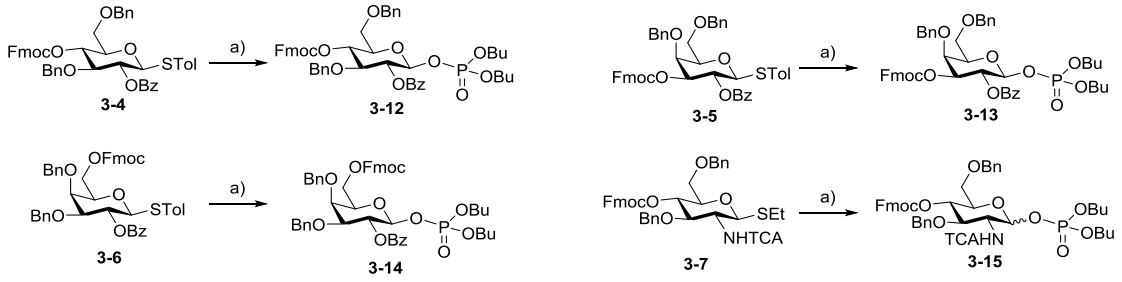
At the end of the synthesis, the resin was removed from the reaction vessel and swollen in DCM, then injected in a UV photoreactor coupled with a syringe pump to perform cleavage of the oligosaccharides from the resin in a flow system.



Scheme 3-2: Automated assembly using thioglycosides. Reagents and conditions: a) building block (6.5 eq), NIS, TfOH, DCM, −20 °C (5 min)→ 0 °C (40 min); b) Ac_2O, MsOH, DCM; c) Piperidine, DMF; d) *hv* (305 nm). HPLC trace: ELSD detection.

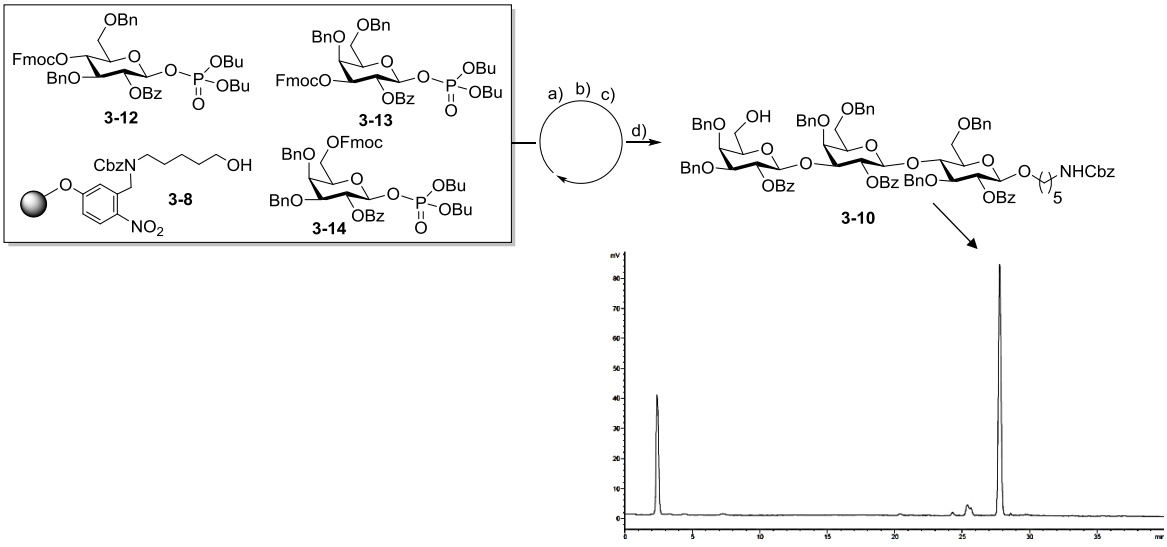
After analyzing the crude product by analytical HPLC and MALDI-MS it was found that an impure mixture was obtained, containing mostly the desired trisaccharide, a disaccharide deletion sequence (from incomplete glycosylation and incomplete capping) and a peak showing an *m/z* value identical to the desired trisaccharide. The presence of the latter peak was of difficult rationalization and a contamination of one of the initial reagents was suspected.

When the same synthetic cycle was repeated twice, attempting to synthesize hexasaccharide **3-11**, HPLC analysis after UV cleavage showed an increase in the complexity of the mixture. After HPLC purification the main peak was found to be the desired hexasaccharide, hardly separable from deletion sequences (penta and tetrasaccharides) and other compounds that were difficult to characterize. It was concluded that these conditions were not suitable to obtain the desired hexasaccharide in acceptable yield and purity. To improve the outcome of the automated synthesis, more reactive phosphate donors were prepared starting from the four initial thioglycosides (Scheme 3-3).



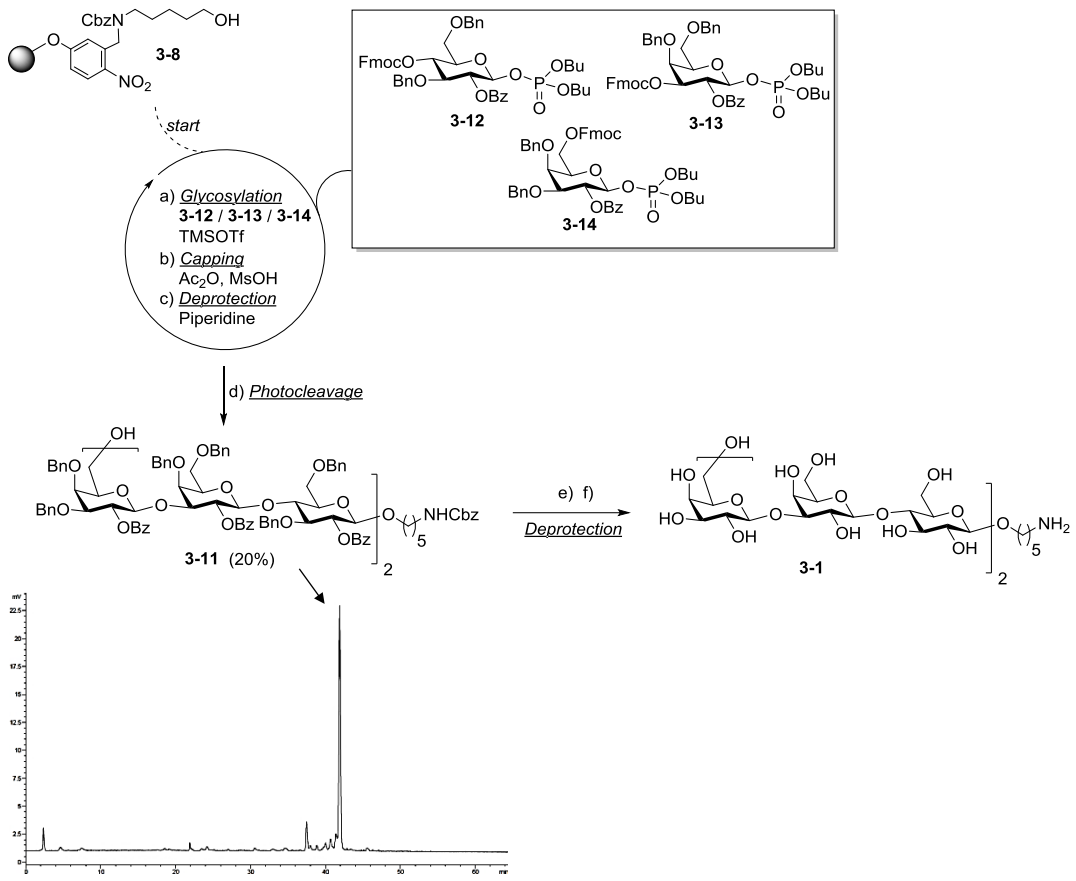
Scheme 3-3: Synthesis of glycosyl phosphate building blocks. Reagents and conditions: a) Dibutyl phosphate, NIS, TfOH, DCM, MS4Å, 0 °C.

Glycosyl phosphates **3-12**, **3-13** and **3-14** were employed in an automated synthesis to obtain trisaccharide **3-10**. Glycosylation conditions were adjusted to optimize activation and couplings with new donors: 5.0 equivalents of donors were employed, and temperature of the reaction was first set at an incubation temperature of −30 °C for ten minutes followed by warming up to −10 °C, and maintained for forty minutes. A TMSOTf solution was used as activator. The crude obtained after resin cleavage showed a much higher purity as almost exclusively the desired product was observed in HPLC analysis (Scheme 3-4).



Scheme 3-4: Automated assembly of trisaccharide using glycosyl phosphate donors. Reagents and conditions: a) building block (5 eq), TMSOTf, −30 °C (5min)→ −10 °C (40 min); b) Ac_2O, MsOH, DCM; c) Piperidine, DMF; d) *hv* (305 nm). HPLC trace: ELSD detection.

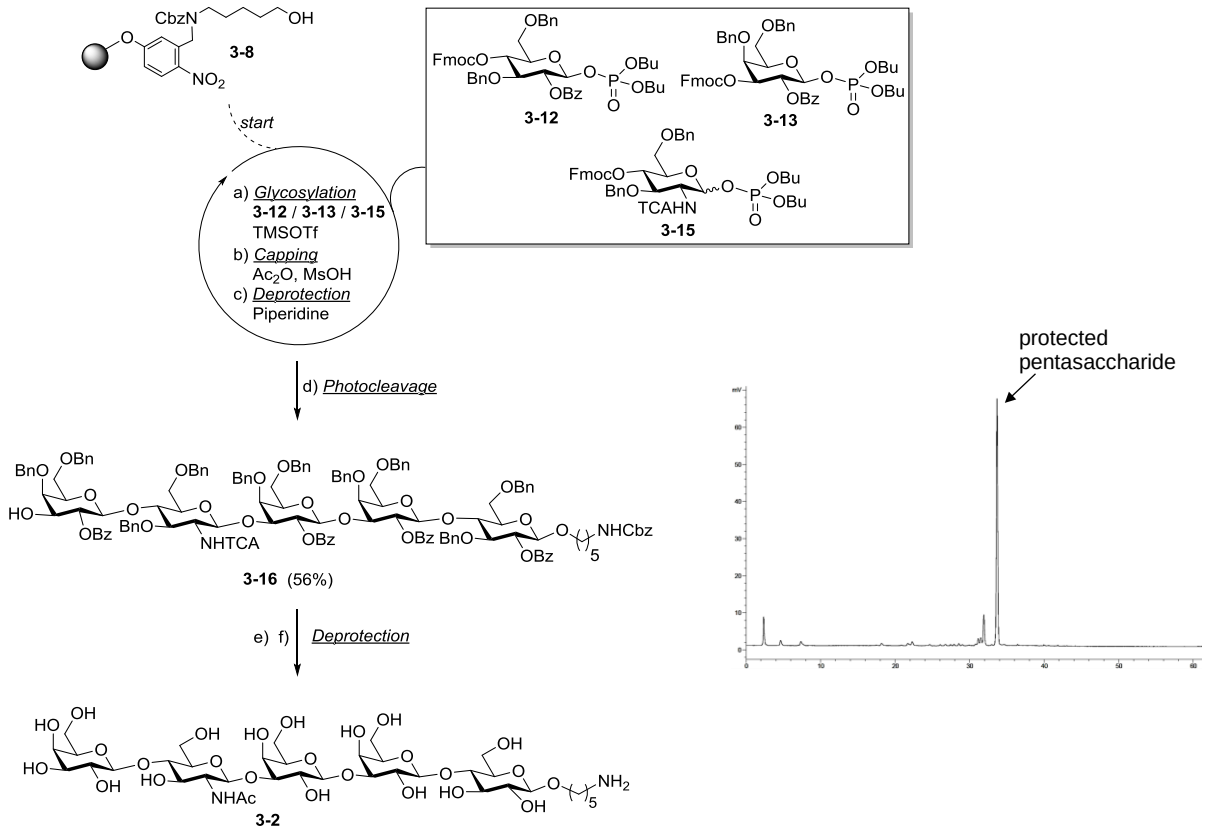
Using the new donors, protected hexasaccharide **3-11** was assembled in a single synthetic sequence and obtained after resin cleavage and NP-HPLC purification in an overall 20% yield (Scheme 3-5). Deprotection was carried out by hydrogenation followed by basic ester hydrolysis. A reversal in the typical order of the deprotection steps was experimentally found to result in faster ester hydrolysis and easier purification of the crude, which nevertheless relied on RP-HPLC since small traces of products of incomplete deacylation were observed. Deprotected hexasaccharide **3-1** was obtained in a satisfying 52% yield over two steps.



Scheme 3-5: Synthesis of hexasaccharide 3-1 using glycosyl phosphate building blocks. Reagents and conditions: a) building block (5 eq), TMSOTf, −30 °C (5min)→ −10 °C (40 min); b) Ac_2O, MsOH, DCM; c) Piperidine, DMF; d) *hv* (305 nm); the yield is based on resin loading; e) H_2, Pd/C, THF/MeOH/AcOH; f) NaOMe, MeOH; 40 °C; 52% over 2 steps. HPLC trace: ELSD detection.

Pentasaccharide **3-2** contains the same sequence included in **3-1**, plus a glucosamine and a galactose. To avoid problems encountered in the assembly of **3-11**, it was decided to perform the synthesis exclusively with phosphate donors. In this case, only three building blocks were needed: one glucose and one galactose, together with donor **3-15** to introduce the galactosamine unit.

By employing the same conditions previously used to synthesize the hexasaccharide, automated assembly proceeded smoothly and pentasaccharide **3-12** was obtained after resin cleavage and NP-HPLC purification in 56% overall yield. Deprotection was again carried out by hydrogenolysis followed by deacylation with sodium methoxide, to afford fully deprotected **3-2** in 53% yield.



Scheme 3-6: Synthesis of pentasaccharide 3-2 using glycosyl phosphate donors. Reagents and conditions: a) building block (5 eq), TMSOTf, –30 °C (5min)→ –10 °C (40 min); b) Ac_2O, MsOH, DCM; c) Piperidine, DMF; d) *hv* (305 nm); the yield is based on resin loading; e) H_2, Pd/C, THF/MeOH/AcOH; f) NaOMe, MeOH; 40 °C; 53% over 2 steps. HPLC trace: ELSD detection.

With pentasaccharide **3-2** in hand, the enzymatic sialylation to obtain hexasaccharide **3-3** could be performed. The choice of sialyltransferase is restricted due to their limited availability, especially for those creating α2→6 linkages. Bacterial sialyltransferases are more tolerant concerning structure differences on acceptors than their human counterparts, and therefore present a broader synthetic scope. Between them, a sialyltransferase that was isolated from the marine bacterium *Photobacterium damsela*[194] (Pd2,6ST) has shown the widest substrate and donor flexibility[195,196], and is presently available as a recombinant protein expressed in bacterial systems. Like other sialyltransferases, this enzyme operates as an inverting glycosyltransferase, resulting in reversion of the stereochemistry of its natural donor, in this case CMP-Neu5Ac (Cytidine-5′-monophosphate-*N*-acetylneuraminic acid) **3-9**, a sugar nucleotide which is commercially available.

To optimize conditions for the enzymatic sialylation of **3-2**, reactions were initially conducted on a 1 nmol scale to limit the consumption of valuable acceptor and enzyme, and analyzed by direct injection into an HPLC-MS. A donor/acceptor ratio of 2:1 was employed and the reaction time was 16 h. As shown in Fig. 3-4A, an investigation of different enzyme amounts (measured as milliunits per micromole of substrate) showed that amounts between 30 and 250 mU/μmol did not result in complete conversion of the initial substrate. It is known that Pd2,6ST can show sialidase activity, breaking the newly formed bond between Neu5Ac and Gal and transferring the sialic acid to cytidine monophosphate, rebuilding the sialyl nucleotide and making the whole process reversible. When this

occurs, yields can be improved if an alkaline phosphatase is added to the reaction[197]. This additional enzyme catalyzes the hydrolysis of the phosphate nucleotide released after donor activation, impeding the sialidase mechanism. However, when the reaction was conducted using 250 mU/μmol of sialyltransferase and calf intenstinal phosphatase (CIP), no significant difference in terms of substrate conversion was observed. Only an increase in the enzyme amount to 500 mU/μmol could lead to complete conversion of the initial substrate. Unfortunately, together with the formation of the desired product (*m/z*=1246.3, [M–H]), considerable amounts of a side-product identifiable as doubly sialylated compound (*m/z*=767.2, [M–2H]) were formed. When the reaction was performed on a 1 μmol scale (approximatively 1 mg of acceptor, Fig. 3-4B), HPLC analysis showed that around 40% of the formed products was disialylated. Due to the low amounts obtained, the side-product could not be further characterized beyond mass spectrometric analysis.

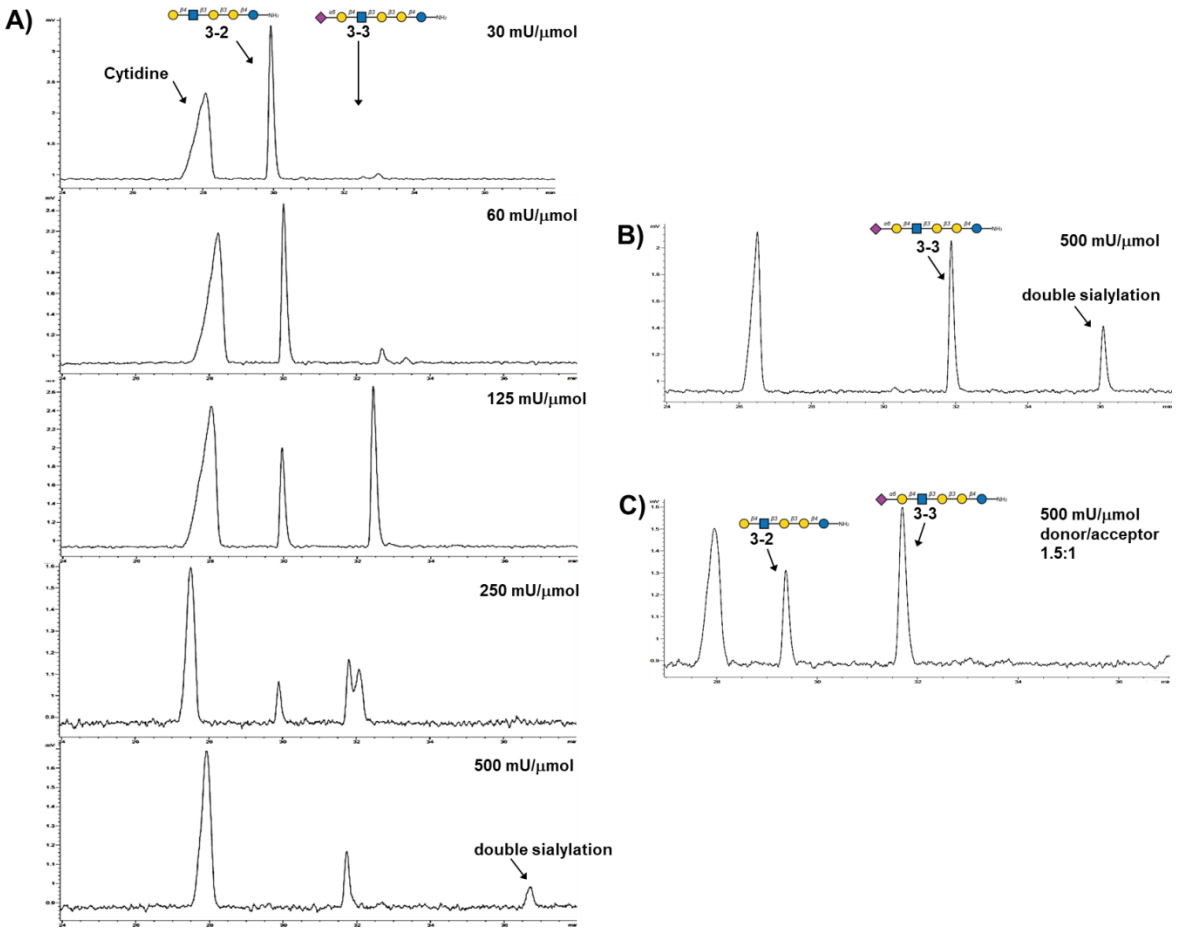


Figure 3-4: Optimization of enzymatic sialylation monitored by HPLC (ELSD detection) at 16 h reaction time. A) Increasing enzyme amount to 500 mU/μmol led to increased substrate conversion at 16 h; B) reaction profile when performed on 1 μmol of acceptor 3-2; C) reaction profile with 1.5 eq initial donor amount.

The observation of such undesired products is not uncommon: it is known that Pd2,6ST can glycosylate also internal galactose residues, either as part of lactose[198] or lactosamine[199] internal sequences. In addition, an α2→3 transferase activity was recently reported with *N*-glycans as acceptors[200], where Pd2,6ST was able to disialylate galactose units both at position 6 and at position

3. Considering structural similarities between **3-2** and compounds included in published studies[198], a double sialylation on an internal galactose seemed more probable.

To limit the side-reaction, the initial donor/acceptor amount was varied (Fig. 3-4C). When 1.5 equiv. of donor were initially present, incomplete conversion was again observed. Finally, it was supposed that only a more careful monitoring of the reaction progress could eventually result in an improved outcome. The reaction was performed on about 1 μmol scale and periodically monitored by HPLC. It was evident that the reaction progressed moderately slow but full donor conversion was reached already at 6 h. Therefore, a shorter reaction time was necessary to maximize substrate conversion and limit side-product formation.

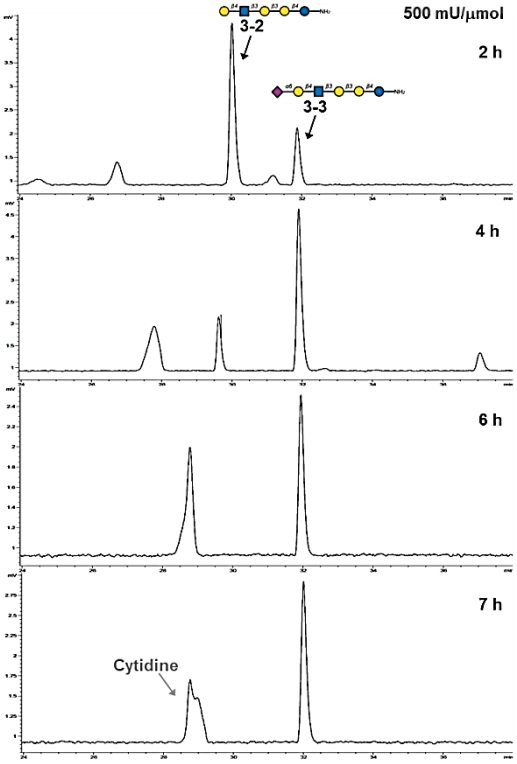
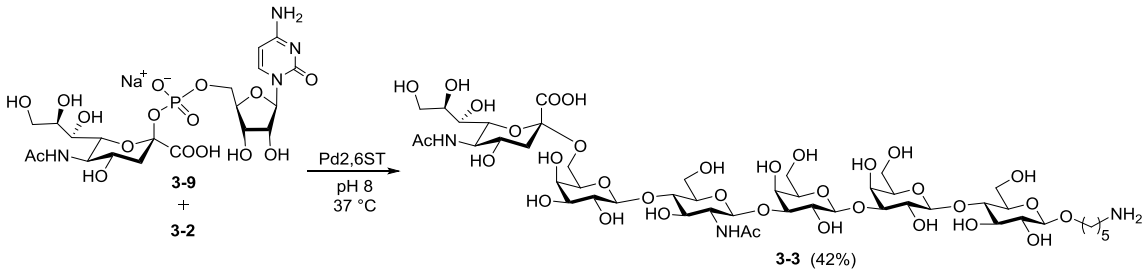


Figure 3-5: Reaction progression monitored by HPLC (ELSD detection).

After 7 h, the reaction was stopped and the product purified by solid-phase extraction and HPLC. Even though ELSD detection did not show peaks other than those given by **3-3** and products of donor hydrolysis, traces of the initial substrate were detected with an MS detector. After purification the desired sialylated hexasaccharide **3-3** was obtained in 42% yield, together with around 10% of the initial substrate.



Scheme 3-7: Enzymatic sialylation on 3-2.

3.3. CONCLUSIONS AND OUTLOOK

A collection of three substructures related to the capsular polysaccharide of *S. suis* serotype 14 was assembled using automated solid-phase synthesis and an enzymatic glycosylation. Two oligosaccharides were obtained through optimized procedures using glycosyl phosphate building blocks, minimizing the formation of unwanted products in the solid-phase process. The third oligosaccharide was obtained through stereoselective introduction of the sialyl residue employing a bacterial sialyltransferase which, in this case, showed a non-optimal specificity and produced unexpected byproducts, thus resulting in modest yield. A different, more acceptor-specific bacterial sialyltransferase could provide better results.

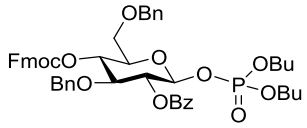
Due to unavailability of serum samples from serotype 14-infected pigs, the synthesized compounds were not screened in glycan arrays experiments. In future studies, these glycans will be printed on microarray slides and the library will be useful for obtaining structural information of serotype-specific epitopes. This will allow for the selection of synthetic oligosaccharides which, after chemical conjugation to a carrier protein, will translate in a semisynthetic glycoconjugate vaccine candidate against *S. suis* serotype 14.

3.4. EXPERIMENTAL SECTION

Commercial grade solvents and reagents were used without further purification. Anhydrous solvents were obtained from a solvent drying system (JCMeyer) or dried according to reported procedures. Analytical TLC was performed on Kieselgel 60 F254 glass (Macherey-Nagel). Spots were visualized with UV light, Sulphuric acid stain [1 mL of 3-methoxyphenol in 1 L of EtOH and 30 mL H_2SO_4] or Ceric ammonium molybdate stain [0.5 g $Ce(NH_4)_4(SO_4)_4 \cdot 2H_2O$, 12 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 15 mL H_2SO_4 in 235 mL H_2O]. Flash chromatography was performed on Kieselgel 60 230-400 mesh (Sigma-Aldrich). Preparative HPLC purifications were performed with an Agilent 1200 Series or Agilent 1260 Infinity II. NMR spectra were recorded on a Varian 400 MHz spectrometer (Agilent), Ascend 400 MHz (cryoprobe, Bruker) or Varian 600 MHz (Agilent) at 25 °C unless indicated otherwise. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CHCl_3$: δ 7.26 in ^1H and 77.16 in ^{13}C; HDO δ 4.79 in ^1H). Bidimensional and non-decoupled experiments were performed to assign identities of peaks showing relevant structural features. The following abbreviations are used to indicate peak multiplicities: *s* (singlet), *d* (doublet) *dd* (doublet of doublets), *t* (triplet), *dt* (doublet of triplets), *td* (triplet of doublets), *q* (quartet), *p* (pentet), *m* (multiplet). Additional descriptors *b* (broad signal) and *app* (apparent first-order multiplet) are also employed when required. Coupling constants (*J*) are reported in Hertz (Hz). NMR spectra were processed using MestreNova 11.0 (MestreLab Research). Specific rotations were measured with a UniPol L1000 polarimeter (Schmidt & Haensch) at λ = 589 nm. Concentration (*c*) is expressed in g/100 mL in the solvent noted in parentheses. IR spectra were measured with a Perkin Elmer 100 FTIR spectrometer. High-resolution mass spectra (ESI-HRMS) were recorded with a Xevo G2-XS Q-Tof (Waters).

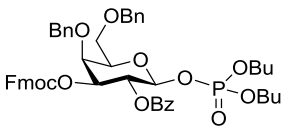
3.4.1. BUILDING BLOCKS SYNTHESES

Dibutylphosphoryloxy 2-*O*-benzoyl-3,6-di-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-D-glucopyranoside (3-12)



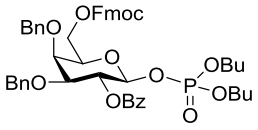
Compound **3-12** was prepared according to reported procedures and spectral data corresponded to those reported in the literature[201] .

Dibutylphosphoryloxy 2-*O*-benzoyl-4,6-di-*O*-benzyl-3-*O*-fluorenylmethoxycarbonyl-β-D-galactopyranoside (3-13)



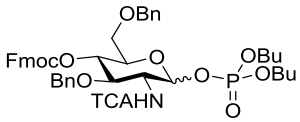
Compound **3-13** was prepared according to reported procedures and spectral data corresponded to those reported in the literature[202].

Dibutylphosphoryloxy 2-*O*-benzoyl-3,4-di-*O*-benzyl-6-*O*-fluorenylmethoxycarbonyl-β-D-galactopyranoside (3-14)



Compound **3-14** was prepared according to reported procedures and spectral data corresponded to those reported in the literature[202].

Dibutylphosphoryloxy 3,6-di-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-2-deoxy-2-trichloroacetamido-α/β-D-glucopyranoside (3-15)



Compound **3-7** (1.044 g; 1.354 mmol) was coevaporated three times with toluene and left under high vacuum overnight. Under Argon atmosphere it was dissolved in DCM (15 mL). Dibutyl phosphate (540 µL; 2.723 mmol) and 4Å Molecular sieves were added to the solution. The suspension was stirred for 60 min and then cooled to 0 °C. NIS (457 mg; 2.03 mmol) and TfOH (12 µL; 0.14 mmol) were added. After 30 min the reaction was carefully quenched with triethylamine, diluted with EtOAc, warmed to r.t. and filtered. The organic solution was washed once with 10% aqueous $Na_2S_2O_3$ and once with water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude material was purified using a RevelerisX2 Flash Chromatography System (Hexane/EtOAc 90:10 to 40:60) to obtain compound **3-15** as a sticky colorless solid (0.577 g; 0.628 mmol; 46%; α/β ~1:3).

^{1}H NMR (400 MHz, $CDCl_3$, α/β mixture) δ 7.79 – 7.71 (m, 8H), 7.60 – 7.52 (m, 8H), 7.44 – 7.37 (m, 8H), 7.35 – 7.26 (m, 24H), 7.24 – 7.17 (m, 24H), 6.88 – 6.82 (m, 4H, NHα/β), 5.74 (dd, *J* = 6.1, 3.3 Hz, 3H, H-1β), 5.33 (d, *J* = 3.6 Hz, 1H, H-1α), 5.11 (dd, *J* = 10.2, 9.2 Hz, 3H, H-3β), 4.96 (dd, *J* = 10.2, 9.1 Hz, 1H, H-3α), 4.64 – 4.55 (m, 8H), 4.54 – 4.46 (m, 8H), 4.39 – 4.17 (m, 17H), 4.15 – 3.93 (m, 22H), 3.71 – 3.55 (m, 9H), 1.70 – 1.51 (m, 16H), 1.44 – 1.23 (m, 16H), 0.98 – 0.79 (m, 22H); ^{13}C NMR (101 MHz, $CDCl_3$, α/β mixture) δ 162.0, 161.8, 154.3, 154.2, 143.32, 143.27, 143.2, 143.1, 141.43, 141.40, 137.6, 137.4, 137.2, 128.6, 128.52, 128.46, 128.10, 128.09, 128.07, 128.04, 127.99, 127.95, 127.93, 127.91, 127.88, 127.33, 127.31, 125.19, 125.17, 125.1, 120.2, 95.5 (d, $^2J_{CP}$ = 6.4 Hz, C-1β), 92.5, 92.2, 91.3 (C-1α), 76.2, 75.5, 74.8, 74.2, 74.1, 73.80, 73.77, 71.0, 70.24, 70.18, 69.3, 69.0, 68.6, 68.48, 68.45, 68.42, 68.39, 54.5, 54.33, 54.25, 46.78, 46.75, 32.32, 32.30, 32.25, 32.23, 29.7, 18.7, 13.72, 13.70; ^{31}P NMR (162 MHz, $CDCl_3$) δ -2.64; HRMS (ESI+) calculated for $C_{45}H_{51}Cl_3NO_{11}PNa$ [M+Na]: 940.2158; found: 940.2151.

3.4.2. PROCEDURES FOR AUTOMATED SOLID-PHASE SYNTHESIS

General materials and methods

The automated syntheses were performed on a home-built synthesizer developed at the Max Planck Institute of Colloids and Interfaces. The synthesizer executes a series of commands combined into modules to achieve specific chemical transformations.

All chemicals used were reagent grade and used as supplied unless otherwise noted. Solvents for preparation of solutions were obtained from an anhydrous solvent system (JC Meyer). Other solvents were HPLC-grade solvents. Building blocks were coevaporated three times with toluene and left under high vacuum for three hours before use. All solutions were freshly prepared and kept under Argon during the automated synthesis. The photocleavable linker was synthesized according to established procedures[135]. Isolated yields are calculated on the basis of resin loading, which was determined as described previously[203]: one glycosylation cycle (Module C) with 10 equiv. of building block was performed, followed by DBU promoted Fmoc-cleavage and determination of Dibenzofulvene production by UV absorbance measure.

The calculated resin loading was 0.32 mmol/g.

All automated syntheses were performed on a 0.0125 mmol scale.

Stock solution preparation

Building blocks: 0.060 mmol of building block in 1 mL of DCM per each glycosylation cycle required.

Acidic wash/activator: 0.45 mL of TMSOTf in 40 mL of DCM (~62 mM)

Pre-capping: 10% (v/v) pyridine in DMF

Capping: 1.2 mL of methanesulfonic acid and 6 mL of acetic anhydride in 52 mL of DCM

Fmoc deprotection: 20% (v/v) piperidine in DMF

Modules for automated synthesis

MODULE A: RESIN PREPARATION BEFORE SYNTHESIS

The resin was placed in the reaction vessel and swollen in DCM for 20 min at room temperature prior to synthesis. During this time, all reagent lines needed for the synthesis were washed and primed.

MODULE B: ACIDIC WASH

The resin was washed with DMF, THF, and DCM (three times each with 2 mL), then swollen in DCM (2 mL). The temperature of the reaction vessel was adjusted to −20 °C. Acidic wash solution (1 mL) was then delivered dropwise to the reaction vessel. After bubbling for 3 min the solution was drained and the resin was washed with DCM (2 mL).

Action	Cycles	Solution	Vol (mL)	T (°C)	Time
Cooling	-			−20	
Deliver	1	DCM	2	−20	
Deliver	1	Acidic wash	1	−20	3 min
Wash	1	DCM	2	−20	25 s

MODULE C: GLYCOSYLATIONS

The temperature was adjusted to the initiation temperature (T1) and then the building block solution (1 mL) was delivered to the reaction vessel. Then the activator solution (1 mL) was added dropwise. After an initiation time (t1) the temperature was raised to incubation temperature (T2). After the incubation time (t2) the solution was drained and the resin was washed with DCM.

Action	Cycles	Solution	Vol (mL)	T (°C)	Time
Cooling	-			−30	
Deliver	1	Building block	1	−30 (T1)	
Deliver	1	Activator	1	−30 (T1)	
Reaction	1			−30 (T1) −10 (T2)	5 min (t1) 40 min (t2)
Warming	-			25	
Wash	6	DCM	2	25	25 s

MODULE D: CAPPING

The resin was washed with DMF twice (2 mL) and then pre-capping solution (2 mL) was delivered. After 1 min the solution was drained and the resin washed with DCM three times (3 mL). Capping solution (4 mL) was delivered into the reaction vessel. After 20 min the reaction solution was drained and the resin washed three times with DCM (3 mL).

Action	Cycles	Solution	Vol (mL)	T (°C)	Time
Wash	2	DMF	2	25	25 s
Deliver	1	Pre-capping	2	25	1 min
Wash	3	DCM	2	25	25 s
Deliver	1	Capping	4	25	20 min
Wash	3	DCM	2	25	25 s

MODULE E: Fmoc DEPROTECTION

The resin was washed three times with DMF (2 mL). Fmoc deprotection solution (2 mL) was delivered into the reaction vessel. After 5 min the reaction solution was drained and the resin washed three times with DMF (2 mL) and five times with DCM (2 mL).

Action	Cycles	Solution	Vol (mL)	T (°C)	Time
Wash	3	DMF	2	25	25 s
Deliver	1	Fmoc deprot	2	25	5 min
Wash	3	DMF	2	25	25 s
Wash	5	DCM	2	25	25 s

3.4.3. POST-AUTOMATED SYNTHESIS STEPS

Photocleavage

After automated synthesis, the oligosaccharides were cleaved from the solid support using a continuous-flow reactor equipped with a UV-150 Medium pressure Mercury lamp, as described previously[135]. The solution was filtered to remove the resin and concentrated.

Purification

The obtained crude products were analyzed and purified using analytical or preparative HPLC on Agilent 1200 Series using Hexane/EtOAC as eluent.

METHOD A (analytical): YMC-Diol-300 column (150 x 4.6mm); flow rate 1.00 mL/min; 10% EtOAc isocratic (5 min), linear gradient to 70% EtOAc (40min), linear gradient to 100% EtOAc (5 min).

METHOD B (preparative): YMC-Diol-300 column (150 x 20.0mm); flow rate 15.00 mL/min; 10% EtOAc isocratic (5 min), linear gradient to 70% EtOAc (50min), linear gradient to 100% EtOAc (5 min).

METHOD C (preparative): YMC-Diol-300 column (150 x 20.0mm); flow rate 15.00 mL/min; 10% EtOAc isocratic (5 min), linear gradient to 70% EtOAc (45min), linear gradient to 100% EtOAc (5 min).

3.4.4. SYNTHESIS OF OLIGOSACCHARIDES – PROTOCOLS AND SPECTRAL DATA

***N*-Benzyloxycarbonyl-5-aminopentyl 2-*O*-benzoyl-3,4-di-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2-*O*-benzoyl-3,6-di-*O*-benzyl-β-D-glucopyranosyl-(1→6)-2-*O*-benzoyl-3,4-di-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2-*O*-benzoyl-3,6-di-*O*-benzyl-β-D-glucopyranoside (3-11)**

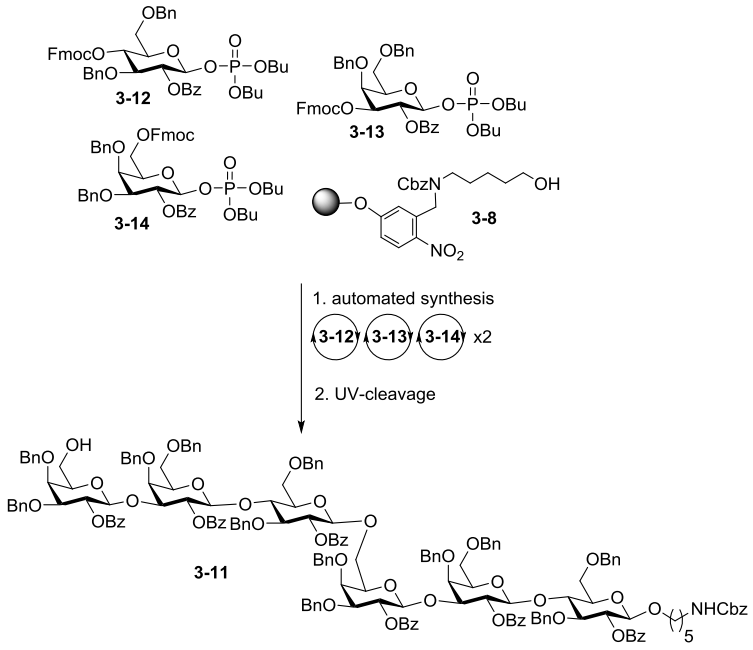


Table 1: automated synthesis protocol for **3-11**	
Steps	**Modules**
1	A
2	B, C (**3-12** ~5 eq), D, E
3	B, C (**3-13** ~5 eq), D, E
4	B, C (**3-14** ~5 eq), D, E
5	B, C (**3-12** ~5 eq), D, E
6	B, C (**3-13** ~5 eq), D, E
7	B, C (**3-14** ~5 eq), D, E

The product was cleaved from the solid support and purified as described in *Post-automated synthesis steps* using METHOD B to obtain **3-11** (7.4 mg; 2.54 μmol; 20% overall yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.90 (m, 4H), 7.70 – 6.87 (m, 104H), 5.65 (dd, *J* = 10.2, 7.8 Hz, 1H), 5.54 – 5.38 (m, 3H), 5.19 – 4.97 (m, 7H), 4.93 – 4.77 (m, 3H), 4.68 – 4.42 (m, 15H), 4.33 – 4.01 (m, 13H), 3.99 – 3.95 (m, 1H), 3.92 – 3.77 (m, 6H), 3.75 – 3.57 (m, 6H), 3.54 – 3.20 (m, 17H), 3.17 – 3.05 (m, 2H), 2.89 – 2.79 (m, 2H), 1.50 – 1.19 (m, 6H), 1.16 – 1.04 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.2, 165.1, 165.0, 164.2, 164.1, 156.4, 139.1, 139.0, 138.8, 138.6, 138.4, 138.3, 138.24, 138.17, 137.6, 137.5, 136.8, 133.3, 133.1, 132.9, 132.7, 132.5, 130.2, 130.01, 129.96, 129.83, 129.76,

129.6, 128.8, 128.72, 128.69, 128.63, 128.59, 128.49, 128.46, 128.4, 128.23, 128.20, 128.17, 128.13, 128.05, 128.02, 127.99, 127.94, 127.91, 127.8, 127.73, 127.67, 127.6, 127.44, 127.39, 127.2, 127.1, 127.0, 120.2, 102.1, 102.0, 101.2, 100.7, 100.5, 80.8, 80.6, 80.0, 79.4, 79.1, 79.0, 76.5, 76.2, 75.7, 75.4, 75.1, 75.0, 74.9, 74.7, 74.5, 74.3, 73.8, 73.53, 73.48, 73.4, 73.0, 72.8, 72.6, 72.4, 72.1, 72.0, 71.8, 71.3, 69.5, 68.8, 68.5, 68.0, 67.5, 66.8, 66.6, 61.9, 40.9, 29.4, 28.9, 23.2; HRMS (ESI+) calculated for $C_{175}H_{175}NO_{39}Na$ [M+Na]: 2937.1633; found: 2937.1758.

***N*-Benzyloxycarbonyl-5-aminopentyl 2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2-*O*-benzoyl-3,6-di-*O*-benzyl-β-D-glucopyranoside (3-16)**

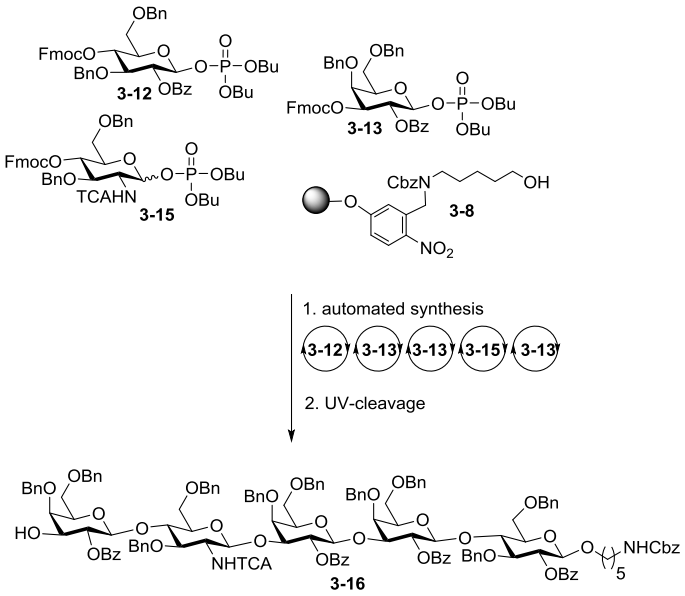


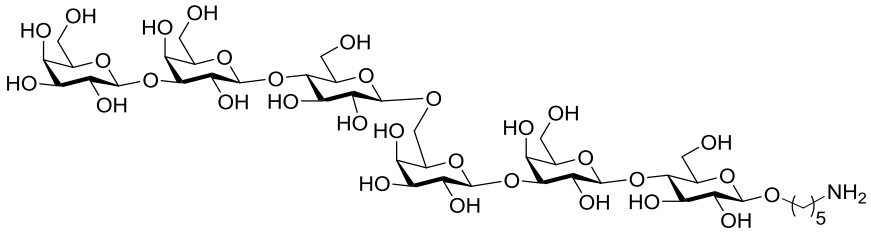
Table 2: automated synthesis protocol for **3-16**	
Steps	**Modules**
1	A
2	B, C (**3-12** ~5 eq), D, E
3	B, C (**3-13** ~5 eq), D, E
4	B, C (**3-13** ~5 eq), D, E
5	B, C (**3-15** ~5 eq), D, E
6	B, C (**3-13** ~5 eq), D, E

The product was cleaved from the solid support and purified as described in *Post-automated synthesis steps* using METHOD C to obtain **3-16** (17.8 mg; 7.10 µmol; 56% overall yield).

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 – 7.98 (m, 2H), 7.95 – 7.91 (m, 2H), 7.63 – 7.58 (m, 3H), 7.53 – 7.10 (m, 70H), 7.07 – 7.01 (m, 3H), 6.99 – 6.94 (m, 2H), 6.30 (d, *J* = 8.2 Hz, 1H), 5.49 (dd, *J* = 10.2, 7.7 Hz, 1H), 5.41 (dd, *J* = 10.1, 7.9 Hz, 1H), 5.18 (dd, *J* = 10.0, 7.9 Hz, 1H), 5.13 – 5.02 (m, 4H), 4.96 (d, *J* = 11.7 Hz, 1H), 4.86 (t, *J* = 11.3 Hz, 2H), 4.68 – 4.30 (m, 19H), 4.28 – 4.24 (m, 3H), 4.19 (d, *J* =

12.2 Hz, 1H), 4.10 (d, *J* = 11.8 Hz, 1H), 4.00 – 3.87 (m, 5H), 3.78 – 3.28 (m, 23H), 3.28 – 3.22 (m, 1H), 3.22 – 3.17 (m, 1H), 3.13 – 3.08 (m, 1H), 2.92 – 2.83 (m, 2H), 1.46 – 1.32 (m, 2H), 1.26 – 1.20 (m, 2H), 1.17 – 1.04 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.2, 165.2, 164.6, 164.0, 161.8, 156.4, 139.2, 138.84, 138.82, 138.5, 138.43, 138.35, 138.2, 138.1, 138.0, 137.9, 137.8, 136.9, 133.5, 133.1, 132.82, 132.76, 130.3, 130.0, 129.9, 129.8, 129.6, 128.9, 128.8, 128.70, 128.66, 128.62, 128.61, 128.49, 128.46, 128.40, 128.38, 128.35, 128.33, 128.31, 128.28, 128.21, 128.18, 128.16, 128.14, 128.11, 128.06, 128.04, 128.01, 127.93, 127.91, 127.89, 127.84, 127.80, 127.6, 127.4, 127.3, 127.0, 102.2, 101.3, 100.7, 100.5, 80.9, 79.2, 78.8, 78.3, 76.6, 76.5, 76.4, 76.3, 75.6, 75.0, 74.9, 74.6, 74.5, 74.4, 74.2, 74.1, 73.9, 73.8, 73.61, 73.59, 73.5, 73.4, 72.9, 72.7, 72.1, 69.4, 69.2, 69.0, 68.3, 67.9, 67.6, 66.6, 57.6, 40.9, 29.5, 29.0, 23.2; HRMS (ESI+) calculated for $C_{143}H_{145}Cl_3N_2O_{32}Na$ [M+Na]: 2529.8738; found: 2529.8655.

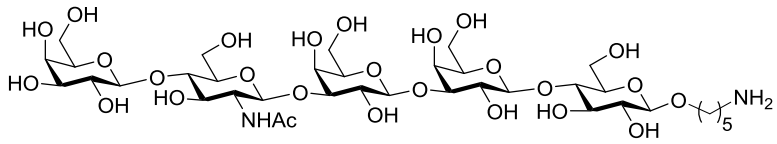
5-Aminopentyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (3-1)



Compound **3-11** (7.4 mg; 2.54 µmol) was dissolved in THF/MeOH 1:3 (2.0 mL) and AcOH (200 µL). Pd/C was added, the solution was purged with argon and hydrogen and left stirring under H_2 atmosphere (3 bars) for 2 days at room temperature. The mixture was then filtered through a PTFE filter (0.45µm) and concentrated. The obtained product was dissolved in MeOH (2.0 mL). Sodium methoxide 0.5 M in MeOH (0.20 mL; 0.10 mmol) was added. The reaction was warmed to 40 °C and stirred for 24 h, then neutralized with the addition of AcOH and purified by size-exclusion chromatography (Sephadex LH-20, H_2O/MeOH 1:1) and HPLC [Hypercarb; 150x4.6mm; flow rate 0.70 mL/min; H_2O (+0.1% Formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (5 min)] to obtain hexasaccharide **3-1** after lyophilization (1.4 mg; 1.30 µmol; 52% over 2 steps).

^{1}H NMR (600 MHz, D_2O) δ 4.66 – 4.61 (m, 2H), 4.57 – 4.49 (m, 4H), 4.25 – 4.20 (m, 2H), 4.06 – 3.59 (m, 37H), 3.37 – 3.30 (m, 2H), 3.02 (t, *J* = 7.6 Hz, 2H), 1.74 – 1.66 (m, 4H), 1.51 – 1.44 (m, 2H); ^{13}C NMR (151 MHz, D_2O) δ 104.3, 104.1, 102.7, 102.51, 102.49, 102.0, 81.8, 78.24, 78.18, 75.00, 74.96, 74.9, 74.70, 74.68, 74.4, 74.3, 73.7, 72.8, 72.7, 72.4, 72.3, 71.0, 70.9, 70.12, 70.09, 70.0, 69.4, 68.6, 68.5, 68.3, 61.0, 60.92, 60.90, 60.04, 60.01, 39.3, 28.1, 26.3, 22.0; HRMS (ESI+) calculated for $C_{41}H_{74}NO_{31}$ [M+H]: 1076.4239; found: 1076.4233.

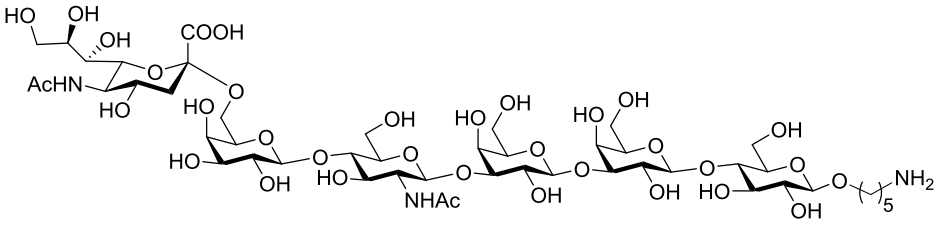
5-Aminopentyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (3-2)



Compound **3-16** (7.9 mg; 3.15 µmol) was dissolved in THF/MeOH 1:3 (2.0 mL) and AcOH (200 µL). Pd/C was added, the solution was purged with argon and hydrogen and left stirring under H_2 atmosphere (3 bars) for 3 days at room temperature. The mixture was then filtered through a PTFE filter (0.45µm) and concentrated. The obtained product was dissolved in MeOH (2.0 mL). Sodium methoxide 0.5 M in MeOH (0.20 mL; 0.10 mmol) was added. The reaction was warmed to 40 °C and stirred for 24 h, then neutralized with the addition of AcOH and purified by size-exclusion chromatography (Sephadex LH-20, H_2O/MeOH 1:1) and HPLC [Hypercarb; 150x4.6mm; flow rate 0.70 mL/min; H_2O (+0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (5 min)] to obtain pentasaccharide **3-2** after lyophilization (1.6 mg; 1.68 µmol; 53% over 2 steps).

^{1}H NMR (700 MHz, D_2O) δ 4.75 (d, J = 8.5 Hz, 1H), 4.63 (d, J = 7.9 Hz, 1H), 4.55 – 4.49 (m, 3H), 4.22 (br s, 1H), 4.17 (br s, 1H), 4.03 – 3.94 (m, 4H), 3.90 – 3.55 (m, 28H), 3.36 – 3.31 (m, 1H), 3.03 – 2.99 (m, 2H), 2.07 (s, 3H), 1.73 – 1.68 (m, 4H), 1.51 – 1.46 (m, 2H); ^{13}C NMR (176 MHz, D_2O) δ 175.0, 104.4, 102.9, 102.8, 102.6, 102.1, 82.1, 82.0, 78.34, 78.32, 75.4, 75.0, 74.8, 74.7, 74.6, 74.5, 72.9, 72.6, 72.2, 71.0, 70.23, 70.19, 70.15, 68.6, 68.39, 68.37, 61.1, 61.0, 60.9, 60.2, 60.0, 55.3, 39.5, 28.2, 26.7, 23.3, 22.2, 22.1; HRMS (ESI+) calculated for $C_{37}H_{67}N_2O_{26}$ [M+H]: 955.3977; found: 955.3987.

5-Aminopentyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (3-3)



Pentasaccharide **3-2** (1.13 mg; 1.18 µmol) was added to a 10 mM CMP-Neu5Ac solution in water (220 µL; 2.20 µmol), followed by 700 µL of 0.1 M Tris Buffer (pH 8.0) and 100 µL of α-2,6-Sialyltransferase from *Photobacterium damsela* (5 mU/µL; 500 mU). The reaction was incubated at 37 °C and shaken (300 rpm) for 7 h. The solution was heated to 90 °C for 2 min, then frozen and lyophilized. The residue was purified using a C-18 SPE-cartdrige (H_2O/ACN) and HPLC [Hypercarb; 150x4.6mm; flow rate 0.7 mL/min; H_2O (+0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (5 min)] to obtain hexasaccharide **3-3** after lyophilization (0.62 mg; 0.50 µmol; 42%).

^{1}H NMR (700 MHz, D_2O) δ 4.75 (d, *J* = 7.2 Hz, 1H), 4.61 (d, *J* = 7.6 Hz, 1H), 4.53 – 4.45 (m, 3H), 4.21 – 4.18 (m, 1H), 4.16 – 4.14 (m, 1H), 4.03 – 3.92 (m, 5H), 3.92 – 3.52 (m, 32H), 3.31 (t, *J* = 8.2 Hz, 1H), 3.03 – 2.99 (m, 2H), 2.68 (dd, *J* = 12.7, 4.7 Hz, 1H), 2.05 (m, 6H), 1.76 – 1.66 (m, 5H), 1.47 (m, 2H); ^{13}C NMR (176 MHz, D_2O) δ 174.9, 173.5, 171.1, 104.4, 103.5, 102.6, 102.5, 102.0, 100.2, 82.1, 81.9, 80.5, 78.3, 75.0, 74.8, 74.7, 74.5, 74.3, 73.7, 72.8, 72.6, 72.4, 72.2, 71.7, 70.8, 70.19, 70.16, 70.1, 68.43, 68.38, 68.3, 68.2, 63.4, 62.7, 61.0, 60.9, 60.2, 60.1, 55.0, 51.9, 40.1, 39.4, 28.2, 26.4, 22.3, 22.1, 22.0; HRMS (ESI+) calculated for $C_{48}H_{84}N_3O_{34}Na$ [M+H+Na]: 634.7411; found: 634.7410.

CHAPTER 4 – SUPPLEMENTARY SECTION

IDENTIFICATION OF THE MINIMAL GLYCOTOPE OF *STREPTOCOCCUS PNEUMONIAE* 7F CAPSULAR POLYSACCHARIDE USING SYNTHETIC OLIGOSACCHARIDES

This Section was adapted in part from the following publication:

Ménová P., Sella M., Sellrie K., Pereira C.L., Seeberger P.H., Identification of the Minimal Glycotope of Streptococcus pneumoniae 7F Capsular Polysaccharide using Synthetic Oligosaccharides, *Chem. Eur. J.*, **2018**, *24*, 4181–4187. https://doi.org/10.1002/chem.201705379

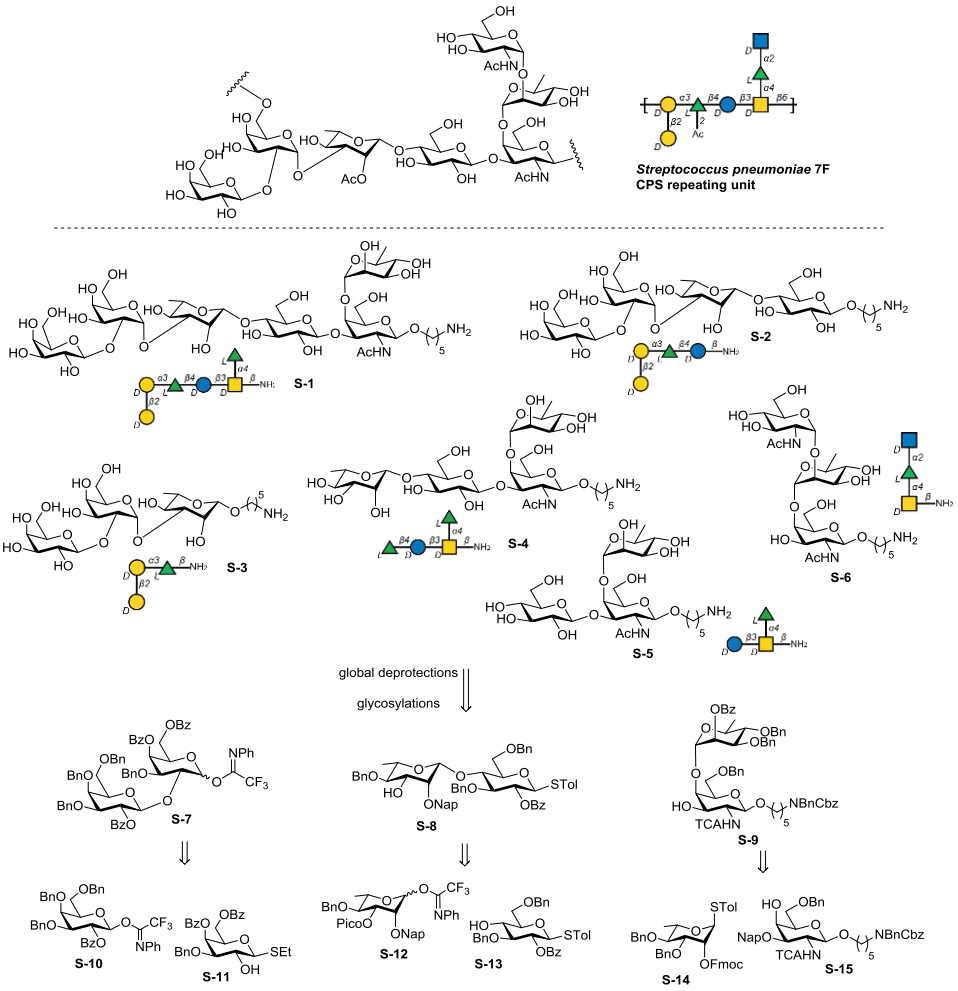
4.1. INTRODUCTION

Streptococcus pneumoniae, a gram-positive, human-specific pathogen and common constituent of the nasopharyngeal flora causes life-threatening invasive diseases such as pneumonia, meningitis, bacteremia and acute otitis media. To date, 97 serotypes of *S. pneumoniae* have been described[204] but less than 20 serotypes account for the majority of infections[205]. Protection against *S. pneumoniae* infections can be achieved by vaccination with a 13-valent pneumococcal conjugate vaccine (PCV13/Prevnar13®) introduced in the US in 2010 and in the EU in 2012. This vaccine added the emerging serotypes 1, 3, 5, 6A, 7F and 19A to those already included in the heptavalent vaccine PCV7/Prevnar7® (4, 6B, 9V, 14, 18C, 19F, 23F) licensed a decade earlier. The capsular polysaccharides are purified from bacterial cultures and conjugated by reductive amination to the carrier protein CRM_{197}. Structural similarities between protective epitopes may account for cross-protection towards other serotypes such as 6C and 7A, that are not included in the PCV13 formulation[206]. Naturally-derived capsular polysaccharides have been the basis for effective pneumococcal vaccines, but little is known about the protective glycotopes for many serotypes. Synthetic oligosaccharides are useful tools for epitope elucidation. Oligosaccharides corresponding to frameshifts of repeating units and sub-units, differing in chain length and monosaccharide composition help to identify antigenic determinants for the creation of semi-synthetic glycoconjugate vaccine candidates. These oligosaccharides may help to explain the observed cross-reactive immune response against other serotypes at the molecular level.

The structure of the ST7F capsular polysaccharide repeating unit (RU) consists of a double-branched heptasaccharide (Figure I)[207]. The linear backbone, composed of [→6)-α-D-Gal-(1→3)-(2-OAc)-β-L-Rha-(1→4)-β-D-Glc-(1→3)-β-D-GalNAc-(1→], contains two branching points to two short side-chains [β-D-Gal(1→] and [α-D-GlcNAc-(1→2)-L-α-Rha-(1→] at two adjacent residues.

To identify the minimal glycotope that can elicit a robust immune response to the CPS, a series of oligosaccharides related to the repeating unit of ST7F CPS were synthesized. Structures of the oligosaccharides were designed to probe the effect of branching, length and acetylation. All

oligosaccharides are equipped with a reducing-end aminopentanol linker to enable printing on glycan arrays and conjugation to a carrier protein (Scheme S-I) (**).



Scheme S-I: Structure of ST7F CPS repeating unit, synthetic antigens library and retrosynthesis of oligosaccharides.

4.2. SYNTHESIS OF OLIGOSACCHARIDES

Six differentially protected monosaccharide building blocks were needed to construct the six oligosaccharides (Scheme S-I). Combination of monosaccharides produced disaccharide building blocks **S-7**, **S-8** and **S-9**. Successive glycosylations involving disaccharide and monosaccharide building blocks provided access, after global deprotection, to various related sequences for glycan array glycotope analysis. Oligosaccharides **S-3**, **S-5** and **S-6** were assembled from the mono- and disaccharide building blocks as outlined in Schemes S-II and S-III.

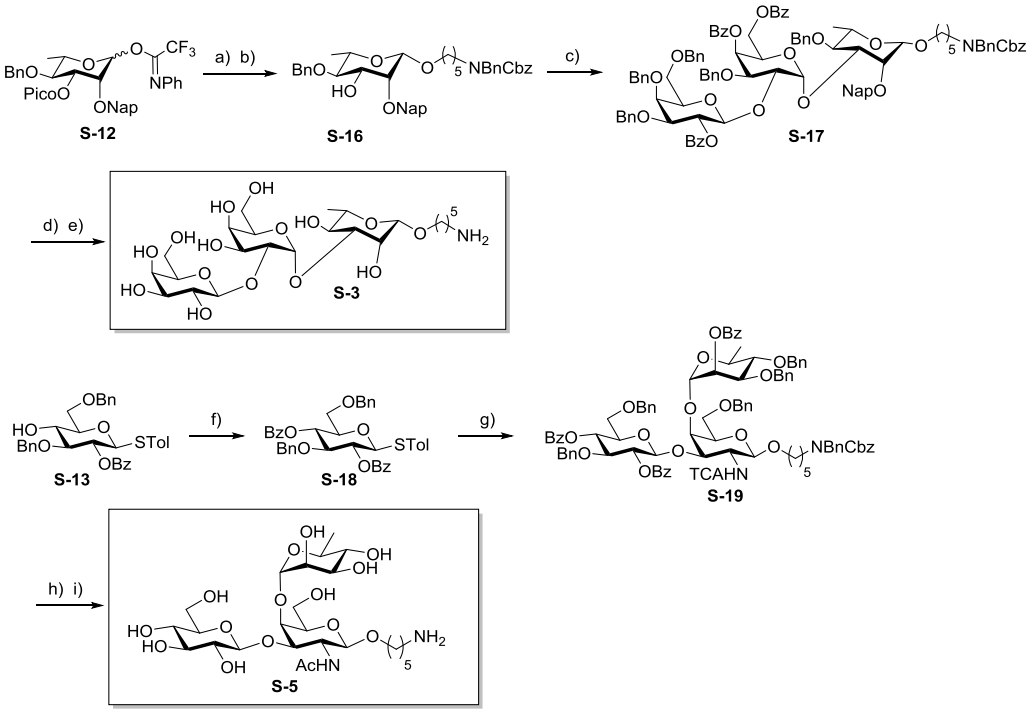
For the synthesis of trisaccharide **S-3** (Scheme II), rhamnose donor **S-12** was first reacted with the *N*-protected aminopentanol linker to provide an inseparable mixture of α and β anomers (1:4.5). Subsequent removal of the Pico group with $Cu(OAc)_2$ and chromatographic separation afforded pure β-linked alcohol **S-16** in 68% overall yield. Glycosylation of trifluoroacetimidate **S-7** with alcohol **S-16** in

** Compounds S-1, S-2, S-4 and building blocks were synthesized by Dr. Petra Ménová.

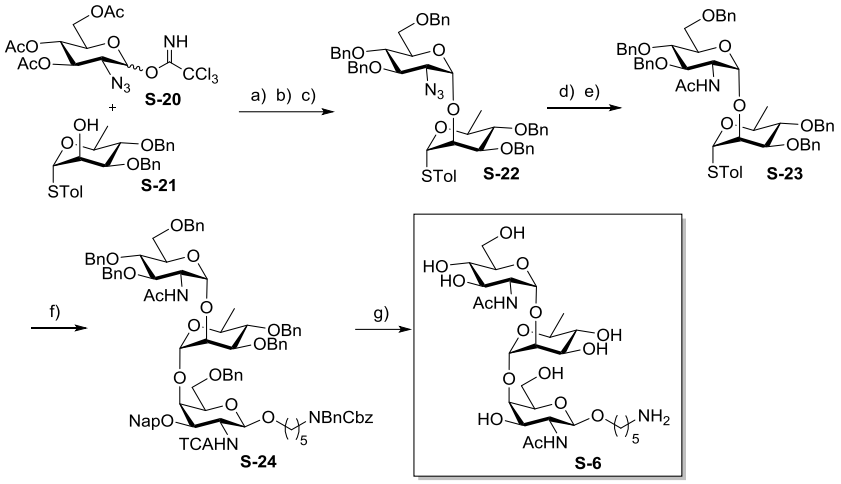
DCM/Et_2O proceeded with good α selectivity (~95:5), presumably due to a combination of solvent effects and remote acyl participation. Debenzoylation of the obtained trisaccharide required harsh conditions (15% aq. NaOH in refluxing methanol for four days) and led to partial decomposition of the starting compound. Finally, hydrogenation gave trisaccharide **S-3** in 15% yield over five steps.

The synthesyis of trisaccharide **S-5** (Scheme II) started with benzoylation of the free hydroxyl group in building block **S-13**. The obtained donor **S-18** was coupled with disaccharide acceptor **S-9**, and subsequent global deprotection provided trisaccharide **S-5** in 47% overall yield.

The synthesis of linear trisaccharide **S-6** proved challenging (Scheme III). All attempts to glycosylate electron-rich 2-azido glucose at various positions failed. Resorting to known peracetylated azidoglucose donor **S-20**[208-210] to install 1,2-*cis* linkages by reaction with rhamnose acceptor **S-21** afforded a mixture of α and β anomers. Deacetylation and subsequent benzylation proceeded cleanly, giving benzylated α-linked disaccharide **S-22**, separable from traces of β-isomer by column chromatography. Azido disaccharide **S-22** was converted to acetamide **S-23** by nickel chloride and sodium borohydride-mediated reduction followed by subsequent acetylation. Final glycosylation of the disaccharide with building block **S-15** proceeded with excellent stereoselectivity and exclusively α-linked product **S-24** was isolated, albeit in modest yield due to extensive thioglycoside hydrolysis. A single-step deprotection by catalytic hydrogenation afforded the desired trisaccharide **S-6** in 5% overall yield.



Scheme S-II: Synthesis of compounds S-3 and S-5. Reagents and conditions: a) $HO(CH_2)_5NBnCbz$, NIS, TfOH, DCM, –40 °C→–20 °C; b) $Cu(OAc)_2{\cdot}H_2O$, DCM/MeOH 2:1, 68% over two steps; c) **S-7**, NIS, TMSOTf, DCM/Et_2O, –15 °C, 55%; d) NaOH, MeOH; e) H_2, Pd/C, EtOAc/tBuOH/H_2O 2:1:1, 40% over two steps; f) Bz_2O, Et_3N, DCM, >95%; g) **S-9**, NIS, TfOH, DCM, –15 °C, 76%; h) NaOMe, MeOH/THF 1:1, 35 °C; i) H_2, Pd/C, EtOAc/tBuOH/H_2O 2:1:1, 62% over two steps.



Scheme S-III: Synthesis of trisaccharide S-6. Reagents and conditions: a) TMSOTf, DCM/Et_2O 1:3, 0 °C; b) NaOMe, MeOH; c) BnBr, NaH, DMF, 32% over three steps; d) $NiCl_2.6H_2O$, $NaBH_4$, MeOH/THF 1:1; e) Ac_2O, Py, DCM, 73% over two steps; f) 2-15, NIS, TfOH, 0 °C, 33%; g) H_2, Pd/C, EtOAc/tBuOH/H_2O 2:1:1, 64%.

4.3. GLYCAN ARRAY EVALUATION OF MINIMAL EPITOPES

Glycan microarray analysis of a human reference serum[211] served to identify epitopes recognized by human antibodies. Synthetic oligosaccharides**,** as well as native ST7F CPS, structurally related serotype 7A (ST7A) CPS[212], pneumococcal cell wall polysaccharide (CWPS) and various synthetic oligosaccharides as negative controls were printed on NHS-activated glass slides (Figure S-I and Section 4.5.2)[213]. Human serum 007sp recognized all oligosaccharides with the exception of trisaccharide **S-5**. An inhibition assay after preincubation with purified ST7F CPS showed a strong decrease in signal intensities for compounds **S-1**, **S-2**, **S-3** and **S-6**. Serotype-specific antibodies in the serum recognize these oligosaccharides, suggesting that both side-chains are important for antibody binding. The low level of binding inhibition to tetrasaccharide **S-4** indicates the presence of other cross-reactive antibodies in the human serum directed against common epitopes. Antibodies recognizing oligosaccharides containing similar substructures have been detected during serum analyses utilizing *S. pneumoniae* serotype 2[171]. Both side chains in the polysaccharide RU are recognized by anti-ST7F antibodies. A synthetic oligosaccharide antigen containing the side-chains or the entire repeating unit would be a good start for the development of a synthetic vaccine candidate.

Due to structural similarities between the CPSs of serotypes 7F and 7A (ST7A has the same repeating unit but for the β-D-Gal*p*(1→ side chain), shared epitopes have been hypothesized and cross-reactivity of a human serum post-PCV13 immunization was observed in an OPA assay[206]. To verify whether our synthetic glycans contain ST7A epitopes, even though these glycans contain an additional galactose not present in serotype 7A, ST7A CPS was printed on glycan arrays and a second inhibition assay was performed. Preincubation of the serum with native ST7A CPS led to partial binding inhibition to ST7F CPS. Binding inhibition to the synthetic glycans showed a similar pattern to that of serotype 7F, although much weaker inhibition was observed for compounds **S-1**, **S-2**, and **S-3**, confirming again the fundamental role of the β-D-Gal residue in the anti-ST7F epitope. A similar high level of inhibition was observed with trisaccharide **S-6**. These results show that an anti-

pneumococcal polyvalent human serum is able to specifically recognize serotype 7A polysaccharide and that antibodies bind to a similar portion of the polysaccharide repeating unit. Based on these results, immunizations using glycoconjugate containing an SP7F-specific synthetic antigen should induce antibodies cross-reactive to serotype 7A.

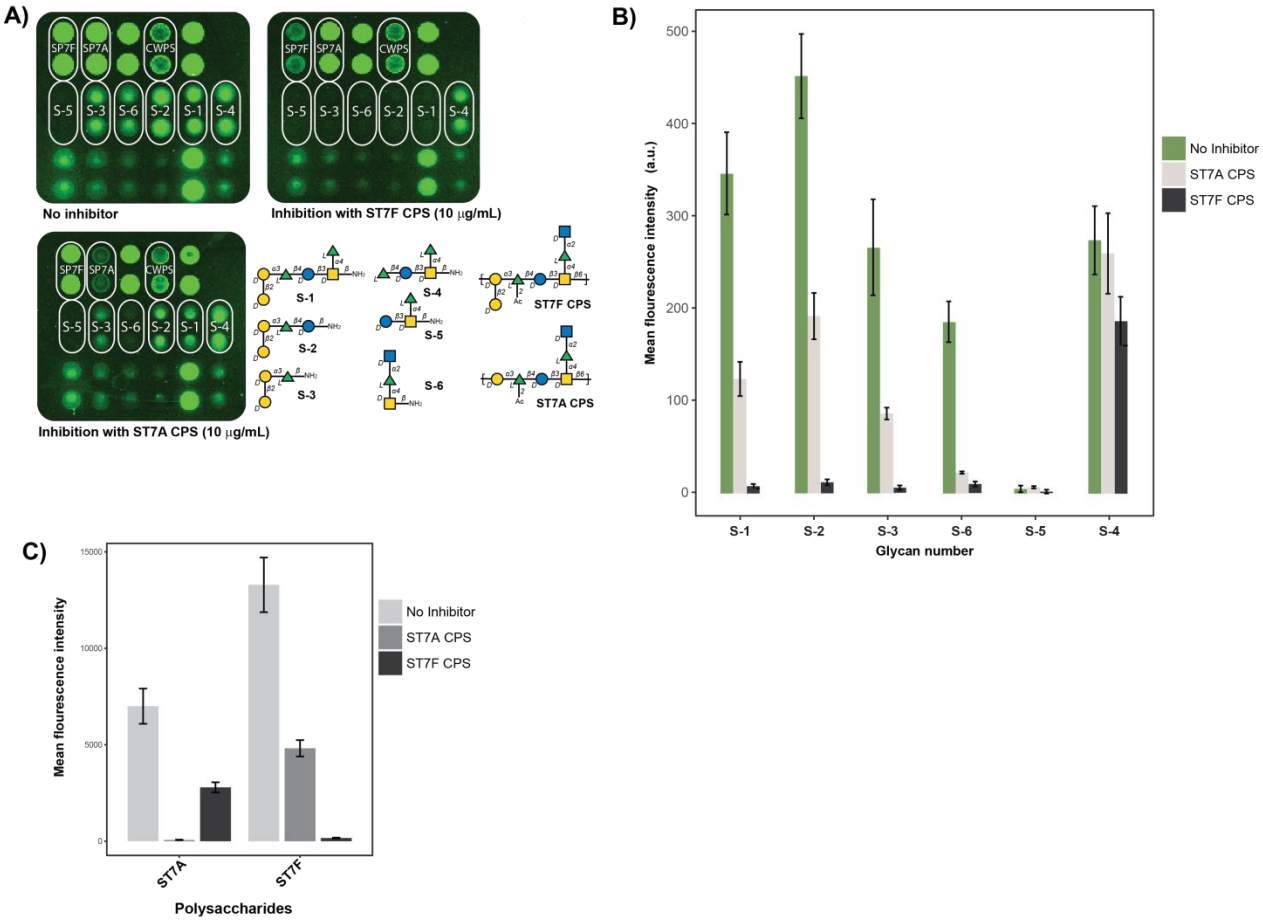


Figure S-I: Identification of minimal epitopes by glycan microarray screening of reference serum. (A) Synthetic glycans and native CPSs were immobilized on microarray slides and the slides were incubated with human reference serum 007sp (1:180 dilution). The bound antibodies were detected using fluorescently labeled secondary antibodies. For the inhibition study, sera were preincubated with ST7F or ST7A CPS (10 µg/mL) and then incubated with the printed arrays. (B, C) Comparison of mean fluorescence intensities (MFI) with synthetic glycans (B) or native polysaccharides (C) in the presence or absence of natural CPS. Data are represented as mean ± SD of duplicate determinations.

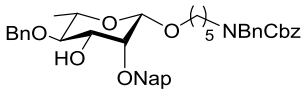
4.4. CONCLUSIONS

Six oligosaccharides representing different subunits of the ST7F CPS repeating unit, equipped with a reducing-end linker, were synthesized. The glycans were printed onto microarray slides to probe human reference sera. The results suggest that both side chains play an important role in antigen recognition and likely are an essential part for the development of a synthetic vaccine antigen. The synthetic antigen should be able to induce antibodies against both serotypes 7F and 7A based on the cross-reactivities observed during the glycan microarray analyses.

4.5. EXPERIMENTAL SECTION

4.5.1. SYNTHETIC PROTOCOLS AND SPECTRAL DATA

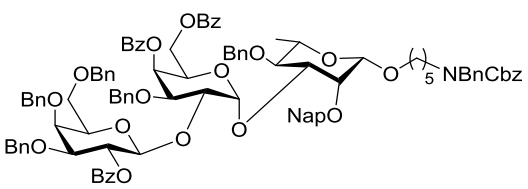
***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 4-*O*-benzyl-2-*O*-naphtylmethyl-β-L-rhamnopyranoside (S-16)**



Trifluoroacetimidate donor **S-12** (31 mg, 0.046 mmol) and linker acceptor (23 mg, 0.070 mmol) were co-evaporated twice with toluene and dried under high vacuum overnight. DCM (1 mL) and 4Å molecular sieves were added and the mixture was stirred at room temperature for 60 min. Then the solution was cooled to −40 °C, TMSOTf (0.1 M in DCM, 45 µL, 4.5 µmol) was added and the reaction mixture was stirred for 60 min at −40 °C to −20 °C. The reaction was quenched with triethylamine, filtered and concentrated. The residue was purified by column chromatography (30% to 40% ethyl acetate in hexanes). The obtained mixture of diastereoisomers (37 mg, 0.045 mmol) was dissolved in DCM/MeOH 2:1 (0.9 mL), $Cu(OAc)_2{\cdot}H_2O$ (9 mg, 0.045 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. The solvents were evaporated and the crude material was purified by column chromatography (20% ethyl acetate in hexanes) to afford compound **S-16** as a white solid (22 mg, 68%) and the corresponding alfa-anomer (5 mg) (α/β 1:4.5 after isolation).

$[\alpha]_D^{20}$ = 34.3° (c = 0.8, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} : 3456, 3033, 2935, 1698, 1605,1498, 1455, 1423, 1366, 1229, 1186, 1073, 1030, 911, 857, 820, 735, 698; ^1H NMR (600 MHz, $CDCl_3$, -6°C) δ 7.89 – 7.75 (m, 4H), 7.56 – 7.44 (m, 3H), 7.39 – 7.22 (m, 15H), 7.19 – 7.12 (m, 1H), 5.24 – 5.11 (m, 3H), 4.91 (d, *J* = 10.8 Hz, 1H), 4.76 (t, *J* = 12.3 Hz, 1H), 4.60 (d, *J* = 10.8 Hz, 1H), 4.52 – 4.46 (m, 2H), 4.42 (d, *J* = 23.9 Hz, 1H), 4.00 – 3.89 (m, 1H), 3.83 (dd, *J* = 17.0, 3.8 Hz, 1H), 3.62 – 3.56 (m, 1H), 3.45 – 3.32 (m, 1H), 3.31 – 3.23 (m, 3H), 3.20 (m, 1H), 1.74 – 1.50 (m, 4H), 1.38 (d, *J* = 5.7 Hz, 3H), 1.36 – 1.27 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.9, 156.3, 138.5, 138.0, 137.0, 136.9, 135.8, 133.4, 133.2, 128.7, 128.58, 128.55, 128.5, 128.2, 128.14, 128.06, 127.94, 127.87, 127.5, 127.3, 126.34, 126.30, 126.2, 101.7, 82.3, 78.2, 75.3, 75.2, 74.2, 71.5, 69.9, 69.8, 67.3, 50.6, 50.3, 47.3, 46.3, 29.5, 28.0, 27.6, 23.5, 18.2; HRMS (ESI+) calculated for $C_{44}H_{49}NNaO_7$ [M+Na]: 726.3401, found: 726.3414.

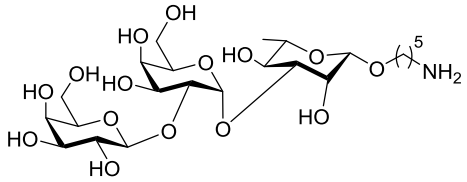
***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→2)-4,6-di-*O*-benzoyl-3-*O*-benzyl-α-D-galactopyranosyl-(1→3)-4-*O*-benzyl-2-*O*-naphtylmethyl-β-L-rhamnopyranoside (S-17)**



Trifluoroacetimidate donor **S-7** (21 mg, 0.017 mmol) and acceptor **S-16** (10 mg, 0.014 mmol) were twice co-evaporated with toluene and then dried under vacuum overnight. They were dissolved in

DCM/Et_2O 1:1 (0.6 mL) and 4Å Molecular sieves were added. The solution was stirred at room temperature for 30 min and subsequently cooled to −15 °C. TMSOTf (0.05 M in DCM, 28 µL, 1.4 µmol) was added dropwise and the mixture was stirred at −15°C for 1 h. The reaction was quenched with triethylamine, filtered and concentrated. Purification by column chromatography (20 to 30% ethyl acetate in hexane) afforded the product (14 mg; 4.2 µmol) as a mixture of anomers. Subsequent HPLC purification (YMC-diol-300NP column, 150 x 20 mm, flow rate 15 mL/ min, 20% AcOEt in Hex (5 min), linear gradient to 55% AcOEt (35 min), linear gradient to 100% AcOEt (5 min)) afforded pure **S-17** as colorless solid (13 mg, 7.64 µmol, 55%) and correspondent β-anomer (0.7 mg, 0.41 µmol).
$[\alpha]_D^{20}$ = 52.7° (c = 0.4, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} : 3033.2, 2926.7, 1727.48, 1700.0, 1603.2, 1497.6, 1453.9, 1422.1, 1365.9, 1268.3, 1176.9, 1096.2, 1070.8, 1027.6, 819.0, 735.5, 711.6, 698.7; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 – 7.87 (m, 6H), 7.73 – 7.43 (m, 8H), 7.42 – 7.00 (m, 42H), 6.87 – 6.80 (m, 2H), 5.58 (dd, *J* = 10.2, 8.0 Hz, 1H), 5.40 (d, *J* = 3.2 Hz, 1H), 5.22 – 5.13 (m, 2H), 5.08 (d, *J* = 13.7 Hz, 1H), 5.01 (d, *J* = 3.5 Hz, 1H), 4.85 – 4.71 (m, 4H), 4.59 – 4.35 (m, 9H), 4.35 – 4.27 (m, 2H), 4.07 – 3.94 (m, 3H), 3.93 – 3.89 (m, 1H), 3.89 – 3.78 (m, 2H), 3.70 – 3.54 (m, 4H), 3.53 – 3.37 (m, 4H), 3.34 – 3.15 (m, 3H), 2.94 (br d, *J* = 9.9 Hz, 1H), 1.76 – 1.61 (m, 4H), 1.49 – 1.29 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2, 165.7, 165.1, 138.6, 138.5, 138.1, 137.93, 137.87, 137.6, 133.2, 133.1, 132.8, 130.2, 130.1, 129.94, 129.88, 129.85, 128.7, 128.62, 128.57, 128.5, 128.43, 128.37, 128.3, 128.2, 128.13, 128.05, 128.04, 128.00, 127.95, 127.9, 127.71, 127.68, 127.5, 127.3, 127.2, 127.1, 126.1, 125.9, 125.7, 101.8, 101.4, 98.6, 81.2, 79.5, 79.4, 77.8, 75.2, 74.7, 74.6, 73.7, 73.7, 72.7, 71.8, 71.7, 71.0, 68.7, 68.4, 67.8, 67.3, 63.5, 50.4, 46.5, 29.9, 28.3, 23.6, 17.8; HRMS (ESI+) calculated for $C_{105}H_{105}NNaO_{20}$ [M+Na]: 1723.7122, found: 1723.7185.

5-Aminopentyl β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-β-L-rhamnopyranoside (S-3)

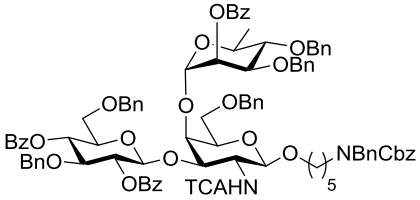


Compound **S-17** (4.0 mg, 2.4 µmol) was dissolved in MeOH (1 mL). 15% NaOH aqueous solution (50 µL) was added and the reaction mixture was stirred at 40 °C for 48 h. Then the temperature was raised to 60 °C and the reaction was stirred for an additional 48 h. The solution was cooled to 0 °C and neutralized with acetic acid, then diluted with water (5 mL) and extracted with ethyl acetate (3x10mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The obtained crude product was dissolved in EtOAc (1 mL), *t*BuOH (0.5 mL) and H_2O (0.5 mL). Pd/C was added, the vial was purged first with argon, then with H_2 and the reaction mixture was stirred under H_2 atmosphere (3 bar) at room temperature for 16 h. The catalyst was filtered off (hydrophobic PTFE filter, 0.45 µm) and the solution was concentrated. The residue was subjected to a second hydrogenation cycle under the same conditions. After 16 h the catalyst was filtered off, the solution was concentrated and purified by HPLC (Hypercarb column, 150 x 10 mm, flow rate of 1.3 mL / min,

H_2O (0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (5 min)) to afford **S-3** after lyophilization (0.60 mg, 0.97 µmol, 40%).

^{1}H NMR (700 MHz, D_2O) δ 8.39 (s,1H, $HCOO^-$), 5.32 (d, *J* = 3.7 Hz, 1H), 4.57 (s, 1H), 4.52 (d, *J* = 7.7 Hz, 1H), 4.21 (d, *J* = 3.0 Hz, 1H), 4.15 (t, *J* = 6.4 Hz, 1H), 4.06 – 4.02 (m, 1H), 4.01 – 3.99 (m, 1H), 3.92 – 3.86 (m, 2H), 3.84 – 3.79 (m, 1H), 3.77 – 3.58 (m, 8H), 3.58 – 3.54 (m, 1H), 3.45 – 3.40 (m, 1H), 3.40 – 3.34 (m, 1H), 2.93 (t, *J* = 7.5 Hz, 2H), 1.66 – 1.57 (m, 4H), 1.43 – 1.36 (m, 2H), 1.27 (d, *J* = 6.1 Hz, 3H). ^{13}C NMR (176 MHz, D_2O) δ 171.03 ($HCOO^-$), 105.0, 99.8, 94.9, 78.3, 77.5, 75.3, 72.5, 72.1, 71.0, 70.5, 70.4, 69.7, 69.1, 68.5, 68.2, 66.6, 60.8 (2C), 39.4, 28.2, 26.6, 22.2, 16.8; HRMS (ESI+) calculated for $C_{23}H_{44}NO_{15}$ [M+H]: 574.2711, found: 574.2712.

***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2,4-di-*O*-benzoyl-3,6-di-*O*-benzyl-β-D-glucopyranosyl-(1→3)-[2-*O*-benzoyl-3,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→4)]-6-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-galactopyranoside (S-19)**

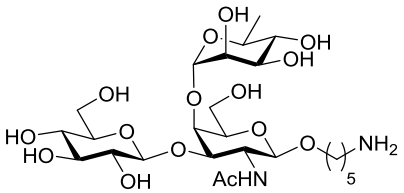


Thioglycoside donor **S-18** (66 mg, 0.098 mmol) and disaccharide acceptor **S-9** (86 mg, 0.074 mmol) were twice co-evaporated with toluene and then dried under high vacuum overnight. DCM (3 mL) and 4Å AW MS were added and the mixture was stirred at room temperature for 30 min. Then the solution was cooled to −15 °C, NIS (27 mg, 0.120 mmol) followed by TfOH (0.11 M in DCM, 70 µL, 7.7 µmol) were added and the reaction mixture was stirred for 30 min at −15 °C. The reaction was quenched with triethylamine and diluted with aqueous $Na_2S_2O_3$ (10%, 5 mL) and DCM (5 mL). The mixture was filtered and the phases were separated. The aqueous phase was extracted with DCM (1 × 10 mL) and the combined organic layer was washed with saturated aqueous $NaHCO_3$ (1 × 10 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The crude material was purified by column chromatography (20% ethyl acetate in hexanes) and HPLC (YMC-diol-300NP column, 150 x 20 mm, flow rate 15 mL/min, 20% AcOEt in Hex (5 min), linear gradient to 55% AcOEt (35 min), linear gradient to 100% AcOEt (5 min)) to afford **S-19** as colorless solid (25 mg, 76%).

$[\alpha]_D^{20}$ = -2.5° (c = 1.0, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} : 3066, 3033, 2927, 2863, 1727, 1453, 1265, 1108, 1069, 1028, 737, 711, 699; ^{1}H NMR (700 MHz, $CDCl_3$) δ 8.24 (d, *J* = 7.6 Hz, 2H), 7.98 – 7.94 (m, 4H), 7.58 – 7.51 (m, 3H), 7.50 – 7.45 (m, 4H), 7.42 (t, *J* = 7.3 Hz, 2H), 7.40 – 7.35 (m, 6H), 7.34 – 7.16 (m, 17H), 7.16 – 7.08 (m, 4H), 7.07 – 7.05 (m, 2H), 7.03 – 6.99 (m, 1H), 6.98 – 6.93 (m, 4H), 6.80 – 6.65 (m, 1H), 6.10 – 6.04 (m, 2H), 5.71 (t, *J* = 9.5 Hz, 1H), 5.26 (br s, 1H), 5.18 – 5.10 (m, 2H), 5.06 – 5.02 (m, 2H), 4.89 – 4.85 (m, 1H), 4.82 (d, *J* = 7.8 Hz, 1H), 4.75 (d, *J* = 11.9 Hz, 1H), 4.71 (d, *J* = 10.8 Hz, 1H), 4.67 (d, *J* = 11.4 Hz, 1H), 4.65 – 4.59 (m, 2H), 4.52 (d, *J* = 11.8 Hz, 1H), 4.48 – 4.42 (m, 3H), 4.30 (br s, 1H), 4.22 – 4.17 (m, 2H), 4.16 – 4.12 (m, 1H), 4.06 – 3.97 (m, 2H), 3.82 – 3.70 (m, 2H), 3.67 (t, *J* = 9.4 Hz, 1H), 3.64 – 3.55 (m, 3H), 3.50 – 3.46 (m, 1H), 3.43 – 3.39 (m, 1H), 3.37 – 3.26 (m, 1H), 3.23 – 3.08 (m, 3H), 1.53 – 1.39 (m, 4H), 1.32 (d, *J* = 6.1 Hz, 3H), 1.25 – 1.13 (m, 2H); ^{13}C NMR (176 MHz, CDCl3) δ 165.6, 165.2, 164.8, 139.0, 138.8, 138.3, 138.1, 137.82, 137.80, 133.3,

133.2, 132.9, 130.6, 130.4, 130.3, 129.97, 129.93, 129.90, 128.66, 128.55, 128.49, 128.46, 128.4, 128.37, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 102.6, 101.3, 98.0, 92.2, 80.4, 79.9, 79.8, 76.4, 75.6, 73.8, 73.60, 73.57, 73.5, 72.5, 72.1, 71.0, 70.3, 69.8, 69.7, 69.6, 68.6, 67.3, 56.8, 50.69, 50.37, 47.25, 46.31, 29.4, 23.4, 18.4; HRMS (ESI+) calculated for $C_{96}H_{97}Cl_3N_2NaO_{20}$ [M+Na]: 1726.5633, found: 1726.5598.

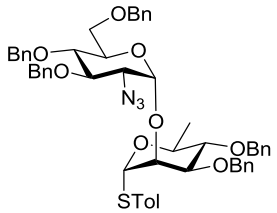
5-Aminopentyl β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→4)]-2-acetamido-2-deoxy-β-D-galactopyranoside (S-5)



Compound **S-19** (4.1 mg, 2.4 µmol) was dissolved in MeOH/THF 1:1 (1 mL). Sodium methoxide (0.5 M in MeOH, 0.20 mL, 0.10 mmol) was added and the reaction mixture was stirred at 35 °C for 48 h. To quench the reaction, Amberlite was added until the solution became neutral. The resin was filtered off and the solution was concentrated. The obtained product was dissolved in EtOAc (1 mL), *t*BuOH (0.5 mL) and H_2O (0.5 mL). Pd/C was added, the vial was purged first with argon, then with H_2 and the reaction mixture was stirred under H_2 atmosphere (3 bar) at room temperature for 48 h. The catalyst was filtered off (hydrophobic PTFE filter, 0.45 µm) and the resulting acidic solution was neutralized with triethylamine. Purification by HPLC (Hypercarb column, 150 x 10 mm, flow rate of 1.3 mL / min, H2O (0.1% formic acid) isocratic (5 min), linear gradient to 30% ACN (30 min), linear gradient to 100% ACN (5 min)) and lyophilization afforded **S-5** (1.0 mg; 1.51 µmol; 62%).

^{1}H NMR (700 MHz, D_2O) δ 8.39 (s, 1H, $HCOO^-$), 5.25 (d, *J* = 1.8 Hz, 1H), 4.45 (d, *J* = 8.6 Hz, 1H), 4.41 (d, *J* = 7.8 Hz, 1H), 4.20 (d, *J* = 2.8 Hz, 1H), 4.09 – 4.07 (m, 1H), 3.96 (dd, *J* = 10.9, 8.6 Hz, 1H), 3.88 – 3.82 (m, 3H), 3.79 (dd, *J* = 9.8, 3.3 Hz, 1H), 3.76 – 3.64 (m, 5H), 3.54 (dt, *J* = 10.2, 6.3 Hz, 1H), 3.42 – 3.38 (m, 2H), 3.35 – 3.33 (m, 2H), 3.22 (dd, *J* = 9.3, 7.8 Hz, 1H), 2.95 – 2.91 (m, 2H), 1.96 (s, 3H), 1.65 – 1.58 (m, 2H), 1.57 – 1.52 (m, 2H), 1.38 – 1.32 (m, 2H), 1.23 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (176 MHz, D_2O) δ 174.8, 171.0, 104.5, 101.4, 101.2, 80.2, 75.7, 75.5, 75.2, 74.7, 73.2, 71.8, 70.2, 70.2, 70.0, 69.6, 69.4, 61.3, 60.8, 51.6, 39.4, 28.1, 26.4, 22.2, 22.1, 16.6; HRMS (ESI+) calculated for $C_{25}H_{47}N_2O_{15}$ [M+H]: 615.2971, found: 615.2969.

***p*-Tolyl 2-azido-2-deoxy-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→2)-3,4,-di-*O*-benzyl-1-thio-α-L-rhamnopyranoside (S-22)**

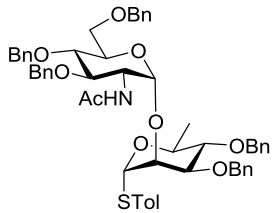


Trichloroacetimidate donor **S-20** (315 mg, 0.663 mmol) and acceptor **S-21** (199 mg, 0.442 mmol) were co-evaporated three times with toluene and dried under vacuum overnight. They were then dissolved

in DCM/Et_2O 1:3 (4.5 mL) under argon atmosphere and 4Å Molecular sieves were added. The solution was stirred at room temperature for 30 min and subsequently cooled to 0 °C. TMSOTf (0.1 M in DCM, 220 µL, 0.044 mmol) was added dropwise over 10 minutes and the mixture was stirred at 0 °C for 2 h. The reaction was quenched with triethylamine, filtered and concentrated. Purification by column chromatography (5 to 10% ethyl acetate in toluene) afforded impure acetylated disaccharide in quantitative yield. The product was directly dissolved in MeOH (4.5 mL). NaOMe (0.5 M in MeOH, 85 µL, 0.043 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. To quench the reaction, Amberlite was added until the solution became neutral. The resin was filtered off and the solution was concentrated. The crude product was dissolved in DMF (2.5 mL) under nitrogen atmosphere and benzyl bromide (163 µL, 1.373 mmol) was added. NaH (60% susp. in mineral oil, 62 mg, 1.550 mmol) was added portionwise. The reaction was stirred for 3 h then it was carefully diluted with MeOH and concentrated. The crude was taken up in DCM (20 mL) and brine (10 mL). After phase separation the aqueous phase was extracted with DCM (2 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. The crude material was purified by column chromatography (10 to 20% ethyl acetate in hexanes) to obtain pure **S-22** as colorless solid (130 mg, 0.143 mmol, 32% over 3 steps).

$[\alpha]_D^{20}$ = 33.1° (c =1.1, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} 3032.9, 2926.8, 2108.0, 1496.5, 1455.1, 1364.7, 1246.3, 1210.1, 1103.0, 1088.6, 1065.1, 1040.5, 912.5, 809.9, 735.4, 697.7; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 – 7.16 (m, 26H), 7.06 (m, 3H), 5.37 (s, 1H), 4.93 (d, *J* = 3.6 Hz, 1H), 4.89 – 4.82 (m, 3H), 4.70 (d, *J* = 10.8 Hz, 1H), 4.67 – 4.64 (m, 2H), 4.61 (d, *J* = 10.8 Hz, 1H), 4.46 (d, *J* = 12.0 Hz, 1H), 4.41 (d, *J* = 10.7 Hz, 1H), 4.34 – 4.31 (m, 1H), 4.28 (d, *J* = 12.0 Hz, 1H), 4.16 – 4.11 (m, 2H), 4.02 (t, *J* = 9.6 Hz, 1H), 3.81 – 3.77 (m, 1H), 3.69 (t, *J* = 9.5 Hz, 1H), 3.57 (t, *J* = 9.5 Hz, 1H), 3.37 – 3.32 (m, 1H), 3.25 – 3.20 (m, 1H), 3.17 – 3.12 (m, 1H), 2.28 (s, 3H), 1.29 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 138.5, 138.3, 138.22, 138.21, 138.1, 137.8, 132.6, 130.5, 130.1, 128.6, 128.54, 128.52, 128.50, 128.2, 128.1, 128.0, 127.93, 127.91, 127.89, 127.8, 127.6, 127.4, 96.6, 85.7, 80.4, 79.2, 79.1, 78.4, 75.6, 75.2, 75.12, 75.10, 73.7, 72.4, 70.9, 69.5, 68.0, 62.9, 21.3, 17.7; HRMS (ESI+) calculated for $C_{54}H_{57}N_3NaO_8S$ [M+Na]: 930.3764, found: 930.3743.

***p*-Tolyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→2)-3,4,-di-*O*-benzyl-1-thio-α-L-rhamnopyranoside (S-23)**

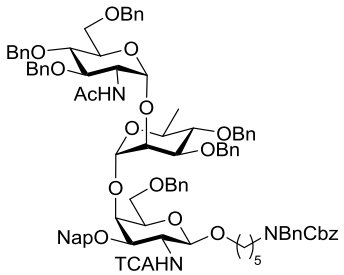


Compound **S-22** (170 mg, 0.187 mmol) was dissolved in MeOH/THF 1:1 (2.0 mL). Nickel chloride hexahydrate (5 mg, 0.019 mmol) was added and the solution was cooled to 0 °C. $NaBH_4$ (9 mg, 0.243 mmol) was added portionwise and the solution was stirred for 30 min. Then it was diluted with MeOH, warmed to room temperature and the solvent was evaporated. The residue was dried under high vacuum and redissolved in DCM (2.0 mL). Pyridine (110 µL, 1.42 mmol) and acetic anhydride (55 µL,

0.561 mmol) were added and the solution was stirred for 1 h. The reaction was diluted with $CHCl_3$ (30 mL) and extracted with 1M HCl (1 × 10 mL), saturated aqueous $NaHCO_3$ (1 × 10 mL) and brine (1 × 10 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude material was purified by column chromatography (20 to 40% ethyl acetate in hexanes) to obtain pure **S-23** as colourless solid (126 mg, 0.136 mmol, 73% over 2 steps).

$[\alpha]_D^{20}$ = 37.1° (c =1.1, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} 3427.7, 3065.0, 3032.1, 2921.9, 2870.9, 1682.6, 1516.4, 1496.6, 1454.7, 1365.0, 1310.4, 1208.9, 1099.6, 1085.7, 1069.9, 1043.24, 1029.4, 990.1, 910.8, 848.5, 810.2, 736.3, 697.8; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.15 (m, 25H), 7.16 – 7.09 (m, 2H), 7.03 (d, *J* = 7.9 Hz, 2H), 5.45 (d, *J* = 9.4 Hz, 1H), 5.04 (d, *J* = 1.9 Hz, 1H), 4.90 (d, *J* = 10.9 Hz, 1H), 4.81 (d, *J* = 11.6 Hz, 1H), 4.79 – 4.71 (m, 2H), 4.66 (d, *J* = 11.9 Hz, 1H), 4.63 – 4.55 (m, 2H), 4.57 – 4.42 (m, 3H), 4.36 (d, *J* = 12.1 Hz, 1H), 4.23 (t, *J* = 2.4 Hz, 1H), 4.20 – 4.01 (m, 3H), 3.77 – 3.67 (m, 2H), 3.60 (m, 1H), 3.51 (dd, *J* = 10.8, 3.5 Hz, 1H), 3.39 – 3.25 (m, 2H), 2.26 (s, 3H), 1.62 (s, 3H), 1.19 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 138.5, 138.3, 138.2, 138.1, 137.97, 137.94, 132.0, 130.5, 130.1, 128.8, 128.59, 128.58, 128.56, 128.53, 128.49, 128.4, 128.2, 128.02, 127.99, 127.97, 127.96, 127.8, 127.7, 127.6, 96.6, 86.0, 80.3, 79.6, 78.6, 78.3, 75.6, 75.3, 75.0, 74.6, 73.6, 71.8, 71.1, 68.9, 68.2, 52.4, 23.3, 21.3, 18.4; HRMS (ESI+) calculated for $C_{56}H_{61}NNaO_9S$ [M+Na]: 946.3965, found: 946.3932.

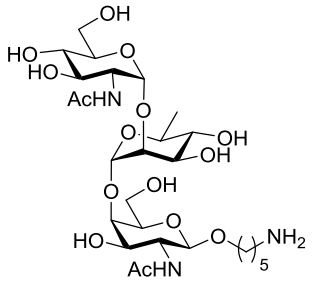
***N*-(Benzyl)benzyloxycarbonyl-5-aminopentyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→2)-3,4,-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→4)-6-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-galactopyranoside (S-24)**



Disaccharide **S-23** (68 mg, 0.074 mmol) and monosaccharide **S-9** (50 mg, 0.058 mmol) were co-evaporated three times with toluene and dried under high vacuum overnight. They were dissolved in DCM (2.5 mL) under Argon atmosphere and 4Å Molecular sieves were added. The solution was stirred at room temperature for 30 min and subsequently cooled to 0 °C. NIS solution (100 mg/mL in DCM/Dioxane, 170 µL, 0.075 mmol) was added, followed by a TfOH solution (0.05 M in DCM/Dioxane, 44 µL, 5.8 µmol) and the mixture was stirred for 1 h at 0 °C and for 30 min at r.t.. The reaction was quenched with triethylamine and diluted with aqueous $Na_2S_2O_3$ (10%, 5 mL) and DCM (5 mL). The mixture was filtered and the phases were separated. The aqueous phase was extracted with DCM (1 × 5 mL) and the combined organic layer was washed with saturated aqueous $NaHCO_3$ (1 × 5 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The crude material was purified by column chromatography (30% to 50% ethyl acetate in hexanes) to afford pure trisaccharide **S-24** as a colorless solid (32 mg, 0.019 mmol, 33%).

$[\alpha]_D^{20}$ = 35.4° (c =1.7, $CHCl_3$); IR (thin film, cm^{-1}): ν_{max} 3286.1, 3065.1, 3032.4, 2928.2, 2868.5, 1670.9, 1605.9, 1524.0, 1497.9, 1455.1, 1423.6, 1365.3, 1303.5, 1210.4, 1102.8, 1051.9, 1029.4, 910.7, 821.5, 736.8, 698.2, 672.0; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.84 – 7.77 (m, 3H), 7.70 (s, 1H), 7.47 – 7.09 (m, 43H), 5.45 – 5.38 (m, 1H), 5.15 (m, 2H), 5.07 (s, 1H), 4.91 (d, *J* = 11.1 Hz, 1H), 4.84 (d, *J* = 11.5 Hz, 1H), 4.79 (m, 2H), 4.73 – 4.57 (m, 7H), 4.55 – 4.43 (m, 4H), 4.31 (d, *J* = 12.2 Hz, 1H), 4.15 (m, 3H), 4.08 – 4.01 (m, 2H), 3.90 (m, 1H), 3.83 – 3.72 (m, 3H), 3.69 – 3.56 (m, 5H), 3.48 – 3.38 (m, 3H), 3.31 (m, 3H), 3.23 (m, 1H), 3.16 (m, 1H), 1.66 (s, 3H), 1.50 (m, 4H), 1.26 (m, 2H), 1.16 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.2, 169.9, 162.0, 138.6, 138.5, 138.3, 138.1, 138.0, 137.9, 137.7, 134.2, 133.4, 133.3, 129.0, 128.83, 128.66, 128.64, 128.60, 128.56, 128.54, 128.52, 128.46, 128.4, 128.31, 128.28, 128.22, 128.20, 128.16, 128.12, 128.05, 127.99, 127.97, 127.94, 127.92, 127.86, 127.80, 127.75, 127.73, 127.68, 127.66, 127.4, 126.43, 126.37, 126.3, 126.2, 99.3, 98.5, 96.7, 80.3, 79.7, 78.3, 77.9, 75.1, 74.5, 73.6, 73.53, 73.49, 73.0, 71.6 (4C), 70.0 (2C), 68.9, 68.7, 68.4, 67.3, 56.0, 52.4, 50.7, 50.4, 47.3, 46.3, 29.3, 28.1, 27.6, 23.4 (2C), 18.7; HRMS (ESI+) calculated for $C_{95}H_{102}Cl_3N_3NaO_{17}$ [M+Na]: 1684.6173, found: 1684.6115.

5-Aminopentyl 2-acetamido-2-deoxy-α-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-galactopyranoside (S-3)



Compound **S-24** (4.0 mg, 2.40 µmol) was dissolved in EtOAc (1 mL), *t*BuOH (0.5 mL), H_2O (0.5 mL) with one drop of acetic acid. Pd/C was added and the vial was purged first with argon, then with H_2. the reaction mixture was stirred under H_2 atmosphere at room temperature for 24 h. The catalyst was filtered off (hydrophobic PTFE filter, 0.45 µm) the solvent evaporated under vacuum. The crude material was purified by size-exclusion chromatography (Sephadex LH-20, 50% MeOH in H_2O). Lyophilization afforded **S-3** as a salt with acetic acid (1.1 mg, 1.54 µmol, 64%).

^{1}H NMR (600 MHz, D_2O) δ 5.14 (d, *J* = 1.7 Hz, 1H), 4.96 (d, *J* = 3.7 Hz, 1H), 4.46 (d, *J* = 7.9 Hz, 1H), 4.17 – 4.14 (m, 1H), 4.05 – 3.97 (m, 3H), 3.95 – 3.85 (m, 5H), 3.84 – 3.73 (m, 6H), 3.62 – 3.51 (m, 3H), 2.99 – 2.95 (m, 2H), 2.05 (d, *J* = 4.9 Hz, 6H), 1.93 (s, 3H, CH_3COO^-), 1.70 – 1.57 (m, 4H), 1.44 – 1.39 (m, 2H), 1.34 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (176 MHz, D_2O) δ 181.5, 174.7, 174.4, 101.8, 98.9, 95.5, 76.0, 75.6, 74.8, 71.9, 71.7, 71.4, 70.5, 70.0, 69.8, 69.7, 69.3, 61.4, 60.3, 53.7, 52.4, 39.5, 28.1, 26.8, 23.3, 22.22, 22.15, 21.8, 16.7; HRMS (ESI+) calculated for $C_{27}H_{50}N_3O_{15}$ [M+H]: 656.3242, found: 656.3245.

4.5.2. GLYCAN ARRAYS PREPARATION AND SCREENING

Glycan microarray slides were prepared by robotically spotting solutions on NHS activated glass slides. In detail, synthetic glycans and polysaccharides were dissolved in printing buffer (50 mM sodium phosphate, pH 8.5) in the concentrations outlined in Figure S-II, i.e. 0.2 mg/mL polysaccharides and 0.1 mM synthetic oligosaccharides. The solutions were transferred to a 384 well V bottom plate (Genetix) and robotically printed onto NHS activated glass slides (CodeLink slides, Surmodics) using an S3 non-contact microarray spotter (Scienion) equipped with a Type 4 coated nozzle (PDC80). Humidity in the printing chamber was kept constant at 45% during the entire print run. Following printing, the slides were incubated overnight at room temperature in a humidity-saturated chamber. Remaining reactive groups were quenched by incubating the slides in quenching solution (50 mM sodium phosphate, 100 mM ethanolamine, pH 9) at room temperature for one hour. The slides were washed twice with water, dried by centrifugation at 300 x g for three minutes (Eppendorf CombiSlide system) and stored dry at 4 °C until use. Directly before the assay, the slides were blocked with a solution of 3% (w/v) BSA in PBS (BSA-PBS) for 60 min at room temperature, washed 3 × 2 min with PBS and dried by centrifugation. A 64 well incubation gasket (FlexWell 64 grid, Grace BioLabs) was attached to the slide. For the inhibition assay, human reference serum 007sp (NIBSC, UK) was diluted in 3% (w/v) BSA-PBS-0.1% Tween containing 10 µg/mL pneumococcal cell wall polysaccharide and either no or 10 µg/mL SP7F-CPS or SP7A-CPS (all polysaccharides obtained from Statens Serum Institut, Denmark), incubated 20 min at 37 °C, and added in duplicates to the glycan arrays. After incubation for 1 h at r.t., slides were washed 3 × 2 min with PBS containing 0.1% (v/v) Tween-20 (PBST) by adding 50 µL to each well. The secondary antibody diluted in 3% (w/v) BSA-PBS-0.1% Tween (goat anti-human IgG-Fc AlexaFluor® 488 1:400, Dianova) was directly added with 25 µL to the wells of the gasket and incubated for 1 h at room temperature in the dark. After incubation the slides were washed twice with PBS-T, twice with PBS, rinsed with deionized water and dried by centrifugation (300 x g, 3 min) prior to scanning with a GenePix 4300A microarray scanner (Molecular Devices). Intensities were evaluated as mean fluorescence intensity of circles of identical diameter for all glycans with local background subtraction using GenePix 7 (Molecular Devices).

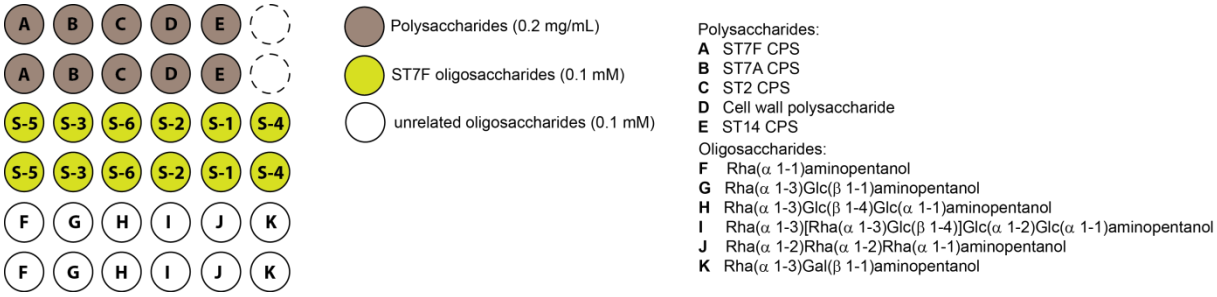


Figure S-II: Microarray slide printing pattern with employed printing concentrations. ST7F oligosaccharides S1–S6 (yellow), natural polysaccharides A–E (grey) and unrelated oligosaccharides F–K (white) were printed according to the above described procedure.

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