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Vuela solo quien se atreve a hacerlo

Luis Sepùlveda

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List of abbreviations

mAb = monoclonal antibody
Ac = Acetyl
ACN = Acetonitrile
Ac_2O = Acetic anhydride
AcOH = Acetic acid
AgOTf = Silver trifluoromethanesulfonate
APC = Antigen Presenting Cell
DP = Degree of Polymerization
$BF_3 \cdot NEt_3$ = Boron trifluoride diethyl etherate
Bn = Benzyl
BnBr = Benzyl bromide
bs = broad singlet
BuOH = butanol
$(tBu)_2Si(OTf)_2$ = Di-tert-butyl silyl bis (trifluoromethanesulfonate)
Bz = Benzoyl
BzCl = Benzoyl chloride
CAN = Ammonium cerium (IV) nitrate
CCl_3CN = Trichloro acetonitrile
CPS = Capsular Polysaccharide
DBU = 1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC = Dicyclohexylcarbodiimide
DCM = Dichloromethane
DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEA = diethanolamine
DMAP = 4-(Dimethylamino)pyridine
DMF = N,N-Dimethylformamide
DMSO = dimethyl sulfoxide
d = doublet
dd = doublet of doublets
dt = doublet of triplets
DT = Diphteria Toxoid
EDTA = ethylenediaminetetraacetic acid
EtOAc = Ethyl acetate
EtOH = Ethanol
Et_3SiH = triethylsilane
Fab = Fragment antigen-binding
Fmoc = Fluorenylmethoxycarbonyl
Fmoc-Cl = Fluorenylmethoxycarbonyl chloride
HSA = Human Serum Albumine
HEPES = (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPAEC-PAD = High-Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection
GBS = Group B streptococcus
IAP = Intrapartum Antibiotic Prophylaxis
K_d = dissociation constant
Lev = levulinoyl ester
m = multiplet
MD = Molecular Dynamics
MeONa = Sodium methoxide
MeOH = Methanol
MeOTf = Methyl Trifluoromethansulfonate
MS = Molecular Sieves
pNPP = *p*-Nitrophenyl Phosphate
NIS = N-iodosuccinimide
OPKA = opsonophagocytic killing assay
OS = oligosaccharide
PBS = phosphate buffered saline
PMP = p-Methoxyphenyl
Ph = phenyl
$PhBCl_2$ = dichlorophenylborane
$PhCH(OMe)_2$ = Benzaldehyde dimethyl acetal
Phth = Phthaloyl
$Phth_2O$ = Phthalic anhydride
PTSA = p-Toluenesulfonic acid
Py = Pyridine
rt = room temperature
RU = repeating unit
s = singlet

SEt = Ethyl-thiol
SIDEA = di-*N*-hydroxysuccinimidyl adipate
SPR = Surface Plasmon Resonance
STD-NMR = Saturation Transfer Difference
Nuclear Magnetic Resonance
TBAI = tetrabutylammonium iodide
TBDPS-Cl = tert-butyl diphenylsilyl chloride
TCA = Trichloroacetimidate
TD = T dependent
TEA = Triethylamine
TFA = Trifluoracetic acid
TfOH = Trifluoromethanesulfonic acid
THF = Tetrahydrofuran
TLC = Thin layer chromatography
TMSOTf = Trimethylsilyl
trifluoromethanesulfonate
Troc = 2,2,2-trichloroethoxycarbonyl
Tol = Toluene
t = triplet
TT = Tetanus Toxoid

Chapter 1

Synthetic glycoconjugate vaccines

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Introduction

Despite the enormous progress achieved by modern medicine, numerous diseases still have a profound impact on public health. Infectious pathologies are a global major concern both in industrialized and developing countries, and the rise of multidrug-resistant bacteria is a great concern. Vaccines are considered among the most cost-effective ways to prevent morbidity and mortality from infectious diseases. Over the last 50 years vaccines have greatly contributed to improve human health by controlling, and in some cases eradicating, many infectious diseases, both viral (for example, smallpox, measles and polio) and bacterial (for example, diphtheria and tetanus), that were the cause of many deaths and disability in the 20th century.

General principles of vaccination

The immune system is an important defense mechanism for our organism, being able to recognize infected cells (*non-self*) and trigger an immune response against them. The immune response can be divided into innate and adaptive immunity. The first fights intruding pathogens in a rapid and non-specific way and can be considered a first line of defense. Onset of adaptive immunity is delayed but directed specifically against the respective intruder. A multitude of pathogen-specific cells are created by clonal expansion of few precursor cells. Once the infection is resolved, some of these cells survive in the body to form immunological memory ready to fight the same pathogen faster when a second encounter occurs. Vaccination takes advantage of the adaptive immunity.
Indeed, the principle of vaccination is that exposure to a small sample of disease-causing microorganism (or to a part or portion of it) teaches the immune system to rapidly recognize the menace and to create memory that enables the body to fight efficiently the real pathogen during a later encounter. Vaccination induces an immune response in the host that leads to the production of immune effectors called antibodies, which are produced by B lymphocytes and are capable to recognize and specifically bind to a toxin or a pathogen (or to a portion representative of it)[1].
Two key players of the immune system are B and T lymphocytes, which both are provided with receptors to recognize specific antigens. When cell-antigen interactions occur, B-lymphocytes generate plasma cells and secrete specific antibodies against a particular antigen. B-lymphocytes are responsible for the humoral response, while T-lymphocytes produce T-cytotoxic and T-helper cells, involved in both humoral and cell-mediated immunity. Cooperation of B- and T-lymphocytes leads to the formation of memory B cells. When a second exposure to the same antigen takes place, memory B cells generate specific antibodies with particular combinatory sites capable of binding the structural-complementary antigens. They usually only recognize a small part of the molecule, called epitope. The activation of a B cell to create memory B cells requires the participation of macrophages or T-helper cells and this can only be stimulated by T-dependent (TD) antigens, like proteins. Following interaction with antigen-presenting cells (APC) like dendritic cells, macrophages and B-cells, protein antigens are internalized and processed into

small peptides which are then re-exposed and presented to T-lymphocytes in association with the MHC class II molecules. Interaction with T cells induces B cells to differentiate into plasma cells and memory B cells, thus initiating downstream adaptive immune responses. Unlike T-independent antigens, TD antigens are immunogenic early in infancy, the immune response induced can be boosted, is enhanced by adjuvants and is characterized by antibody class switch and production of antigen-specific IgG. The long polysaccharide chains found on bacterial capsule are T-independent antigens as they do not require T-cell activation for the induction of specific B-cell (antibody) response. Polysaccharide antigens directly activate polysaccharide-specific B cells which differentiate then into plasma cells to produce antibodies, but memory B cells are not formed. Moreover a pre-existing Memory B-cell pool can be depleted by immunization with unconjugated polysaccharide, with risk of hypo-responsiveness on subsequent immunizations[2].

Polysaccharide-based and glycoconjugate vaccines

Polysaccharides are important virulence factor especially for encapsulated bacteria that present these carbohydrate structure on their surface complex.

Around the 1930s the protective role of antibodies (Abs) induced by pneumococcal polysaccharides started to be investigated and in 1945 the first vaccine composed of purified polysaccharides from selected pneumococcal serotypes was tested in humans[3].

The research on vaccines development was subsequently slowed down by the introduction of antibiotics. However, with the emergence of drug resistant strains, the development of polysaccharide vaccines started again and several of them have been studied in large clinical studies. Polysaccharide vaccines against meningococcus serogroups ACWY, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b were licensed between the seventies and the eighties[4, 5]. These bacteria possess polysaccharides which are either polymers formed by one monosaccharide unit (homopolymers) or more complex oligosaccharide repeats (heteropolymers), that can be charged or neutral.

Polysaccharides, although efficacious in adults, fail to evoke immunological memory or long-lived antibody production as they are T-independent antigen. Conjugate vaccines, obtained by covalent linkage of bacterial polysaccharides to carrier proteins, have allowed to overcome limitations of unconjugated polysaccharide vaccines.

The T cell help provided by protein epitopes present in glycoconjugates imparts the capacity to induce a long lasting and boostable IgG antibody production to the appended carbohydrates also in children below the age of two[6, 7]. Also, vaccination with glycoconjugates has been proven more effective than the one with polysaccharides in adult population[8, 9]. Finally, conjugate vaccines have been shown to reduce carriage or impact on transmission of meningococci, while there is some evidence that plain polysaccharide vaccines cannot[10].

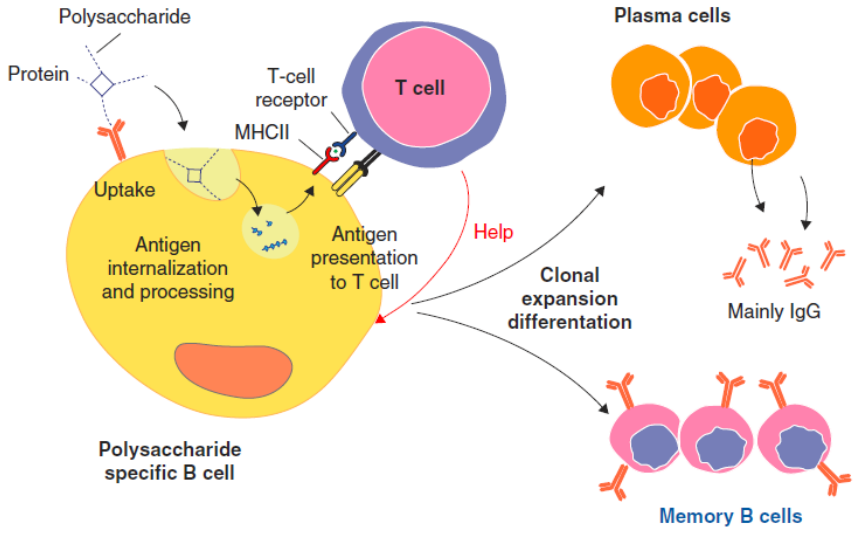

Figure 1. Mechanism of action of glycoconjugates vaccines[2]

Even though Avery and Goebel demonstrated already in 1929 that non-immunogenic sugar become able to induce antibodies in animal model after conjugation to a carrier protein, the first application of this concept to a vaccine for human use started in 1980 with the development of the first glycoconjugate vaccine against *Haemophilus influenzae* type b (Hib)[11]. Since then, glycoconjugates have been proven efficacious and cost effective to combat many life-threatening bacteria, such as *Streptococcus pneumoniae* (23 serotypes)[12], *Neisseria meningitidis* (A, C, W and Y)[13] and *Salmonella Typhi*[14-16].

A list of licensed glycoconjugate vaccines is reported in Table 1.

Table 1. Licensed glycoconjugate vaccines

Vaccine indication	Type of conjugate	Manufacturer
Haemophilus influenzae type b	PRP-TT	Sanofi-Pasteur
	PRP-OMPC	Merck
	PRP-CRM_{197}	Pfizer
	Hib-CRM_{197}	GSK
Haemophilus influenzae type b/Neisseria meningitidis group C	MenC/Hib-TT	GSK
Neisseria meningitidis serogroups $ACW_{135}Y$	MenA-TT	Serum Institute India
	MenC-CRM_{197}	Pfizer, GSK
	MenC-TT	Baxter
	MenACWY-DT	Sanofi-Pasteur
	MenACWY-CRM_{197}	GSK
	MenACWY-TT	Pfizer
Streptococcus pneumoniae serotypes	7 valent-CRM197 (4, 6B, 9V, 14, 18C, 19F, 23F)	Pfizer
	13 valent-CRM197 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F)	Pfizer
	10 valent-DT/TT Protein D (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)	GSK

Classical approaches for glycoconjugate vaccines preparation

As for other classes of vaccines, development of glycoconjugate vaccines is a long, complex and economically demanding process[17], that can last 10-15 years[18]. Increased complexity of future vaccines and need of reduced time to release have guided towards the development of faster approaches for glycoconjugate vaccines design.

Traditionally, polysaccharides are obtained by bacterial growths followed by harvesting and several purification steps, differing according to the nature of the carbohydrate (length, charge, necessity to preserve substitution patterns).

With long polysaccharides, chemical conjugation to the carrier protein is performed through random linkage along the saccharide chain, resulting in the formation of very high molecular weight cross-linked and rather undefined and heterogeneous structures[2, 19, 20]. Six proteins are used as carriers in licensed vaccines: Tetanus toxoid (TT), Diphtheria toxoid (DT), CRM_{197}, the Outer Membrane Protein Complex of meningococcus B (OMPC), Protein D from *H. influenzae* and recombinant Exotoxin A of *P. aeruginosa*[2, 21, 22].

Glycan-protein conjugation can take place using the carbohydrate functional moieties (e.g. hydroxyl, carboxyl groups) or alternatively derivatizing the polysaccharide with linkers to allow insertion of specific functionalities (e.g. thiols, bromide) and reduce the steric hindrance between protein and saccharide. One of the most employed transformations for conjugation entails the use of a $NaIO_4$ oxidation which generates aldehydes from *cis*-diols. These chemical groups can be directly linked to the ε-amine of the protein lysine residues by reductive amination, or further derivatized before linkage to the protein[23-26]. CDAP chemistry (1-Cyano-4-dimethylaminopyridinium tetrafluoroborate) is also randomly applied to commonly use hydroxyl groups of polysaccharides for condensation with the amines of the protein[27]. Recently, pneumococcal polysaccharides, pre-activated with sodium periodate, have been conjugated to CRM_{197} carrier protein by an in vacuo glycation method, and capped using sodium borohydride to prepare a 13-valent vaccine which was shown to give an immune response comparable to Prevnar for 10 out of 13 serotypes[28]. Dry-glycation (or in vacuo glycation) is a water free conjugation method consisting of colyophilization of the protein with the activated sugar, and then subjecting the lyophilized mixture to a vacuum at high temperature.

On a manufacture standpoint, use of shorter oligosaccharides facilitates consistency of production, sterile filtration, purification of the resulting conjugate, particularly from unreacted polysaccharide, and its characterization. Oligosaccharides can be linked to the protein through the end reducing sugar directly, or via a spacer. Production of well-defined and uniform lower molecular weight polysaccharide populations is typically achieved by chemical or mechanical polysaccharide fragmentation followed by size fractionation[29-31]. Depending on the polysaccharide structure, $NaIO_4$ oxidation results in simultaneous fragmentation and generation of terminal aldehyde groups available for conjugation (e.g. *H. influenzae* type b CPS). Alternatively, the polysaccharides can be fragmented by treatment with hydrogen peroxide[32] or via acid hydrolysis, and sized by chromatography or ultrafiltration techniques to isolate populations more defined in length for conjugation[31, 33]. Individual bacterial strains may also express a wide range of saccharide sizes, as it is usually the case of *O*-polysaccharides, that, in some instances, may not include the desired target size for vaccine development. Also, in this case, isolation of the sugar with desired length is required, impacting on overall yield and complexity of the process.

Synthetic and enzymatic approaches for glycoconjugate vaccines

Although isolation of polysaccharides from bacterial growth is the primary approach used for manufacturing licensed vaccines, approaches avoiding large scale pathogen fermentation have emerged over the last decades[34]. Specifically, organic synthesis has shown it can meet the demand of bacterial related oligosaccharides, with the advantages of a more defined structure, ease of characterization, lack of bacterial contaminants, higher batch-to-batch reproducibility, and more robust correlation of the elicited immune response with the oligosaccharide chemical structure[35]. Feasibility of large scale production of synthetic carbohydrates for vaccine manufacturing has been shown for the *H. influenzae* type b Quimi-Hib vaccine[36], which was produced by a polymerization approach based on H-phosphonate chemistry. Recently, a synthetic oligosaccharide based vaccine to combat *S. flexneri* serotype 2a has been tested in Phase 1 clinical trials[37]. Despite these examples, chemical synthesis of oligosaccharides remains challenging and time consuming, especially compared to the synthesis of other biopolymers such as peptides and oligonucleotides. The difficulty of oligosaccharides assembly is due to the presence of multiple hydroxy groups on the sugar monomers that must be discriminated during the synthesis; moreover, the union of two saccharides through formation of a new glycosidic linkage generates a new stereocenter[38]. A variety of methods, both in solution and in solid phase, have been pursued to accelerate glycans assembly and provide faster access to oligosaccharide well-defined structures[39].

One-pot multi-step strategies (Figure 2A) to assemble complex oligosaccharides integrate several glycosylation steps into one synthetic operation to furnish target oligosaccharides in a short period of time and without the need for protecting group manipulation and intermediate isolation[40, 41]. Databases based on the relative reactivities values (RRVs) of a variety of building blocks have been developed to aid the generation of the target oligosaccharides using reactivity guided synthesis design[42].

Automated synthetic approaches have now become available (Figure 2B). Starting from an adapted peptide synthesizer, via multiple home-built systems, different versions of the Glyconeer automated synthesizer (currently 2.1) have been developed to assemble synthetic glycans of increasing length and complexity by repeating cycles of glycosylation, capping and deprotection steps on a polystyrene Merrifield resin[43-45]. This system has been shown applicable for multimilligram synthesis of diverse glycans, varying from a homopolymeric mannans up to a 30-mer[46] to complex structures, such as alginates forming the *P. aeruginosa* exopolysaccharide featuring the chemically challenging 1,2-*cis*-mannosidic bond[47], as well as biologically relevant oligosaccharides bearing various *cis*-galactosidic and *cis*-glucosidic linkages[45].

Automation is also feasible via a HPLC system[48] (Figure 2C) or a combination of automated solution phase synthesis and use of fluorous tags[49].

At the moment, fragments obtained by automated glycan assembly (AGA) or other fast assembling procedures are prepared in a quantity sufficient for structural characterization through

methods such as Saturation Transfer Difference Nuclear Magnetic Resonance (STD-NMR), Surface Plasmon Resonance (SPR), or X-ray crystallography which individually or in combination allow selection of structures to be further investigated in animal models as vaccine candidates, but we are still far from the scale required for process development. Among the techniques to study interactions of carbohydrates with protective antibodies, glycan microarray enables fast screening of synthesized glycans against animal or human antibodies requiring minimal quantities of analytes[34]. The combined approach of AGA and glycoarray has led to identification of crucial epitopes from polysaccharide antigens and to the selection of synthetic vaccine candidates. For this reason, it has been shown as a valid and rapid alternative to surface polysaccharide isolation for many glycan antigens, including capsular polysaccharide from carbapenem resistant *K. pneumoniae*[50], *Clostridium difficile* PS-I[51], and *S. aureus* teichoic acids[52]. AGA has been also applied to *S. pneumoniae* vaccine development, in particular to the identification of optimal oligosaccharide epitopes from different serogroups of *S. pneumoniae* which are not included in the currently marketed anti-pneumococcal vaccines[53-55]. The synthetic approach has been recently shown able to provide antigens for the formulation of a pentavalent glycoconjugate vaccine comprising *S. pneumoniae* serogroups 2, 3, 5, 8 and 14. Such synthetic formulation elicited antibodies in rabbits with strong opsonophagocytic activity. Furthermore, coformulation of the anti-pneumococcal licensed vaccines Prevnar-13 and Synflorix with synthetic glycoconjugates from serotypes currently not covered by vaccination proved effective and led to improved 15- and 13-valent vaccines, respectively[56].

Modern synthetic techniques are, therefore, becoming powerful tools to accelerate identification of carbohydrate antigens without the need for polysaccharide purification from a biological matrix.

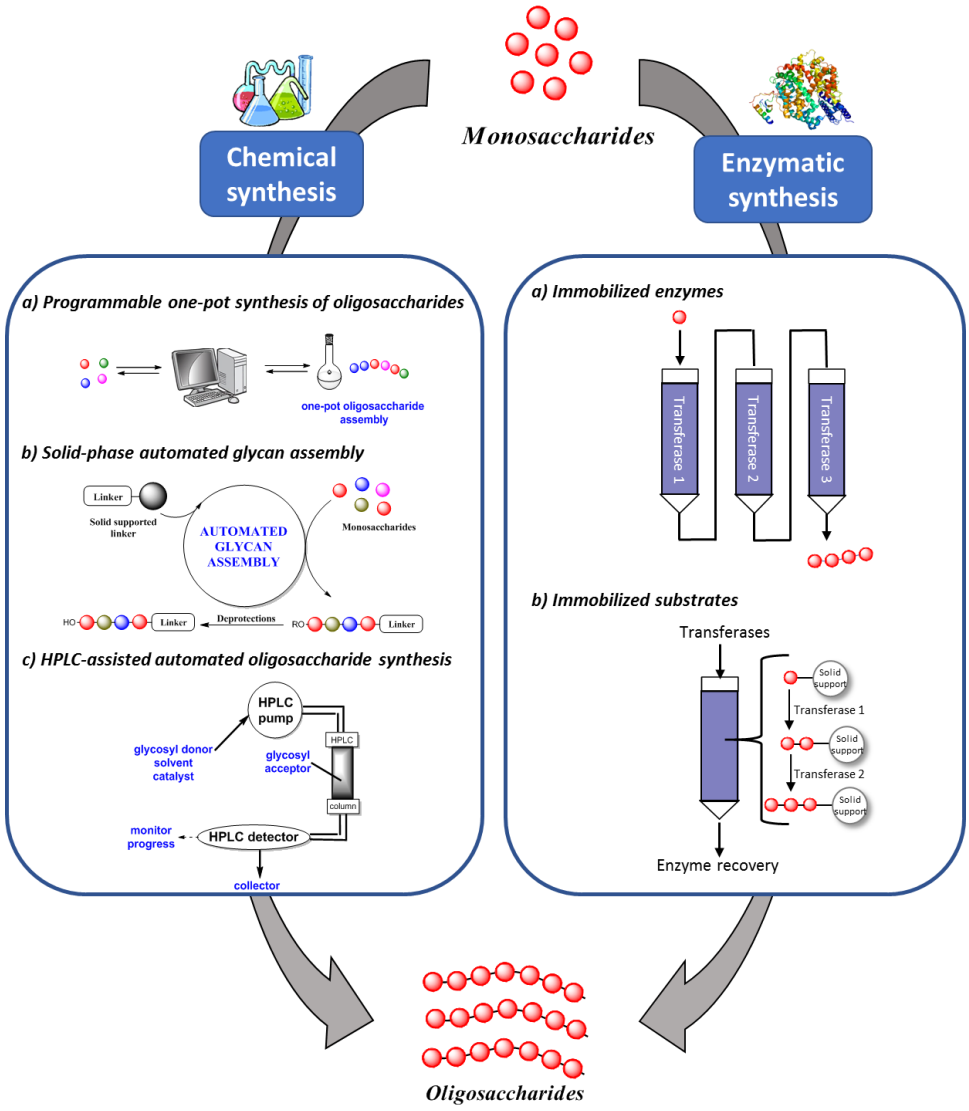

Figure 2. Automated chemo-enzymatic approaches to oligosaccharide antigens preparation

Enzyme-catalyzed oligosaccharide assembly is an interesting alternative to chemical synthesis of complex bacterial glycans since enzymatic glycosylation by glycosyltransferases allows stereo- and regioselectivity without the need to undergo tedious protecting group manipulation. Enzymatic reactions occur in aqueous solutions and only require to control a few reaction conditions such as temperature, system pH, and in certain cases metal catalysis. Also, there are no toxic byproducts produced in this glycosylation process, making the enzymatic synthesis of oligosaccharides an environmentally friendly approach[57]. These advantages make enzyme-based carbohydrate production attractive for industrialization.

A first automated carbohydrate synthesizer employing enzymatic synthesis has recently been reported[58] (Figure 2).

Recent progress made with the cloning and functional expression of capsule polymerases and pioneering studies that demonstrate the suitability of recombinant enzymes for the *in vitro* production of capsular polysaccharides (CPS) have opened new perspectives for the economic and safe production of conjugate vaccines[59, 60]. For example, Gerardy-Schahn's and coworkers have developed enzyme-based approaches focusing on the synthesis of meningococcal capsular polysaccharides. In 2016, they described a scalable *in vitro* synthesis of CPS serogroup X from a chemically pure precursor using recombinant *Neisseria meningitidis* X capsule polymerase[61].

As for serogroup X, a soluble version of the polymerase has been developed also for *in vitro* synthesis of *N. meningitidis* serogroup A polysaccharide[59].

A chemoenzymatic method using recombinant sialyl-transferases to develop well-defined glycoconjugates has been reported for the *N. meningitidis* serogroup C by Vann's group[62, 63].

The biosynthesis of the other two relevant *N. meningitidis* polysaccharides, serogroup W and Y, involves different catalytic strategies for sialic acid and hexose transfer, and enzymes responsible for these processes have been identified[64, 65]. Therefore, fully-chemoenzymatic manufacturing of polysaccharides needed for MenACWY vaccine is potentially feasible.

The combination of chemical and enzymatic methods can also be exploited for fast and efficient production of oligosaccharides as vaccine components. An example of this combined strategy is the use of a sialyltransferase isolated from rat liver to achieve a double α-sialylation at the position 3 of galactose in a near quantitative yield for the assembly of dimeric repeating units of the type III Group B *Streptococcus* polysaccharide[66]. Production of the pentasaccharide hapten for a vaccine candidate against endemic shigellosis has also been shown feasible by combined chemical and enzymatic steps[67].

Currently, two main approaches are under investigation for the automated enzyme-mediated bacterial oligosaccharide synthesis, using immobilizing enzymes or substrates (Figure 2) on an appropriate support or resin[68]. Chemical and enzymatic steps have been combined for the preparation of *N. meningitidis* serogroup X fragments[69, 70] by using immobilized enzyme. Starting from a fully synthetic trisaccharide acceptor and a truncated immobilized construct of *N. meningitidis* X capsular polymerase (CsxA), an on-column method was developed to provide rapidly conjugation-ready oligosaccharides with a size optimal for immunogenicity[70]. Enzyme-immobilization is a mature technology but it has the limitation that only immobilized enzymes can be removed at the end of the sugar elongation process, while the oligosaccharide product is still mixed with other reagents[57]. To overcome this, a different strategy has been recently developed where glycosyltransferase-catalyzed reactions are performed to generate oligosaccharides equipped with sulfonate tags for purification by a selective catch and release from adequate resins[58]. Although this method has been applied so far only to mammalian glycans, it has the potential to be extended to microbial oligosaccharides.

Automated chemical and enzymatic synthesis appear highly complementary and the combination of both will greatly expand the chemical space that can be rapidly accessed in the coming years. While in the absence of structural information, long oligosaccharides have to be employed to fully cover all of the relevant glycotopes, an understanding of the structural basis for the immune recognition of carbohydrate epitopes and the elucidation of minimal saccharide antigens responsible for an effective immune response is relevant to support the rational design of modern glycoconjugate vaccines with short oligosaccharides obtained by synthetic, chemoenzymatic, or bioengineering methods[71, 72]. An interesting concept that could simplify the use of synthetic carbohydrates is the identification of glycotopes expressed across multiple microorganisms. This is the case of Poly-N-acetyl glucosamine (PNAG), a polysaccharide found in a large number of bacterial, fungal and eukaryotic pathogens[73]. Natural antibodies to PNAG are poorly effective at mediating *in vitro* microbial killing or *in vivo* protection. However, synthetic deacetylated glucosamine 5-mer or 9-mer have been shown to elicit, after conjugation to carrier proteins, broad-based immunity (among others, *Mycobacterium tuberculosis*, *Burkholderia cenocepacia*, *A. baumannii*, *N. meningitidis* serogroup B, *Streptococcus pyogenes*)[74]. The safety profile and potential interactions with microbiota and off-target toxicity in humans, however, are aspects that still need to be clarified for this broadly active conjugate.
A synthetic lipoteichoic acid fragment from *Enterococci faecalis* and *faecium* has also been shown to induce functional and cross-reactive antibodies against *S. aureus*, suggesting that this class of molecules can induce broadly protective response across different Gram-positive bacteria[75].

Perspectives

Glycoconjugate vaccines have been proven cost-effective tools to prevent dramatic infectious diseases such as pneumonia and meningitis. Historically they have been developed to overcome limitations of plain polysaccharides and confer protection and boostable response in children. Lifecyle management of existing vaccines requires incessant monitoring of possible serotype replacement and mutable epidemiology which can demand increased vaccine coverage. In addition, the use of an increased number of glycoconjugate vaccines with a relatively small number of carrier proteins (primarily TT, DT and CRM_{197}) and the complexity of the immunization schedules have highlighted in certain cases reduced immunogenicity against the polysaccharide hapten due to preexisting immunity towards the protein (so called "carrier epitope suppression")[76]. Technologies enabling the use of alternative carriers in conjunction with simplified vaccine formulations are, therefore, requested for the future vaccines.
Several additional factors encourage the development of novel and faster methods for glycoconjugates design and production. A large part of the world composed is constituted by low and middle-income countries, where infectious diseases account for around half of all deaths with around 90% of this mortality being attributed to diarrheal and respiratory diseases, AIDS, tuberculosis and malaria[77]. Methodologies for fast and affordable vaccine production combined

with sustainable models for capacity building and technology transfer are necessary to render vaccination accessible to these populations. The success of the MenAfriVac vaccine and program for extension to a multivalent ACWXY conjugate is generating a model for development and implementation of vaccination in low income countries[78].

Also, the need of novel therapeutic approaches to tackle antimicrobial resistant pathogens has been clearly underscored by recent analyses from WHO and CDC[79, 80]. In addition, fungal infections are a concern for immunocompromised patients, such those with immune systems impaired by cancer chemotherapy, or hospitalized individuals[81]. Finally, increased life expectation in the developed countries and increased numbers of travelers are also expanding the request for vaccination of adult and elder population in the developed countries[82].

Among the emerging approaches for carbohydrate production, chemical and enzymatic strategies, and their automation, hold the potential to enable the pathogen-free production of carbohydrates and rationally selected glycoepitopes for vaccine design. These methodologies are now giving access to a large variety of complex glycans including those deriving from pathogen for which vaccines are not available (e.g. *S. flexneri* type 2a O-antigen, *K. pneumoniae* CPS, *S. aureus* PNAG). Advancement of mass spectrometry approaches for sugar fingerprint analysis coupled with automated chemical or enzymatic synthesis is expected to further progress the field in the near future[83]. Proof of feasibility for a multivalent pneumococcal conjugate vaccine based on synthetic oligosaccharides has been recently shown. Nevertheless, these methods at the present still require rather extensive case-by-case optimization of the synthetic strategy and large-scale production might constitute a challenge for multivalent vaccines. Therefore, effort is expected to focus in adapting these novel approaches for manufacturing.

As an emerging technology, combination of nanoparticle scaffolds with chemical and enzymatic methodologies for carbohydrate synthesis appears very appealing in the design of totally synthetic constructs. Whereas this approach is at a very early stage of development, it could represent the future frontier of pathogen contaminant-free, highly characterized, totally synthetic carbohydrate-based vaccines. Its advancement will depend on how quickly accessible the synthetic carbohydrate production will be, as discussed above, and how consistent the manufacturing for large scale production will be.

In parallel with these novel technologies to obtain glycoconjugate vaccines, advancements of techniques for deciphering surface polysaccharide structure[84] and carbohydrate-protein interactions[85], as well as progresses in the field of analytics and immunoassays, including novel tools and high-throughput methodologies for rapid analysis of sera specificity[86] and functional activity[87, 88], are contributing to make antigen identification and structure guided vaccine design faster.

Outline of the thesis

This Chapter has provided an overview of glycoconjugate vaccines, generally describing their mechanism of action compared to polysaccharide-based vaccines. Classical approaches to produce glycoconjugate vaccines were described, with a focus on chemical and enzymatic methods to provide fast access to synthetic oligosaccharide antigens.
In **Chapter 2** Group B streptococcus (GBS) epidemiology is outlined, underlining the correlation between different serotypes and clinical symptoms to highlight the need for a multivalent vaccine. The synthesis of GBS repeating units from different serogroups is also reported, showing feasibility of a GBS conjugate vaccine based on synthetic oligosaccharides. In **Chapter 3** a regioselective glycosylation approach to the disaccharide synthon GlcNAcβ(1→3)Galβ is introduced, with the aim to obtain branched oligosaccharides using fewer protecting groups and therefore reducing the number of steps to the final target compounds. In particular, the impact of the protecting groups on the glycosyl acceptor and donor as well as the leaving group on the glycosyl donor were evaluated and a combination of building blocks to achieve a fully regioselective glycosylation was identified. Disaccharide synthons bearing temporary protecting groups at C-3 and C-4 were synthesized to be used as intermediates for the synthesis of GBS type Ia and Ib related glycans. In **Chapter 4** the appropriate disaccharide synthon prepared through regioselective glycosylation was used as starting material for elongation to obtain a panel of GBS Ia related oligosaccharides, ranging from a linear tetrasaccharide up to a heptasaccharide to be used for epitope mapping of relevant glyco epitopes of the corresponding polysaccharide. Each fragment was prepared with an aminopropyl linker at the terminal sugar for future conjugation to carrier proteins and immunological studies. The regioselective reaction to the GlcNAcβ(1→3)Galβ disaccharide also has a central role in **Chapter 5**, where the first synthesis of GBS type Ib repeating units, represented by two frameshifts, a linear and a branched structure, is described. These structures differ from GBS type Ia glycans only for the connection of the side chain Neu5Acα(2→3)-Galβ to the glucosamine, which is a β(1→3) linkage instead of β(1→4) as in type Ia. To overcome low reactivity of the glucosamine 3-OH, the bulky, electron withdrawing NPhth protecting group on the glucosamine was replaced by the Troc group and a more flexible silylidene ring was inserted to block glucosamine 4- and 6-hydroxyls. The synthetic repeating units of GBS Ia and Ib were used to validate a structural model aiming to elucidate the conformation of the corresponding polysaccharides. In **Chapter 6**, structural studies on the panel of Ia and Ib related fragments synthesized are reported. In particular, the synthetic fragments were used as inhibitors in competitive Surface Plasmon Resonance experiments of the binding of PSIa and Ib to the specific mAbs. To investigate the interactions between the glycans and the mAb better, STD-NMR experiments were undertaken. Finally, the fragments were conjugated to carrier proteins for immunogenicity evaluation *in vivo*. **Chapter 7** presents the synthesis of a hexasaccharide from GBS type III CPS corresponding to the minimal epitope recognized by a protective mAb according to the published crystal structure of the derived Fab with a

semisynthetic DP2 fragment. This oligosaccharide was first used to map the relevant interactions between the sugar and the mAb, and then conjugated to CRM_{197} and tested in vivo. Immunological results showed that the hexasaccharide was able to elicit an IgG titer comparable to the CRM_{197}-PSIII conjugate, directly related to a high glycosylation degree. **Chapter 8** summarizes the findings of this thesis and outlines future plans and strategies.

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Chapter 2

Group B Streptococcus

Group B *Streptococcus* epidemiology overview

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is one of the major causes of sepsis, pneumonia and meningitis in neonatal and infants in the first three months after birth[1]. It is a Gram-positive beta-hemolytic coccus which appears in pairs or chains, that colonizes the urogenital and gastrointestinal tracts of more than 30% of the healthy population and, in particular, the vagina of 25-40% of healthy women[2, 3]. It is an etiologic agent also in adults, in particular elderly or immunocompromised persons, diabetics and patients affected by others chronic pathology of liver and kidney.

GBS clinical isolates are classified into ten serotypes, according to the chemical nature of capsular polysaccharides (PS): Ia, Ib, II, III, IV, V, VI, VII, VIII and IX[4-6]. However, around 8-14% of the clinical isolates in the Europe and USA are non-typeable strains because they cannot be distinguished on the basis of PS antigenicity. All GBS serotypes contain, in different combinations, these carbohydrates: galactose, glucose, rhamnose, *N*-acetylglucosammine and sialic acid (*N*-acetylneuraminic acid) (Figure 1).

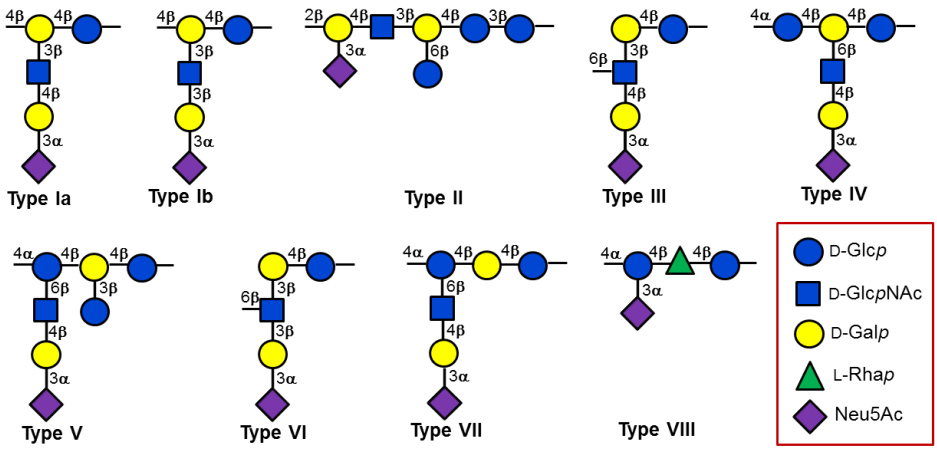


Figure 1. Chemical structure of GBS strains capsular polysaccharides

In most cases infection is transferred vertically from asymptomatic mothers and every year 8000-12000 infected babies and 2000 deaths are recorded in the US alone. Infection by contact of throat, ear, nostril, umbilicus and respiratory ways with amniotic liquid or infected vaginal fluids occurs in uterus or at delivery. After the aspiration/ingestion of bacteria, neonatal lungs become the starting focus of infection. From here it can rapidly arrive to blood flow, continue towards other tissues and organs like blood-brain barrier.

Neonatal disease can occur in two different forms: **Early-Onset Disease** (EOD) and **Late-Onset Disease** (LOD).

EOD occurs during the first seven days of life, with the vast majority of cases (approximately 90%) happening during the first 24 hours of life[7]. Neonates with EOD present respiratory disease, sepsis without focus and meningitis. Serotype Ia and III account together for more than 70% of EOD.

LOD occurs beyond seven days of life and can develop up to three months of age. In 50% of the cases it has a maternal origin, while in the other half of the cases infection is acquired nosocomially or from community sources. Neonates with LOD present sepsis, meningitis, urinary infection, osteoarthritis, respiratory disease and cellulitis. Over 20% of survivors of GBS meningitis have permanent sequelae, including hearing loss, mental retardation, cortical blindness and seizures[8]. LOD is mostly caused by serotype III, with an incidence until beyond 70% depending on the geographic area.

GSB can cause diseases also in non-pregnant adults, and studies performed on non-pregnant adults with GBS associated invasive disease revealed that GBS serotypes Ia, III and V accounted for more than two-third of cases. In particular, more than 25% of the subjects had invasive GBS disease caused by type V strains (Table 1)[9, 10].

Table 1. Disease spectrum and serotype distribution of Group B streptococcal infections according to the age of the patient[9]

	Common manifestation	**Risk factors**	**Case fatality rate**	**Serotype distribution**	
Early-onset diseases	Sepsis with unknown source (80-85%) Pneumoniae (10%) Meningitis (7%)	Maternal obstetric complications, maternal genital GBS colonization, GBS bacteriuria	5-10%	Ia (27.7%) Ib (7.3%) II (4.2%) III (46.2%) IV (1.5%) V (12.9%)	
Late-onset diseases	Primary bacteremia (65%) Meningitis (25-30%) Bone and joint infection (5%) Cellulitis and/or adenitis (4%)	Prematurity	2-6%	Ia (11.3%) Ib (5.4%) II (0.7%) III (76.0%) IV (0%) V (6.6%)	
Pregnant women	Genitourinary tract infection (50%) Chorioamnionitis (4%) Endometritis (8%) Primary bacteremia (31%) Pneumonia (2%) Puerperal sepsis (2%)	Genital GBS colonization	Not available	*Infection* Ia (32.7%) Ib (4.0%) II (14.9%) III (28.7%) IV (0%) V (19.8%)	*Colonization* Ia/Ib (36.0%) II (14.5%) III (24.0%) IV (1.2%) V (202%)
Non-pregnant adults	Primary bacteremia (24%) Skin and soft tissue infection (20%) Respiratory tract infection (12%)	Central nervous system infection, old age, diabetes mellitus, liver cirrhosis, stroke, cancer,	3.7% (up to 15% in old adults)	Ia (24.3%) Ib (12.2%) II (11.9%) III (16.5%) IV (0.3%) V (27.5%)	

	Urinary tract infection (10%) Bone and joint infection (5%) Endocarditis (4%)	neurogenic bladder, decubitus ulcer		

GBS prevention strategies

Intrapartum Antibiotic Prophylaxis (IAP) is the most widely used strategy for GBS prevention in developed countries and is applied after the identification of GBS carriers through a screening-based approach or by identification of clinical risk factors, such as prematurity (<37 weeks' gestation), prolonged rupture of membrane, intraamniotic infection, temperature (>38°C) or previous births of baby with GBS disease. The screening approach recommends that all pregnant women should be screened between 35 and 37 weeks gestation for GBS vaginal and rectal colonization. Both approaches recommend antibiotics administration during labor to women who were GBS positive at the screening time and/or presenting one of the risk factors[8, 11]. Although this approach led to decrease in EOD infections, no impact on LOD disease was observed. Furthermore, the IAP approach presents a number of drawbacks that prevent its further implementation: the screening method for early identification of GBS carriers is complex in terms of speed/accuracy of prenatal screening culture, women delivering preterm or showing adverse antibiotic events can't be subjected to antimicrobial prophylaxis and concerns about resistance to β-lactam antibiotic are raised[12].

For this reason, vaccination against GBS is an appealing alternative to IAP. Effective vaccines would stimulate the production of functionally active antibodies that could cross the placenta and provide protection against neonatal GBS infection.

GBS capsular polysaccharide represents a main virulence factor, which helps GBS to escape host defense mechanism by interfering with phagocytic clearance.

The first evidence of the protective nature of CPS-specific antibodies was in 1930s when Rebecca Lancefield *et al.* demonstrated that polyclonal antibodies from rabbit sera, able to recognize PS epitopes, conferred protection against GBS infection in animal models[13, 14]. During the last two decades, plain GBS polysaccharides have been extensively studied as vaccines in preclinical and clinical human studies. However, the first human clinical trial conducted in the 1980s showed that the purified native PS from serotype III was not sufficient to induce a robust IgG response in adults and induced an insignificant response in neonates. Subsequently, conjugation of PSs to immunogenic proteins, such as tetanus toxoid (TT)[15, 16], was shown to dramatically increase the immune response in children, eliciting the differentiation of memory cells associated to a long-term protection. Following these findings, PS-TT conjugate vaccines prepared with GBS type-specific CPS (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) were produced and tested pre-clinically in animal models. These studies showed the higher immunogenicity of CPS-TT compared to the uncoupled CPS. This success constituted the rationale to proceed with the clinical studies in humans.

Although the immunogenicity in humans of GBS type-specific CPS antigens was successfully increased through conjugation to TT, the immune responses were serotype-specific.
Further studies demonstrated that glycoconjugate vaccines constituted by serotypes Ia, Ib, II, III, and V PS linked to TT were safe, well-tolerated and highly immunogenic in human adults, but cross-protection between serotypes was still lacking[17]. Therefore, a multivalent vaccine was required to obtain a broad coverage of the vaccine against the prevalent circulating GBS serotypes. A tetravalent combination of PS–TT conjugates (serotypes Ia, Ib, II, and III) was successfully tested in a mouse model and further human trials were performed using the combination of two PS TT-conjugates (serotypes II and III). Results showed that the combination had the same immunogenicity and reactogenicity of each monovalent PS vaccine[17].
However, although recent epidemiological studies[18] suggest that a tetravalent combination of serotypes Ia, Ib, III and V would be sufficient to achieve a coverage against the majority of GBS strains circulating in Europe and North America, there are other geographical areas where such combination would not be effective due to a different serotypes distribution.
An additional obstacle to the licensure of vaccine against GBS is the difficulty of conducting efficacy clinical trials in human: large sample size would be required, but the use of intrapartum antibiotic prophylaxis (IAP) reduces the incidence of neonatal disease.
To overcome this difficulty, Lin and coworkers suggested a ways to estimate the maternal GBS-PSs antibody levels needed to give protection to neonates from EOD[19]. This work demonstrated that it is possible to define thresholds of anti-GBS PSIa specific IgG levels in the mothers which are predictive for the protection of newborns, since the probability of developing EOD declined with increasing maternal levels of anti-PSIa IgG. More recently, thresholds have also been set for the levels of maternal anti GBS-PSIII IgGs required to protect newborns from EOD caused by GBS serotype III[20]. These results suggest that complex clinical trials required for a GBS vaccine registration might be replaced with an *in vitro* correlate of protection based on the quantification of vaccine induced antibodies.

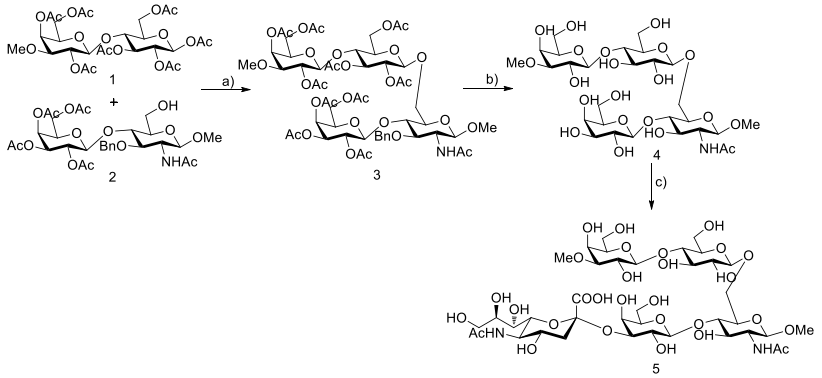
GBS glycans synthesis

GBS capsular polysaccharides represent promising targets for vaccine development. When long polysaccharides are used, correlation between chemical structure and immunogenicity is difficult to establish. Indeed, natural polysaccharides used for vaccine manufacturing are heterogeneous mixtures, and the impact of variables like length or substitution pattern cannot be easily determined[21]. Moreover, conjugation implies chemical manipulations of sugar residues, or the downstream terminal aldehyde, which are coupled to the protein. The understanding of the parameters impacting their immune-activity is better achieved by means of synthetic carbohydrates, because of their defined length and composition of glycoconjugates. Furthermore, synthetic glycans are coupled to the protein using a linker preinstalled at the downstream end of the oligosaccharides, allowing preservation of the integrity of sugar epitopes. Thus, synthetic

glycans are not only used to elucidate the biological function of CPSs but also to develop fully synthetic or semi-synthetic vaccines[22].

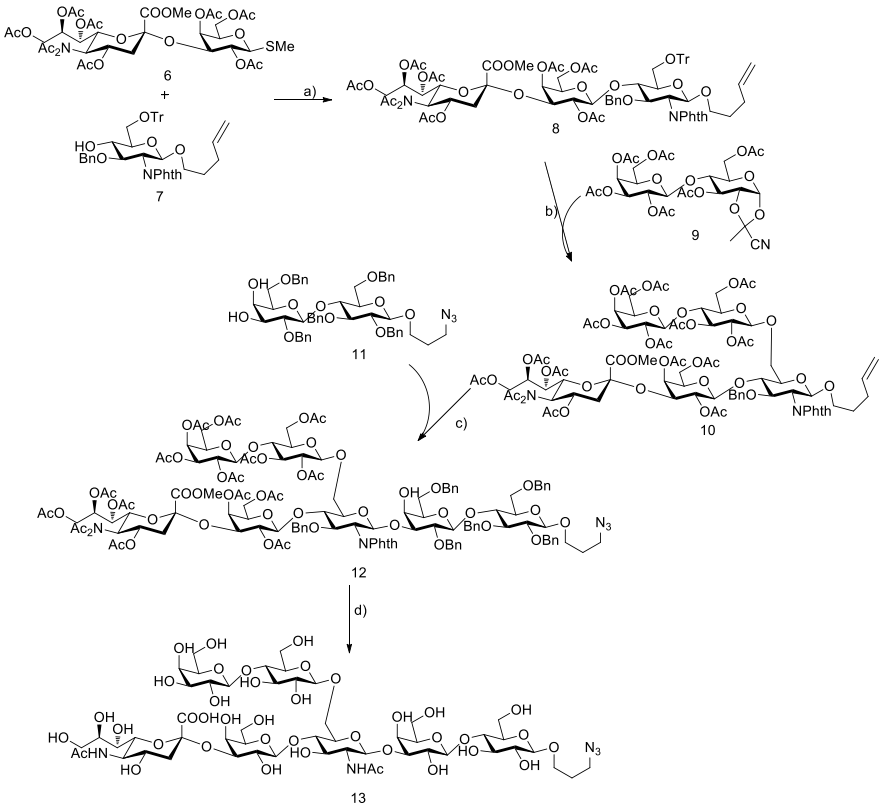
The availability of short defined oligosaccharides related to GBS PS repeating units is an important prerequisite to explore interactions between CPS and specific antibodies and to map the corresponding GBS PS epitopes. For this reason, different approaches have been proposed for the synthesis of GBS oligosaccharide fragments. Serotype III is by far the most studied GBS strain and approaches to produce short PSIII fragments have been based on either depolymerization or chemical synthesis.

Among the synthetic approaches, Poszgay et al. reported the chemical synthesis of desialylated tetrasaccharide fragment **4**[23], that was subsequently sialylated with a specific rat liver sialyltransferase to obtain the branched repeating unit **5** of GBS type III CPS (Scheme 1).



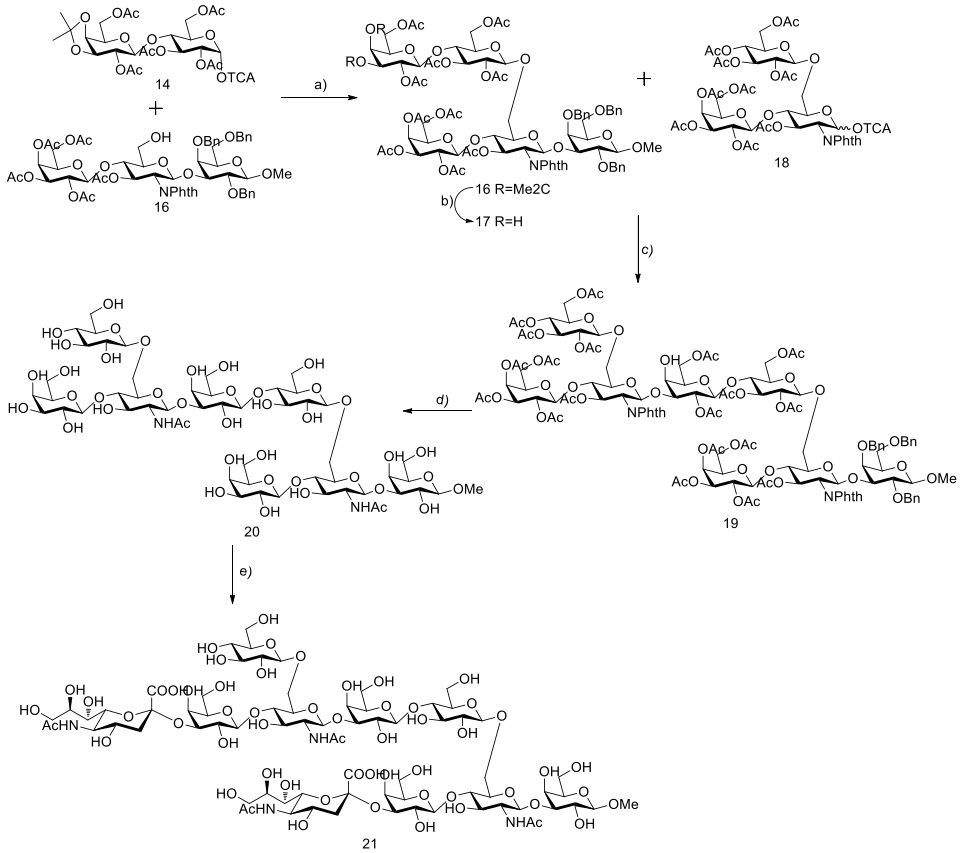
Scheme 1. Chemo-enzymatic synthesis of GBS type III repeating unit[23]. *Reagents and conditions*: a) TMSOTf, DCM, 24h, 25°C, 42.7%; b) NaOMe, MeOH, 24h, 25°C; H_2, Pd-C, EtOH, AcOH, 48h, 25°C, 77.9%; c) CMP-Neu5Ac, Galβ1,3(4)GlcNAc-α(2,3)-sialyltransferase, pH 6.5, 24h, 37°C, 33.8%.

Fully chemical assembly of a heptasaccharide related to PSIII was described by Demchenko et al.[24] that used a highly convergent strategy based on incorporation of the protected α-Neu5Ac-(2→3)-Galp methylthioglycoside **6** previously reported[25] to the GlcpNAc acceptor **7**. Further glycosylation of the 6-OH position of this residue by lactose donor **9** gave the pentenyl pentasaccharide **10**, which was in turn used as donor for glycosylation of the lactose acceptor **11** affording the target protected fragment **12** (Scheme 2). The related desialylated hexasaccharide fragment was also described by the same authors[26].



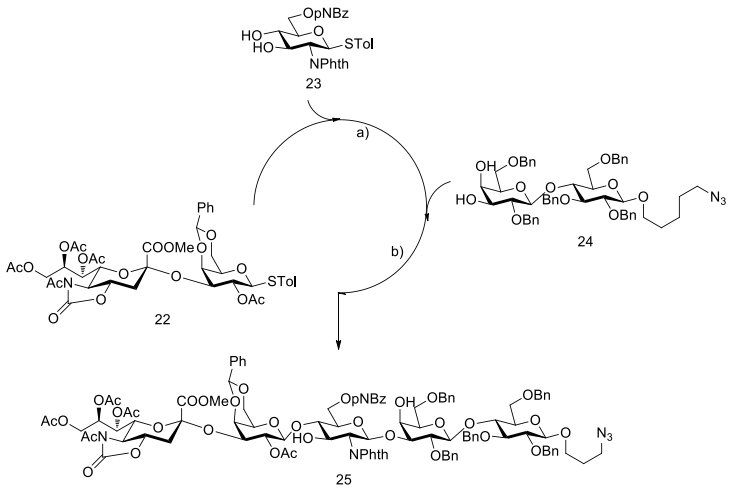
Scheme 2. Synthesis of GBS III heptasaccharide **13**. *Reagents and conditions*: a) MeOTf, MS 3Å, DCM, 96%; b) $TrCO_4$, DCM, 82%; c) NIS/TMSOTf, MS 3Å, DCM, 62%; d) LiI, Pyridine; $(NH_2CH_2)_2$, *n*-BuOH; Ac_2O/MeOH; Pd-C, EtOH/H_2O; 1N aq NaOH, 31%.

A chemo-enzymatic approach was also used by Zou et al. to assemble a decasaccharide, representing two repeating units, of GBS III[27]. The octasaccharide precursor **20** was obtained from the reaction between the acetylated trisaccharide donor **18** and the pentasaccharide acceptor **17** and subsequently enzymatically sialylated by reaction with α-(2→3)-sialyltransferase and CMP-Neu5Ac as donor (Scheme 3). Using the same octasaccharide, a *N*-propionyl substituted sialic acid analog of the GBSIII dimer was also prepared.

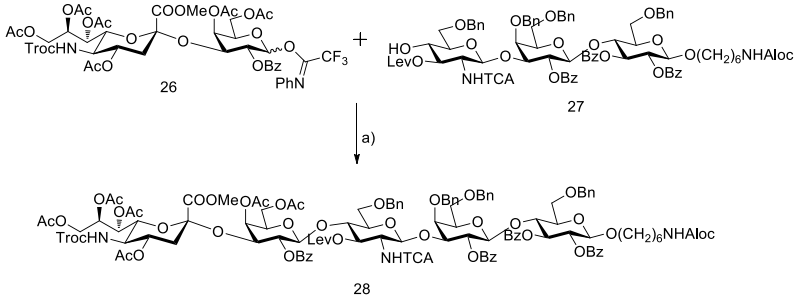


Scheme 3. Chemo-enzymatic route to GBS III decasaccharide. *Reagents and conditions*: a) TMSOTf, DCM, -45°C, 2h, 49%; b) 1:10 TFA/DCM, 0°, 2h, 89%; c) TMSOTf, DCM, -45°C, 2h, 69%; d) $NH_2NH_2 \cdot H_2O$ in 95% EtOH, reflux, 16h; Ac_2O, MeOH/H_2O, overnight, 35%; e) CMP-Neu5Ac, α(2→3)-sialyltransferase, 80%.

A similar enzymatic approach has been also utilized to transform oligosaccharides from S. pneumoniae type 14 to PSIII counterparts[28]. Syntheses of structures related to the linear repeating unit of GBS III, a glycan that is commonly found in nature[29], have also been reported. For instance, Hsu et al. used a 5-*N*,4-*O*-oxazolidinone α-Neu5Ac-(2→3)-Gal*p* tolylthioglycoside donor to achieve the one-pot assembly of oligosaccharide **25** (Scheme 4)[30], whereas the *N*-Trichloroethoxycarbonyl protected α-Neu5Ac-(2→3)-Galp trifluoroacetimidate donor **26** was utilized by Hanashima et al. for glycosylation of the trisaccharide acceptor **27**[31] (Scheme 5).

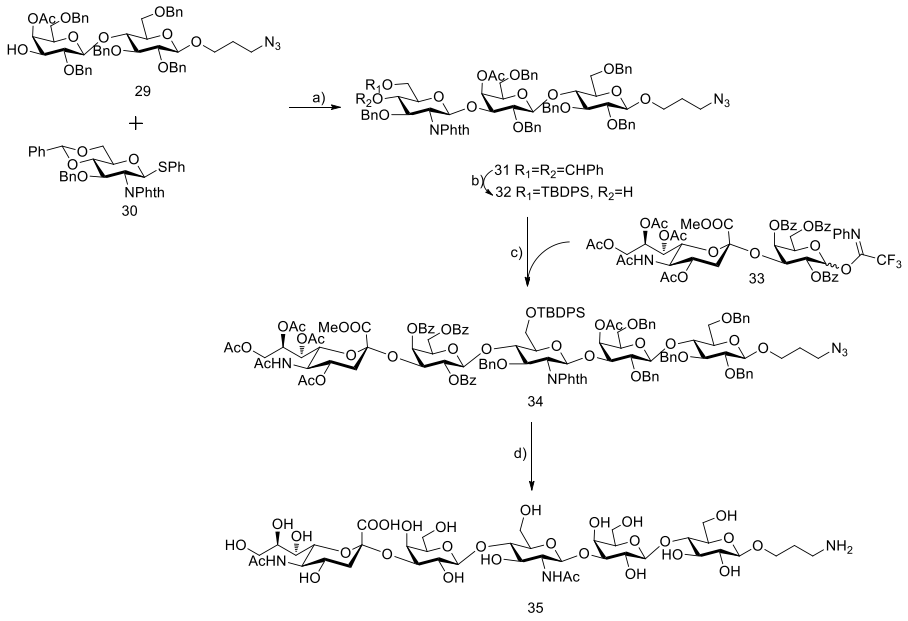


Scheme 4. Reactivity-based one-pot glycosylation of pentasaccharide **25**. *Reagents and conditions*: a) NIS, TfOH, MS 4Å, DCM, -78°C; b) NIS, TfOH, -20°C to rt, 48% over two steps performed one-pot.



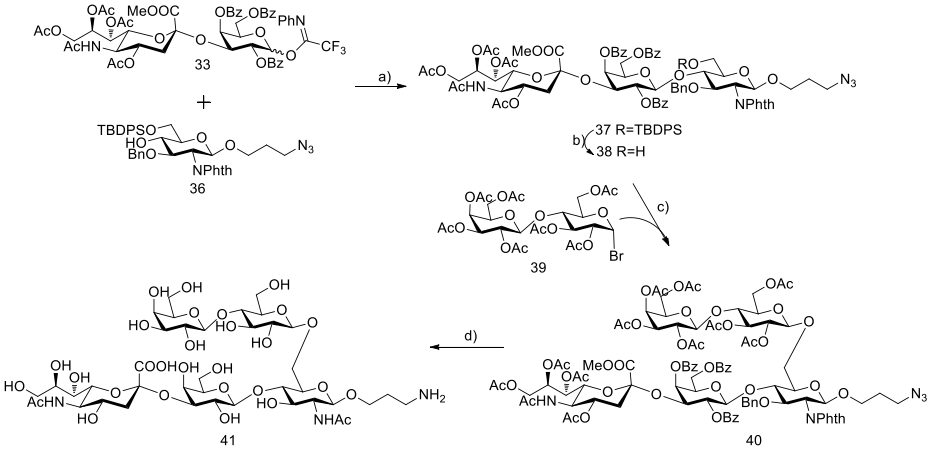
Scheme 5. Hanashima's synthesis of the linear repeating unit of GBS III. *Reagents and conditions*: a) TMSOTf, DCM, 89%.

Finally, in 2017 Cattaneo et al. described the synthesis of the three different frameshifts (linear pentasaccharide **35**, branched pentasaccharide **41** and "Y-shape" pentasaccharide **47**) of GBS PSIII repeating unit bearing a linker at the end-terminal sugar for conjugation to carrier proteins[32]. All pentasaccharide fragments were synthesized through a [3+2] convergent approach employing the sialogalactoside imidate donor **33**, obtained by simple manipulation of a commercially available building block. This disaccharide was used to glycosylate the linear trisaccharide acceptor **32** to obtain the protected linear repeating unit **34**, which after standard deprotection reactions afforded the target structure **35** (Scheme 6).



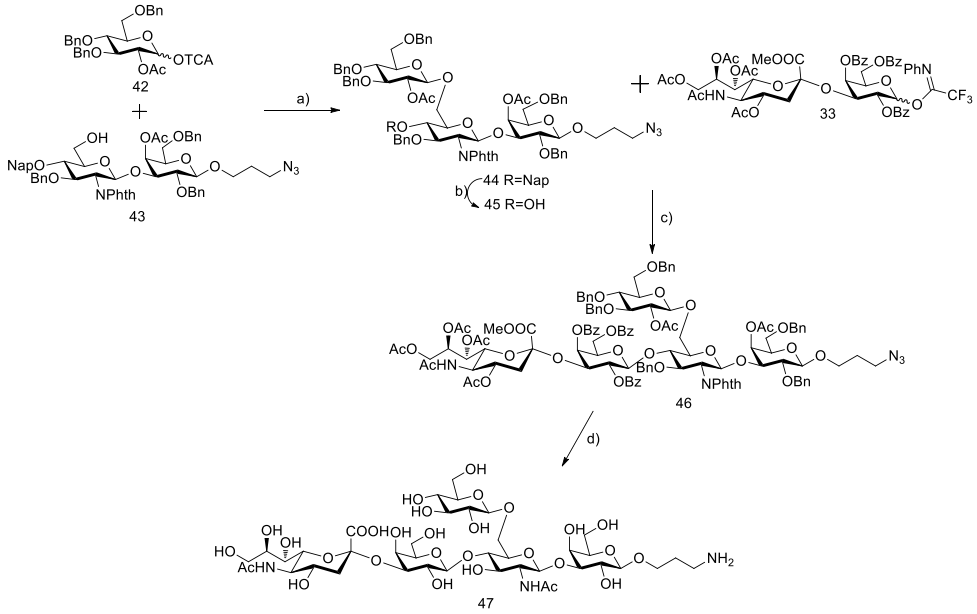
Scheme 6. Synthesis of the GBS III linear repeating unit. *Reagents and conditions:* a) NIS, TfOH, -20°C, 72%; b) 4:1 AcOH/H_2O, 70°C; TBDPS-Cl, DMAP, Py, 60°C, 80% over two steps; c) TMSOTf, DCM, 55%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 33%.

The branched repeating unit **41** was again prepared with a [3+2] convergent route, where the sialylated trisaccharide acceptor **38** was glycosylated with peracetylated lactose bromide **39** to obtain the protected pentasaccharide **40**, precursor of the target oligosaccharide **41** (Scheme 7).



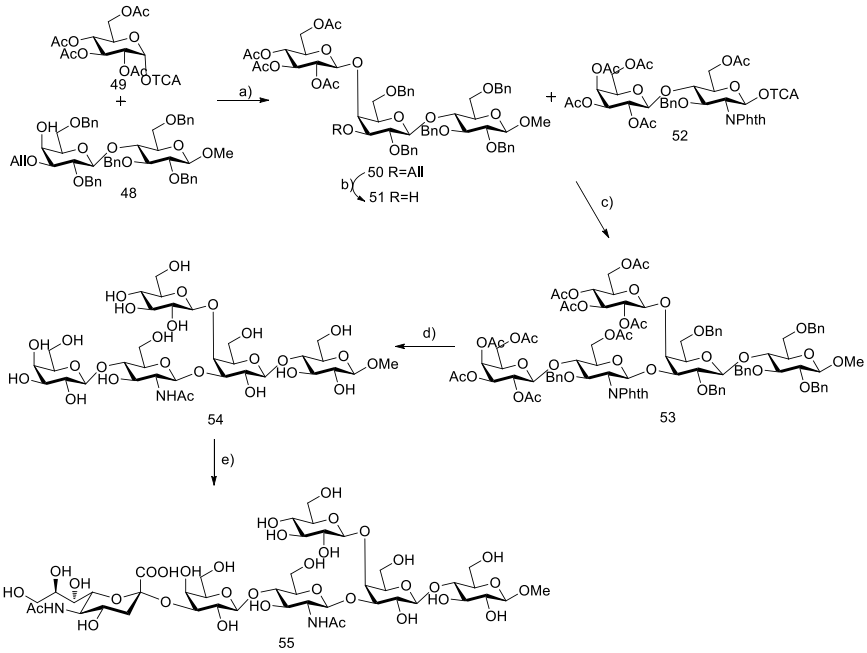
Scheme 7. Synthesis of the GBS III branched repeating unit. *Reagents and conditions*: a) TMSOTf, DCM, 70%; b) HF·Py, 4:1 THF/Py, 0°C to rt, 78%; c) AgOTf, DCM, 68%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 42%.

Finally, synthesis of the Y-shape repeating unit **47** was accomplished starting from the trisaccharide acceptor **45**, obtained after Nap removal from compound **44**, which in turn derived from the glycosylation of disaccharide **43** with the glucose imidate donor **42**. Trisaccharide **45** was then glycosylated with the known disaccharide donor **33**, affording the pentasaccharide **46**, tha was deprotected to give target **47** (Scheme 8).



Scheme 8. Synthesis of the GBS III Y-shape repeating unit. *Reagents and conditions*: a) TMSOTf, DCM, 72%; b) DDQ, 4:1 DCM/MeOH, 85%; c) TMSOTf, DCM, 65%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 55%.

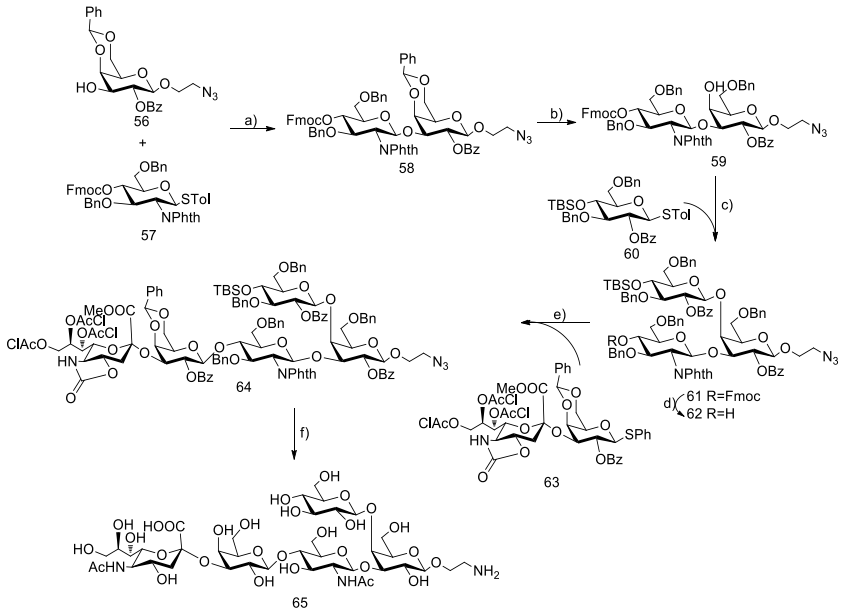
In recent years, syntheses of glycans from other GBS serotypes have been reported, with a particular attention to serotype Ia. A chemo-enzymatic synthesis was described by Zou and Jennings to deliver a GBS Ia related hexasaccharide starting from a synthetic pentasaccharide, which was sialylated with a recombinant α-(2→3)-sialyltransferase using CMP-Neu5Ac as donor (Scheme 9)[33].



Scheme 9. *Reagents and conditions*: a) TMSOTf, DCM, -45°C, 75%; b) $PdCl_2$, MeOH, 71%; c) TMSOTf, DCM, -45°C, 41%; d) H_2NNH_2, 95% EtOH, reflux, 16h; Ac_2O/ Py, overnight; NaOMe, MeOH, 2h; H_2, Pd-C, 79%; e) CMP-Neu5Ac, α(2→3)sialyltransferase, 59%.

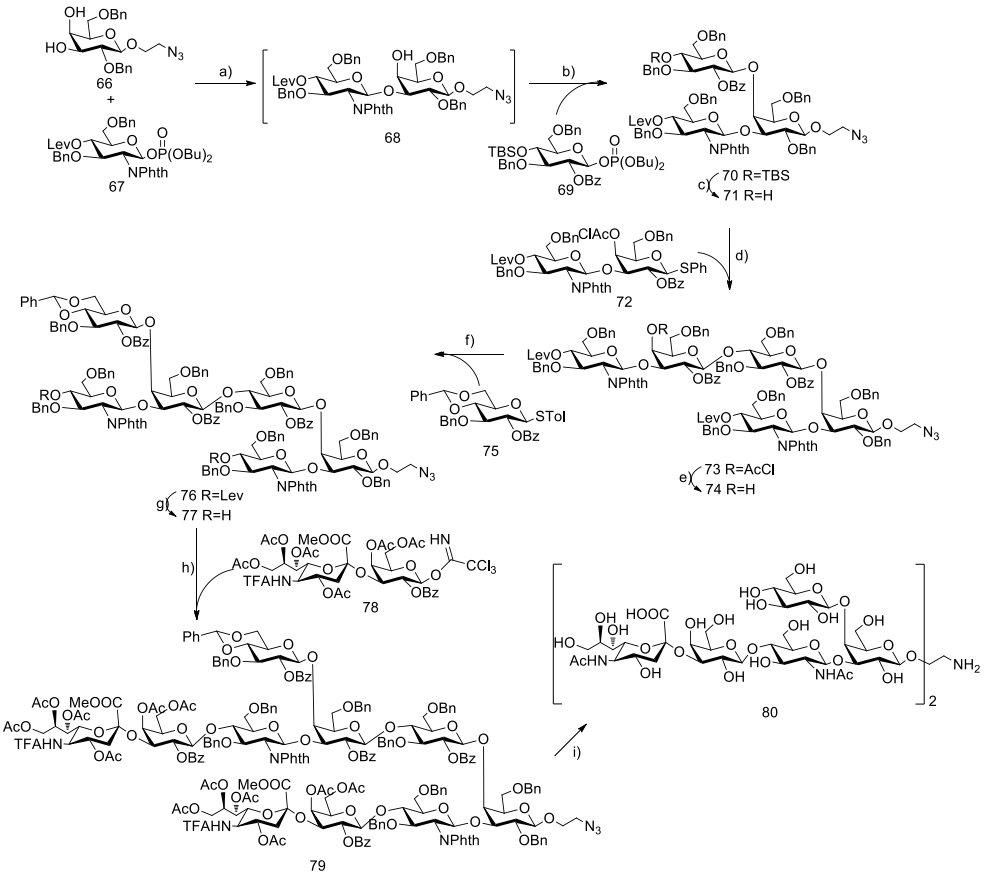
In 2015 the total synthesis of pentasaccharide **65** corresponding to the branched repeating unit of GBS Ia has been accomplished by Guo and coworkers, using a convergent [2+3] route between a sialo-galactose thioglycoside donor and a branched trisaccharide acceptor (Scheme 10)[34].

An investigation on the reactivity of the galactose acceptor in the glycosylation reaction showed that the presence of a sugar at the 4-*O*-position had a big impact on the reactivity of the 3-hydroxy group, since it made the 3-*O* position inaccessible for further glycosylation. Glycosylating the 3-*O*-position had a smaller impact on the 4-*O*-position; for these reasons the proper sequence for installation around galactose 3-/4-OH was to glycosylate the 3-*O*-position prior to 4-*O*-glycosylation. To this purpose, galactose **56** bearing a benzylidene protective group to block the C4 and C6 positions was used in the first glycosylation to obtain the fully protected Glc*N*Ac-β-(1→4)-Gal disaccharide **58**. At this point, the regioselective opening of the benzylidene ring afforded the disaccharide acceptor **59** with the 4-hydroxy group available for the second glycosylation to obtain the branched trisaccharide **61**. Deprotection of the Fmoc protecting group yielded trisaccharide **62**, which was glycosylated with the sialogalactoside donor **63** to afford pentasaccharide **64**. Full deprotection using a six steps procedure gave the target compound **65**, corresponding to the repeating unit of GBS serotype Ia CPS.



Scheme 10. Synthetic route to GBS serotype Ia repeating unit. *Reagents and conditions*: a) NIS, AgOTf, MS 4Å, -30°C to -25°C, DCM, 75%; b) $NaBH_3CN$, HCl, MS 4Å, DCM, 0°C to rt, 95%; c) NIS, AgOTf, MS 4Å, -40°C to -10°C, DCM, 72%; d) Et_3N/DCM 1:1, rt, 100%; e) NIS, AgOTf, MS 4Å, -30°C to -25°C, DCM, 55%; f) TEA-3HF, THF, rt, 48h; LiOH, MeOH/H_2O (1:1), NH_2NH_2-H_2O, reflux, 6d; Ac_2O/Py, rt, 12h; NaOMe/MeOH, rt, 12h; H_2, Pd-C, MeOH, 24h, 67%.

Recently, this approach was extended to the synthesis of GBS Ia decasaccharide **80** corresponding to two repeating units, using a highly convergent strategy (Scheme 11). First, the key intermediate trisaccharide **70** was constructed via a one-pot iterative glycosylation. This trisaccharide was elongated to provide hexasaccharide **77** to which two side chains were attached by dual glycosylation with the sialogalactoside donor **78**[35].



Scheme 11. Synthesis of GBS type Ia dimer. *Reagents and conditions*: a) TMSOTf, DCM, -78°C to -40°C; b) TMSOTf, DCM, -78°C to -40°C, 88%; c) $NEt_3 \cdot HF$, DCM, rt, 12h, 95%; d) NIS, TfOH, MS 4Å, DCM, -40°C, 1h, 85%; e) Et_3N, MeOH, rt, 10 min, 88%; f) NIS, TfOH, MS 4Å, DCM, -40°C, 1h, 92%; g) NH_2NH_2, DCM/MeOH, rt, 10 min, 89%; h) TMSOTf, MS 4Å, DCM, 0°C, 1h, 55%; i) LiOH, MeOH, H_2O, 12h; NH_2NH_2, EtOH, reflux, 24h; Ac_2O, Py, 12h; NaOMe, MeOH, 6h; H_2, Pd-C, 24h, 56%.

The synthesis of the repeating unit of other GBS serotypes which are not the topic of this thesis have also been described. It is worth to mention that Gao et al. described the first synthesis of GBS type V repeating unit, consisting of an heptasaccharide fragment, which was prepared by a preactivation-based one-pot [2+1+4] glycosylation strategy which not only resulted in the reduction of the number of steps to the final oligosaccharide, but also in avoiding many manipulations to the glycosyl donor[36]. In 2017, the same group described the synthesis of GBS type II branched heptasaccharide corresponding to a frameshift of the repeating unit, which was achieved by a convergent [4+2+1] glycosylation strategy[37]. Finally, recently Guo and coworkers assembled both a hexasaccharide repeating unit of GBS type VII, and the corresponding dimer. The synthesis was achieved by means of a preactivation-based iterative one-pot glycosylation protocol, which was made possible by optimization of the challenging stereoselective α-glucosylation reaction[38].

These synthetic efforts have paved the way towards preparation of more complex GBS oligosaccharides from different serotypes. These structurally defined oligosaccharides can be used either to map the relevant epitopes expressed on the capsular polysaccharides or as potential antigens in GBS glycoconjugate vaccines.

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Chapter 3

Regioselective Strategies to GlcNAcβ1-3Gal Disaccharide Synthons

Novel intermediates for preparation of Group B Streptococcus polysaccharide antigens, Adamo, R.; Del Bino, L. *PCT Int. Appl.*, **2019**, Patent n° WO 2019012476 A1 20190117

Introduction

Despite the enormous possible variation in carbohydrate structures, some motifs are recurrently expressed both by prokaryotic and eukaryotic cell. One example is the disaccharide GlcNAc-β-(1→3)Galβ, which is present in several bacterial carbohydrates, including the Group B *Streptococcus* (GBS) type Ia, Ib and III capsular polysaccharides, *Streptococcus pneumoniae* type 14 (Pn14), and *Neisseria meningitidis* lipooligosaccharide (Figure 1). This motif is also a component of mammalian glycans, such as the milk oligosaccharide lacto-*N*-tetraose.

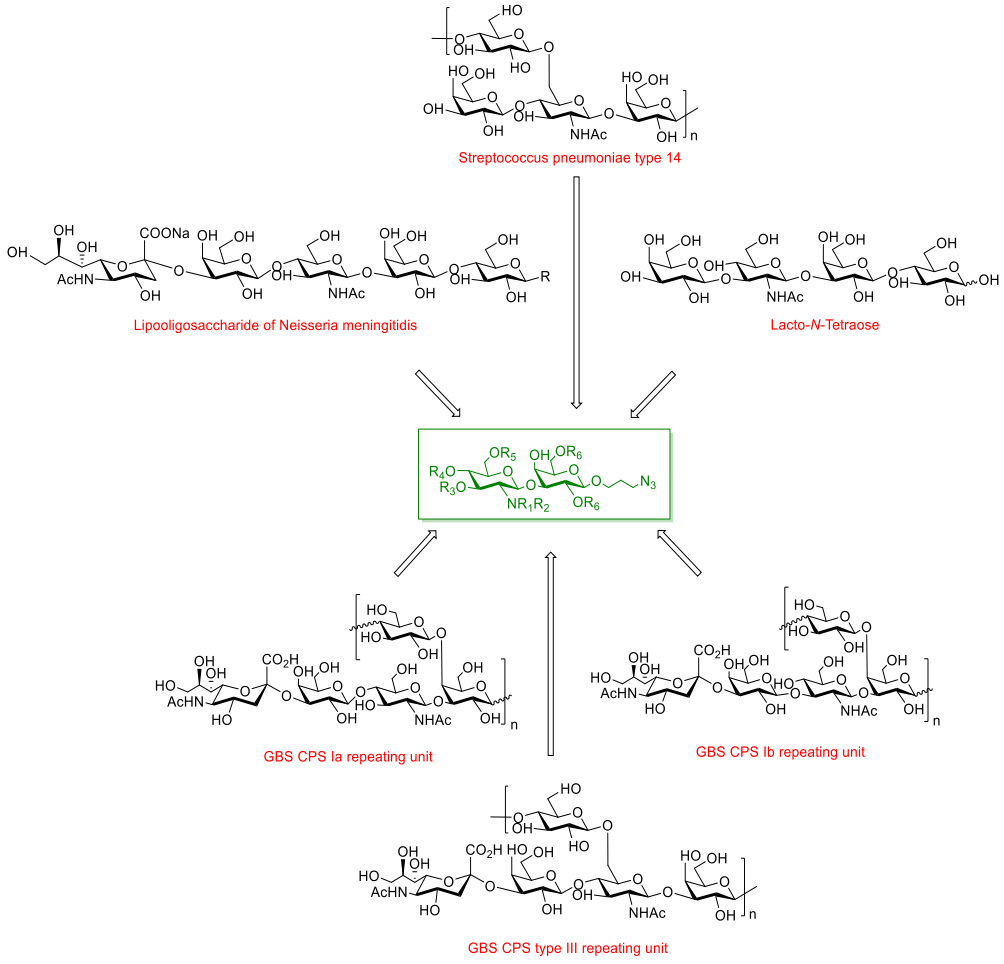


Figure 1. Example of bacterial and mammalian structures containing the GlcNAc-β-(1→3)Galβ disaccharide moiety

Interest towards the synthesis of CPS Ia and Ib oligosaccharide fragments is based on the willingness to investigate the structural features regulating the interactions with serotype specific mAbs and start delineating potential oligosaccharide epitopes. While approaching the synthesis,

the formation of the disaccharide GlcNAcβ1-3Gal motif was envisaged as a key step to enable convergent syntheses of a variety of structures depicted in Figure 1. Typically, installation of the GlcNAcβ1-3Gal disaccharide within more complex glycans has been achieved with the 4-hydroxyl group of the Gal acceptor either protected or already engaged in a glycosidic linkage. For instance, Craft et al.[1] recently observed that a 4-*O*-acetyl group was needed in the Gal acceptor to achieve glycosylation at position 3 with an *N*-Trichloroethoxycarbonyl protected glucosamine donor. This finding was in line with previous reports[2, 3] where the position 3 of a 4-*O*-acetyl Gal acceptor was glycosylated with a *N*-phthalimido protected glucosamine thioglycoside donor during the synthesis of GBS PSIII fragments. Demchenko et al[4] synthesized a heptasaccharide fragment of GBS PSIII via a [5+2] block coupling in which 3-OH of a lactose acceptor was glycosylated regioselectively in the presence of a 4-OH. The regioselective glycosylation of the 3-OH of Gal in the presence of 4-OH has also been described in the synthesis of fragments from *Streptococcus pneumoniae* type 14, although by the use of a 3,4,6-tri-*O*-acetylated GlcNAc donor further chemical elongation of this residue was not foreseen. A similar approach was used for the preparation of a pentasaccharide from *N. meningitidis* LOS, where the sialic acid was inserted by enzymatic reaction with the deprotected Pn14-like fragment[5].

Finally, in the recently described preparation of a CPS Ia repeating unit[6], a 4,6-*O*-benzylidene protected Gal acceptor was used for glycosylation with a glucosamine trichloroacetimidate donor, subsequent regioselective ring opening preceded the glycosylation of position 4, thereby resulting in the construction of the trisaccharide GlcNAcβ1-3[Glcβ1-4)]Gal.

There is a growing demand of expeditious procedures for the construction of complex glycans, and regio- and stereoselective reactions distinguishing among diverse deprotected hydroxyls are highly desirable to simplify the oligosaccharide assembly[7-9].

It was therefore reasoned that regioselective glycosylation of Gal 3-OH would be key for accelerating the synthesis of the GlcNAcβ1-3Gal disaccharide and rendering the 4-OH available for further glycosylation without the need of tedious protection/deprotection sequences[10].

In this chapter, tactics to achieve regioselective syntheses of protected GlcNAcβ1-3Gal building blocks are described. These key synthons can be used then in convergent routes towards a series of fragments from CPS Ia and Ib capsular polysaccharides with a built-in aminopropyl linker amenable for future conjugation to carrier proteins.

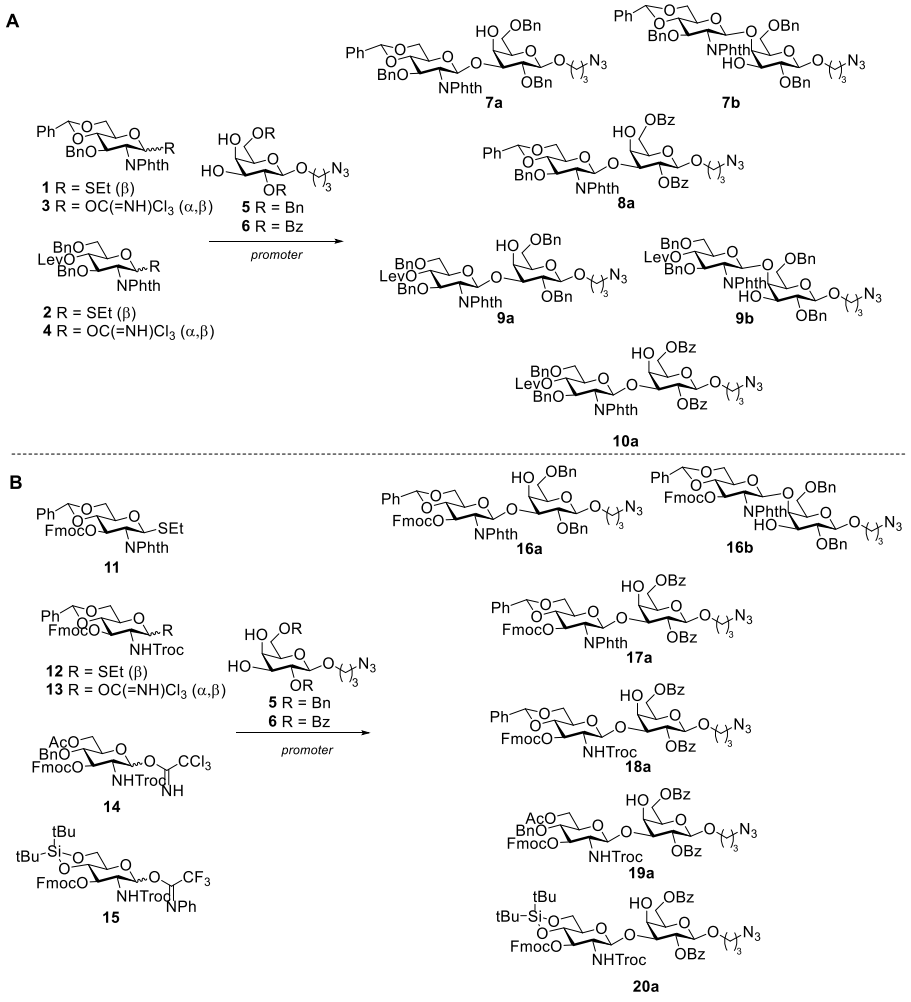
Results and Discussion

Protecting groups play a key role in oligosaccharides assembly[11-13]. They are employed to mask specific hydroxyl and amino groups to differentiate their reactivity and allow for selective modification, but they also have a large impact on the regio- and stereoselective control of the glycosylation. For example, the nature of the protective group installed at the C-2 position of the donor has a major impact on the stereoselectivity of glycosylation. When a participating protective group is installed at the C-2 position of a glycosyl donor, the glycosylation proceeds through an

acyloxonium intermediate, by which the nucleophilic attack takes place preferentially from the opposite face of the sugar ring, leading to the stereoselective formation of the 1,2-*trans* glycosidic linkage[14]. In additon, the reactivity of glycosyl donors and acceptors can be tuned by the employment of electron-withdrawing (disarming) or electron-donating (arming) protecting groups, such as benzoyl or benzyl groups, respectively[12, 15].

To achieve a regioselective synthesis of the key target GlcNAcβ1-3Gal disaccharides, the effect of arming benzyl and disarming benzoyl groups[15-17] at position 2 and 6 of the Gal acceptors on the reactivity of the 3- and 4-OH, was investigated, in combination with various protecting and leaving groups in the GlcN donors. Accordingly, a series of glucosamine thioglycoside and trichloroacetimidate donors in which the amine was protected by either the phthalimido (Phth) or trichloroethyl carbamate (Troc) participating groups was synthesized. The levulinoyl (Lev) and fluorenylmethyloxycarbonyl (Fmoc) were selected for temporary protection of either position 3 or 4. Alternatively, a 4,6-*O*-benzylidene was used to lock the 4 and 6 hydroxyls in GlcN to be subjected to regioselective ring opening delivering the 4-OH at a later stage of the synthesis. The glucosamine and galactose building blocks necessary for evaluating the glycosylation conditions were all prepared from commercially available starting materials and all intermediates were fully characterized when not already described in the literature (See Experimental section for details).

The prepared donors and acceptors were coupled under several glycosylation conditions to optimize the synthesis of the GlcNAcβ1-3Gal building block. In Table 1 and Scheme 1 the conditions for the preparation of a GlcNAcβ1-3Gal synthon with a temporary group at its C4'-OH, to allow the ensuing assembly of GBS CPSIa fragments are described.



Scheme 1. A) Preparation of disaccharide intermediates **7-10** for the synthesis of the GBS CPS Ia repeating unit. Promoters and conditions are described in Table 1. B) Preparation of disaccharide intermediates **16-20** for the synthesis of the GBS PSIb repeating unit. Promoters and conditions are described in Table 2.

When the ethylthioglycoside **1** was coupled with acceptors **5** and **6** using NIS/TfOH as promoters at -30°C, no product formation was observed (Entry 1 and 6, Table 1) due to donor decomposition. In the presence of the milder Lewis acid AgOTf at -30°C, NIS promoted condensation of **1** or **2** with **5** afforded along with the desired product **7a** and **8a**, respectively, the 4-*O*-glycosylated regioisomers (Entry 2-3, Table 1). The formation of the β-(1→4) linkage in disaccharide **8b** was confirmed by acetylation and NMR analysis of the acetylated product. In the ^{1}H NMR spectrum a shift from 3.32 to 4.69 ppm of the H-3 signal of Gal, appearing as a doublet of doublets with $J_{2,3}$ = 10.3 Hz and $J_{3,4}$ = 2.5 Hz was observed, confirming occurrence of glycosylation at position 4.

Table 1. Reactions of GlcN donors **1-4** with Gal acceptors **5-6**

Entry	Donor	Acceptor	Product	Yield
1	**1**	**5**	NIS/TfOH, - 30 °C	nd[a]
2	**1**	**5**	NIS/Ag(OTf), 30°C	**7a** (43%), **7b** (26%)
3	**2**	**5**	NIS/Ag(OTf), - 30°C	**8a** (40%), **8b** (28%)
4	**3**	**5**	TMSOTf, - 10°C	**7a** (31%)
5	**4**	**5**	TMSOTf, -10°C	**8a** (45%)
6	**1**	**6**	NIS/TfOH, - 30°C	nd[a]
7	**1**	**6**	NIS/Ag(OTf), - 30°C	**9a** (53%)
8	**2**	**6**	NIS/Ag(OTf), - 30°C	**10a** (65%)
9	**4**	**6**	TMSOTf, -10°C	**10a** (33%)
10	**3**	**6**	TMSOTf, - 10°C	**9a** (77%)

a. DCM was the solvent in all tested conditions. b. nd = not determined, product could not be detected.

Reaction of the 4,6-*O*-benzylidene glucosamine trichloroacetimidate **3** with **5** in presence of TMSOTf at -10°C (Entry 4, Table 1) provided the desired product **7a** in 31% yield, due to concomitant conversion of the donor into the anomeric acetamide byproduct. On the other hand, the imidate **4** and acceptor **5** exclusively gave **8a** with a higher yield (45%, Entry 5, Table 1), showing that the combination of 4,6-*O*-benzylidene protection and trichloroacetoimidoyl leaving group resulted in improved regioselectivity, but still moderate yield.

When the di-*O*-benzoyl acceptor **6** was exploited, reaction with both donor **1** and **2** under NIS/AgOTf activation regioselectively afforded compound **9a** (53%) and **10a** (65%), respectively (Entry 7-8, Table 1). The imidate **4** (Entry 9, Table 1) also solely gave compound **10a**, but in a lower yield (33%). Finally, combination of the 4,6-*O*-benzylidene **3** (Entry 10, Table 1) and acceptor **6** enabled to attain **9a** in satisfactory 77% yield.

To summarize, these findings indicated that 2,6-di-*O*-Bz Gal acceptor (**6**) generally lead to higher regioselectivity and yields compared to the 2,6-di-*O*-benzyl derivative (**5**). Trichloroacetimidate donors **3** and **4** showed higher regioselectivity with both the 2,6-di-*O*-Bz and 2,6-di-*O*-Bn Gal acceptors, but with variable yields. The most efficient routes to GlcNAc-β-(1→3)Gal were achieved by combination of the 2,6-di-*O*-Bz acceptor **6** with either 4-*O*-levulinoyl ethylthiol **2** under NIS/AgOTf mediated activation or the 4,6-*O*-benzylidene GlcN imidate **3** in presence of TMSOTf, which led to disaccharides **10a** and **9a**, respectively. Furthermore, the 3,6-di-*O*-benzyl ether **2** with NIS/AgOTf activation performed better than the trichloroacetimidate counterpart **4**.

On the basis of these results, the same strategy to attain regioselectivity was transferred to glucosamine donors with an orthogonal protection at C3'-OH, in order to obtain a disaccharide synthon, following the preparation of the repeating unit of the GBS PS Ib. Fmoc was chosen as temporary protecting group, and the corresponding glucosamine donors protected as *N*-phthalimido or *N*-trichloroethoxycarbamoyl derivatives were tested. In order to evaluate the effect of the torsional constrain of the donor on the glycosylation outcome, glucosamine trichloroacetimidate **14** presenting the 4-OBn, 6-OAc protecting group pattern was tested, as well as the trifluoroacetimidate donor **15** where the 4,6 hydroxyls are locked in a silylidene ring[18].

Table 2. Reaction of GlcN donors **11-15** with Gal acceptors **5-6**

Entry	Donor	Acceptor	Product	Yield
1	**11**	**5**	NIS/TfOH, - 30°C	**16a** (30%), **16b** (<5%)
2	**11**	**5**	NIS/AgOTf, - 30°C	**16a** (38%), **16b** (26%)
3	**11**	**6**	NIS/TfOH, - 30°C	**17a** (40%)
4	**11**	**6**	NIS/AgOTf, - 30°C	**17a** (68%)
5	**12**	**6**	NIS/TfOH, - 30°C	nd[a]
6	**12**	**6**	NIS/AgOTf, - 30°C	**18a** (65%)
7	**13**	**6**	TMSOTf, - 10°C	**18a** (70%)
8	**14**	**11**	TMSOTf, - 10°C	**19a** (50%)
9	**15**	**11**	TMSOTf, - 10°C	**20a** (62%)

a. DCM was the solvent in all tested conditions. b. nd = not determined, product could not be detected.

Table 2 summarizes the results obtained with these donors and acceptors.

The glycosylation of di-*O*-benzyl acceptor **11** with donor **5** using NIS with either TfOH or AgOTf as co-promoters gave variable mixtures of the β-(1→3) **16a** and β-(1→4) **16b** disaccharides (Entry 1-2, Table 2). Switching to the use of di-*O*-benzoyl acceptor **6** in combination with donor **11** in the presence of NIS/TfOH allowed the attainment of the desired product **17a** (40%, Entry 3, Table 2), although it was obtained as a mixture with a non-identified byproduct. The use of NIS/AgOTf activation at -30°C further improved the yield up to 68% (Entry 4, Table 2), confirming the better capacity of benzoyl protection to control the regioselectivity of the reaction. These conditions were proven to be also efficient for the GlcNTroc donor **12** which gave **18a** in 65% yield (Entry 6, Table 2). When the corresponding trichloroacetimidate **13** was exploited, the yield was increased up to 70% (Entry 7, Table 2), corroborating the potential of this type of donors for the regioselective control of the reaction. On the other hand, the yield dropped to 50% when the imidate glucosamine **14** was used to glycosylate the 2,6-di-*O*-benzoylated Gal **6** (Entry 8, Table 2). Finally, trifluoroacetimidate glucosamine **15** bearing a 4,6-*O*-silylidene protection in presence of TMSOTf as promoter afforded the target disaccharide **20a** with a yield (62%, Entry 9, Table 2) slightly lower compared to the benzylidene-protected counterpart **13**, suggesting that the glycosylation reaction might benefit from torsional disarming effect in the donor[19]. Trichloroacetimidate donor **13** and the 2,6-di-*O*-benzoylated acceptor **6** proved to be the best coupling partners to obtain the target GlcNAc-β-(1→3)-Gal motif for further elongation in position 3 of glucosamine.

Overall, these results can be rationalized as the regioselectivity of the glycosylation benefits from the more pronounced electron withdrawing effect of the 2,6- di-*O*-benzoyl in comparison with the 2,6-di-*O*-benzyl substituents in the Gal acceptor. Indeed, the benzoyl groups further decrease the intrinsically lower nucleophilicity of the axial 4-hyodroxyl with respect to the 3-hydroxyl group. In addition, mild activation conditions (NIS/AgOTf) for the thioglycoside donor or the torsional

disarming effect of the benzylidene group for the trichloroacetimidate donor appear to favour the glycosylation reaction over side product formation.

Conclusion

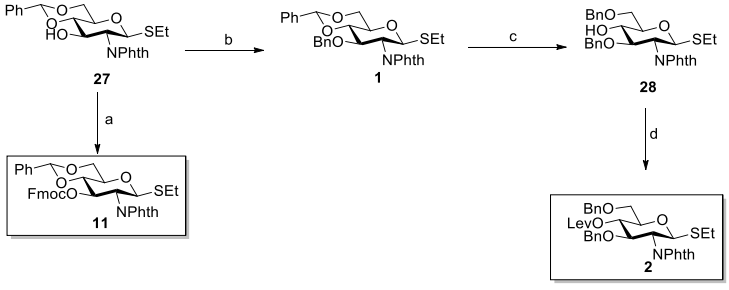
This chapter has introduced the concept of regioselective β(1→3) glycosylation of galactose by using a number of glucosamine donors with a different pattern of protecting groups; the aim of this methodological work was to identify the best combinations of donor and acceptor to prepare the biologically relevant GlcNAc-β-(1→3)Galβ disaccharide with high yield and complete regioselectivity. It was demonstrated that not only the leaving group and the protective groups on the glycosyl donor are important factors in determining the outcome of the glycosylation reaction, but also the stereoelectronic effect of the protecting groups on the glycosyl acceptor plays a crucial role. The use of the electron-withdrawing benzoyl protecting group on the galactose C2 and C6 hydroxyls instead of electron-neutral benzyl groups, resulted in effective deactivation of the galactose C4-OH towards the glycosylation. Therefore, only the β(1→3) linkage was formed during the glycosylation with the selected glucosamine building blocks.
Thus, conditions for an efficient and completely regioselective β-(1→3)-glycosylation of galactose were identified employing glucosamine donors with orthogonal protective groups both at 4-OH or 3-OH. This gave access to key disaccharide synthons which are intermediates to the synthesis of linear and branched oligosaccharide fragments from several bacterial carbohydrates.
The elongation of selected disaccharides at the galactose C4-OH and at glucosamine C3 or C4-OH can give access to biologically relevant oligosaccharide fragments from GBS serotype Ia, Ib and III capsular polysaccharide, to be used for determining the structure-immunogenicity relationship of the corresponding glycoconjugate vaccines, as will be described in following chapters of this thesis.

Experimental

General Methods and procedures. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50ºC on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm) or using pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (Biotage, silica 0.050 nm). Unless otherwise specified, a gradient 0-100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) or Biotage Isolera instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{1}H values are reported in ppm, relative to internal Me_4Si (^{1}H = 0.00, $CDCl_3$); solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{13}C values are reported in ppm relative to the signal of $CDCl_3$ (^{13}C = 77.0, $CDCl_3$). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Assignment of ^{13}C NMR spectra of some compounds was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters. Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data and *m/z* values found in their mass spectra.

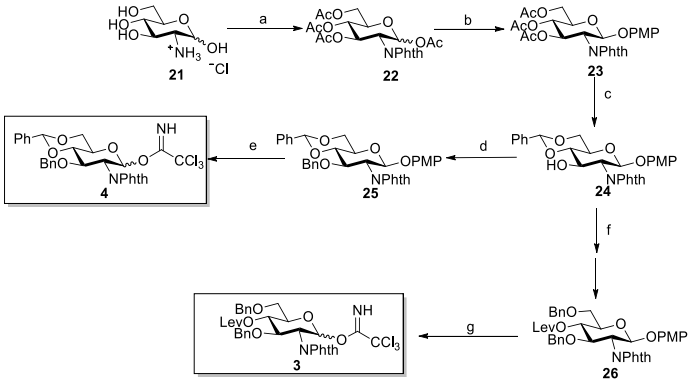
Synthetic schemes

Syntheses of the thioglycoside donors **1**, **2** and **11**[20]



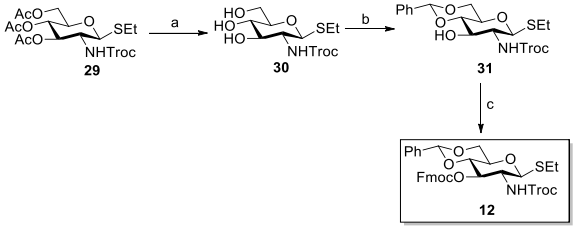
Scheme 2. *Reagents and conditions:* a) Fmoc-Cl, Py, DCM, 64%; b) BnBr, NaH, TBAI, DMF, 0°C, 82%; c) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C; 78% d) Levulinic acid, DCC, DMAP, dry DCM, 77%.

Syntheses of the trichloroacetimidate donors **3** and **4**



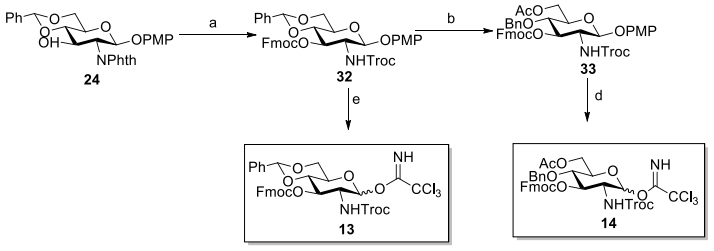
Scheme 3. *Reagents and conditions:* a) $(Phth)_2O$, TEA, MeOH; Ac_2O, Py, DMAP; 75% over two steps; b) *p*-methoxyphenol, $BF_3{\cdot}Et_2O$, DCM dry, 0°C, 89%; c) NaOMe, MeOH; $PhCH(OMe)_2$, PTSA, CH_3CN, 84% over two steps; d) BnBr, NaH, TBAI, DMF, 0°C, 82%; e) CAN, CH_3CN/H_2O, 0°C; CCl_3CN, DBU, DCM dry, 70% over two steps; f) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C; Levulinic acid, DCC, DMAP, dry DCM, 70% over two steps; g) CAN, CH_3CN/H_2O, 0°C; CCl_3CN, DBU, DCM dry, 70% over two steps.

Synthesis of the thioglycoside donor **12**[21]



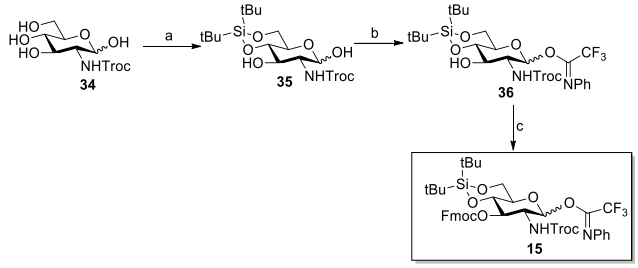
Scheme 4. *Reagents and conditions:* a) MeONa, MeOH, quantitative; b) $PhCH(OMe)_2$, PTSA, CH_3CN, 84%; c) Fmoc-Cl, Py, DCM, 94%.

Syntheses of the trichloroacetimidate **13** and **14**



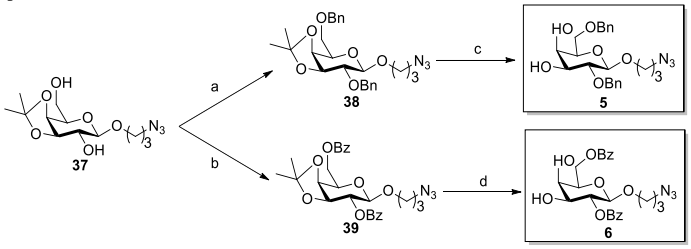
Scheme 5. *Reagents and Conditions*: a) $H_2NCH_2CH_2NH_2$, EtOH, 70° C; $NaHCO_3$, Troc-Cl, H_2O/Et_2O; Fmoc-Cl, DCM/Py 10:1, 60% yield over three steps; b) $PhBCl_2$, Et_3SiH, DCM dry, -78°C; Ac_2O/Py, 0° to rt, 50% yield over two steps; c) CAN, CH_3CN/H_2O, 0°C; CCl_3CN, DBU, DCM dry, 50% over two steps; d) CAN, CH_3CN/H_2O, 0°C; CCl_3CN, DBU, DCM dry, 41% over two steps.

Synthesis of the trifluoroacetimidate **15**[22]



Scheme 6. *Reagents and conditions:* a) $(tBu)_2Si(OTf)_2$, DMF, -30°C, 90% b) 2,2,2-Trifluoro-N-phenylacetimidoyl chloride, Cs_2CO_3, DCM dry, 85% c) Fmoc-Cl, Py, DCM, 94%.

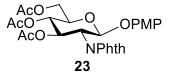
Syntheses of acceptors **5** and **6**[23]



Scheme 7. *Reagent and conditions*: a) BnBr, NaH, DMF, 0°C to rt, 90%; b) BzCl, Py, 0° to rt, 95%; c) $AcOH/H_2O$, 70°C, 92%; d) $AcOH/H_2O$, 70°C, 92%.

Synthesis of building blocks

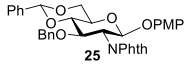
***p*-Methoxyphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-*β*-D-glucopyranoside 23.** Compound **22** (17 g, 35.6 mmol) was dissolved in dry DCM (60.0 mL) at 0°C with 4Å activated molecular sieves (40 g) and stirred for 10 min under nitrogen. *p*-Methoxyphenol (25 g, 201.4 mmol) and boron trifluoride etherate (24 mL, 194.5 mmol) were added at 0°C. After 1 h the mixture was allowed to warm up to rt. Stirring was continued for further 24 h, when TLC showed complete reaction (7:3 cyclohexane:EtOAc). TEA was added, solid was filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (cyclohexane:EtOAc) giving **23** (18 g, 89%) as a brown oil. $[\alpha]_D^{25}$ = +63.04° (c 1.3, $CHCl_3$). ESI HR-MS ($C_{27}H_{27}NO_{11}$) *m/z* $[M+Na]^+$ found 564.1473; calcd 564.1482.



^{1}H NMR (400 MHz, CDCl3) δ 7.80-6.66 (m, 8H, H-Ar), 5.81 (m, 2H, H-1, H-3) , 5.19 (t, *J* = 9.7, 1H, H-4), 4.50 (dd, $J_{1,2}$ = 8.6 Hz, $J_{2,3}$ = 10.6 Hz, 1H, H-2), 4.29 (dd, $J_{5,6a}$ = 5.3 Hz, $J_{6a,6b}$ = 12.3 Hz, 1H, H-6a) , 4.16 (dd, $J_{5,6b}$ = 1.9, 1H, H-6b), 3.90-3.86 (m, 1H, H-5), 3.66 (s, 3H, O*CH*$_3$) , 2.04, 1.98, 1.82 (3 x s, 3H each, 3 x C*H*$_3$CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.2, 169.5 (3 x CO), 134.4-114.4 (C-Ar), 97.5 (C-1), 72.0 (C-5), 70.7 (C-3), 68.9 (C-4), 62.0 (C-6), 55.6 (O*C*H$_3$), 54.5 (C-2), 20.8, 20.7, 20.5 (3 x CO*C*H$_3$).

***p*-Methoxyphenyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside 25.**



Sodium methoxide was added to a stirred mixture of compound **23** (18.0 g, 35.6 mmol) in methanol (40 mL) until pH 9. After 20 h the reaction was quenched with Dowex 50WX2. After the filtration of the resin, the filtrate was evaporated under reduced pressure.

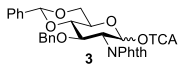
To the crude material acetonitrile (30 mL), benzaldehyde dimethyl acetal (6.9 mL, 68 mmol) and *p*-toluenesulfonic acid (0.470 g, 2.73 mmol) were added. After 3 h the reaction was quenched with triethylamine (4.7 mL), and the mixture was evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane: EtOAc) to afford **24** (6.3 g, 84 % yield) as a yellow solid.

Sodium hydride (0.148 g, 3.7 mmol) was added to a stirred solution of compound **24** (0.930 g, 1.85 mmol) in N,N-dimethylformamide (7.0 mL) at 0°C under nitrogen. After 15 min benzyl bromide (0.66 mL, 5.55 mmol) was added, and the mixture was allowed warming to rt. After 2 h methanol (10 mL) was added, and the mixture was evaporated under reduced pressure. The product was dissolved in EtOAc and washed with $NaHCO_3$ (x2), dried (Na_2SO_4) and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane: EtOAc) to afford **25** (0.900 g, 82% yield) as a yellow solid. $[\alpha]_D^{25} = +65.17^\circ$ (c 1.1, $CHCl_3$). ESI-HR MS ($C_{35}H_{31}NO_8$) m/z $[M+Na]^+$ found 616.1866; calcd 616.1947.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78- 6.74 (m, 18H, H-Ar), 5.77 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1), 5.68 (s, 1H, C*H*Ph), 4.86 (d, ^2J=12.4 Hz, 1H, C*H*HPh), 4.57 (d, ^2J = 12.4 Hz, 1H, CH*H*Ph), 4.53-4.48 (m, 2H, H-3, H-2), 4.45 (dd, $J_{5,6a}$ = 4.9 Hz, $J_{6a,6b}$ = 10.4 Hz, 1H, H-6a), 3.98-3.88 (m, 2H, H-4, H-6b), 3.80-3.74 (m, 1H, H-5), 3.73 (s, 3H, OCH_3)

^{13}C NMR (101 MHz, $CDCl_3$) δ 134.00-114.53 (C-Ar), 101.40 (*C*HPh), 98.00 (C-1), 83.00 (C-4), 74.20 (*C*H_2Ph), 74.51 (C-3), 68.74 (C-6), 55.74 (C-2), 66.30 (C-5), 55.60 (O*C*H_3).

3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-*α,β*-D-glucopyranosyl trichloroacetimidate 3.

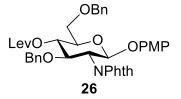


3

Cerium ammonium nitrate (5.110 g, 9.32 mmol) was added to a stirred solution of compound **25** (3 g, 4.66 mmol) in 4:1 acetonitrile: water (50 mL) at 0°C. After 3 h, TLC (7:3 cyclohexane:EtOAc) showed the disappearance of the starting material and the formation of one major spot. The reaction was washed with a solution of $NaHCO_3$ (x 2) and the combined organic phases were dried with Na_2SO_4 and evaporated under reduced pressure. The crude (1.681 g, 3.45 mmol) was dissolved in DCM (10 mL) dry under nitrogen and trichloroacetonitrile (1.730 mL, 17.25 mmol) and 1,8-diazobicyclo[5.4.0]undec-7-ene (0.152 mL, 1.03 mmol) were added. After stirring for 2h at rt, TLC (7:3 cyclohexane:EtOAc) showed complete reaction. The solvent was removed at reduced pressure and the crude was purified by flash chromatography (cyclohexane:EtOAc) to afford **3** (1.524 g) in 70% yield in 2:1 α/β ratio. $[\alpha]_D^{25} = +64.25^\circ$ (c 4.15, $CHCl_3$). ESI MS ($C_{30}H_{25}Cl_3N_2O_7$) *m/z* $[M+H]^+$ found 632.06; calcd 631.89.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.5 (s, 1H, NH), 7.62-6.80 (m, 14H, H-Ar), 6.42 (d, $J_{1,2}$ = 8.4 Hz, H-1_β), 6.30 (d, $J_{1,2}$ = 3.8 Hz, H-1_α), 5.60 (s, 1H, C*H*Ph$_\alpha$), 5.57 (s, 1H, C*H*Ph$_\beta$), 5.46 (t, J = 9.0 Hz, H-3_α), 4.95 (d, ^2J= 11.1 Hz, 1H, C*H*HPh$_\alpha$), 4.75 (d, ^2J = 12.4 Hz, 1H, C*H*HPh$_\beta$), 4.62 (d, ^2J = 11.1 Hz, 1H, CH*H*Ph$_\alpha$), 4.58-4-52 (m, H-2_α) , 4.94-4.36 (m, H-2_β, H-3_β, CH*H*Ph$_\beta$, H-$6a_\beta$), 4.33-4.31 (m, H-$6a_\alpha$), 4.18-4.12 (m, H-5_α), 3.86-3.76 (m, H-4_α, H-$6b_\alpha$, H-4_β, H-5_β, H-$6b_\beta$)

***p*-Methoxyphenyl 3,6-di-*O*-benzyl-2-deoxy-4-*O*-levulinoyl-2-phthalimido-*β*-D-glucopyranoside 26.**



26

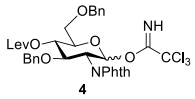
A solution of **24** (0.500 g, 0.9 mmol) in ACN (5 mL) was cooled at 0°C. $Me_3N{\cdot}BH_3$ (274 mg, 3.76 mmol) and $BF_3{\cdot}O(Et)_2$ (0.464 mL, 3.76 mmol) were added, and the reaction was stirred for 2 h under nitrogen. TLC (7:3 cyclohexane:EtOAc) showed complete reaction. TEA was added until neutral pH, followed by MeOH. The solvent was removed at reduced pressure and the crude was purified by flash chromatography (cyclohexane:EtOAc).

To the obtained product (0.400 g, 0.67 mmol) dissolved in DCM (5 mL), N-N-ethylcarbodiimide hydrochloride (0.206 g, 1.0 mmol), 4-dimethylaminopyridine (0.122 g, 1.0 mmol) and levulinic acid (0.156 g, 1.34 mmol) were added. The mixture was stirred overnight at rt. The solvent was removed by rotary evaporation, and the resulting crude material was purified by flash chromatography (cyclohexane:EtOAc) to give the compound **26** (325 mg) in 70% yield. $[\alpha]_D^{25} = +78.37^\circ$ (c 3.25, $CHCl_3$). ESI HR-MS ($C_{40}H_{39}NO_{10}$) *m/z* $[M+Na]^+$ found 716.2447; calcd 716.2472.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62-6.58 (m, 18H, H-Ar), 5.57 (d, $J_{1,2}$ =8.8 Hz, H-1), 5.14 (t, J = 8.9 Hz, 1H, H-4), 4.62 (d, ^2J = 11.9 Hz, 1H, C*H*Ph), 4.46 -4.42 (m, 3H, CH_2PH, H-3, H-2), 4.28 (d, ^2J = 11.9 Hz, 1H, C*H*HPh), 3.82-3.67 (m, 1H, H-5), 3.61 (s, 3H, OCH_3), 3.58-3.54 (m, 2H, H-6), 2.59 (t, J = 6.4 Hz, 2H, CH_2CO), 2.41 (t, J = 6.4Hz, 2H, CH_2COO), 2.07 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 206.2, 171.5, 160.8 (3 x CO), 137.0-114.0 (C-Ar), 97.4 (C-1), 72.9 (C-4), 74.2 (CH_2Ph), 73.59 (CH_2Ph), 77.24 (C-3), 55.41 (C-2), 73.8 (C-5), 69.56 (C-6), 55.55 (OCH_3), 37.77 (CH_2CO), 29.83 (CH_3), 27.94 (CH_2COO).

3.6-Di-*O*-benzyl-2-deoxy-4-*O*-levulinoyl-2-phthalimido-*β*-D-glucopyranosyl trichloroacetimidate 4.



4

Cerium ammonium nitrate (515 mg, 0.94 mmol) was added to a stirred solution of compound **26** (325 mg, 0.47 mmol) in 4:1 acetonitrile:water (25 mL) at 0°C. After 3 h, a TLC (cyclohexane: EtOAc 1:1) showed the disappearance of the starting material and the formation of one major spot. The reaction was washed two times with a solution of $NaHCO_3$ and the organic phase was dried with Na_2SO_4 and evaporated under reduced pressure.

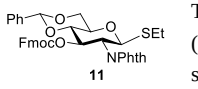
The crude was dissolved in DCM dry (10 mL) under nitrogen and trichloroacetonitrile (0.368 g, 2.55 mmol) and 1,8-diazobicyclo[5.4.0]undec-7-ene (0.023 g, 0.153 mmol) were respectively added. After stirring for 2h at rt, TLC showed complete reaction (1:1 cyclohexane:EtOAc). The solvent was removed at reduced pressure and the crude was purified by flash chromatography (cyclohexane:EtOAc) to afford **4** (261 mg, yield 70%). $[\alpha]_D^{25}$ = +68.49° (c 0.55, $CHCl_3$). ESI HR-MS ($C_{35}H_{33}Cl_3N_2O_9$) *m/z* $[M+Na]^+$ found 732.0040; calcd 732.0035.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62-6.85 (m, 14H, H-Ar), 6.36 (d, $J_{1,2}$ = 7.6 Hz, H-1$_\beta$), 5.22 (t, J = 9.0 Hz, 1H, H-4), 4.62 (d, ^2J = 12.3 Hz, 1H, C*H*HPh$_b$), 4.53-4.39 (m, 4H, H-2, H-3, C*H*HPh$_a$, CH*H*Ph$_a$), 4.30 (d, ^2J = 12.3 Hz, 1H, CH*H*Ph$_b$), 3.92-3.85 (m, 1H, H-5), 3.66-3.53 (m, 2H, H-6), 2.65-2.48 (m, 2H, CH_2CO), 2.47-2.29 (m, 2H, CH_2COO), 2.06 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 206.4, 171.6, 160.8 (3 x CO), 133.9-123.3 (C-Ar), 93.9 (C-1), 76.7 (C-3), 74.44 (C-5), 74.11, 73.48, 72.17 (C-4), 68.89 (C-6), 54.46 (C-2), 37.69 (*C*H_2CO), 33.96, 29.79 (CH_3), 27.91 (*C*H_2COO), 25.62, 24.95.

^{13}C NMR (101 MHz, $CDCl_3$) δ 134.0-123.4 (C-Ar), 101.4 (*C*HPh$_\beta$), 101.3 (*C*HPh$_\alpha$), 95.4 (C-1$_\alpha$), 94.3 (C-1$_\beta$), 83.4, 82.5 (C-4), 74.7, 74.3, 74.2 (C-3$_\beta$), 72.4 (C-3$_\alpha$), 68.5, 66.9 (C-5$_\beta$), 65.4 (C-5$_\alpha$), 54.7 (C-2).

Ethylthio 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-phthalimido-*β*-D-glucopyranoside 11.

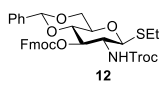


11

The known compound **27** (200 mg, 0.45 mmol) was dissolved in dry DCM (10 mL). Fmoc-Cl (351 mg, 1.36 mmol) and pyridine (0.182 mL, 2.25 mmol) were added at 0°C, and the reaction stirred at rt for 1 h. TLC (4:1 cyclohexane:EtOAc) showed complete reaction, the solvent was removed under reduced pressure and the crude was purified by flash chromatography (8:2 cyclohexane:EtOAc) to afford **11** (202 mg) in 64% yield as pale yellow oil. $[\alpha]_D^{25}$= +13.53° (c 2.5, $CHCl_3$). ESI HR-MS ($C_{38}H_{33}NO_8S$) *m/z* $[M+Na]^+$ found 686.1807; calcd 686.1825.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16-7.95 (m, 17H, H-Ar), 5.88 (t, J= 9.5 Hz, 1H, H-3), 5.59-5.65 (m, 2H, C*H*Ph, H-1), 4.58 (t, J= 10.3 Hz, 1H, H-2), 4.47-4.52 (m, 1H, CH_{2a}^{Fmoc}), 4.09-4.17 (m, 2H, H-6), 3.92-4.00 (m, 2H, CH^{Fmoc}, H-4), 3.84-3.92 (m, 2H, CH_{2b}^{Fmoc}, H-5), 2.65-2.85 (m, 2H, SCH_2), 1.25 (t, J = 7.3 Hz, 3H, SCH_2CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.9, 167.2, 154.5 (3 x CO), 143.1-119.9 (C-Ar), 101.8 (*C*HPh), 81.9 (C-1), 79.2 (C-4), 74.4 (C-3), 70.5 (C-5), 70.3, 68.6 (C-6), 55.4, 54.1 (C-2), 46.3, 26.9, 24.4 (S*C*H_2), 14.9 (SCH_2CH_3).

Ethylthio 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside 12.



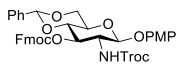
12

Compound **31** (219 mg, 0.45 mmol) was dissolved in 5.0 mL of dry DCM and the resulting solution was cooled down to 0°C. Pyridine (0.250 mL) was added, followed by Fmoc-Cl (0.310 mL, 1.125 mmol). The reaction mixture was stirred for 30 min. The crude was purified by column chromatography (7:3 cyclohexane:EtOAc) to afford **12** (300 mg, 94% yield) as a white solid. $[\alpha]_D^{25}$= -14.18° (c 0.025, $CHCl_3$). ESI HR-MS ($C_{33}H_{32}Cl_3NO_8S$) *m/z* $[M+Na]^+$ found 730.0852; calcd 730.0812.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81-7.19 (m, 13H, Ar-H), 5.59 (s, 1H, C*H*Ph), 5.31 (d, $J_{N,H}$ = 9.2 Hz, 1H, NH), 5.20 (t, *J* = 9.8 Hz, 1H, H-3), 4.7 (d, $J_{1,2}$ = 10.2 Hz, 1H, H-1), 4.65 (d, ^2J = 2.54 Hz, 2H, Cl_3CCH_2), 4.45-4.35 (m, 3H, H-6, CH_2^{Fmoc}), 4.26 (t, *J* = 7.5 Hz, 1H, CH^{Fmoc}), 3.92 (t, *J* = 9.9 Hz, 1 H, H-2), 3.88-3.80 (m, 2H, H-6', H-4), 3.65-3.57 (m, 1H, H-5), 2.70-2.78 (m, 2H, CH_2SEt), 1.28 (t, 3H, *J* = 7.4 Hz, CH_3SEt).

^{13}C NMR (101 MHz, $CDCl_3$) δ 129.3-120.2 (C-Ar), 101.8 (*C*HPh), 85.5 (C-1), 78.7 (C-4), 76.5 (C-3), 74.8 (Cl_3CCH_2), 71.0 (CH_2^{Fmoc}), 70.7 (C-6), 68.7 (C-5), 56.1 (C-2), 46.7 (CH^{Fmoc}), 24.6 (CH_2 SEt), 14.9 (CH_3SEt).

***p*-Methoxyphenyl 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside 32.**



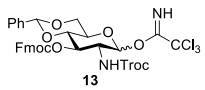
32

Compound **24** (2.014 g, 3.64 mmol) was dissolved in 20.0 mL of ethanol and ethylenediamine (1.2 mL, 18.22 mmol) was added. The resulting reaction mixture was stirred at 70°C for 6 h. After 6 h TLC (cyclohexane:EtOAc 1:1) showed full conversion of the starting material and formation of a new spot on the baseline. The reaction mixture was evaporated under reduced pressure affording a white solid. The crude was resuspended in methanol and filtered under vacuum. The filtrate was evaporated to dryness affording a yellow syrup, which was redissolved in 20.0 mL of a 1:1 Et_2O/H_2O mixture. The resulting solution was cooled down to 0°C and $NaHCO_3$ (3.06 mg, 36.4 mmol) was added, followed by Troc-Cl (2.5 mL, 18.2 mmol). The resulting reaction mixture was stirred at 0°C for 45 min, then checked by TLC (7:3 cyclohexane/EtOAc), which showed complete consumption of the starting material. The reaction mixture was quenched by addition of aqueous $NaHCO_3$, then extracted with DCM (3 x 10 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure affording a pale solid as a crude. The crude was purified by flash column chromatography using a gradient from 0 to 100% of EtOAc in cyclohexane. Clean fractions were collected and evaporated under reduced pressure affording the product as a white solid (1.419 g, 66% yield). The compound (1.2 g, 2.03 mmol) was dissolved in 10.0 mL of dry DCM. The resulting solution was cooled down to 0°C in an ice-water bath, then Fmoc-Cl (1.3 g, 5.078 mmol) and pyridine (0.5 mL) were added. The reaction mixture was stirred for 1 h at rt, then checked by TLC (cyclohexane/EtOAc), which showed full conversion of starting material. The crude was evaporated under reduced pressure and purified by flash column chromatography. Pure fractions were collected and evaporated to dryness affording compound **32** (1.3 g, 60% yield over three steps). $[\alpha]_D^{25}$ = -1.19˚ (c 1.1, $CHCl_3$). ESI HR-MS ($C_{38}H_{34}Cl_3NO_{10}$) *m/z* $[M+NH_4]^+$ found 787.1596; calcd 787.1587.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60-2.22 (m, Ar-H), 5.58 (s, 1H, C*H*Ph), 5.48 (d, $J_{N,H}$ = 8.7 Hz, 1H, NH), 5.33 (t, *J* = 10.0 Hz, 1H, H-3), 5.15 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1), 4.70 (d, ^2J = 11.8 Hz, 1H, C*H*HCCl$_3$), 4.63 (d, 1H, CH*H*CCl$_3$), 4.47-4.33 (m, C*H*H^{Fmoc}, 3H, H-6), 4.25 (t, *J* = 7.2 Hz, 1H, CH^{Fmoc}), 3.97 (t, *J* = 8.9 Hz, 1H, H-2), 3.90-3.82 (m, 2H, C*H*H^{Fmoc}, H-4), 3.77 (s, 3H, OCH_3), 3.64-3.56 (m, 1H, H-5).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.8, 155.1 (2 x CO), 154.2 (C_q), 151.0 (C_q), 144.3-114.6 (C-Ar), 101.6 (*C*HPh), 100.9 (C-1), 78.5 (C-4), 74.9 (C-3), 74.5 (CH_2CCl_3), 70.5 (C-6), 68.5 (CH_2^{Fmoc}), 66.3 (C-5), 57.1 (C-2), 55.7 (OCH_3), 46.5 (CH^{Fmoc}).

4,6-*O*-benzylidene-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-*α,β*-D-glucopyranosyl trichloroacetimidate 13.



13

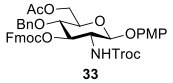
Compound **32** (1.550 g, 2.06 mmol) was dissolved in 15 mL of a 4:1 ACN/H_2O mixture and the resulting suspension was cooled down to 0°C. Cerium ammonium nitrate (2.16 g, 4.12 mmol) was added and the reaction was stirred for 2 h at 0°C. After 2 h, TLC (6:4 cycloexane:EtOAc) showed disappearance of the starting material. The reaction mixture was diluted with DCM and washed twice with iced aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was purified by column chromatography (cyclohexane/EtOAc). Pure fractions were collected and evaporated affording the target 1-OH intermediate (1.1 g, 80% yield) as colorless oil.

The 1-OH intermediate (0.212 g, 0.319 mmol) was dissolved in a 1:1 DCM/CCl_3CN mixture under nitrogen atmosphere and cooled down to 0°C. NaH (1.28 mg, 0.032 mmol) was added and the resulting solution was stirred for 3 h and let slowly to go to rt. After 3 h TLC (6:4 cyclohexane/EtOAc) showed full conversion of the starting material; the reaction mixture was evaporated under reduced pressure and the crude was purified on column chromatography with a gradient from 0 to 100% of EtOAc in hexane (containing 3% of TEA). Pure fractions were collected and evaporated under reduced pressure affording the glucosamine imidate **13** as a colorless oil (0.125 g, 50% yield,) primarily as α anomer. $[\alpha]_D^{25} = +31.33^\circ$ (c 0.2, $CHCl_3$). ESI HR-MS ($C_{33}H_{28}Cl_6N_2O_9$) *m/z* $[M+H]^+$ found 806.0878; calcd 806.9926.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H, C=NH); 7.54-7.14 (m, 13H, Ar-H); 6.39 (d, $J_{1,2}$ = 3.6 Hz, 1H, H-1); 5.57 8s, 1H, C*H*Ph); 5.38 (d, $J_{N,H}$ = 9.7 Hz, 1H, NH), 5.28 (t, J = 10.1 Hz, 1H, H-3), 4.67 (d, ^2J = 12.0 Hz, 1H, C*H*HCCl$_3$), 4.48 (d, 1H, CH*H*CCl$_3$), 4.41-4.30 (m, 3H, incl. H-2, H-6); 4.25-4.19 (m, 1H, C*H*^{Fmoc}), 4.09-4.02 (m, 1H, H-5), 3.91 (t, 1H, J = 9.7 Hz, H-4), 3.79 (t, J = 10.4 Hz, 1H, H-6b).

^{13}C NMR (101 MHz, $CDCl_3$) δ 160.7 (CO), 143.1, 143.0, 129.6-120.0 (C-Ar), 101.7 (*C*HPh), 95.1 (C-1), 78.3 (C-4), 77.2, 74.7 (*C*H$_2$CCl$_3$), 73.4 (C-3), 70.7 (*C*H$_2^{Fmoc}$), 70.1 (*C*H$_2$Ph), 68.5 (C-6), 65.4 (C-5), 65.2, 54.6 (C-2), 50.4, 46.4 (*C*H^{Fmoc}).

***p*-Methoxyphenyl 6-*O*-acetyl-4-*O*-benzyl-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside 33.**



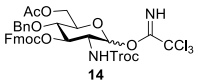
33

Compound **32** (370 mg, 0.49 mmol) was dissolved in 5.0 mL of dry DCM under nitrogen atmosphere in presence of 4 Å activated molecular sieves. The resulting suspension was cooled down to -78°C, when dichlorophenylborane (0.229 mL, 1.764 mmol) and Et_3SiH (0.549 mL, 3.43 mmol) were added. The resulting reaction mixture was stirred and allowed to slowly reach rt. TLC (6:4 cylcohexane:EtOAc) showed consumption of the starting material, the reaction was quenched by addition of saturated aqueous $NaHCO_3$ and extracted with DCM (3 x 10 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was dissolved in a 1:1 solution of Ac_2O/pyridine. The resulting reaction mixture was stirred at rt. After 16 h TLC (6:4 cyclohexane/EtOAc) showed complete reaction, then the crude mixture was coevaporated with toluene and the residue was purified by column chromatography (cyclohexane/EtOAc). Pure fractions were collected and evaporated to dryness affording glucosamine **33** (185 mg, 50% yield over two steps). $[\alpha]_D^{25} = -11.07^\circ$ (c 0.025, $CHCl_3$). ESI HR-MS ($C_{41}H_{40}Cl_3NO_{10}$) *m/z* $[M+Na]^+$ found 834.1625; calcd 834.1615.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80-6.76 (m, 22H, Ar-H); 5.36 (d, $J_{N,H}$ = 9.0 Hz, 1H, NH), 5.18 (dd, $J_{3,4}$ = 10.5 Hz, $J_{2,3}$ = 8.8 Hz, 1H, H-3), 4.99 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1), 4.72-4.62 (m, 3H, C*H*$_2$CCl$_3$, C*H*HPh), 4.54 (d, ^2J = 11.5 Hz, 1H, CH*H*Ph), 4.51-4.44 (m, 1H, C*H*H^{Fmoc}), 4.41-4.32 (m, 2H, H-6a, CH*H*^{Fmoc}), 4.27-4-20 (m, 2H, CH^{Fmoc}, H-6b), 3.94 (t, 1H, J = 8.9 Hz, H-2), 3.79-3.65 (m, 2H, H-4, H-5), 3.76 (s, 3H, OC*H*$_3$), 2.04 (s, 3H, CH$_3$CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 155.8, 155.3 (3 x CO), 154.2-114.5 (C-Ar), 100.4 (C-1), 78.7 (C-3), 75.3 (C-5), 74.8 (*C*H$_2$CCl$_3$), 72.8 (C-4), 70.5 (*C*H$_2^{Fmoc}$), 62.6 (C-6), 56.5 (C-2), 55.7 (O*C*H$_3$), 46.7 (CH^{Fmoc}), 20.8 (*C*H$_3$CO).

6-*O*-Acetyl-4-*O*-benzyl-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*α,β*-D-glucopyranosyl trichloroacetimidate 14.



14

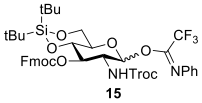
Compound **33** (185 mg, 0.227 mmol) was dissolved in 10 mL of a 4:1 acetonitrile/water mixture and the resulting solution was cooled down to 0°C using a water/ice bath. Cerium ammonium nitrate (373 mg, 0.680 mmol) was added and the resulting reaction mixture was stirred at 0°C. After 2 h TLC (3:2 cycloexane:EtOAc) showed full consumption of the starting material. The reaction mixture was poured into iced $NaHCO_3$ and extracted with DCM (3x20 mL). The organic phases were dried over Na_2SO_4 and evaporated under reduced pressure affording a crude product which was directly dissolved in 2.0 mL of a 1:1 CCl_3CN/DCM mixture. The resulting solution was cooled down to 0°C, NaH (60% dispersion in mineral oil, 1.0 mg, 0.0212 mmol) and trichloroacetonitrile (2 mL) were added and the resulting reaction mixture was stirred for 2 h, letting slowly reach rt.

After 2 h, TLC (6:4 cyclohexane:EtOAc) showed full conversion of the starting material, so the reaction was quenched by addition of Et_3N and the reaction mixture was evaporated to dryness. The crude was purified by column chromatography with a gradient of EtOAc in cyclohexane (containing 1% of TEA). Pure fractions were collected and evaporated to dryness affording the target compound **14** as a clear oil (0.085 g, 41% yield over two steps, only α). $[\alpha]_D^{25} = +9.63^\circ$ (c 0.85, $CHCl_3$). ESI HR-MS ($C_{36}H_{34}Cl_6N_2O_9$) *m/z* $[M+Na]^+$ found 871.0300; calcd 871.0293.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H, $NHCCl_3$); 7.84-7.24 (m, 13H, Ar-H); 6.42 (d, $J_{1,2}$ = 3.4 Hz, 1H, H-1), 5.37 (d, $J_{N,H}$ = 9.2 Hz, 1H, NH), 5.30 (dd, $J_{3,4}$ = 11.4 Hz, $J_{2,3}$ = 9.6. 1H, H-3); 4.78 (d, ^2J = 11.0 Hz, 1H, C*H*HPh), 4.71-4.50 (m, 4H, CH_2CCl_3, CH*H*Ph, CH^{Fmoc}), 4.56-4.24 (m, 5H, H-2, H-6, CH_2^{Fmoc}), 4.09 (dt, 1H, $J_{4,5}$ =9.9 Hz, $J_{5,6}$ = 2.9 Hz, H-5); 3.99 (t, 1H, *J* = 9.5 Hz, H-4), 2.06 (s, 3H, CH_3CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6 (CNH), 163.7, 160.6, 155.6 (CO), 143.2-120.1 (C-Ar), 100.0 (Cq), 94.8 (C-1), 77.2 (C-3), 75.1 (*C*H_2Ph), 74.6 (*C*H_2CCl_3), 74.1 (C-4), 71.4 (C-5), 70.7 (*C*H_2^{Fmoc}), 62.0 (C-6), 54.2 (C-2), 46.5 (*C*H^{Fmoc}), 20.8 (*C*H_3CO).

4,6-*O*-di-*tert*-butylsilylidene-2-deoxy-3-*O*-(9*H*-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*α,β*-D-glucopyranosyl *N*-phenyl-trifluoroacetimidate 15.



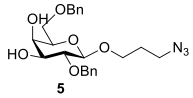
15

Compound **36** (2.278 g, 3.42 mmol) was dissolved in 10.0 mL of dry DCM and the resulting solution was cooled down to 0°C. Pyridine (1.0 mL) was added, followed by Fmoc-Cl (2.2 g, 8.55 mmol). The reaction mixture was stirred for 1 hour at rt. The crude was purified by column chromatography (9:1 cyclohexane:EtOAc) to afford **15** (2.0 g, 70% yield) as a white solid. $[\alpha]_D^{25} = +27.87^\circ$ (c 0.05, $CHCl_3$). ESI HR-MS ($C_{40}H_{44}Cl_3F_3N_2O_9Si$) *m/z* $[M+Na\text{-}CNPhCF_3]^+$ found 737.3012; calcd 738.1430.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82-6.78 (m, 13H, Ar-H); 5.99 (bs, 1H, H-1), 5.26 (d, $J_{N,H}$ = 8.9 Hz, 1H, NH), 5.10-5.01 (m, 1H, H-3), 4.73 (d, ^2J = 12.1 Hz, 1H, C*H*$HCCl_3$), 4.62 (d, 1H, CH*H*CCl_3); 4.47 (dd, *J* = 9.9, 7.9 Hz, 1H, C*H*H^{Fmoc}), 4.40-4.33 (m, 1H, CH*H*^{Fmoc}), 4.31-4.15 (m, 3H, H-2, CH^{Fmoc}, H-6a); 4.10-3.91 (m, 3H, H-6b, H-4, H-5), 1.05 (s, 9H, tBu), 0.95 (s, 9H, tBu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.3, 154.0 (CO), 143.2 (C_q), 141.3 (C_q), 129.4-119.2 (C-Ar), 94.9 (CCl_3), 93.6 (C-1), 77.6 (C-3), 74.6 (C-4, C-5), 75.4 (*C*H_2CCl_3), 71.4 (C_q), 70.6 (*C*H_2^{Fmoc}), 65.9 (C-6), 55.6 (C-2), 46.5 (*C*H^{Fmoc}), 27.3 (tBu), 26.8 (tBu), 22.7 (*C*$(CH_3)_3$), 20.0 (*C*$(CH_3)_3$).

3-Azidopropyl 2,6-di-*O*-benzyl-*β*-D-galactopyranoside 5.

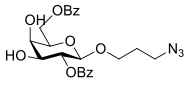


5

A suspension of compound **38**[23] (3.0 g, 5.7 mmol) in 80% aqueous AcOH (20 mL) was stirred at 70°C for 2 h when TLC (7: 3 cyclohexane:EtOAc) showed complete conversion of the starting material to a slower moving spot. Solvents were evaporated in vacuo, coevaporated with toluene to remove traces of AcOH. The residue was purified by flash chromatography using cyclohexane:EtOAc as eluent to give the pure product **10** (2.5 g, 92%) as yellow oil. $[\alpha]_D^{25} = +12.78^\circ$ (c 1.05, $CHCl_3$). ESI HR-MS ($C_{23}H_{29}N_3O_6$) *m/z* $[M+Na]^+$ found 466.2023; calcd 466.1954.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46-7.25 (m, 10H, H-Ar), 4.95 (d, ^{2}J= 11.6 Hz, 1H, C*H*HPh), 4.69 (d, ^{2}J = 11.6 Hz, 1H, CH*H*Ph), 4.62 (s, 2H, CH_2Ph), 4.38 (d, $J_{1,2}$ = 7.7 Hz, 1H, H-1), 4.07-4.00 (m, 2H, OCH_{2b}, H-4), 3.83-3.73 (m, 2H, H-6), 3.69-3.58 (m, 3H, OCH_{2a}, H-3, H-2), 3.55-3.49 (m, 1H, H-5), 3.44 (t, J = 5.4 Hz, 2H, CH_2N_3), 1.93 (m, 2H, C$H_2CH_2N_3$).
^{13}C NMR (101 MHz, $CDCl_3$) δ 128.6-127.7 (C-Ar), 103.6 (C-1), 79.16 (C-5), 74.73 (*C*H_2Ph), 73.72 (*C*H_2Ph), 73.28 (C-2), 73.13 (C-3), 69.34 (C-6), 68.94 (C-4), 66.54 (O*C*H_2), 48.37 (*C*H_2N_3), 29.27 (*C*$H_2CH_2N_3$).

3-Azidopropyl 2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 6.



6

A suspension of compound **39**[23] (3.0 g, 5.7 mmol) in 80% aqueous AcOH (20 mL) was stirred at 70°C for 2 h when TLC (7:3 cyclohexane: EtOAc) showed complete conversion of the starting material to a slower moving spot. Solvents were evaporated in vacuo, coevaporated with toluene to remove traces of AcOH. The residue was purified by flash chromatography using cyclohexane:EtOAc as eluent to give the pure product **6** (2.5 g, 92%). $[\alpha]_D^{25}$ = -3.94° (c 0.45, $CHCl_3$). ESI HR-MS ($C_{23}H_{25}N_3O_8$) *m/z* $[M+Na]^+$ found 494.1591; calcd 494.1539.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06-7.38 (m, 10H, H-Ar), 5.14 (t, J = 8.9 Hz, 1H, H-2), 4.69-4.64 (m, 1H, H-6a), 4.56-4.50 (m, 2H, H-1, H-6b), 3.98 (d, $J_{3,4}$ = 2.5 Hz, 1H, H-4), 3.97-3.88 (m, 1H, OCH_{2a}), 3.86-3.83 (m, 1H, H-5), 3.81-3.78 (m, 1H, H-3), 3.59-3.53 (m, 1H, OCH_{2b}), 3.21 (t, J = 6.5 Hz, 2H, CH_2N_3), 1.82-1.65 (m, 2H, C$H_2CH_2N_3$).
^{13}C NMR (101 MHz, $CDCl_3$) δ 167.29, 166.62 (2 x CO), 133.7-128.4 (C-Ar), 101.09 (C-1), 99.9, 74.31 (C-2), 72.78 (C-3), 72.21 (C-5), 68.59 (C-4), 66.38 (O*C*H_2), 62.77 (C-6), 47.95 (*C*H_2N_3), 29.03 (*C*$H_2CH_2N_3$).

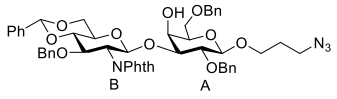
Preparations of disaccharides

Procedure A for glycosylation with thioglycoside donors with NIS/TfOH.
Donor (0.11 mmol) and acceptor (0.1 mmol) with activated 4 Å molecular sieves (0.1 g) were added at the solution of dry DCM (5 mL) and stirred for 20 min under nitrogen. NIS (0.2 mmol) and TfOH (0.02 mmol) were added at –30°C. The reaction was stirred for 2 and then allowed to warm up to rt. Stirring was continued for 12 h, monitoring by TLC (Tol:EtOAc or cyclohexane:EtOAc). The reaction was stirred for 12 h monitoring by (Tol:EtOAc or cyclohexane:EtOAc). the reaction was quenched with TEA, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (cyclohexane:EtOAc) to give the purified products.

Procedure B for glycosylation with thioglycoside donors with NIS/AgOTf.
A solution of donor (0.11 mmol) and acceptor (0.1 mmol) with activated 4 Å molecular sieves (0.1 g) in dry DCM (5 mL) was stirred for 20 min under nitrogen. NIS (0.2 mmol) and AgOTf (0.02 mmol) were added at –30°C. The reaction was stirred in the dark allowing to warm up to rt. After TLC (Tol:EtOAc or cyclohexane:EtOAc) showed complete reaction, the mixture was quenched with TEA, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (cyclohexane:EtOAc) to give the purified products.

Procedure C for glycosylation with trichloroacetimidate/trifluoroacetimidate donors
A solution of donor (0.11 mmol) and acceptor (0.1 mmol) with activated 4 Å molecular sieves (0.1 g) in dry DCM (5 mL) was stirred for 20 min under nitrogen. TMSOTf (0.02 mmol) was added at –10°C. After 4 h (TLC; Tol:EtOAc or cyclohexane:EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol:EtOAc or cyclohexane:EtOAc) to afford the purified products.

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 7a.



7a

Protocol A: **7a** not detected

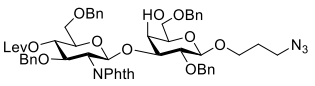
Protocol B. After flash chromatography (cyclohexane:EtOAc) a 3:2 mixture (61% yield) of disaccharide **7a** and the β-(1→4) product, which could not be isolated as a clean compound, was obtained.

Protocol C **7a**, 31% yield. $[\alpha]_D^{25}$ = +14.39° (c 0.25, $CHCl_3$). ESI HR-MS ($C_{51}H_{52}N_4O_{12}$) *m/z* [*M*+ Na]$^+$ found 935.3396; calcd 935.3479.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-6.87 (m, 24H, 24H-Ar) 5.65 (s, 1H, C*H*Ph), 5.48 (d, $J_{1,2}$ = 10.2 Hz, 1H, H-1^B), 4.81 (d, ^2J = 12.2 Hz, 1H, C*H*HPh), 4.59 (s, 2H, C*HH*Ph), 4.50 (d, ^2J = 12.2 Hz, 1H, CH*H*Ph), 4.45-4.43 (m, 1H, C*H*HPh), 4.34-4.32 (m, 2H, H-6a^B, H-2^B), 4.23-4.20 (m, 2H, CH*H*Ph, H-1^A), 4.05 (d, $J_{3,4}$ = 2.8 Hz, 1H, H-4^A), 3.89-3.78 (m, 4H, H-6b^B,OCH_{2a}, H-6a^A, H-5^B), 3.74-3.67 (m, 2H, H-6b^A, H-3^B), 3.60-3.57 (m, 3H, H-4^B, H-3^A, H-5^A), 3.48-3.40 (m, 2H, OCH$_{2b}$, H-2^A), 3.16 (dt, *J* = 3.2, 6.5 Hz, 2H, CH_2N$_3$), 1.70 (dt, *J* = 6.6, 13.3 Hz, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 129.13-123.28 (C-Ar), 103.34 (C-1^A), 101.40 (*C*HPh), 99.68 (C-1^B), 82.86, 82.79 (C-5^B), 77.59 (C-2^A), 74.44, 74.30 (*C*H$_2$Ph), 74.16 (*C*H$_2$Ph), 73.67 (*C*H$_2$Ph), 69.07 (C-6^A), 68.67 (C-6^B), 68.19 (C-4^A), 66.42 (O*C*H$_2$), 66.22 (C-3^B), 55.87 (C-2^B), 48.12 (*C*H$_2$N$_3$), 29.07 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 3,6-*O*-benzyl-2-deoxy-4-*O*-levulinoyl-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 9a.



9a

Protocol A. No reaction observed.

Protocol B. **9a** and **9b** were obtained in 40% and 28% yield, respectively.

Protocol C. **9a** was purified in 31% yield.

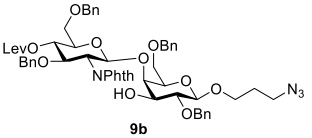
$[\alpha]_D^{25}$ = + 43.28° (c 0.65, $CHCl_3$). ESI HR-MS ($C_{56}H_{60}N_4O_{14}$) *m/z* [*M*+ Na]$^+$ found 1035.3871; calcd 1035.7878.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51-6. 85 (m, 24H, H-Ar), 5.41 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1^B), 5.12 (t, *J* = 9.3 Hz, 1H, H-4^B), 4.66 (d, J= 12.4 Hz, 1H, C*H*HPh$_a$), 4.59-4.36 (m, 7H, 5 x C*H*Ph, H-3^B, H-2^B), 4.33 (d, ^2J = 12.4 Hz, 1H, CH*H*Ph), 4.23 (d, ^2J = 11.1 Hz, 1H, C*H*HPh), 4.20 (d, $J_{1,2}$ = 7.5 Hz, 1H, H-1^A), 4.08 (d, $J_{3,4}$ = 2.9 Hz, 1H, H-4^A), 3.90-3.80 (m, 1H, OCH$_{2a}$),3.73-3.42 (m, 9H, H-5^B, H-6a,b^B, H-6a,b^A, H-2,5,3^A, OCH$_{2b}$) 3.18 (t, *J* = 6.9 Hz, CH_2N$_3$), 2.71-2.68 (m, 2H, CH$_2^{Lev}$), 2.59-2.43 (m, 2H, CH$_2^{Lev}$), 2.18 (s, 3H, CH$_3^{Lev}$), 1.76-1.67 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 206.2, 171.6, 128.42-123.29 (C-Ar), 103.15 (C-1^A), 98.59 (C-1^B), 83.55 (C-3^A), 77.5 (C-5^A), 76.9 (C-3^B), 74.47 (CH$_2$Ph), 74.10 (CH$_2$Ph), 74.16 (CH$_2$Ph), 73.57 (CH$_2$Ph), 73.45 (C-5^B), 73.18 (C-2^A), 72.45 (C-4^B), 69.66 (C-6^B), 69.46 (C-6^A), 67.84 (C-4^A), 66.32 (O*C*H$_2$), 55.42(C-2^B) , 48.17 (*C*H$_2$N$_3$), 37.70 (*C*H$_2^{Lev}$), 29.79 (*C*H$_3^{Lev}$), 29.10 (*C*H$_2$CH$_2$N$_3$), 27.88 (*C*H$_2^{Lev}$).

Azidopropyl 3,6-*O*-benzyl-2-deoxy-4-*O*-levulinoyl-2-phthalimido-*β*-D-glucopyranosyl-(1→4)-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 9b

$[\alpha]_D^{25}$ = + 18.87° (c 1.9, $CHCl_3$). ESI HR-MS ($C_{56}H_{60}N_4O_{14}$) *m/z* [*M*+ Na]$^+$ found 1035.3914; calcd 1035.3878.



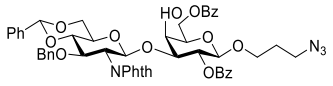
9b

^{1}H NMR (400 MHz, $CDCl_3$) δ) δ 7.23-6.83 (m, 24H, H-Ar), 5.23 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1^B), 5.09 (t, *J* = 9.7 Hz, 1H, H-4^B), 4.59 (d, *J* = 9.5 Hz, 1H, C*H*HPh), 4.54-4.22 (m, 9H, 7 x each C*H*HPh, H-3^B, H-2^B), 4.07 (d, $J_{1,2}$ = 7.7 Hz,1H, H-1^A), 3.84 (d, $J_{3,4}$ = 2.7 Hz,1H, H-4^A), 3.80-3.42 (m, 8H, OCH$_2$, H-6a,b^A, H-6a,b^B, H-5^A, H-5^B), 3.32 (dd, $J_{2,3}$ = 9.7 Hz, 1H, H-3^A), 3.24 (t, *J* = 6.8 Hz, 2H, CH_2N$_3$), 2.87 (t, *J* = 8.6 Hz, 1H, H-2^A), 2.54-2.35 (m, 4 H, CH$_2$CH$_2^{Lev}$), 2.08 (s, 1H, CH$_3^{Lev}$), 1.80-1.67 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.6-122.9 (C-Ar), 103.08 (C-1^A), 99.70(C-1^B), 79.89 (C-2^A), 76.8 (C-3^B), 76.6 (C-4^A), 73.79 (C-5^A), 73.46 (*C*H$_2$Ph) ,73.34 (2 x *C*H$_2$Ph), 72.86 (*C*H$_2$Ph), 72.70 (C-3^A), 72.64 (C-5^B), 69.84 (C-6$^{A/B}$),

69.71 (C-6$^{A/B}$), 66.02 (O*C*H$_2$), 55.68 (C-2^B), 48.35 (*C*H$_2$N$_3$), 37.74 (*C*H$_2^{Lev}$), 29.80 (*C*H$_2^{Lev}$), 29.17 (*C*H$_2$CH$_2$N$_3$), 27.95 (*C*H$_3^{Lev}$).

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 8a



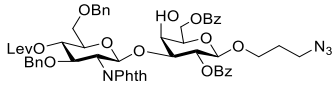
8a

Protocol B. **8a,** 53% yield.
Protocol C. **8a**, 77% yield.
[α]$_D^{25}$ = + 44.98° (c 0.4, CHCl$_3$). ESI HR-MS (C$_{51}$H$_{48}$N$_4$O$_{14}$): *m/z* = [*M*+Na]$^+$ found 963.3200; calcd 963.3065.

^{1}H NMR (400 MHz, CDCl$_3$) δ 8.08-6.64 (m, 24H, H-Ar), 5.53 (s, 1H, C*H*Ph), 5.32 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1^B), 5.22 (t, *J* = 8.9 Hz,1H, H-2^A), 4.67-4.49 (m, 3H, H-6^A, C*H*HPh), 4.37-4.29 (m, 2H, CH*H*Ph, H-1^A), 4.29-4.22 (m, 2H, H-6a^B, H-3^B), 4.18 (dd, $J_{1,2}$ = 8.3 Hz, $J_{2,3}$ = 10.1 Hz, 1H, H-2^B), 4.11 (dd, 1H, $J_{3,4}$ = 2.8 Hz, H-4^A), 3.83-3.68 (m, 5H, H-5^A, H-3^A, H-4^B, H-6b^B, OCH$_{2a}$), 3.62-3.53 (m, 1H, H-5^B), 3.31 (dt, *J* = 4.3, 8.6 Hz, 1H, OCH$_{2b}$), 3.01-2.85 (m, 2H, C*H*$_2$N$_3$), 1.66-1.42 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) δ 166.4-164.5 (2 x C=O), 137.7-122.7 (C-Ar), 101.4 (*C*HPh), 101.2 (C-1^A), 99.9 (C-1^B), 82.7 (C-4^B), 80.8 (C-3^A), 74.2 (C-3^B), 74.0 (*C*H$_2$Ph), 71.9 (C-5^A), 70.5 (C-2^A), 68.6 (C-6^B), 68.5 (C-4^A), 66.3 (C-5^B), 65.9 (O*C*H$_2$), 63.5 (C-6^A), 55.5 (C-2^B), 47.8 (*C*H$_2$N$_3$), 28.9 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 3,6-*O*-benzyl-2-deoxy-4-*O*-levulinoyl-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 10a.



10a

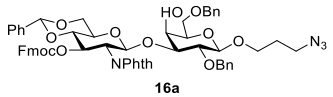
Protocol A. No product formation.
Protocol B. **10a,** 65% yield.
Protocol C. **10a**, 33% yield.
[α]$_D^{25}$ = + 62.78° (c 1.4, CHCl$_3$). ESI HR-MS (C$_{56}$H$_{56}$N$_4$O$_{16}$) *m/z* [*M*+Na]$^+$ found 1063.3577; calcd 1063.3589.

^{1}H NMR (400 MHz, CDCl$_3$) δ 8.05-6.84 (m, 24H, H-Ar), 5.36 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1^B), 5.31 (d, *J* = 8.9 Hz, 1H, H-4^B), 5.08 (t, *J* = 9.1 Hz, 1H, H-2^A), 4.61-4.44 (m, 5H, H-6^A, 3 x C*H*HPh), 4.39 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1^A), 4.36-4.34 (m, 1H, H-3^B), 4.31-4.29 (m, 1H, H-2^B), 4.27-4.24 (m, 2H, H-4^A, CH*H*Ph), 3.86-3.79 (m, 4H, OCH$_{2a}$, H-3,5^A, H-5^B), 3.60-3.58 (m, 2H, H-6^B), 3.41 (dt, *J* = 4.5, 9.2 Hz, 1H, OCH$_{2b}$), 3.20 (t, *J* = 6.5 Hz, 2H, C*H*$_2$N$_3$), 3.10-2.65 (m, 4H, *CH*$_2$*CH*$_2^{Lev}$), 2.16 (s, 3H, CH$_3^{Lev}$), 1.64-1.56 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) δ 166.8, 164.0 (C=O), 133.1-127.3 (C-Ar), 101.13 (C-1^A), 101.40, 98.82 (C-1^B), 81.05, 73.91, 73.49, 72.33, 72.04 (C-2^A), 70.49 (C-4^B), 69.52, 68.07, 65.84 (O*C*H$_2$), 63.73, 55.10 (C-2^B), 47.79 (*C*H$_2$N$_3$, *C*H$_2^{Lev}$), 37.68 (*C*H$_2^{Lev}$), 29.78 (*C*H$_3^{Lev}$), 28.86 (*C*H$_2$CH$_2$N$_3$), 27.84 (*C*H$_2^{Lev}$).

3-Azidopropyl 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9-fluorenylmethyloxycarbonyl)-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 16a.

Protocol A. After flash chromatography (Tol:EtOAc) **16a** and **16b** were purified in 30% and <5% yield, respectively.



16a

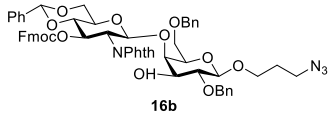
Protocol B. **16a**, 38% yield; **16b**, 26% yield.
[α]$_D^{25}$ = +10.37° (c 0.9, CHCl$_3$). ESI HR-MS (C$_{59}$H$_{56}$N$_4$O$_{14}$) *m/z* [*M*+Na]$^+$ found 1067.3629; calcd 1067.3691.

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63-6.85 (m, 27 H, H-Ar), 5.71 (t, *J* = 10.1 Hz, 1H, H-3^B), 5.62 (d, $J_{1,2}$ = 8.7 Hz, 1H, H-1^B), 5.51 (s, 1H, C*H*Ph), 4.51, 4.48 (2 d, ^2J = 12.3 Hz, 2H, 2 x C*HH*Ph), 4.47 (dd, $J_{2,3}$ = 10.4 Hz, 1H, H-2^B), 4.38 (d, ^2J = 11.7 Hz, 1H, C*H*HPh), 4.31 (dd, $J_{5a,6a}$ = 4.6 Hz, $J_{6a,6b}$ = 10.2 Hz, 1H, H-6^A), 4.17-4.13 (m, 2H, H-1^A, CH*H*Ph), 4.01-3.99 (m, 2H, H-4^A, CH$_2^{Fmoc}$), 3.87-3.81 (m, 2H, H-4^B, CH^{Fmoc}), 3.79-3.61 (m, 5H, H-6$^B_{a,b}$, H-6b^A, OCH$_{2a}$, H-5^B), 3.57 (dd, $J_{3,4}$ = 3.3 Hz, $J_{2,3}$ = 9.5 Hz, 1H, H-3^A), 3.51 (t, *J* = 6.0 Hz,

1H, H-2^{A}), 3.39 (dd, $J_{4,5}$ = 7.7 Hz, $J_{5,6a}$ = 9.2 Hz, 1H, H-5^{A}), 3.36-3.33 (m, 1H, OCH$_{2b}$), 3.06 (dt, *J* = 3.5, 6.8 Hz, 2H, C*H*$_2$N$_3$), 1.64-1.57 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) δ 134.04-119.88 (C-Ar), 103.42 (C-1^{A}), 101.83 (*C*HPh), 99.45 (C-1^{B}), 83.06 (C-3^{A}), 78.91 (C-4^{B}), 77.55 (C-5^{A}), 74.27 (*C*H$_2$Ph), 73.69 (C-3^{B}), 73.49 (*C*H$_2$Ph), 73.38, 72.73 (C-2^{A}), 70.36, 69.02 (C-6^{B}), 68.57 (C-6^{A}), 68.20 (C-4^{A}), 66.48 (O*C*H$_2$), 66.29 (C-5^{B}), 60.42, 55.25 (C-2^{B}), 48.36, 48.12 (C*H*$_2$N$_3$), 46.32 (*C*H^{Fmoc}), 29.08 (C*H*$_2$CH$_2$N$_3$), 28.25, 21.07, 14.21.

3-Azidopropyl 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9-fluorenylmethyloxycarbonyl)-2-phthalimido-*β*-D-glucopyranosyl-(1→4)-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 16b.



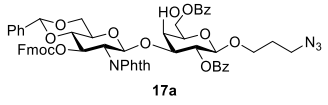
16b

[α]$_D^{25}$ = -8.99° (c 0.85, CHCl$_3$). ESI HR-MS (C$_{59}$H$_{56}$N$_4$O$_{14}$) *m/z* [*M*+Na]$^+$ found 1067.3680; calcd 1067.3691.

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.70-6.94 (m, 27 H, H-Ar), 5.89 (t, *J* = 9.3Hz, 1H, H-3^{B}), 5.51 (s, 1H, C*H*Ph), 5.47 (d, $J_{1,2}$ = 7.8 Hz,1H, H-1^{B}), 4.55-4.48 (m, 3H, 2 CH*H*Ph, H-2^{B}), 4.16-4.08 (m, 3H, 2 CH*H*Ph, includ. d, 4.12, $J_{1,2}$ = 7.7 Hz, H-1^{A}), 3.95 (m, 2H, CH^{Fmoc}, H-4^{A}), 3.89-3.62 (m, 7H, includ. H-4,5,6^{B}, H-6^{A}, OCH$_{2a}$), 3.58 (m, 1H, OCH$_{2b}$), 3.47 (m, 1H, H-5^{A}), 3.36 (dd, $J_{3,4}$ = 2.9, $J_{2,3}$ = 7.2 Hz, 1H, H-3^{A}), 3.30 (m, 2H, CH$_2$N$_3$), 3.00 (dd, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 9.8 Hz, 1H, H-2^{A}), 1.80 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) δ 134.0-119.9 (C-Ar), 103.27 (C-1^{A}), 101.7 (C*H*Ph), 100.4 (C-1^{B}), 79.9 (C-2^{A}), 79.1 (C-4^{B}), 77.2 (C-4^{A}), 74.9 (*C*H$_2$Ph), 73.6 (C-3^{B}), 73.4 (*C*H$_2$Ph), 72.8 (C-3^{A}), 70.2 (C-5$^{A/B}$), 68.8, 68.6 (C-6^{A,B}), 66.2 (O*C*H$_2$), 65.4 (C-5$^{A/B}$), 55.3 (C-2^{B}), 48.4 (*C*H$_2$N$_3$), 46.4 (*C*H^{Fmoc}), 29.2 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-3-*O*-(9-fluorenylmethyloxycarbonyl)-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 17a.



17a

Protocol A. **17a**, 40% yield.

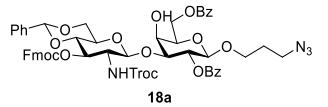
Protocol B. **17a**, 68% yield.

[α]$_D^{25}$ = + 36.44° (c 0.65, CHCl$_3$). ESI HR-MS (C$_{59}$H$_{52}$N$_4$O$_{16}$) *m/z* ([*M*+Na]$^+$ found 1095.3247; calcd 1095.3276.

^{1}H NMR (400 MHz, CDCl$_3$) δ 8.01-7.07 (m, 27 H, H-Ar), 5.62-5.57 (m, 1H, H-3^{B}), 5.56 (d, $J_{1,2}$ = 8.5 Hz, 1H, H-1^{B}), 5.50 (s, 1H, C*H*Ph), 5.27 (t, *J* = 9.1 Hz, 1H, H-2^{A}), 4.63 (dd, $J_{5,6a}$ = 11.0 Hz, $J_{6a,6b}$ = 5.0 Hz, 1H, H-6a^{A}), 4.55 (dd, $J_{5,6a}$ = 11.0 Hz, $J_{6a,6b}$ = 4.9 Hz, 1H, H-6b^{A}), 4.41 (t, *J* = 9.4 Hz, 1H, H-2^{B}), 4.34 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1^{A}), 4.31-4.27 (d, $J_{6a,6b}$ = 12.0 Hz, 1H, H-6a^{B}), 4.15 (d, $J_{3,4}$ = 3.2 Hz, 1H, H-4^{A}), 3.93 (d, *J* = 7.8 Hz, 2H, CH$_2^{Fmoc}$), 3.85-3.59 (m, 7H, H-3,5^{A}, H-4,5^{B}, H-6b^{B}, CH^{Fmoc}, OCH$_{2a}$), 3.36-3.30 (m, 1H, OCH$_{2b}$), 3.01-2.87 (m, 2H, C*H*$_2$N$_3$), 1.63-1.43 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) δ 166.4, 164.6, 154.3 (3 x C=O), 133.78-119.84 (C-Ar), 101.84 (*C*H$_2$Ph), 101.24 (C-1^{A}), 99.67 (C-1^{B}), 81.09 (C-4^{B}), 78.77 (C-3^{A}), 73.23, 71.88 (C-5^{A}), 70.48 (*C*H$_2^{Fmoc}$), 70.33 (C-2^{B}), 68.52 (C-4^{A}), 68.47 (C-6^{B}), 66.34 (C-5^{B}), 65.99 (O*C*H$_2$), 63.38 (C-6^{A}), 54.90 (C-2^{B}), 47.76 (*C*H$_2$N$_3$), 46.25 (*C*H^{Fmoc}), 28.86 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 4,6-*O*-benzylidene-2-deoxy-3-*O*-(9H-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 18a.



18a

Protocol B. **18a,** 65% yield.

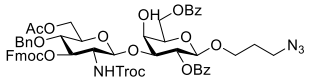
*Protocol C.***18a**, 70% yield.

[α]$_D^{25}$ = -10.29° (c 0.55, CHCl$_3$). ESI HR-MS (C$_{54}$H$_{51}$Cl$_3$N$_4$O$_{16}$) *m/z* ([*M*+ Na]$^+$ found 1134.2173; calcd (1134.2138).

^{1}H NMR (400 MHz, CDCl$_3$) δ 8.17-7.10 (m, 23H, Ar-H), 5.56-5.48 (m, 2H, C*H*Ph, H-2^{A}), 5.24 (t, *J* = 10.0 Hz, 1H, H-3^{B}), 5.09 (d, $J_{N,H}$ = 8.3 Hz, 1H, NH), 4.99 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1^{B}), 4.73 (dd, *J* = 4.9, 11.4 Hz, 1H, C*H*HCCl$_3$),

4.65 (dd, 1H, CH*H*CCl$_3$), 4.55 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1^A), 4.37-4.25 (m, 4H, CH$_2^{Fmoc}$, H-6a^A, H-6a^B), 4.25-4.15 (m, 2H, CH^{Fmoc}, H-5^B), 4.09 (d, $J_{6a,6b}$ = 12.0 Hz, 1H, H-6b^A), 4.03-3.91 (m, 3H, incl. OCH$_{2a}$, H-3^A, H-4^A), 3.86-3.70 (m, 3H, H-2^B, H-6b^B, H-4^B), 3.69-3.48 (m, 2H, H-5^A ,OCH$_{2b}$), 3.26-3.13 (m, 2H, CH$_2$N$_3$), 2.05-1.51 (m, 2H, C*H*$_2$CH$_2$N$_3$). ^{13}C NMR (101 MHz, CDCl$_3$) δ 165.3, 155.0 (2 x CO), 143.0-120.1 (C-Ar), 101.6 (*C*HPh), 101.6 (C-1^A), 101.3 (C-1^B), 80.6 (C-4^A), 78.3 (C-4^B), 74.1 (C-3^B), 73.9 (C-6^A), 72.1 (C-3^A), 70.9 (C-2^A), 70.4 (*C*H$_2^{Fmoc}$), 68.7 (C-5^B), 68.4 (C-6^B), 66.4 (O*C*H$_2$), 63.4 (*C*H$_2$CCl$_3$), 57.2 (C-5^A), 47.9 (*C*H$_2$N$_3$), 46.5 (*C*H^{Fmoc}), 29.6 (*C*H$_2$CH$_2$N$_3$).

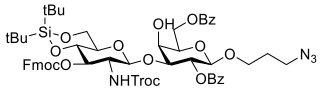
3-Azidopropyl 6-*O*-acetyl-4-*O*-benzyl-2-deoxy-3-*O*-(9H-fluoren-9-ylmethyl carbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 19a.



19a

Protocol C: **19a**, 50% yield.
[α]$_D^{25}$ = +2.96° (c 0.6, CHCl$_3$). ESI HR-MS (C$_{56}$H$_{55}$Cl$_3$N$_4$O$_{17}$) *m/z* ([*M*+ K]$^+$ found 1199.2210; calcd 1199.2259.
^{1}H NMR (400 MHz, CDCl$_3$) δ 8.14-7.31 (m, 23H, Ar-H), 5.52 (dd, $J_{2,3}$ = 9.4 Hz, $J_{3,4}$ = 8.0 Hz, 1H, H-2^A), 5.19-5.08 (m, 2H, NH, H-3^B), 4.86 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1^B), 4.76-4.63 (m, 4H, C*H*$_2$Ph, C*H*HCl$_3$), 4.56 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1^A), 4.51 (d, *J* = 11.3 Hz, 1H, C*H*HCl$_3$), 4.44-4.30 (m, 4H), 4.24 (d, $J_{3,4}$ = 2.6 Hz, 1H, H-4^A), 4.18-4.12 (m, 2H), 4.04 (d, $J_{6a,6b}$ = 12.3 Hz, 1H, H-6), 4.00-3.90 (m, 3H), 3.66-3.51 (m, 4H), 3.25-3.17 (m, 2H, CH$_2$N$_3$), 2.06 (s, 3H, CH$_3$CO), 1.83-1.66 (m, 2H, C*H*$_2$CH$_2$N$_3$).
^{13}C NMR (101 MHz, CDCl$_3$) δ 133.4-120.2 (C-Ar), 101.1 (C-1^A), 100.6 (C-1 ^{B}), 81.4, 78.6 (C-2^A), 74.7, 73.9, 72.3, 70.8 (C-3^B), 70.3, 68.3 (C-4^A), 66.2, 63.5, 62.5, 62.0, 48.1 (OCH$_2$), 46.7 (CH^{Fmoc}), 29.2 (*C*H$_2$CH$_2$N$_3$), 21.6 (COCH$_3$).

3-Azidopropyl 4,6-*O*-di-*tert*-butylsilylidene-2-deoxy-3-*O*-(9H-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl-(1→3)-2,6-di-O-benzoyl-*β*-D-galactopyranoside 20a.



20a

A solution of donor **20** (0.330 g, 0.38 mmol) and acceptor **11** (150 mg, 0.316 mmol) with activated 4 Å molecular sieves (0.2 g) in dry DCM (5 mL) was stirred for 20 min under nitrogen. TMSOTf (11.4 µL, 0.063 mmol) was added at 0°C. After 4 h (TLC; Tol:EtOAc 8:2) the reaction was quenched with TEA, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol:EtOAc 8:2) to afford disaccharide **20a** (220 mg, 62% yield) as a pale solid.
[α]$_D^{25}$ = -33.62° (c 0.2, CHCl$_3$). ESI HR-MS (C$_{55}$H$_{63}$Cl$_3$N$_4$O$_{16}$Si) *m/z* [*M*+ Na]$^+$ found 1191.2927; calcd 1191.2966. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.12-7.28 (m, 18H, Ar-H), 5.49 (dd, $J_{1,2}$ = 9.6 Hz, $J_{2,3}$ = 8.1 Hz, 1H, H-2^A), 5.09-5.00 (m, 2H, H-3^B, NH), 4.99-4.81 (m, 2H, incl. H-1^B, C*H*HCCl$_3$), 4.73-4.56 (m, 3H, incl. CH$_2^{Fmoc}$ CH*H*CCl$_3$), 4.53 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1^A), 4.41-4.19 (m, 4H, incl. CH^{Fmoc}, H-6a^B, H-6b^B), 4.17 (d, $J_{3,4}$ = 2.9 Hz, 1H, H-4^A), 4.13 (dd, 1H, $J_{5,6a}$ = 5.4 Hz, $J_{6a,6b}$ = 10.6 Hz, H-6), 4.06-3.81 (m, 5H, incl. H-3^A, CH$_{2a}$), 3.66-3.41 (m, 3H, incl. H-2^B, CH$_{2b}$), 3.31-3.15 (m, 2H, CH$_2$N$_3$), 1.86-1.62 (m, 2H, C*H*$_2$CH$_2$N$_3$), 1.03 (s, 9H, tBu), 0.92 (s, 9H, tBu).
^{13}C NMR (101 MHz, CDCl$_3$) δ 166.4, 165.2, 155.1, 154.6 (4 x CO), 143.4-120.1 (C-Ar), 101.3 (C-1^A), 101.1 (C-1^B), 100.0 (C$_q$), 80.3, 77.9, 77.2, 75.5, 75.1, 74.9, 73.8, 73.7, 72.0, 71.9, 70.6, 70.4, 68.6, 66.4, 66.2, 66.0, 63.4, 56.6 (C-2^B), 47.9 (O*C*H$_2$), 46.5 (*C*H^{Fmoc}), 29.0 (*C*H$_2$CH$_2$N$_3$), 27.3 (tBu), 26.8 (tBu), 22.7 (*C*(CH$_3$)$_3$), 19.9 (*C*(CH$_3$)$_3$).

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Chapter 4

Synthesis of GBS CPS serotype Ia oligosaccharides library

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Introduction

GBS is Gram positive bacterium causing infections in infants and newborns, having as main clinical manifestations sepsis, pneumonia and meningitis, as extensively described in Chapter 2.[1] Intrapartum Antibiotic Prophylaxis (IAP) is the chosen strategy to combat GBS infections in developed countries, but there are rising concerns about antibiotic resistance[2]. For this reason, a GBS vaccine represents an appealing alternative. The GBS capsular polysaccharide (CPS) is a main virulence factor and is a promising target for vaccine development[1, 3, 4].

On the basis of the serology and the structure of the capsular polysaccharide, ten GBS serotypes have been described (Ia, Ib, II-IX)[4-6]. All identified CPSs are high-molecular weight polymers with a sialic acid residue on the side chain; among them, type Ia, Ib, and III CPS share all the same sugar residues and differ only for one linkage position[7]. The most studied of GBS polysaccharides is type III. This CPS is known to form a helical structure[8, 9], and this feature has an impact on epitope exposure[10].

In the past, serotypes Ia, Ib, II, and III were equally prevalent in normal vaginal carriage and early-onset sepsis (developing at less than 7 days of age). However, recent European studies (2008-2010) showed that serotype Ia, III and V together accounted for 88%, 96%, and 67% of strains isolated from neonates with early-onset disease, neonates with late-onset disease, and vaginal-rectal swabs of colonized pregnant women, respectively[11]. These results reaffirm the clinical importance of types Ia and III[12-14].

Furthermore, serotype Ia, together with serotype V, is responsible of most GBS infections in elderly and immunocompromised patients[14]. Given its clinical relevance, a monovalent GBS Ia polysaccharide CRM-conjugate vaccine has been tested in clinical trials[15, 16], as well as a trivalent formulation including type Ia, Ib and III polysaccharide[17]. These vaccines proved to be safe, well-tolerated and able to induce serotype-specific IgGs.

Despite this, GBS polysaccharide vaccines, as many other CPS-based glycoconjugate vaccines, present some issues. Among them, the difficult analytical characterization due to their heterogeneous composition and the potential presence of bacterial contamination which generates quality control issues and safety concerns[18, 19].

Structurally defined glycoconjugate vaccines composed of synthetic oligosaccharide antigens represent an attractive solution to address this problem. Furthermore, availability of synthetic fragments representing parts of the bacterial CPS allows to investigate the structure-immunogenicity relationship for vaccine optimization.

In 2015, Guo et al. published the first synthesis of the repeating unit of GBS type Ia[20]. Recently, the same group extended the synthetic approach to the synthesis of the dimer (DP2) and prepared CRM_{197} conjugates of both oligosaccharide structures in order to evaluate their immunogenicity[21]. However, in order to design efficacious well-defined semisynthetic vaccines, information on the identity of protective glycotopes expressed on the bacterial CPS are required[18, 22]. So far, no structural studies of GBS type Ia oligosaccharides have been carried out to shed lights on the

carbohydrate epitopes expressed on the corresponding CPS. Indeed, such study relies on the availability of structurally defined oligosaccharides, but neither enzymatic nor chemical methods are currently available to depolymerize type Ia CPS in order to obtain short fragments. Chemical synthesis is therefore the only tool able to provide the target glycans from GBS type Ia capsular polysaccharide. It is to be noted that in a polysaccharide there is a plethora of potential glycotopes and this challenges traditional chemical synthesis because a number of different structures are needed[23]. Having an expeditious and efficient approach to the synthesis of these fragments is key to determine the structure-immunogenicity relationship.

To this aim, this Chapter describes the application of the regioselective synthesis of the key synthon GlcNAc-β-(1→3)-Gal*p* (Chapter 3) to prepare a library of GBS serotype Ia related oligosaccharides. In particular, using an innovative synthetic tactic the repeating unit (both in the linear and branched frameshifts) was prepared, together with fragments longer than the single repeating unit.

These fragments can be used in structural studies, comprising competitive Surface Plasmon Resonance (SPR), STD-NMR and competitive ELISA, to gain molecular insights into the sugar-mAb interactions and help to identify glycotopes expressed on the bacterial capsular polysaccharide. Moreover, availability of oligosaccharide structures from both GBS type Ia and Ib (whose synthesis will be described in Chapter 5) could also help understanding what are the features that cause (despite the very close similarities of these two repeating units as they only differ for the connection between a galactose and the glucosamine) the productions of serotype-specific monoclonal antibodies in response to monovalent and multivalent conjugated vaccines[7] (immunospecificity).

Results and Discussion

Synthesis of oligosaccharide fragments

The repeating unit of GBS serotype Ia is a pentasaccharide structurally very similar to the one of GBS type Ib and III, with a α-(2→3)-sialylated disaccharide side chain and a trisaccharide backbone[7].

On the basis of the structure of the CPS, a small library of target glycans with an increasing structural complexity was designed (Figure 1). This library comprises: the pentasaccharide repeating unit of GBS type Ia both in the branched (**1**) and in the linear frameshift forms (**2**), the non-sialylated tetrasaccharide **3**, the two hexasaccharide structures **4** and **5** (which will be called Y-shape and branched hexasaccharide from now on), and the heptasaccharide **6**. All glycans were designed with an aminopropyl linker at the reducing end amenable for future conjugation to a carrier protein.

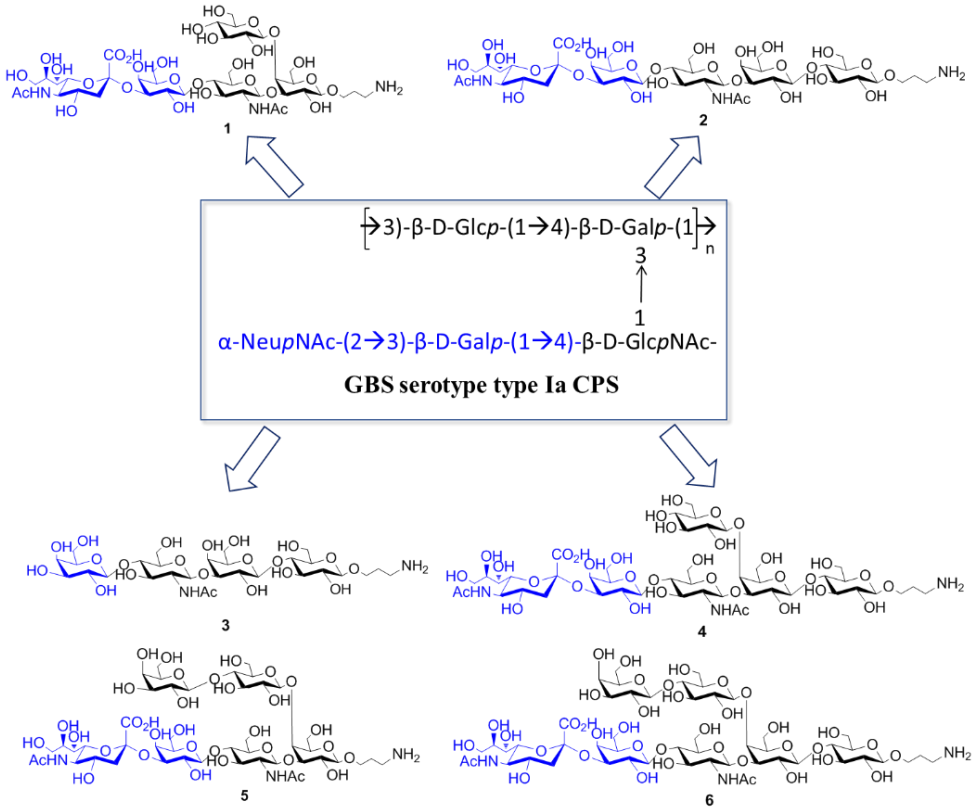
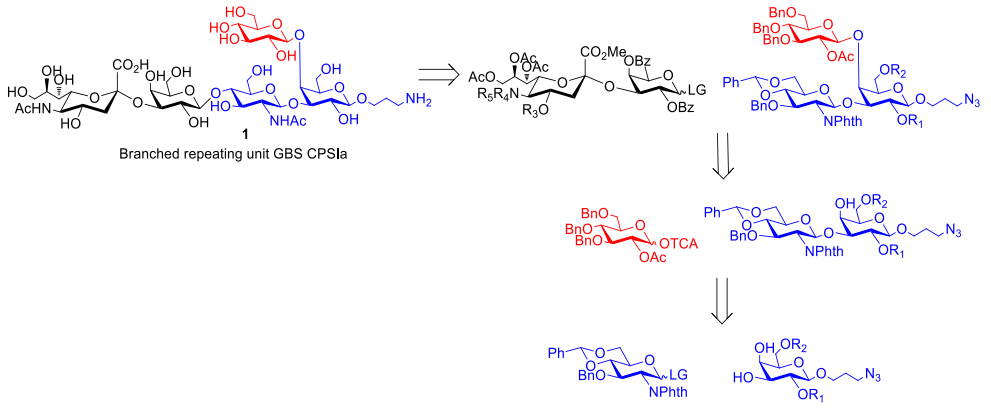


Figure 1. Structure of the GBS type Ia capsular polysaccharide and the library of related glycans to be synthesized.

Starting from the preparation of the pentasaccharide **1**, two main challenges were identified:
(i) the installation of the α-sialic acid connection to the upstream Gal residue. To address this, a convergent [3+2] regioselective approach employing a sialogalactoside donor and a trisaccharide acceptor was chosen.
(ii) the synthesis of the branched trisaccharide Glc*p*NAc-β-D-(1→3)-Gal*p*-β-D-(1→4)-Glc*p* acceptor in an efficient and expeditious way. To this end, the trisaccharide acceptor was prepared starting from the disaccharide synthon Glc*p*NAc-β-D-(1→3)-Gal*p* obtained by means of a regioselective β-(1→3) galactose glycosylation (see Chapter 3). Using this approach allowed to reduce the number of protecting groups employed in the synthesis, as well as the protection/deprotection steps. The glycosylation partners to provide the disaccharide were chosen from the glucosamine donors bearing a temporary protecting group at C-4 and galactose acceptors screened in Chapter 3.



Scheme 1. Retrosynthetic approach to pentasaccharide **1**

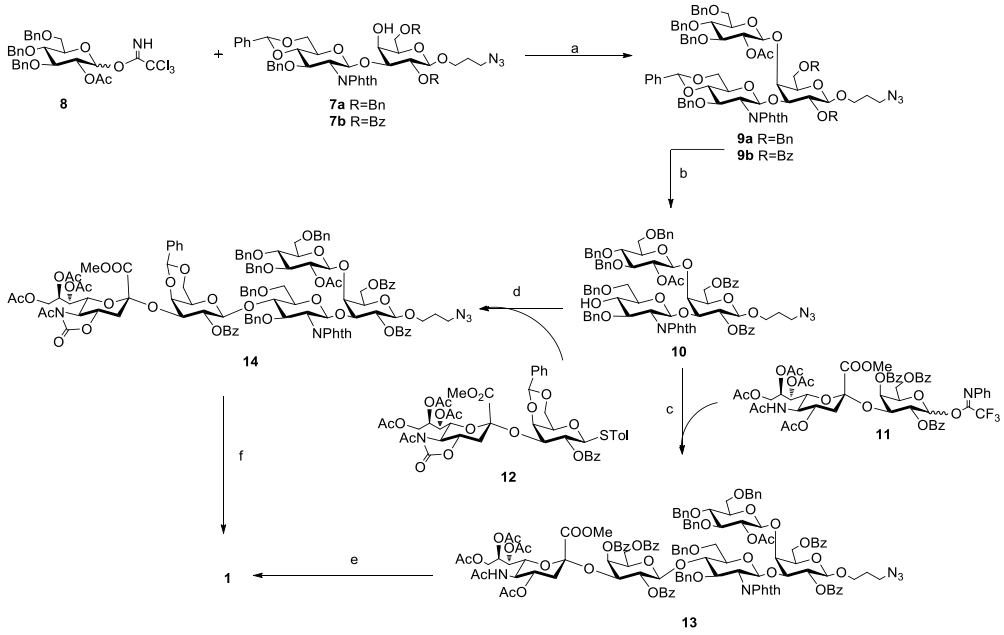
According to our retrosynthetic design (Scheme 1) the target glycan **1** can be obtained through a [2+3] convergent strategy based on the glycosylation of a suitable trisaccharide acceptor with a Neu5Acα2-3Gal donor. This approach envisages the challenging stereoselective α-sialylation of the upstream galactose at an early stage of the synthesis. In this design fast and efficient access to a GlcNAcβ1-3Gal disaccharide building block plays a central role to obtain the trisaccharide acceptor without a temporary protection at Gal 4-OH for further assembly of GBS CPS Ia fragments.

Having identified two disaccharide synthons obtained through an optimized regioselective glycosylation (Chapter 3), the retrosynthesis to the pentasaccharide repeating unit of GBS CPS Ia could be put into practice (Scheme 1). To this end, glucose donor **8**[24] was reacted with benzylated disaccharide **7a** and the benzoylated counterpart **7b** to furnish trisaccharides **9a** and **9b** in 75% and 68% yield, respectively (See Scheme 2).

Despite the deactivating effect of the 6-*O*-benzoyl ester compared to the 6-*O*-benzyl ether, the reaction proceeded with almost identical efficiency using the imidate donor **8**. In contrast, a peracetylated trichloroacetimidate glucose donor with TMSOTf activation was ineffective for glycosylation of the 4-OH. Considering the higher regioselectivity and yield achieved in making disaccharide **7b**, the resulting trisaccharide **9b** was advanced for the construction of the GBS CPS Ia repeating unit and subjected to regioselective opening of the 4,6-*O*-benzylidene acetal with $BF_3{\cdot}Et_2O$ and $Me_3N{\cdot}BH_3$ to provide the acceptor **10** (70%). In order to complete the pentasaccharide construction, the elongation of trisaccharide acceptor **10** with the known donors sialogalactosyl trifluoroacetimidate **11**[25, 26] and thioglycoside **12**[27] were tested. Of these two disaccharides, **12** can be prepared with a higher stereoselective control, whereas **11** is easily accessible from a commercial disaccharide precursor[25].

Glycosylation of trisaccharide **10** with **11** under TMSOTf activation gave the protected pentasaccharide **13** in 75% yield. The use of disaccharide **12** in presence of NIS/TfOH led to the protected pentasaccharide **14** in a similar yield (73%). Pentamer **13** was deprotected by a five-step procedure[28], including (i) removal of the methyl ester of Neu5Ac with lithium iodide in pyridine;

(ii) reaction with ethylenediamine in refluxing ethanol for concomitant removal of the *O*-acetates and the *N*-Phth protecting group; (iii) reacetylation with acetic anhydride/pyridine to install the acetamide group of the GlcNAc residue along with acetyl esters; (iv) methanolysis and (v) final catalytic hydrogenation over Pd/charcoal to provide the crude pentasaccharide **1**. After purification by size-exclusion chromatography, target pentasaccharide **1** was isolated in 40% yield. Pentamer **14** was deprotected by a three steps procedure comprising i) saponification with NaOH in refluxing THF, ii) amine reacetylation using a 2:3 acetic anhydride/methanol mixture, iii) hydrogenation over Pd/charcoal to afford crude pentasaccharide **1**. After purification by size exclusion chromatography, the target pentasaccharide **1**, equipped with an aminopropyl linker, was isolated in a yield of 45%. Yields of **1** obtained via fully protected **13** and **14** was estimated by spectrophotometric quantification of the sialic acid content[29].

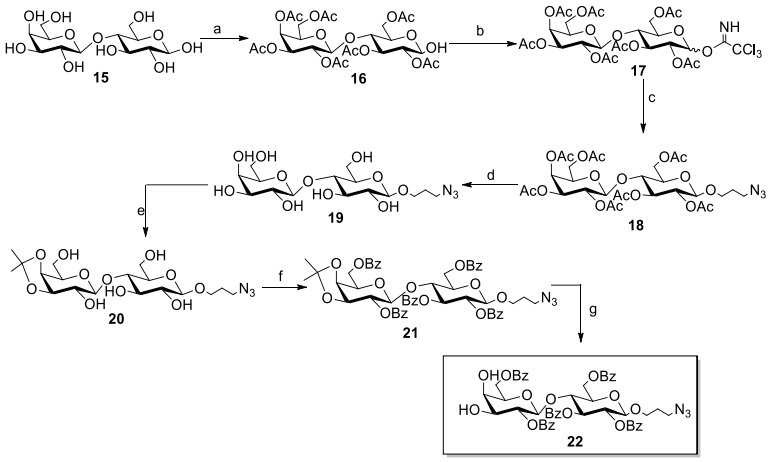


Scheme 2. Assembly of GBS CPSIa repeating unit. *Reagents and conditions*: a) TMSOTf, DCM dry, -10°C, 75% from **7a**; 68% from **7b**; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 70% ; c) TMSOTf, DCM dry, 0°C, 75%; d) TfOH, NIS, DCM dry, -40°C, 73%; e) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 40% (over five steps) ; f) 3M NaOH, THF, reflux; Ac_2O, MeOH, H_2, Pd-C, 45% (over three steps).

Next, a similar regioselective approach was employed for the synthesis of the linear frameshift of the serotype Ia repeating unit **2** and to its non-sialylated tetrasaccharide counterpart **3** (Scheme 4). To achieve this, the benzoylated lactose acceptor **22** and the glucosamine donor **23** were chosen as glycosylation partners to obtain a linear trisaccharide synthon common to both target structures. Lactose **22** was selected as acceptor because the benzoyl protecting groups present decreases the nucleophilicity of the axial 4-OH of galactose, making this hydroxyl group completely unreactive

towards the glucosamine donors, thereby enhancing the regioselectivity of the glycosylation reaction at C-3, as previously demonstrated (Chapter 3).

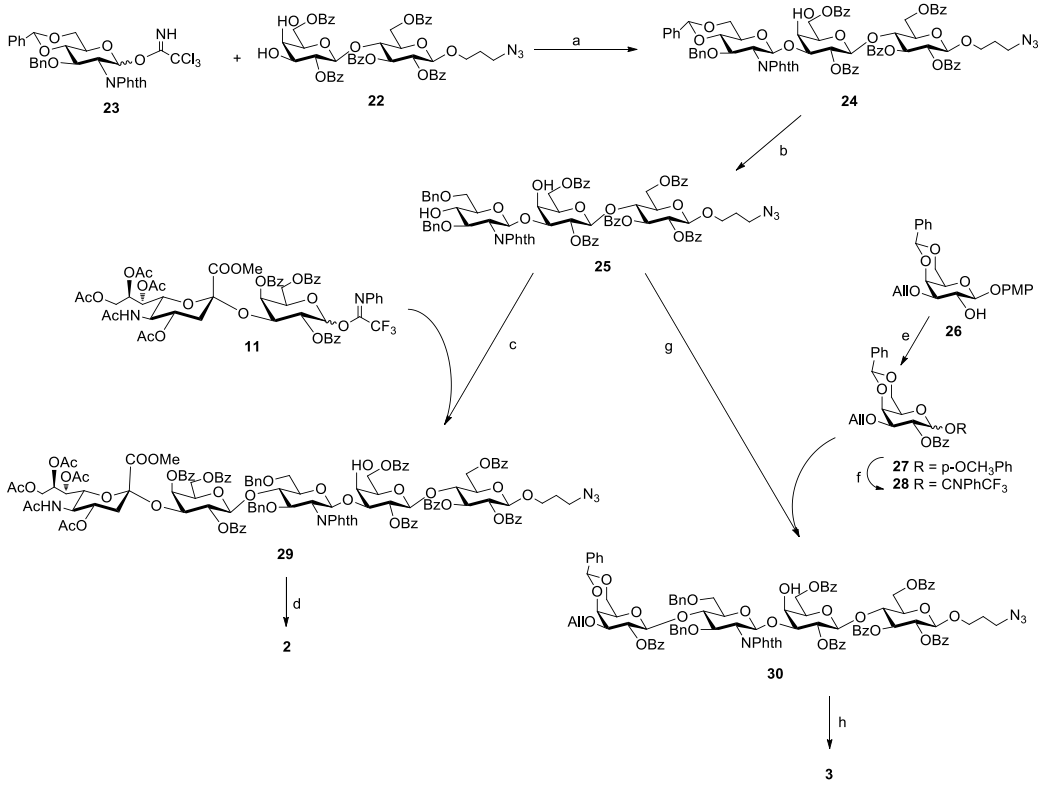
The preparation of lactose acceptor **22** is depicted in Scheme 3 and starts with the acetylation of commercially available D-Lactose (**15**) with acetic anhydride and pyridine, followed by the selective removal of the anomeric acetyl group with ethylenediamine and acetic acid in THF. Conversion of obtained **16** in trichloroacetimidate **17** (2:1 α/β mixture) and subsequent TMSOTf mediated coupling of **17** with the azidopropanol linker afforded acetylated dimer **18**. De-*O*-acetylation using Zemplen conditions and installation of the isopropylidene protective group by dissolving **19** in 2,2-dimethoxypropane in the presence of catalytic amount of *p*-toluenesulfonic acid followed by treatment with 9:1 MeOH: H_2O at 90°C gave compound **20**. Benzoylation of compound **20** using benzoyl chloride and pyridine and, finally, removal of the isopropylidene group afforded the target lactose building block **22** with the free 3-OH and 4-OH positions of the galactose.



Scheme 3. Synthetic pathway to lactose **22**. *Reagents and conditions*: a) Ac_2O/Py; ethylenediamine, AcOH, THF, 75% over two steps; b) CCl_3CN, DBU, DCM dry, 57%; c) azidopropanol, TMSOTf, DCM dry, 74%; d) MeONa/MeOH, 85%; e) *p*-toluenesulfonic acid, 2,2-dimethoxypropane, DMF; MeOH/H_2O, 90°C, 57% over two steps; f) BzCl/Py, 71%; g) AcOH/H_2O, 90°C, 70%.

In line with previous results, glycosylation of lactose **22** and glucosamine **23** afforded the linear trisaccharide acceptor **24** in 68% yield with complete regioselectivity (Scheme 4). Following benzylidene opening in **24**, the coupling of trisaccharide acceptor **25** with both donor **11**[26] and **12**[27] was tested. Reaction of **11** with **24** under influence of TMSOTf at 0°C afforded the target linear pentasaccharide **29** in 65% yield. To demonstrate the regioselectivity the presence of the free galactose 4-OH throughout all stages of the synthesis, from trisaccharide **24** to pentasaccharide **29**, was monitored by following the signal of the Gal H-4, which appeared at 3.97 ppm (d, J = 2.7 Hz) in the ^{1}H NMR and HSQC of all synthetic intermediates. Unexpectedly,

reaction of **25** with the tolyl thioglycoside **12** under NIS/TfOH activation at -40°C was unsuccessful and yielded only traces of the corresponding pentasaccharide, while mainly decomposition of the glycosyl donor was observed, as revealed by LC-MS analysis. The linear pentasaccharide **29** was subjected to the five-step deprotection protocol previously described for compound **13** and after purification by size exclusion chromatography the target oligosaccharide **2** was obtained in 33% overall yield (Scheme 4). Acceptor **25** was also used to obtain a non-sialylated CPS Ia linear fragment for future epitope mapping studies by glycosylation (72% yield) with the trifluoroacetimidate **27**, prepared from the known lactol **26**[28]. After global deprotection tetrasaccharide **3** was obtained in 42% yield (Scheme 4).



Scheme 4. Assembly of linear GBS PSIa fragments **2** and **3**. *Reagents and conditions:* a) TMSOTf, DCM dry, -10°C, 68%; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 65%; c) TMSOTf, DCM dry, 0°C, 65%; d) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 33% (over five steps); e) BzCl, Py, 0° to rt, 40%; f) CAN, ACN/water 4:1, 0°C, 77%; TFACl, Cs_2CO_3, DCM, 61%; g) TMSOTf, -20°C, DCM, 72%; h) $PdCl_2$, MeOH; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 42% (over five steps).

NMR data of the synthesized fragments **1** and **2** showed an excellent agreement with CPS Ia samples (see Table 1 and Figure 2)[30].

Table 1. Chemical shift (ppm) of ^{1}H and ^{13}C NMR signals of compound **1-2** in D_2O

Residue		**Compound 1**		**Compound 2**		**PS Ia**[a]	
		^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
Gal	1	4.39/ *J* 8.1 Hz	103.1	4.43/J 8.2	103.8	4.44	102.3
	2	3.66	70.0	3.57	70.7	3.61	71.2
	3	3.78	82.2	3.72	82.9	3.73	82.5
	4	4.37	74.8	4.16	69.1		
	5	3.68	74.2	3.66	75.1	3.65	73.6
	6	3.72	60.8	3.65	63.2		
	6’	3.72		3.88			
GlcNAc	1	4.69/ *J* 8.4 Hz	103.1	4.69/J 8.2 Hz	103.7	4.70	102.6
	2	3.79	55.3	3.81	55.9	3.75	56.0
	3	3.71	72.1	3.73	72.8	3.62	72.9
	4	3.73	78.2	3.76	78.5	3.63	78.7
	5	3.58	74.6	3.72	75.7	3.48	75.3
	6	3.98	60.1	3.95	68.2		
	6’	3.85		3.95			
Glc	1	4.88/ *J* 7.7 Hz	102.0	4.50/J 8.5 Hz	102.7	4.89	103.2
	2	3.26	73.6	3.32	73.5	3.20	74.2
	3	3.51	75.9	3.64	75.4	3.45	75.3
	4	3.37	69.7	3.65	78.6	3.52	78.8
	5	3.42	75.6	3.66	75.4	3.59	75.0
	6	3.89	60.7	3.81	60.6		
	6’	3.74		3.96			
Gal’	1	4.54/ *J* 8.4 Hz	102.5	4.56/J 9.0 Hz	103.0	4.56	102.4
	2	3.55	69.3	3.57	70.2	3.46	70.1
	3	4.09	75.5	4.12	76.2	4.06	76.2
	4	3.94	67.5	3.92	68.8	3.84	68.2
	5	3.70	75.2	3.71	75.6	3.61	75.9
	6	3.66	60.8	3.74	61.9		
	6’	3.66		3.71			
Neu5Ac	3	2.74	39.6	2.76	40.4	2.72	40.1
	3’	1.78		1.80		1.77	
	4	3.67	68.3	3.68	69.1	3.57	69.0
	5	3.83	51.7	3.85	52.4	3.79	52.2
	6	3.61	72.9	3.62	73.7	3.53	73.7
	7	3.57	68.1	3.65	68.8	3.49	68.8
	8	3.88	71.8	3.87	72.6	3.84	72.6
	9	3.84	62.6	3.88	63.3	3.77	63.3
	9’	3.62		3.65		3.54	

[a]. Not assigned peaks are due to overlapping in the HSQC spectrum of the polysaccharide

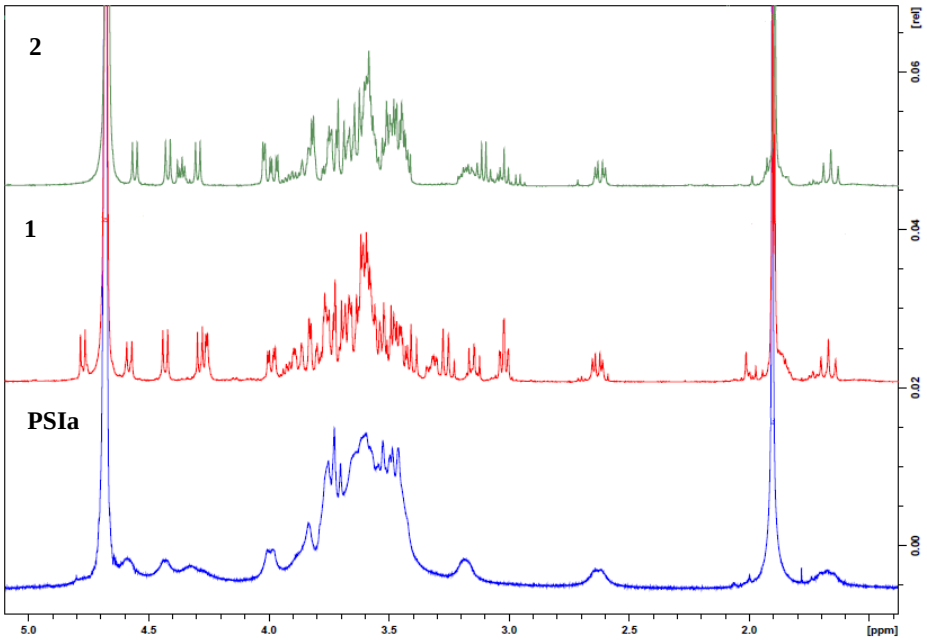
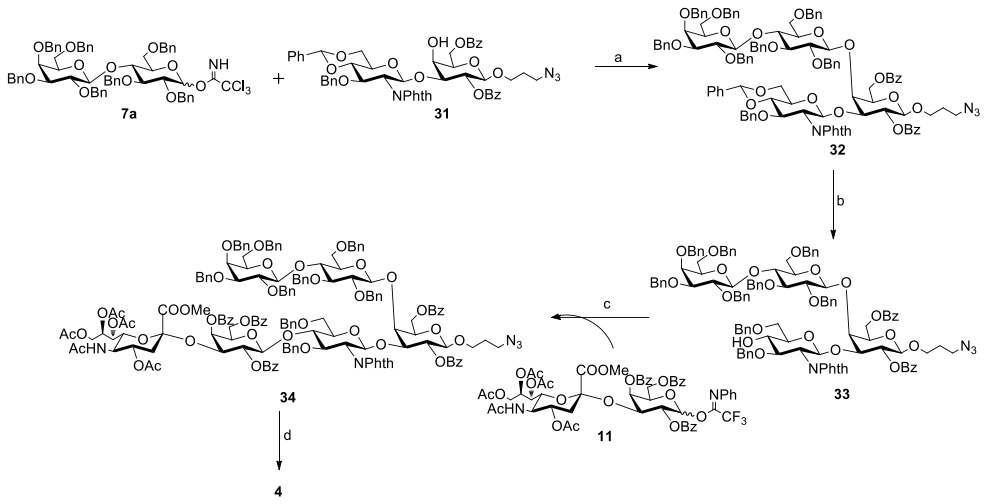


Figure 2. ^{1}H NMR spectra of pentasaccharides **1**–**2** in comparison to PSIa (D_2O, 400 MHz, 298 k)

Once the conditions for the synthesis of GBS PSIa repeating unit were optimized, the key synthons, disaccharide **7a** and trisaccharide **24**, were used to prepare the hexasaccharides **4** and **5** and the heptasaccharide **6** (Scheme 5 and 6).

To prepare the branched hexasaccharide **4,** disaccharide **7a** was condensed with lactose acceptor **31**, affording tetrasaccharide **32** in 60% yield (Scheme 5). Regioselective opening of the benzylidene ring in **32** to give tetrasaccharide acceptor **33**, with the unmasked glucosamine 4-OH, was followed by glycosylation with imidate donor **11** affording the protected hexasaccharide **34** in 78% yield. Compound **34** was subjected to the previously described 5-step deprotection protocol to provide the target branched hexasaccharide **4** in 35% yield.

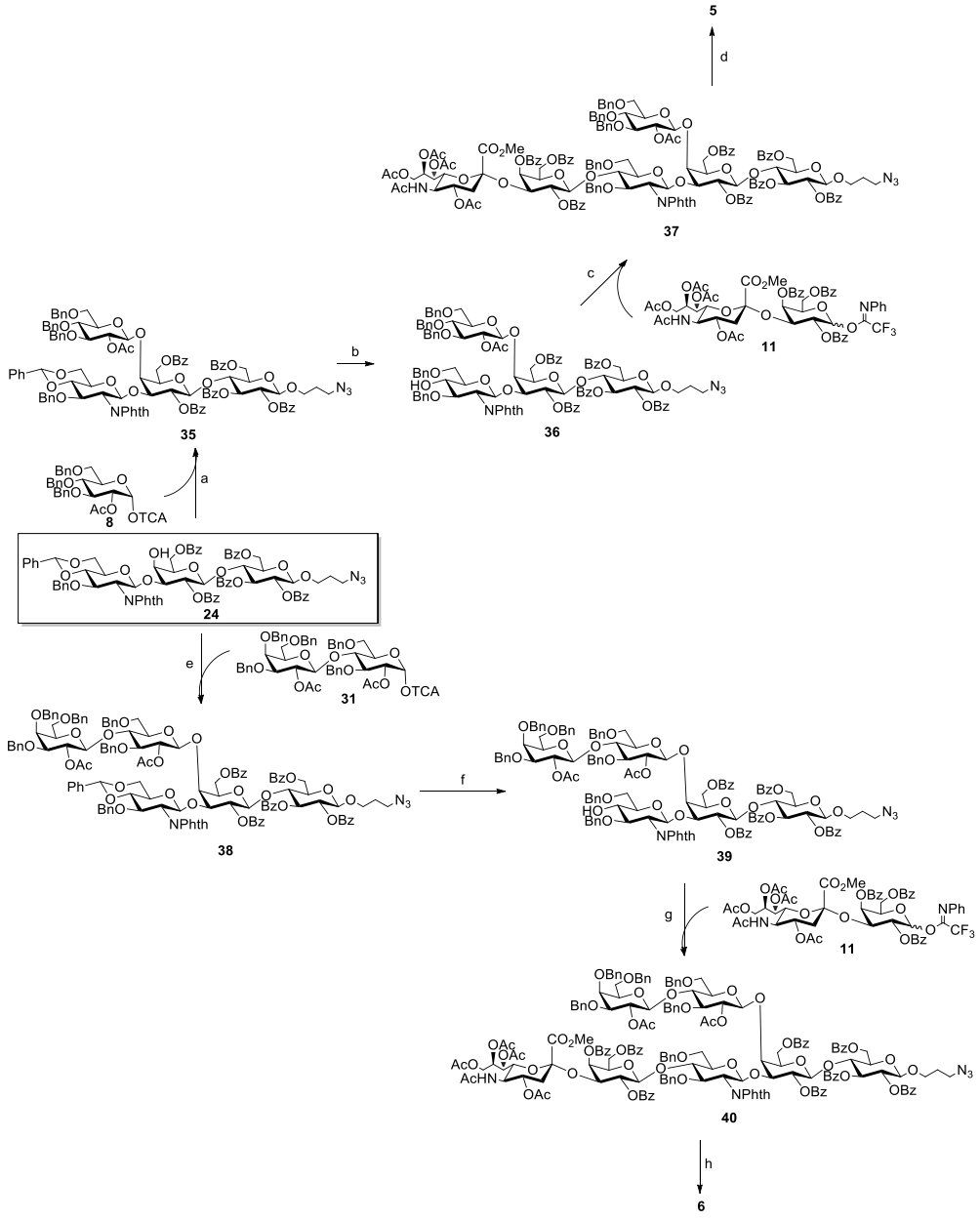


Scheme 5. Synthetic pathway to hexasaccharide **4**. *Reagents and conditions*: a) TMSOTf, DCM dry, 60%; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 52%; c) TMSOTf, DCM dry, 78%; d) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 35% (over five steps).

Finally, the synthesis of the Y-shape hexasaccharide **5** and of the heptasaccharide **6** was accomplished starting from the common linear trisaccharide synthon **24** as depicted in Scheme 6. To obtain Y-shaped **5**, the trisaccharide was glycosylated with glucosyl imidate **8**, affording tetrasaccharide **35** in 65% yield. After regioselective opening of the benzylidene ring, glycosylation of glucosamine 4-OH with the sialogalactoside imidate donor **11** afforded the target protected hexasaccharide **37** in 60% yield. On the other hand, to obtain the heptasaccharide **6**, trisaccharide **24** was glycosylated with the armed lactose trichloroacetimidate **31**, yielding the intermediate pentasaccharide **38** (55%). After treatment with trimethylaminoborane and boron trifluoride etherate, the obtained pentasaccharide acceptor **39** was elongated by glycosylation with the disaccharide donor **11** to the protected heptasaccharide **40** (60% yield).

Both compound **37** and **40** were deprotected, according to the previously described protocol, to afford oligosaccharides **5** and **6** in multimilligram quantities (40% yield)[29].

The ^{1}H NMR spectra of compounds **3**-**6** are depicted in Figure 3 and confirmed the integrity of the obtained structures.



Scheme 6. Assembly of oligosaccharides **5** and **6**. *Reagents and conditions*: a) TMSOTf, DCM dry, 65%; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 55%; c) TMSOTf, DCM dry, 60%; d) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 40% (over five steps); e) TMSOTf, DCM dry, 55%; f) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 55%; g) TMSOTf, DCM dry, 60%; h) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 40% (over five steps).

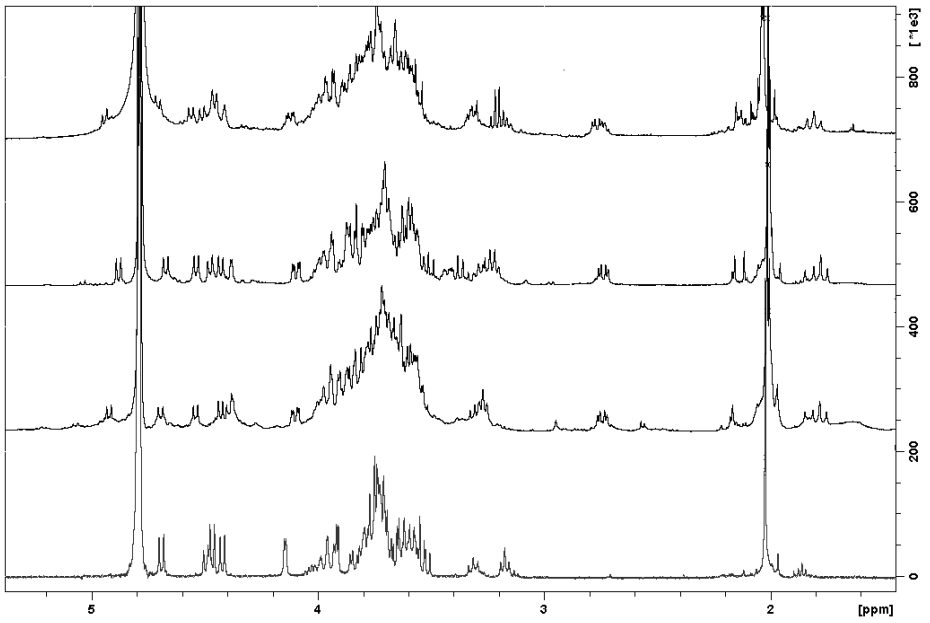


Figure 3. ^{1}H NMR spectra of synthetic oligosaccharides. From the bottom to the top: 1H NMR of: the linear tetrasaccharide **3**, the branched hexasaccharide **4**, the Y-shape hexasaccharide **5** and the heptasaccharide **6**.

Conformational studies

The branched pentasaccharide repeating unit of GBS serotype Ia was used to investigate the conformational behaviour of the type Ia capsular polysaccharide and compare its structure to that of the Ib polymer. Indeed, these two capsular polysaccharides differ exclusively in the connection of the sialogalactoside disaccharides to the GlcNAc residue, which is β-(1→4) in type Ia and β-(1→3) in type Ib. This small difference is crucial to the immunospecificity (anti PSIa and Ib serotype specific mAbs have been isolated and have shown no cross-reactivity).

By a combination of NMR and modelling experiments[31], a model of the natural CPS was built (10 repeating units, 50 monosaccharides) and compared to compound **1**, the branched repeating unit of PSIa. On the basis of this model, the impact of the GlcNAcβ1-3Gal *vs* GlcNAcβ1-4Gal connectivity in the orientation of the Neu5Acα2-3Gal branching was shown and a different shape for the Ia polysaccharide as compared to Ib was revealed. The main difference was the presentation of the protruding Neu5Ac-α-(2→3)-Gal moieties, with a major *exo-anti-Φ* population for Ia and a *exo-syn-Φ* conformation for Ib (see Figure 4, 5 and Chapter 5). The Neu5Acα2-3Gal branches of GBS PSIII have been shown to be strongly engaged in antibody recognition[22], therefore these unique structural features are expected to influence antibody recognition and immunospecificity also in case of GBS PSIa and PSIb.

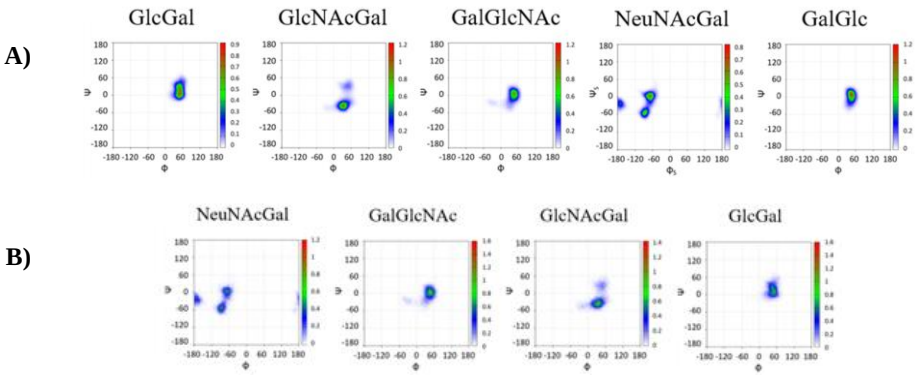


Figure 4. A) Glycosidic linkage analysis for GBS Ia polysaccharide: φ/ψ plots for representative glycosidic bonds of a 10 repeating unit model along the 2.5 μs MD simulation B) Glycosidic linkage analysis for GBS Ia pentasaccharide 1: φ/ψ plots for representative glycosidic bonds along 200 ns of MD simulation.

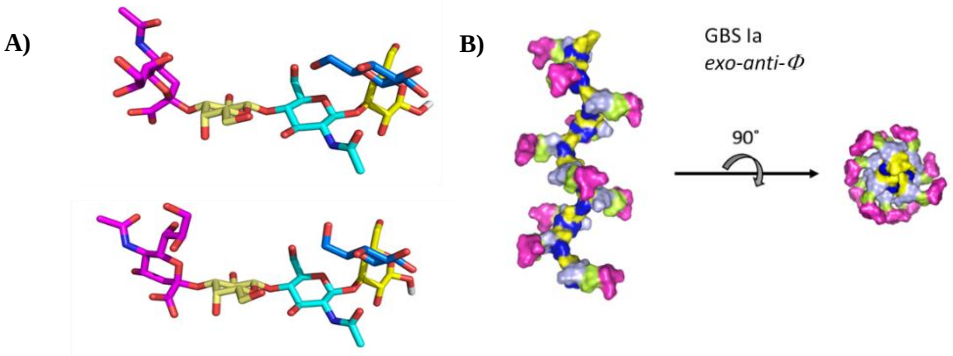


Figure 5. A) Perspectives of the two populations, deduced by ROESY NMR experiments, which define the conformational behaviour of the pentasaccharide repeating unit of GBS Ia. (a) the φ_S torsion angle adopts the major trans (t) geometry. (b) the φ_S torsion angle shows the minor -g conformation. B) Model structures for the GBS Ia polysaccharide with the major exo-anti-φ conformation around all the Neu5Acα2-3Gal linkages

Conclusion

In this Chapter the optimized regioselective β-(1→3)-glycosylation of appropriate galactose synthons was employed to synthesize oligosaccharide fragments from Group B streptococcus serotype Ia capsular polysaccharide. In particular, a small library of six fragments with an increasing degree of complexity (from a non-sialylated linear tetrasaccharide to a Y-shaped heptasaccharide) was designed. Thanks to the regioselective approach described in Chapter 3, the synthesis of the GBS PSIa repeating unit was optimized, reducing the number of synthetic steps and protecting groups employed. Furthermore, fragments longer than one single repeating unit were prepared with high efficiency and using a limited number of building blocks.

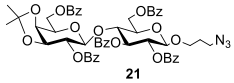
Having access to structurally defined GBS type Ia related glycans is a key step forward to investigate the structural-immunogenicity relationship of the capsular polysaccharide and to gain insights into its conformation.

To this aim, the different conformational behaviour of GBS PSIa compared to the structurally close PSIb was established. The GBS type Ia repeating unit **1** was used to probe the conformation and molecular dynamics of the corresponding capsular polysaccharide, highlighting the different presentation of the protruding Neu5Acα2-3Gal moieties and a reduced flexibility of Ia polymer compared to Ib. These differences are expected to impact epitope exposure.
The synthesized fragments **1**-**6** will be employed to confirm this prediction and to investigate the structural features determining the antibody recognition (Chapter 6).
Moreover, since all the oligosaccharides have been designed and synthesized with an aminopropyl chemical handle, they will be conjugated to carrier proteins for evaluation of their immunogenicity *in vivo*.

Experimental

General Methods and Procedures. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50ºC on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm) or using pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (Biotage, silica 0.050 nm). Unless otherwise specified, a gradient 0-100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) or Biotage Isolera instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{1}H values are reported in ppm, relative to internal Me_4Si (^{1}H = 0.00, $CDCl_3$); solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{13}C values are reported in ppm relative to the signal of $CDCl_3$ (^{13}C = 77.0, $CDCl_3$). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Assignment of ^{13}C NMR spectra of some compounds was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters. Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data and *m/z* values found in their mass spectra.

3-Azidopropyl 2,6-di-*O*-benzoyl-3,4-di-*O*-isopropylidene-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 21.



21

Lactose Linker perOAc **18** (5.4 g, 7.6 mmol), prepared according to the literature[28], was dissolved in MeOH (40 mL) and then MeONa/MeOH was added dropwise until pH = 9. After 16 h (TLC 9:1 DCM/MeOH) the reaction was quenched by addition of Dowex, then the solution was filtered and the solvent evaporated under reduced pressure, affording the lactose linker deacetylated. (2.7 g, 6.4 mmol, 85%).

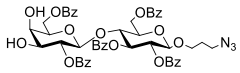
The resulting compound (2.7 g, 6.4 mmol) was dissolved in 30 ml of 9:1 mixture of 2,2-dimethoxypropane:DMF. Catalytic PTSA (0.7 g, 4.5 mmol) was added and the reaction warmed at 50°C for 3 h. After 3 h (TLC 9:1 DCM:MeOH) the reaction was quenched with TEA until neutral pH, and the solvent removed under reduced pressure. The crude was redissolved in 55 ml of 9:1 MeOH/H_2O and stirred at 90°C for 2 h, when the presence of one major spot was detected on TLC. The solvent was removed under reduced pressure, and the crude purified by chromatography (DCM/MeOH) to give the isopropylinated lactose **72** in 57% yield (1.7 g, 3.7 mmol).

Lactose linker isopropylidene **20** (1.7 g, 3.7 mmol) was dissolved in 20 mL of pyridine, then the resulting solution was cooled down to 0°C in a water/ice bath and BzCl (4.3 mL, 37.2 mmol) was added dropwise. The reaction was stirred at rt for 2 h (TLC 6:4 cyclohexane/EtOAc), then the crude mixture was purified on silica gel affording the desired compound **21** (2.6 g, 2.6 mmol, 71% yield). $[\alpha]_D^{25}$ = +39.86° (c 1.7, $CHCl_3$). ESI HR-MS ($C_{53}H_{51}N_3O_{16}$) *m/z* $[M+Na]^+$ found 1008.3178; calcd 1008.3167.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.11-7.91 (m, 10H, H-Ar) 7.66-7.26 (m, 15H, H-Ar), 5.71 (t, J = 9.4 Hz, 1H, H3^A), 5.39 (dd , $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.7 Hz, 1H, H-2^A), 5.13 (t, J = 7.6 Hz, 1H, H2^B), 4.64- 4.57 (m, 3H, H6a^A, H1^A, H1^B) 4.45 (dd, $J_{5,6a}$ = 4.6 Hz, $J_{6a,6b}$ = 12.1 Hz, 1H, H6b^A) 4.27-4.08 (m, 3H, H5^A, H3^B, H6a^B), 4.07 (dd, $J_{3,4}$ = 2.0, $J_{4,5}$ =5.5, 1H, H-4^A), 3.91-3.77 (m, 3H, H4^B, H5^B, OCH_{2a}), 3.66 (dd, $J_{5,6b}$ = 7.4 Hz, $J_{6a,6b}$= 11.4 Hz, 1H, H6b^B), 3.53-3.43 (m, 1H, OCH_{2b}), 3.21-3.13 (m, 2H, CH_2N$_3$), 1.82-1.45 (m, 5H, CH_2CH$_2$N$_3$, CH$_3$), 1.26-1.23 (m, 3H, CH$_3$).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 166.0, 165.9, 165.6, 165.2, 164.9 (5 x CO), 133.5-128.6 (C-Ar), 100.6 (C1^A, C1^B), 76.9 (C-3^B), 75.4 (C-5^A), 73.6 (C-2^B),73.0 (C-4^A), 72.6 (C-3^A, C-5^B), 71.9 (C-2^A), 71.7 (C-4^B), 66.8 (O*C*H$_2$), 62.8 (C-6^B), 62.5 (C-6^A), 47.9 (*C*H$_2$N$_3$), 29.2 (*C*H$_2$CH$_2$N$_3$), 27.4 (*C*H$_3$), 26.2 (*C*H$_3$).

3-Azidopropyl 2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 22.



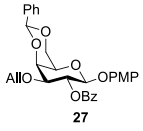
22

Lactose 3,4-isopropylidene 2,6-OBz **21** (2.6 g, 2.6 mmol) was dissolved in 20.0 mL of a 4:1 AcOH/H_2O mixture and the resulting reaction mixture was heated to 90°C. After 2 h (TLC 8:2 Toluene/EtOAc) the solvent was coevaporated with toluene. The crude was purified by column chromatography affording compound **22** (1.6 g, 1.7 mmol, 70% yield). $[\alpha]_D^{25}$ = +37.04° (c 0.7, $CHCl_3$). ESI- HR MS **(**$C_{50}H_{47}N_3O_{16}$) *m/z* $[M+H]^+$ found 946.30, calcd 946.30.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.11-7.29 (m, 25H, H-Ar), 5.69 (t, *J* = 9.4 Hz, 1H, H-3^A), 5.44-5.36 (dd, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.8 Hz, 1H, H-2^A), 5.30-5.23 (dd, $J_{1,2}$ = 7.9 Hz, $J_{3,4}$ = 9.7 Hz, 1H, H-2^B), 4.65-4.56 (m, 3H, H-1^A, H-1^B, H-6a^A), 4.54-4.48 (dd, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 12.2 Hz, 1H, H-6b^A), 4.16-4.09 (t, *J* = 8.8 Hz, 1H, H-4^A), 4.06-3.99 (dd, $J_{5,6a}$ = 6.6 Hz, $J_{6a,6b}$ =11.3 Hz, 1H, H6$^B{}_a$), 3.90-3.76 (m, 3H, OCH$_{2a}$, H-5^A, H-4^B), 3.71-3.63 (d, *J* = 9.3 Hz, 1H, H-3^B), 3.61-3.43 (m, 3H, OCH$_{2b,}$ H-5^B, H-6b^B), 3.29-3.11 (m, 2H, C*H*$_2$N$_3$), 1.85-1.59 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 166.5, 166.2, 166.0, 165.8, 165.2 (5 x CO), 133.4-128.5 (C-Ar), 101.0, 100.8 (C-1^A, C-1^B), 76.1 (C-4^A), 73.7 (C-2^B), 73.0, 72.9 (C-3^A, C-4^B), 72.5, 72.6 (C-3^B, C-5^B), 71.6 (C-2^A), 68.4 (C-5^A), 66.6 (O*C*H$_2$), 62.5 (C-6^A), 61.7 (C-6^B), 47.8 (*C*H$_2$N$_3$), 28.9 (CH$_2$*C*H$_2$N$_3$).

***p*-Methoxyphenyl 3-*O*-allyl-2-*O*-benzyl-4,6-*O*-benzylidene-*β*-D-galactopyranoside 27.**



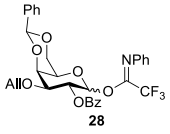
27

Compound **26** (1.0 g, 2.4 mmol) was dissolved in 20 mL of 1:4 pyridine and DCM mixture, then the resulting solution was cooled down to 0°C in a water/ice bath and BzCl (1.0 g, 7.2 mmol) was added dropwise. The reaction stirred at rt overnight, then the reaction was checked by TLC (4:6 cyclohexane:EtOAc), which showed full conversion of the starting material. The solvent was co-evaporated with toluene and purified by column chromatography (cyclohexane:EtOAc) affording **27** (500 mg, 40% yield). $[\alpha]_D^{25}$ = +2.69° (c 0.2, $CHCl_3$). ESI HR-MS ($C_{30}H_{30}O_8$) *m/z* $[M+H]^+$ found 519.20; calcd 519.20.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.15-6.7 (m, 14H, H-Ar), 5.86-5.73 (m, 2H, H-2, C*H*=CH$_2$), 5.59 (s, 1H, C*H*Ph), 5.21 (dd, 1H, *J* = 1.5 Hz, *J* =17.1 Hz, C*H*$_2$=CH), 5.09 (dd, 1H, *J* = 1.3 Hz, *J* = 10.4 Hz, C*H*$_2$=CH), 5.05 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.44-4.33 (m, 2H, H-4, H-6a), 4.20-4.05 (m, 3H, C*H*$_2$=CH, H-6b), 3.81 (dd, $J_{3,4}$ = 3.5, $J_{2,3}$ = 10.1 Hz, 1H, H-3), 3.71 (s, 3H, OCH$_3$), 3.57 (s, 1H, H-5).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 171.6 (CO), 165.1-126.5 (C-Ar), 119.34 (Cq), 117.5 (CH=*C*H$_2$), 114.33 (*C*H=CH$_2$), 101.53 (C-1), 101.31 (*C*H$_2$Ph), 77.10 (C-3), 73.58 (C-4), 70.81 (*C*H$_2$-CH=CH$_{2,}$ C-2), 69.12 (C-6), 66.88 (C-5), 55.59 (O*C*H$_3$).

***p*-Methoxyphenyl 3-*O*-allyl-2-*O*-benzyl-4,6-*O*-benzylidene-*β*-D-galactopyranosyl trifluoroacetimidate 28.**



28

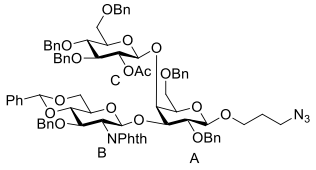
Compound **27** (0.4 g, 0.8 mmol) was dissolved in 10 mL of a 4:1 mixture of acetonitrile and water and the resulting solution was cooled down to 0°C. Cerium ammonium nitrate (0.8 g, 1.5 mmol) was added and the resulting reaction mixture stirred for 2 h at 0°C. After two hours analytical TLC (3:2 cyclohexane/EtOAc) showed full conversion of the starting material and formation of a new spot with lower R_f. The reaction mixture was poured into iced aqueous $NaHCO_3$ and extracted with DCM (5 x). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was purified by column chromatography (cyclohexane/EtOAc), affording the target compound in 77% yield.

Then the obtained compound (0.2 g, 0.5 mmol) and 2,2,2-trifluoro-*N*-phenyl-acetoimidoyl-chloride (0.4 g, 1.8 mmol) were dissolved in 30.0 mL of dry DCM under nitrogen atmosphere. The resulting solution was cooled down to 0°C and Cs_2CO_3 (0.2 mmol, 0.6 mmol) was added. The resulting reaction mixture was stirred at rt for 2 hours, then analytical TLC (6:4 cyclohexane/EtOAc) showed complete conversion of the starting material and formation of a new spot with higher R_f. The reaction mixture was quenched with TEA and the solid was filtered off, then the reaction mixture was concentrated in vacuum and applied to a chromatography column (cyclohexane/EtOAc). Pure fractions were collected and evaporated under reduced pressure affording the trichloroacetimidate **28** (0.260 g, 55%). $[\alpha]_D^{25}$ = +60.93° (c 1.65, $CHCl_3$). ESI HR-MS ($C_{31}H_{28}F_3NO_7$) *m/z* $[M\text{-}CNPhCF_3+Na]^+$ found 435.1413; calcd 435.1414.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.13-7.01 (m, 14H, H-Ar), 6.72 (s, 2H, H-1, H-Ar), 5.86-5.72 (m, 2H, H-2, C*H*=CH_2), 5.58 (s, 1H, C*H*Ph), 5.22 (dd, 1H, *J* = 1.2 Hz, *J* = 17.3 Hz, C*H*$_2$=CH), 5.12 (dd, 1H, *J* = 0.8 Hz, *J* = 9.3 Hz, C*H*$_2$=CH), 4.44-4.29 (m, 2H, H-4, H-6a), 4.21-4.04 (m, 3H, C*H*$_2$-CH=CH_2, H6b), 3.91-3.41 (m, 2H, H-3, H-5).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.9 (C=O), 143.38-124.30, (C-Ar), 119.28 (*C*H=CH_2), 117.80 (CH=*C*H$_2$), 101.27 (*C*H$_2$Ph), 73.30 (C-4), 70.94 (*C*H$_2$-CH=CH_2), 69.93 (C-2), 68.79 (C-6), 67.69 (C-5).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl)-(1→4)-[3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzyl-*β*-D-galactopyranoside 9a.



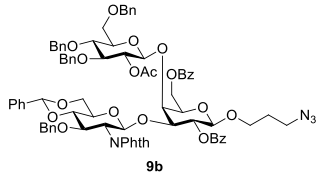
9a

A solution of donor **8** (50 mg, 0.08 mmol) and disaccharide acceptor **7a** (61 mg, 0.067) with activated 4 Å molecular sieves in dry DCM (4 mL) was stirred for 20 min under nitrogen. TMSOTf (3 μL, 0.013 mmol) was added at –10°C. After 4 h (TLC 9:1 Tol:EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Tol:EtOAc) to afford the trisaccharide **9a** in 75% yield (68 mg) as a pale yellow solid. $[\alpha]_D^{25}$ = +24.86° (c 0.8, $CHCl_3$). ESI HR-MS ($C_{80}H_{82}N_4O_{18}$): *m/z* = $[M+ Na]^+$ found 1409.5419; calcd 1409.5522.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-6.85 (m, 39H, H-Ar), 5.66 (s, 1H, C*H*Ph), 5.56 (d, $J_{1,2}$ = 8.8Hz, 1H, H-1^B), 5.00-4.98 (m, 2H, H-1,2^C), 4.91-4.81 (m, 3H, 3 C*H*HPh), 4.60 (d, ^2J = 10.3 Hz, 1H, C*H*HPh), 4.53-4.36 (m, 8H, 7 C*H*HPh, H-6a^C), 4.28 (t, *J* = 9.2 Hz, 1H, H-2^B), 4.19 (d, $J_{1,2}$ = 7.6 Hz, 1H, H-1^A), 4.16 (d, $J_{3,4}$ = 2.3 Hz, 1H, H-4^A), 4.11 (d, ^2J = 11.5 Hz, 1H, C*H*HPh), 3.94-3.89 (m, 1H, H-3^C), 3.86-3.49 (m, 13H, H-3^{A,B}, H-4^{B,C}, H-5^{A-C}, H-6^{A,B}, H-6b^C, OCH$_{2a}$), 3.42-3.37 (m, 1H, OCH$_{2b}$), 3.30 (t, *J* = 8.8 Hz, 1H, H-2^A), 3.13-3.10 (m, 2H, CH_2N_3), 1.79 (s, 3H, CH_3CO), 1.73-1.62 (m, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.9 (CO), 133.7-123.1 (C-Ar), 103.5 (C-1^A), 101.3 (PhCH), 100.3 (C-1^C), 100.2 (C-1^B), 83.4 (C-3^C), 83.1, 81.2, 78.6 (C-2^A), 77.8, 75.2, 75.0, 74.9 (3 x PhCH$_2$), 74.5, 74.4 (C-4^A), 74.1, 73.7, 73.5, 73.4, 73.1 (3 PhCH$_2$), 69.8, 69.1, 68.7 (C-6^{A-C}), 66.3 (OCH$_2$), 65.9, 56.3 (C-2^B), 48.2 (*C*H$_2$N$_3$), 29.1 (*C*H$_2$CH$_2$N$_3$), 20.8 (*C*H$_3$CO).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)-[4,6-*O*-benzylidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 9b.



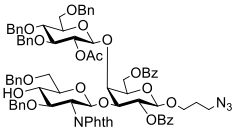
9b

A solution of donor **8** (48 mg, 0.077 mmol) and disaccharide acceptor **7a** (60m g, 0.064 mmol) with activated 4 Å molecular sieves (0.100 g) in dry DCM (4 mL) was stirred for 20 min under nitrogen. TMSOTf (2 μL, 0.013 mmol) was added at –10°C. After 4 h (TLC 9:1 Tol:EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Tol:EtOAc) to afford the trisaccharide **9b** in 68% yield (58 mg) as a pale yellow solid. $[\alpha]_D^{25}$ = +18.18° (c 1.45, $CHCl_3$). ESI HR-MS ($C_{80}H_{78}N_4O_{20}$) *m/z* $[M+Na]^+$ found 1437.5027; calcd 1437.5107.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-6.80 (m, 39H, H-Ar), 5.66 (s, 1H, C*H*Ph), 5.43 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1^B), 5.15 (t, *J* = 8.3 Hz, 1H, H-2^A), 5.03 (t, *J* = 7.4 Hz, 1H, H-2^C), 4.99 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1^C), 4.91 (br. s, 2H, 2 C*H*HPh), 4.86 (d, ^2J = 10.7 Hz, C*H*HPh), 4.75 (d, ^2J = 10.7 Hz, C*H*HPh), 4.71 (d, $J_{5,6a}$ = 4.5 Hz, 12.3 Hz, 1H, H-6a^A), 4.62-4.37 (m, 6H, 4 C*H*HPh, H-6b^A, H-6a^B), 4.35 (d, *J* = 2.7 Hz, 1H, H-4^A), 4.32 (t, *J* = 8.5 Hz, 1H, H-3^B), 4.22 (t, *J* = 9.3 Hz, 1H, H-2^B), 3.94 (t, *J* = 9.5 Hz, 1H, H-3^C), 3.90-3.61 (m, 9H, H-3^A, H-4^{B,C}, H-5^{A,B}, H-6b^B, H-6^C, OCH_{2a}), 3.55-3.52 (m, 1H, H-5^C), 3.44-3.39 (m, 1H, OCH_{2b}), 3.08-2.96 (m, 2H, CH_2N_3), 2.02 (s, 3H, CH_3CO), 1.71-1.55 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 166.4, 164.7 (3 x CO), 133.5-122.9 (C-Ar), 101.3 (Ph*C*H), 101.1 (C-1^A), 100.4 (C-1^C), 100.3 (C-1^B), 83.4 (C-3^C), 83.0 (C-4^B), 80.0 (C-3^A), 78.0, 75.5 (C-5^C), 75.4, 75.3, 74.3 (3 x *C*H$_2$Ph), 74.2 (C-3^B), 74.0 (C-4^A), 73.5 (Ph*C*H$_2$), 73.3 (C-2^C), 72.2, 70.6 (C-2^A), 69.2, 68.7 (C-6^{A,B}), 66.1 (C-5^A), 65.3 (O*C*H$_2$), 64.4 (C-6^C), 55.9 (C-2^B), 47.9 (*C*H$_2$N$_3$), 28.9 (*C*H$_2$CH$_2$N$_3$), 20.7 (CH$_3$CO).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)-[3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 10.



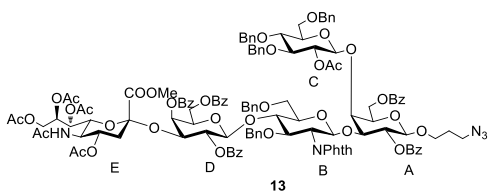
10

A solution of trisaccharide **9b** (258 mg, 0.181 mmol) in acetonitrile was cooled down to 0°C. Trimethylamino borane complex (53 mg, 0.72 mmol) was added, followed by $BF_3{\cdot}Et_2O$ (0.090 mL, 0.72 mmol). The reaction mixture was stirred at 0°C for 3 h, when TLC (4:1 Tol/EtOAc) showed formation of a new spot with lower R_f. The reaction was quenched by addition of Et_3N and MeOH, then evaporated under vacuum. The crude was purified by column chromatography (Tol:EtOAc). Clean fractions were collected and evaporated to dryness affording trisaccharide **10** (0.180 g, 70% yield) as a colorless oil. $[\alpha]_D^{25}$ = + 204.32° (c 0.39, $CHCl_3$). ESI HR-MS ($C_{80}H_{80}N_4O_{20}$) *m/z* ([*M*+ Na]$^+$ found 1439.5051; calcd (1439.5264)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15-6.54 (m, 39H, H-Ar), 5.29 (d, $J_{1,2}$ = 7.4 Hz, 1H, H-1^B), 5.08 (t, *J* = 9.1 Hz, 1H, H-2^A), 5.18-4.9 (m, 2H, incl. H-1^C, H-2^C), 4.81 (s, 2H, C*H*$_2$Ph), 4.76 (d, ^2J = 11.1, 1H, PhC*H*H), 4.66-4.56 (m, 2H, incl. PhC*H*H, H-6a^A), 4.56-4.33 (m, 7H, incl. H-6b^A), 4.32-4.26 (m, 2H, incl. H-1^A, H-4^A), 4.13-4.01 (m, 2H, incl. H-2^B, H-3^B), 3.87-3.66 (m, 6H, incl. H-3^C, H-3^A, H-5^A, H-4^B, H-6^B$_{a,b}$, OCH_{2a}), 3.66-3.52 (m, 4H, incl. H-4^C, H-5^B, H-6a,b^C), 3.49-3.40 (m, 1H, H-5^C), 3.40-3.29 (m, 1H, OCH_{2b}), 3.02-2.85 (m, 2H, CH_2N_3), 1.87 (s, 3H, CH_3CO), 1.66-1.42 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 166.4, 164.5 (3 x CO), 138.7-122.9 (C-Ar), 101.0 (C-1^A), 100.5 (C-1^C), 99.7 (C-1^B), 83.4 (C-3^C), 79.7 (C-3^A), 78.5 (C-3^B), 77.9 (C-4^C), 75.3 (C-5^C), 75.2, 75.0, 74.6 (3 x *C*H$_2$Ph), 74.3 (C-4^A), 74.2 (C-5^A), 73.7 (C-5^B), 73.7, 73.5 (2 x *C*H$_2$Ph), 73.1 (C-2^C), 72.3 (C-4^B), 70.7 (C-6^B), 70.6 (C-2^A), 69.2 (C-6^C), 65.1 (O*C*H$_2$), 64.8 (C-6^A), 55.5 (C-2^B), 47.9 (*C*H$_2$N$_3$), 28.9 (*C*H$_2$CH$_2$N$_3$), 20. 6 (*C*H$_3$CO).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)-[2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-*N*-5-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 13.



13

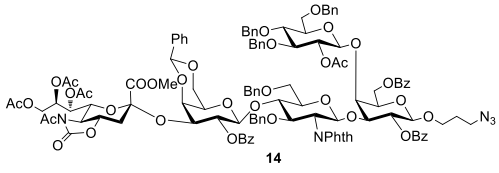
A solution of disaccharide donor **11** (124 mg, 0.109 mmol) and acceptor **10** (110 mg, 0.078 mmol) with 4 Å molecular sieves (0.200 g) in dry DCM (5.0 mL) was stirred for 20 min under nitrogen. TMSOTf (2.8 μL, 0.0156 mmol) was added at -20°C. After 4 h (TLC; 6:4 Tol:Acetone) the reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:Acetone) to afford pentasaccharide **13** in 75% yield

(138 mg). $[\alpha]_D^{25}$ = +41.05° (c 0.75, $CHCl_3$). ESI HR-MS ($C_{127}H_{129}N_5O_{40}$) *m/z* ([*M*+ Na]$^+$ found 2386.8462; calcd 2386.8106.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39-6.56 (m, 54H, Ar-H), 5.77-5.70 (m, 1H, H-8^E), 5.51 (dd, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ =9.8 Hz, 1H, H-2^D), 5.34 (d, $J_{3,4}$ = 3.0 Hz, 1H, H-4^D), 5.26-5.19 (m, 2H, H-2^C, H-1^C), 5.12 (d, 1H, H-1^D), 5.09 (t, *J* = 9.8, 8.3 Hz, 1H, H-2^A), 4.97-4.72 (m, 7H, incl. H-1^B, C*H*HPh), 4.70-4.42 (m, 7H, C*H*HPh), 4.42-4.30 (m, 2H, C*H*HPh), 4.30-4.09 (m, 7H, incl. H-1^A, H-6^E), 4.08-3.92 (m, 2H), 3.92-3.55 (m, 14, incl. $COOCH_3$, H-5^E, H-2^B, OCH_{2a}), 3.54-3.32 (m, 3H, incl. OCH_{2b}), 3.14-2.88 (m, 2H, CH_2N_3), 2.51-2.41 (dd, 1H, $J_{3e,4}$ = 12.7 Hz, $J_{3a,3e}$ = 4.3 Hz, H-3e^E), 2.13, 2.01, 1.92, 1.87, 1.79 (5 x s, 3H each, 5 x CH_3CO), 1.73-1.57 (m, 3H, C*H*$_2$CH$_2$N$_3$, H-3a^E) , 1.50 (s, 3H, CH_3CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.7, 170.5, 170.4, 170.3, 170.2, 168.2, 167.6, 167.0, 165.6, 165.5, 165.0, 164.5 (14 x CO), 138.1-122.8 (C-Ar), 100.9, 100.7, 100.4, 99.4, 97.0, 83.4, 79.5, 77.8, 75.2, 75.1, 74.9, 74.7, 74.6, 74.4, 73.5, 73.4, 73.1, 72.7, 72.3, 72.0, 71.9, 71.8 (C-2^A), 71.6, 71.5, 71.3, 70.6, 70.6, 69.4, 68.9, 68.7, 68.1 (C-4^D), 67.5, 67.1 (C-8^E), 66.7 (C-2^D), 65.0, 64.8, 62.5, 61.6, 55.9, 53.2, 48.8, 47.9 (*C*H$_2$N$_3$), 37.4 (C-3^E), 28.9 (*C*H$_2$CH$_2$N$_3$), 21.5, 21.3, 20.8, 20.7, 20.6, 20.4 (6 x CO*C*H$_3$).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-O-benzyl-*β*-D-glucopyranosyl-(1→4)-[4,6-*O*-benzylidene-2-*O*-benzoyl-3-*O*-(methyl 7,8,9-tri-*O*-acetyl-5-*N*-acetamido,4-*O*-oxazolidinone-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 14.



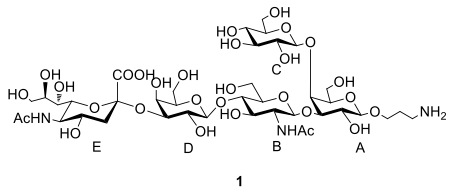
14

A solution of donor **12** (60 mg, 0.063 mmol) and acceptor **10** (60 mg, 0.042 mmol) with 4 Å molecular sieves in dry DCM (3.0 mL) was stirred for 20 min under nitrogen. *N*-Iodosuccinimide (0.019 g, 0.084 mmol) and TfOH (0.7 µL, 0.0084 mmol) were added at -40°C. After 3 h (TLC: 6:4 Toluene/Acetone) the reaction was quenched with TEA, the solid was filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:Acetone) to afford pentasaccharide **14** in 73% yield (68 mg). $[\alpha]_D^{25}$ = +0.70° (c 0.25, $CHCl_3$). ESI HR-MS ($C_{119}H_{121}N_5O_{38}$): *m/z* = ([*M*+ K]$^+$ found 2267.7361; calcd 2266.7321.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17-6.57 (m, 49H, Ar-H), 5.61-5.55 (m, 2H), 5.52 (dd, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 9.8, Hz, 1H, H-2^D), 5.33 (s, 1H, C*H*Ph), 5.21 (d, $J_{1,2}$ = 7.2 Hz, 1H, H-1^B), 5.05 (dd, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 9.9, Hz, 1H, H-2^A), 5.02-4.95 (m, 2H, incl. H-2^C), 4.91 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1^C), 4.91 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1^D), 4.83 (s, 2H, C*H*$_2$Ph), 4.78 (d, ^2J = 10.9 Hz, 1H, C*H*HPh), 4.58-4.51 (m, 2H), 4.50-4.42 (m, 6H), 4.42-4.29 (m, 4H, incl. H-1^A), 4.28-4.23 (m, 2H, incl. H-2^B), 4.21-3.95 (m, 7H), 3.90-3.61 (m, 9H), 3.58-3.31 (m, 10H), 3.05-2.85 (m, 3H, H-3e^E, OCH_2), 2.46 (s, 3H, $NCOCH_3$), 2.18, 1,99, 1,90, 1.83 (5 x s, 3H each, 5 x CH_3CO), 1.79, (t, $J_{3a,3e}$ = 6.2 Hz, 1H, H-3a^E), 1.63-1.46 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 170.9, 170.5, 170.3, 170.0, 168.4, 167.5, 166.9, 166.2, 164.9, 164.4, 153.5 (12 x CO), 137.7-122.7 (C-Ar), 100.9 (*C*HPh), 100.9 (C-1^B), 100.7 (C-1^C), 100.4 (C-1^A), 99.6 (C-1^D), 97.2, 83.4, 79.9, 77.9, 77.8, 77.2, 75.3, 75.2, 75.1, 75.0, 74.9, 74.9, 74.6, 74.4, 73.5, 73.0, 72.9, 72.8, 72.2, 71.4, 71.1, 70.4, 68.9, 68.8,68.7, 67.9, 66.0, 64.9, 63.7, 58.9, 55.9, 52.9 (COO*C*H$_3$), 47.9 (*C*H$_2$N3), 37.0 (C-3^E), 28.9 (*C*H$_2$CH$_2$N$_3$), 24.7, 21.4, 20.8, 20.7, 20.6 (5 x *C*H$_3$CO).

2-Aminopropyl *β*-D-glucopyranosyl-(1→4)-[3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-2-nonulopyranosyl)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)]-*β*-D-galactopyranoside 1.



1

Protocol A: A mixture of protected pentasaccharide **13** (0.1 mmol) and LiI (3 mmol) in pyridine (5 ml) was heated for 24h at 120°C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 ml), and ethylenediamine (400 µl) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuum, and the residue was coevaporated from toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (5 ml), and acetic anhydride (5 ml) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH=13. After 48 h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **1** as an amorphous powder (40% yield).

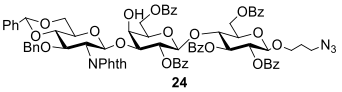
Protocol B: Protected pentasaccharide **14** (0.03 mmol) was dissolved in THF (3 ml) to which 3 M NaOH (0.3 ml) was added. After refluxing for 2 days, the mixture was neutralized with 0.1% HCl and concentrated. The residue was re-dissolved in 2:3 Ac_2O-MeOH (5 ml) and stirred overnight, when C18-TLC (2:3 H_2O/MeOH) showed disappearance of the starting material. After concentration, the residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were freeze-dried to afford the deprotected oligosaccharide **1** as an amorphous powder (45% yield).

$[\alpha]_D^{25}$ = +1.24° (c 0.2, H_2O). ESI HR -MS ($C_{40}H_{69}N_3O_{29}$) *m/z* $[M-H]^-$ 1054.3971; found 1054.3866.

^{1}H NMR (400 MHz, D_2O) δ 4.88 (d, $J_{1,2}$ = 7.7 Hz, 1H, H-1^C), 4.69 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1^B), 4.54 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1^D), 4.39 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^A), 4.37-4.35 (m, 1H), 4.09 (dd, $J_{3,4}$ = 2.9 Hz, $J_{2,3}$ = 9.7 Hz, 1H, H-3^A), 4.02-3.47 (m, 30H), 3.47-3.32 (m, 2H), 3.26 (dd, 1H, $J_{2,3}$ =9.4 Hz, H-2^C), 3.13 (dt, *J* = 8.2, 6.7 Hz, 1H), 2.74 (dd, $J_{3e,4}$ = 12.3 Hz, $J_{3e,3a}$ = 4.6, Hz, 1H, H-3e^E), 2.02 (s, 3H, $NCOCH_3$), 2.01 (s, 3H, CH_3CO), 2.04-1.93 (m, 2H, $CH_2CH_2NH_2$), 1.78 (t, 1H, H-3a^E).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 175.0, 174.8, 173.8 (3 x CO), 103.1 (C-1^A), 102.9 (C-1^D), 102.5 (C-1^C), 102.0 (C-1^B), 82.1, 78.1, 75.8, 75.6, 75.5, 75.2, 74.8, 74.6, 74.2, 73.6, 72.8, 72.1, 71.7, 70.0, 69.7, 69.3, 68.3, 68.0, 67.9, 67.4, 62.5, 61.0, 60.6, 60.1, 55.3, 51.6, 39.6 (CH_2N_3), 37.6 (C-3^E), 26.7 ($CH_2CH_2N_3$), 22.2, 22.0 (2 x CH_3CO). See also Table 1.

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 24.



24

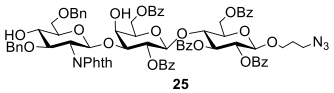
A solution of donor **23** (450 mg, 0.7 mmol) and lactose acceptor **22** (518 mg, 0.548 mmol) with activated 4 Å molecular sieves (500 mg) in dry DCM (7 mL) was stirred for 20 min under nitrogen. TMSOTf (19 µL, 0.1 mmol) was added at –10°C. After 4 h (TLC: 9:1 DCM/EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was

purified by flash chromatography (DCM/EtOAc) to afford the trisaccharide **24** in 68% yield. $[\alpha]_D^{25}$ = + 36.64° (c 0.1, $CHCl_3$). ESI HR-MS ($C_{78}H_{70}N_4O_{22}$) *m/z* $[M+H]^+$ found 1415.46; calcd 1415.45.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.14-6.72 (m, 39H, H-Ar), 5.62 (t, *J* = 9.5 Hz, 1H, H-3^A), 5.59 (s, 1H, C*H*Ph), 5.35 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.6 Hz, H-2^A), 5.31 (d, *J* = 7.5 Hz, 1H, H-1^C), 5.26 (dd, $J_{2,3}$ = 8.1 Hz, $J_{3,4}$ = 9.5 Hz, 1H, H-2^B), 4.70 (d, ^2J = 12.4, 1H, C*H*HPh), 4.55 (d, $J_{1,2}$ = 7.7 Hz, 1H, H-1^A), 4.45 (d, $J_{1,2}$ =8.0 Hz, 1H, H-1^B), 4.39 (d, *J* = 12.4 Hz, 1H, CH*H*Ph), 4.42-4.10 (m, 6H, incl. H-6a^A, H-6b^A, H-6a^B, H-6a^C, H-3^C, H-2^C), 4.05 (t, *J* = 9.5 Hz, 1H, H-4^A), 3.97 (d, *J* = 3.0 Hz, 1H, H-4^B), 3.85-3.72 (m, 3H, incl. H-5^A, H-6b^C, OCH_{2a}), 3.69 (dd, J = 3.3, 9.8 Hz, 1H, H-3^B), 3.66-3.55 (m, 3H, incl. H-4^C, H-6^{B}b, H-5^C), 3.55-3.49 (m, 1H, H-5^B), 3.49-3.41 (m, 1H, OCH_{2b}), 3.21-3.09 (m, 2H, CH_2N_3), 1.80-1.58 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 166.0, 165.8, 165.5, 165.2, 164.0 (5 x CO), 137.6-126.1 (m, C-Ar), 101.3 (CHPh), 101.0 (C-1^A), 100.6 (C-1^B), 99.8 (C1-^{C}), 82.6, 80.6, 75.3, 74.2, 74.0, 72.9, 72.5, 72.1, 71.7, 70.6, 68.5, 68.3, 66.5, 66.1, 62.6, 62.3, 55.5 (C-2^C), 47.8 (*C*H$_2$N$_3$), 28.9 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 3,6-di-*O*-benzyl-2-deoxy-2-phtalimido-*β*-D-glucopyranosyl-(1→3)-[2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)]-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 25.



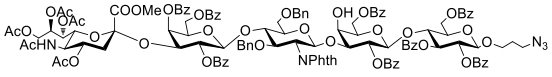
25

Trisaccharide **24** (100 mg, 0.071 mmol) was dissolved in 5.0 mL of acetonitrile and the resulting solution was cooled down to 0°C. Trimethylamino borane complex (26 mg, 0.355 mmol) was added, followed by $BF_3{\cdot}Et_2O$ (43 µL, 0.355 mmol). The reaction mixture was stirred at 0°C for 3 h (TLC 4:1 Tol:EtOAc), then quenched by addition of Et_3N and MeOH, evaporated and purified by flash chromatography (Tol:EtOAc) affording trisaccharide **25** (65% yield). $[\alpha]_D^{25}$ = -4.99° (c 0.05, $CHCl_3$). ESI HR-MS ($C_{78}H_{72}N_4O_{22}$) *m/z* $[M+Na]^+$ found 1439.45; calcd 1439.45.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.14-6.87 (m, 39H, H-Ar), 5.62 (t, *J* = 9.4 Hz, 1H, H-3^A), 5.34 (dd, $J_{1,2}$ = 7.8 Hz, 1H, H-2^A), 5.28-5.21 (m, 2H, H-2^B, H-1^C), 4.64-4.50 (m, 2H, H-1^A, C*H*$_2$Ph), 4.50-4.34 (m, 6H, H-6a^A, 2 x C*H*$_2$Ph, H-1^B), 4.22 (dd, ^2J = 4.7 Hz, 12.2 Hz, 1H, H-6b^A), 4.18-4.01 (m, 4H, including H-2^C, H-6a^B, H-4^A), 3.98 (d, 1H, $J_{3,4}$ = 3.3 Hz, H-4^B), 3.84-3.76 (m, 1H, OCH_{2a}), 3.75-3.40 (m, 9H, including H-3^{B,C}, H-5^B, H-6^C, H-6b^B, OCH_{2b}), 3.20-3.10 (m, 2H, CH_2N_3), 1.79-1.58 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.9, 165.8, 165.4, 165.2, 163.9, 137.8 (7 x CO), 133.4-125.3 (C-Ar), 101.0 (C-1^A), 100.0 (C-1^B), 98.9 (C-1^C), 80.9, 78.4, 75.2, 74.2 (*C*H$_2$Ph), 73.8 (*C*H$_2$Ph), 73.6, 73.5 (*C*H$_2$Ph), 72.9, 72.5 (C-3^A), 72.1, 71.7 (C-2^A), 70.6 (C-2^B), 70.2 (C-6^C), 67.8 (C-4^B), 66.5 (O*C*H$_2$), 62.8 (C-6^B), 62.3 (C-6^A), 54.9 (C-2^C), 47.8 (*C*H$_2$N$_3$), 29.7 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranoside-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 29.



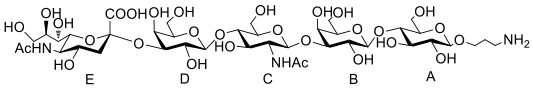
29

A solution of trisaccharide acceptor **25** (103 mg, 0.073 mmol) and disaccharide donor **11** (124 mg, 0.117 mmol) with activated 4 A molecular sieves (100 mg) in DCM (5 mL) was stirred for 20 min under nitrogen. TMSOTf (2.65 µL, 0.015 mmol) was added at 0 °C. After the reaction mixture was stirred for 2h at rt, TLC (6:4 Tol:Acetone) showed complete reaction. TEA was added until neutral pH, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol:Acetone) to afford **29** (112 mg, 65% yield). $[\alpha]_D^{25}$ = +23.9° (c 0.1, $CHCl_3$). ESI HR-MS *m/z* ($C_{125}H_{121}N_5O_{42}$) *m/z* $[M+Na]^+$; found 2387.34, calcd: 2387.73.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.29-6.55 (m, 54H, H-Ar), 5.73-5.66 (m, 1H, H-8^E), 5.60-5.53 (m, 1H, H-3^A), 5.47 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{3,4}$= 9.9 Hz, H-2^D), 5.31-5.25 (m, 2H, H-2^A, H-7^E), 5.22-5.15 (m, 2H, H-2^B, H-4^D), 5.09 (d,

$J_{1,2}$ = 7.5, 1H, H-1^{D}), 5.05 (t, $J_{1,2}$ = 7.5, 1H, H-1^{C}), 4.87-4.77 (m, 3H, incl. CH_2Ph, H-3^{D}), 4.51-4.42 (m, 3H, incl. CH_2Ph, H-1^{A}), 4.37-4.28 (m, 4H, H-1^{B}), 4.19-43.93 (m, 11H, include. H-2^{C}, H-4^{B}, H-4^{A}), 3.85-3.74 (m, 6H, include. COOCH_3, H-5^{E}), 3.61-3.53 (m, 4H), 3.49-3.45 (dd, 1H, $J_{3,4}$ = 3.3 Hz, $J_{2,3}$ = 9.6 Hz, H-3^{B}), 3.44-3.29 (m, 3H), 3.15-3.05 (m, 2H, CH_2N$_3$), 2.45-2.39 (m, 1H, H-3$_{eq}^{E}$), 2.14 (s, 3H, CH_3CO), 2.06-2.03 (m, 3H, CH_3CO), 1.97 (s, 3H, CH_3CO), 1.89 (s, 3H, CH_3CO), 1.76 (s, 3H, CH_3CO), 1.68-1.58 (m, 3H, CH_2CH_2N$_3$), 1.50 (s, 3H, CH_3CO).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.78-163.87 (CO), 138.17-125.31 (C–Ar), 101.01 (C-1^{A}), 100.48 (C-1^{B}), 100.37 (C-1^{C}), 98.75 (C-1^{D}), 96.94, 80.99 (C-3^{B}),75.11, (C-4^{A}), 74.44, 74.34 (*C*H_2Ph), 72.98, 72.78, 72.42 (C-3^{A}), 72.17, 71.90 (C-2^{D}), 71.83, 71.78 (C-7^{E}), 71.63 (C-3^{D}), 70.72 (C-4^{B}), 70.52, 69.38, 68.17 (C-2^{A}), 68.04, 67.61, 67.10 (C-8^{E}), 66.66 (C-2^{B}), 66.50, 63.06, 62.58, 62.31, 61.79, 55.23 (C-2^{C}), 53.22 (C-5^{E}), 48.77 (COO*C*H_3), 47.81 (*C*H_2N$_3$), 37.37 (C-3^{E}), 29.70, 28.86 (*C*H_2CH_2N$_3$), 23.15, 21.38, 20.74, 20.67, 20.43 (6 × *C*H_3CO).

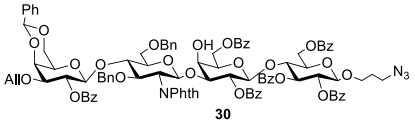
3-Aminopropyl 3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosyl)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside 2.



2

A mixture of protected pentasaccharide **28** (0.1 mmol) and LiI (3 mmol) in pyridine (5 ml) was heated for 24 h at 120°C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 ml), and ethylenediamine (400 µl) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuum, and the residue was coevaporated from toluene (2 x 10 mL) and EtOH (2 x 5 ml). The crude mixture was re-dissolved in pyridine (5 ml), and acetic anhydride (5 ml) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH=13. After 48h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in MeOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **10** as an amorphous powder (33% yield). $[\alpha]_D^{25}$ = +46.0° (c 0.03, H_2O). ESI HR-MS *m/z* $C_{40}H_{69}N_3O_{29}$ $[M+H]^+$ 1056.3971; found 1056.3966. The compound was identical to the one previously reported in the literature[26].

3-Azidopropyl 3-*O*-allyl-4,6-*O*-benzylidene-3-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 30.

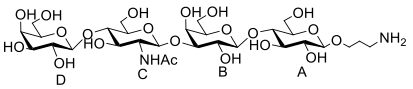

30

A solution of donor **28** (37 mg, 0.063 mmol) and acceptor **25** (60 mg, 0.042 mmol) with activated 4 Å molecular sieves (50 mg) in dry DCM (3 mL) was stirred under nitrogen. TMSOTf (1.5 µL, 0.008 mmol) was added at –5°C. After 2 h the analytical TLC (8:2 Toluene/EtOAc) showed the presence of a lower spot. The reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by column chromatography (Tol/EtOAc) to afford the tetrasaccharide **30** (56 mg, 0.031 mmol) in 72% yield. $[\alpha]_D^{25}$ = +11.2° (c 0.15, $CHCl_3$). ESI HR-MS **(**$C_{101}H_{94}N_4O_{28}$) *m/z* $[M+Na]^+$ found 1834.60, calcd 1834.69. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.14-6.56 (m, 49H, H-Ar), 5.83-5.68 (m, 1H, C*H*=CH_2), 5.60-5.50 (m, 2H, H-3^{D}, H-3^{A}), 5.46 (s, 1H, C*H*Ph), 5.28 (t, *J* = 9.0 Hz, 1H, H-2^{A}), 5.22-5.14 (m, 2H, H-2^{B}, C*H*H=CH), 5.09 (d, 2H, C*H*H=CH, incl. $J_{1,2}$ = 8.6 Hz, H-1^{C}), 4.93 (d, ^2J = 12.0 Hz, 1H, *CH*H-CH=CH_2), 4.72 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1^{D}),

4.49 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1^{A}), 4,43 (d, J = 12.0 Hz, 1H, *CH*H-CH=CH$_2$), 4.42 (d, $J_{1,2}$ = 12.8 Hz, 1H, CH*H*Ph), 4.37 (d, $J_{1,2}$ = 8.2 Hz, 1H, H-1^{B}), 4.33-4.05 (m, 9H, including H-2^{C}), (m, 7H, including H-4^{A}), 3.84 (s, 1H, H-4^{B}), 3.78-3.71 (m, 1H, OCH$_{2a}$), 3.60-3.48 (m, 5H, incl. H-3^{B}, H-3^{D}), 3.39-3.33 (m, 3H, incl. OCH$_{2b}$), 3.27 (s, 1H, OH-4^{B}), 3.13-3.07 (m, 2H, CH$_2$N$_3$), 1.69-1.54 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (101 MHz, CDCl$_3$) 165.89-163.90 (CO), 138.48-126.45 (C-Ar, *CH*=CH$_2$), 117.38 (*C*H$_2$CH=CH$_2$), 101.24 (*C*HPh), 101.07 (C-1^{D}), 100.99 (C-1^{A}), 100.45 (C-1^{B,C}), 98.80, 80.99, 78.31, 77.36, 77.27, 76.97, 75.21, 75.10 (*C*H$_2$Ph), 74.60, 73.34, 73.25, 72.96, 72.49, 72.07 (C-2^{B}, C-3^{A}), 71.76 (C-2^{A}), 71.42, 70.58, 70.46 (C-2^{D}), 67.55 (C-4^{B}), 66.50, 66.47, 62.86, 55.32 (C-2^{C}), 47.81 (*C*H$_2$N$_3$), 29.71, 28.86 (CH$_2$CH$_2$N$_3$).

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



5

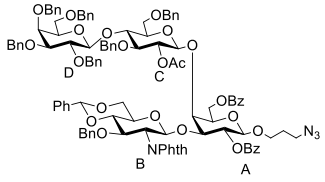
Compound **33** was treated with $PdCl_2$ (2 eq) in methanol (2.5 mL) for 6 h. After filtration, the crude material was dissolved in ethanol (3 mL), and ethylenediamine (400 µL) was added. After being stirred for 16 h at 110 °C, the reaction mixture was concentrated under vacuum, and the residue was coevaporated with toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (1.5 mL), and acetic anhydride (1.5 mL) was added at 0°C. After stirring for 16 h at rt, the reaction mixture was quenched with MeOH and concentrated under reduced pressure. The mixture was dissolved in MeOH (2 mL) and MeONa was added until pH = 12. After 48h the reaction was neutralized and the solvent removed under vacuum. The compound was purified by flash chromatography in silica C-18 (MeOH/H_2O), and the obtained product was dissolved in *t*BuOH (2 mL), to which Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off, the filtrate concentrated under reduced pressure and the crude was purified by size exclusion column chromatography affording compound **5** with a yield of 42%.

$[\alpha]_D^{25}$ = -28.20˚ (c 0.1, $CHCl_3$). ESI-HR MS (**(**$C_{29}H_{52}N_2O_{21}$) *m/z* $[M+Na]^+$ found 765.3136, calcd 765.3135.

^{1}H-NMR (400 MHz, D_2O) δ 4.57 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^{C}), 4.37 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1^{B}), 4.34 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1^{A}), 4.30 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1^{D}), 4.02 (d, 1H, J = 3.1 Hz), 3.95-3.77 (m, 4H), 3.76-3.36 (m, 20H, includ. H-3^{A}, OC*H*$_2$), 3.19 (t, J = 8.2 Hz, 1H, H-2^{A}), 3.05 (t, 1H, J = 7.2 Hz, C*H*$_2$N$_3$), 1.9 (s, 3H), 1.96-1.81 (m, 1H, C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (101 MHz, D_2O) δ 102.9 (C-1^{D}), 102.8 (C-1^{C}), 102.7 (C-1^{A}), 102.0 (C-1^{B}), 81.9, 78.5, 78.4, 75.2, 74.7, 72.8, 72.6, 72.1, 70.7, 70.1, 68.5, 68.2, 67.8, 61.0, 60.1, 55.2, 37.5, 25.8, 22.2.

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[(2,3,4,6-tetra-*O*-benzyl-*β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 32.



32

A solution of donor **30** (0.900 g, 0.85 mmol) and disaccharide acceptor **7a** (0.500 g, 0.53 mmol) with activated 4 Å molecular sieves (0.500 g) in dry DCM (7 mL) was stirred for 20 min under nitrogen. TMSOTf (19 µL, 0.109 mmol) was added at –10°C. After 4 h (TLC; 9:1 DCM: EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Tol:EtOAc) to afford the tetrasaccharide **32** in 60% yield (0.590 g) as a white amorphous solid.

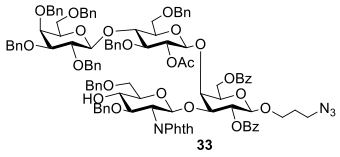
$[\alpha]_D^{25}$ = +11.27˚ (c 0.5, $CHCl_3$). ESI HR-MS ($C_{107}H_{106}N_4O_{25}$) *m/z* $[M-Na]^+$ 1869.7146; found 1869.7047.

^{1}H NMR (400 MHz, CDCl3) δ 8.17-6.74 (m, 54H, H-Ar), 5.59 (s, 1H; CHPh), 5.44 (d, 1H, J=8.3 Hz, H-1^{B}), 5.21 (dd, 1H, J=8.3, 10.1, H-2^{C}), 5.18 (d, 1H, J=11.6 Hz, C*H*HPh), 5.08-5.00 (m, 3H, incl. H-1^{C}, H-2^{C}), 4.95 (d, 2H, J=2.5 Hz, CH$_2$Ph), 4.82-4.74 (m, 4H), 4.70 (dd, 1H, J=3.3, 12.0 Hz, H-6$^{A}_{a}$), 4.65-4.31 (m, 10H, incl. H-6$^{A}_{b}$, H-1^{D},

H-1^{A}), 4.30-4.22 (m, 2H, incl. H-2^{B}), 4.07-3.72 (m, 11H, incl. H-3^{A}, H-3^{C}, OC*H*H), 3.70-3.61 (m, 1H), 3.58-3.49 (m, 3H), 3.48-3.34 (m, 4H, incl. OCH*H*), 3.14-2.95 (m, 2H, CH_2N_3), 1.93 (s, 3H, $COCH_3$), 1.74-1.53 (m, 2H, $CH_2CH_2N_3$)

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 167.6, 166.9, 166.4, 164.5 (5xCO), 139.6-122.9 (m, 68C, C-Ar), 103.1 (C-1^{D}), 101.4 (CHPh), 100.9 (C-1^{A}), 100.3 (C-1^{C}), 99.8 (C-1^{B}), 83.1, 82.6, 81.4, 80.1, 79.2, 75.8, 75.3, 74.7, 74.6, 74.5, 74.3, 73.8, 73.7, 73.5, 73.2, 73.1, 72.6 (C-2^{C}), 72.6, 72.3, 70.7 (C-2^{A}), 68.7, 68.5, 68.2 (CH_2N_3), 66.1, 65.0, 64.6 (C-6^{A}), 55.9 (C-2^{B}), 47.9 (OCH_2), 28.9 ($CH_2CH_2N_3$), 20.5 (CH_3).

3-Azidopropyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[(2,3,4,6-tetra-*O*-benzyl-*β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 33.



33

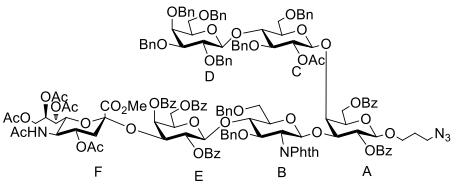
A solution of **32** (0.380 g, 0.21 mmol) in ACN (10 mL) was cooled at 0°C. Me_3NBH_3 (0.075 g, 1.03 mmol) and $BF_3{\cdot}OEt_2$ (0.127 mL, 1.03 mmol) were added and the reaction was stirred for 2 h under nitrogen. TLC showed complete reaction (8:2 toluene:EtOAc). First TEA and then MeOH were added until neutral pH. The solvent was removed at reduced pressure and the crude was purified by flash chromatography (toluene:EtOAc) to afford **33** in 52% yield (0.200 g).

$[\alpha]_D^{25}$ = +29.32° (c 0.25, $CHCl_3$). ESI HR -MS ($C_{107}H_{108}N_4O_{25}$) *m/z* $[M+Na]^+$ 1871.7303; found 1871.7026.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-6.77 (m, 54H, H-Ar), 5.31 (d, 1H, J=7.7 Hz, H-1^{B}), 5.18-5.08 (m, 2H, incl. H-2^{A}), 5.04-4.84 (m, 6H, incl. H-1C, H-2C, 3xCH_2Ph), 4.78-4.26 (m, 16H, incl.H-1D, H-6^{A}a, H-6^{A}b,), 4.26-4.05 (m, 3H), 3.99 (t, 1H, J=9.4 Hz), 3.95-3.89 (m, 2H), 3.89-3.69 (m, 7H), 3.69-3.54 (m, 2H), 3.54-3.27 (m, 6H), 3.10-2.91 (m, 2H), 1.77 (s, 3H), 1.66-1.49 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 166.3, 164.6 (3xCO), 139.7-126.8 (m, 68 C, C-Ar), 102.9 (C-1^{D}), 100.9 (C-1^{A}), 100.3 (C-1^{C}), 99.1 (C-1^{B}), 82.6, 81.3, 80.0, 78.7, 78.6, 75.5, 75.1, 74.7, 74.6, 74.5, 74.3, 74.0, 73.7, 73.6, 73.6, 73.4, 73.2, 73.0, 72.6, 72.5, 72.4 (C-2^{C}), 70.8, 70.6 (C-2^{A}), 68.5, 68.2 (CH_2N_3), 64.9, 64.8, 55.6 (C-2^{B}), 48.0 (OCH_2), 28.9 ($CH_2CH_2N_3$), 20.4 (CH_3).

3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosyl)-2,4,6-tri-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-benzyl-*β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl)-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 34.



34

A solution of donor **11** (0.162 g, 0.143 mmol) and tetrasaccharide acceptor **33** (0.176 g, 0.095 mmol) with activated 4 Å molecular sieves (0.200 g) in dry DCM (3 mL) was stirred for 20 min under nitrogen. TMSOTf (3.4 µL, 0.019 mmol) was added at –10°C. After 4 h (TLC; 6:4 Toluene:acetone) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Tol:acetone) to afford the tetrasaccharide **34** in 78% yield (0.206 g) as a white amorphous solid.

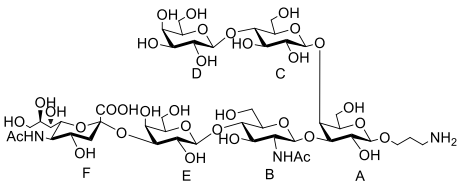
$[\alpha]_D^{25}$ = +26.62° (c 0.25, $CHCl_3$). ESI HR -MS ($C_{154}H_{157}N_5O_{45}$) *m/z* $[M+Na]^+$ 2819.0151; found 2819.0154.

^{1}H NMR (400 MHz, CDCl3) δ 8.36-6.54 (m, 69H, H-Ar), 5.74-5.66 (m, 1H, H-8^{F}), 5.48 (dd, 1H, J=7.4, 9.6 Hz, H-2^{E}), 5.33 (d, 1H, J=3.2 Hz, H-4^{E}), 5.22 (dd, 1H, J=2.3, 9.6 Hz, H-7^{F}), 5.18-5-04 (m, 4H, incl. H-2^{A}, H-1^{E}), 4.99-4.90 (m, 3H, incl. H-3^{E}, H-1^{B}), 4.90-4.77 (m, 4H), 4.75 (s, 2H, OCH_2Ph), 4.72-4.63 (m, 3H), 4.61-4.38 (m, 8H, incl. H-1^{A}), 4.35-4.08 (m, 10H), 4.09-3.93 (m, 4H, incl. H-9^{F}), 3.92-3.64 (m, 12H, COOCH3), 3.64-3.31 (m, 8H, incl.

C*H*HN$_3$), 3.30-3.24 (m, 1H, CH*H*N$_3$), 3.10-2.89 (m, 2H, OCH$_2$), 2.43 (dd, 1H, J=4.5, 12.4 Hz, H-3$^F_{eq}$), 2.14 (s, 3H, COCH$_3$), 1.98 (s, 3H, COCH$_3$), 1.91 (s, 3H, COCH$_3$), 1.78 (s, 3H, COCH$_3$), 1.70-1.57 (m, 7H, incl. C*H*$_2$CH$_2$N$_3$, H-3$^F_{ax}$, COCH$_3$), 1.50 (s, 3H, COCH$_3$).

^{13}C NMR (101 MHz, CDCl$_3$) 170.8, 170.7, 170.5, 170.1, 168.1, 167.5, 167.1, 166.2, 165.6, 165.5, 164.9, 164.7 (12xC=O), 139.8-122.9 (C-Ar), 102.9 (C-1^A), 100.8, 100.5, 100.4 (C-1^B), 100.0, 98.6 (C-1^E), 97.0, 82.7, 81.2, 79.9, 77.6, 77.5, 77.2, 76.9, 76.8, 75.3, 75.1, 74.7, 74.5, 74.3, 73.7, 73.4, 73.1, 72.9, 72.7, 72.5, 72.4, 71.9, 71.8 (C-2^E), 71.7 (C-2^A), 70.9 (C-3^E), 70.5, 69.4, 68.1 (C-4^E), 67.2 (C-8^F), 66.5 (C-7^F), 64.8, 64.7, 62.4, 61.5, 55.9 (C-2^B), 53.2 (COOCH$_3$), 48.8, 47.9 (OCH$_2$), 37.4 (C-5^F), 28.9 (*C*H$_2$CH$_2$N$_3$), 23.2, 21.4, 20.8, 20.4, 20.2 (6xCOCH$_3$).

2-Aminopropyl *β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→4)-[3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-2-nonulopyranosyl)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)]-*β*-D-galactopyranoside 4.



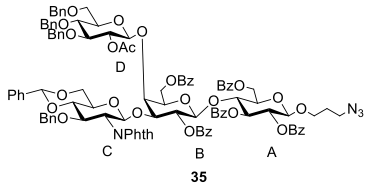
4

A mixture of protected hexasaccharide **34** (0.1 mmol) and LiI (3 mmol) in pyridine (5 ml) was heated for 24h at 120°C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 ml), and ethylenediamine (400 μl) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuum, and the residue was coevaporated with toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (5 ml), and acetic anhydride (5 ml) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH=13. After 48 h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **4** as an amorphous powder (40% yield).

^{1}H NMR (400 MHz, D$_2$O) δ 4.93 (d, 1H, $J_{1,2}$= 8.0 Hz, H-1^C), 4.70 (d, 1H, $J_{1,2}$=8.1 Hz, H-1^B), 4.54 (d, 1H, $J_{1,2}$=7.9 Hz, H-1^E), 4.43 (d, 1H, $J_{1,2}$=7.6 Hz, H-1^D), 4.40 (d, 1H, $J_{1,2}$=8,1 Hz, H-1^A), 4.38 (d, 1H, $J_{1,2}$=2.7 Hz, H-4^A), 4.10 (dd, 1H, $J_{1,3}$=9.3 Hz, $J_{1,2}$=2.7 Hz, H-3^E), 4.03-3.51 (m, 36H), 3.30 (t, 1H, J=8.6 Hz, H-2^C), 3.27 (t, 2H, J=7.1 Hz, C*H*$_2$NH$_2$), 2.74 (dd, 1H, $J_{3e,4}$ = 12.3 Hz, $J_{3e,3a}$ = 4.6, Hz, H-3e^F), 2.07-1.95 (m, incl. 2xNHCH$_3$, C*H*$_2$CH$_2$NH$_2$), 1.78 (t, 1H, $J_{3e,4}$ =12.2 Hz, H-3a^F).

^{13}C NMR (101 MHz, D$_2$O) δ 103.1 (C-1^B), 102.9 (C-1^A), 102.8 (C-1^D), 102.5 (C-1^E), 101.7 (C-1^C), 82.1 (C-4^A), 78.4, 78.1, 75.5, 75.3, 75.2, 74.7, 74.4, 74.2, 73.3 (C-2^C), 72.9, 72.5, 72.1, 71.7, 70.9, 70.0, 69.3, 68.5, 68.3, 68.1, 67.5, 67.4 (OCH$_2$), 67.2, 62.6 (C-9^F), 61.0, 60.7, 60.1, 55.8 (CH$_2$NH$_2$), 55.3 (C-2^B), 51.6 (C-5^F), 39.6 (C-3^F), 24.3 (*C*H$_2$CH$_2$NH$_2$), 22.2 (NHCH$_3$), 22.0 (NHCH$_3$).

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[(2-*O*-*acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-*tri-*O*-benzoyl-*β*-D-glucopyranoside 35.



35

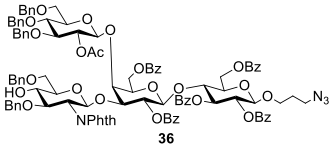
A solution of donor **8** (0.303 g, 0.476 mmol) and trisaccharide acceptor **24** (0.450 g, 0.317 mmol) with activated 4 Å molecular sieves (0.400 g) in dry DCM (7 mL) was stirred for 20 min under nitrogen. TMSOTf (11 µL, 0.063 mmol) was added at –10°C. After 4 h (TLC; 9:1 Toluene:EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Toluene:EtOAc) to afford tetrasaccharide **35** in 65% yield (0.380 g) as a white amorphous solid.

$[\alpha]_D^{25}$ = +19.14° (c 1.5, $CHCl_3$). ESI HR -MS ($C_{107}H_{102}N_4O_{28}$) *m/z* $[M+Na]^+$ 1913.6681; found 1913.6892.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17-6.73 (m, 54H, H-Ar), 5.66-5.57 (m, 2H, incl CHPh, H-3A), 5.38 (dd, 1H, J=7.9, 9.6 Hz, H-2^A), 5.33 (d, 1H, J=8.2 Hz, H-1^C), 5.08 (dd, 1H, J=7.9, 10.0, H-2^B), 4.96-4.83 (m, 5H, incl. H-1^D, H-2^D, 3xCH_2OBn), 4.75-4.63 (m, 3H, incl. 3xCH_2OBn), 4.55 (d, 1H, J=7.7 Hz H-1^A), 4.51 (d, 1H, J=12.0 Hz, 1xCH_2OBn), 4.44, 4.04 (m, 8H, incl. 1xCH_2OBn, H-4^B, H-2^C, H-3^C), 4.01-3.87 (m, 2H, H-4^A, H-3^D), 3.85-3.65 (m, 7H, incl. C*H*HN$_3$, H-5^B, H-5^A, H-3^B, 2xCH_2OBn), 3.64-3.51 (m, 3H), 3.51-3.38 (m, 3H, incl. CH*H*N$_3$), 3.23-3.07 (m, 3H, incl. OCH_2), 2.09 (s, 3H, $COCH_3$), 1.81-1.58 (m, 2H, C*H*$_2$CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 167.5, 166.8, 165.93, 165.8, 165.3, 164.0, 163.4 (8 x CO), 138.6-122.8 (m, C-Ar), 101.3 (CHPh), 101.1 (C-1^A), 100.6 (C-1^B), 100.1 (s, 2C, C-1^C, C-1^D), 83.4 (C-3^D), 82.9 (C-5^B), 79.7, 77.9, 75.6 (C-4^A), 75.4, 75.3, 75.1, 74.3, 74.2 (C-4^B), 73.5 (C-3^C), 73.4, 73.1 (C-2^D), 72.9, 72.4 (C-3^A), 72.3, 71.5 (C-2^A), 70.9 (C-3^B), 69.1, 68.5, 66.6 (CH_2N_3), 66.0, 63.1, 62.4, 55.8 (C-2^C), 47.9 (OCH_2), 28.9 ($CH_2CH_2N_3$), 20.6 (CH_3).

3-Azidopropyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[(2-O-acetyl-3,4,6-tri-O-benzyl-β-D- glucopyranosyl)-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside 36



36

A solution of **35** (0.370 g, 0.195 mmol) in ACN (10 mL) was cooled at 0°C. Me_3NBH_3 (0.071 g, 0.98 mmol) and $BF_3{\cdot}OEt_2$ (0.120 mL, 0.98 mmol) were added and the reaction was stirred for 2 h under nitrogen. TLC showed complete reaction (8:2 toluene:EtOAc). First TEA and then MeOH were added until neutral pH. The solvent removed at reduced pressure and the crude was purified by flash chromatography (toluene:EtOAc) to afford **36** in 55% yield (0.200 g).

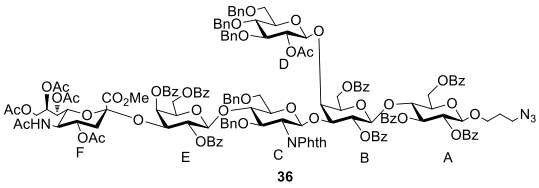
$[\alpha]_D^{25}$ = +20.07° (c 1.5, $CHCl_3$). ESI HR -MS ($C_{107}H_{100}N_4O_{28}$) *m/z* $[M+Na]^+$ 1911.6524; found 1911.6460.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36-6.63 (m, 54H Ar-H), 5.59 (t, 1H, J=9.4 Hz, H-3^A), 5.37 (t, 1H, J=8.2 Hz, H-2^A), 5.24 (d, 1H, 7.3 Hz, H-1^C), 5.05 (dd, 1H, J=8.6, 9.8 Hz, H-2^B), 4.93-4.80 (m, 5H, incl. H-1^D, H-2^D, 3xOCH_2Ph), 4.70-4.58 (m, 2H, 2xOCH_2Ph), 4.58- 4.23 (m, 8H, incl. H-1^A, H-1^B, H-6$^A{}_a$, 5xCH_2OPh), 4.23-4.00 (m, 6H, incl. H-6$^A{}_b$, H-2C), 3.95 (t, 1H, J=9.1 Hz, H-4^A), 3.91-3.82 (m, 1H, H-3^D), 3.83-3.32 (m, 14H, incl. CH_2N_3, H-4^D, H-5^A, H-3^B), 3.23-3.01 (m, 3H, incl.OCH_2), 2.00 (s, 3H. $COCH_3$), 1.80-1.55 (m, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 165.9, 165.8, 165.5, 165.3, 164.0 (6xCO), 138.7-122.9 (m, C-Ar), 101.1 (C-1^A), 100.6 (C-1^B), 100.1 (C-1^D), 99.5 (C-1^C), 83.4 (C-3^D), 79.4, 78.4, 77.8, 75.5 (C-4^A), 75.4, 75.2, 75.0, 74.2, 73.6, 73.5, 73.4, 73.0 (C-2^D), 72.4 (C-3^A), 71.5 (C-2^A), 71.0 (C-2^B), 70.6, 69.1, 66.6 (CH_2N_3), 63.4, 62.5 (C-6^A), 55.4 (C-2^C), 47.9 (OCH_2), 28.9 (*C*H$_2$CH$_2$N$_3$), 20.5 (CH_3).

3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosyl)- 2,4,6-tri-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-*N*-phthalimido-*β*-D-glucopyranosyl-(1→3)-

[*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl}-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 37.



36

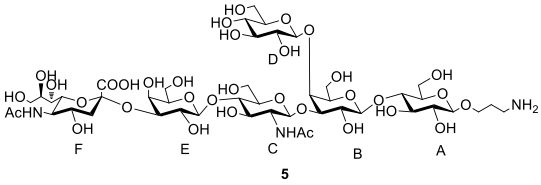
A solution of donor **11** (0.900 g, 0.85 mmol) and disaccharide acceptor **36** (0.500 g, 0.53 mmol) with activated 4 Å molecular sieves (0.500 g) in dry DCM (7 mL) was stirred for 20 min under nitrogen. TMSOTf (19 μL, 0.109 mmol) was added at –10°C. After 4 h (TLC; 9:1 Toluene:EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Toluene:EtOAc) to afford the tetrasaccharide **37** in 60% yield (0.590 g) as a white amorphous solid.

$[\alpha]_D^{25}$ = +40.07° (c 0.2, $CHCl_3$). ESI HR -MS ($C_{154}H_{151}N_5O_{48}$) *m/z* $[M+Na]^+$ 2860.9529; found 2860.9487.

^{1}H NMR (400 MHz, CDCl3) δ 8.28-6.55 (m, 69H, H-Ar), 5.71-5.63 (m, 1H, H-8^F), 5.54 (t, 1H, J=9.0 Hz, H-3^A), 5.46 (dd, 1H, J=8.0, 10.0 Hz, H-2^E), 5.34 (t, 1H, J=8.5 Hz, H-2^A), 5.29 (d, 1H, J=3.3 Hz, H-4^E), 5.20 (dd, 1H, J=2.2, 9.7, H-7^F), 5.07 (dd, 2H, J=5.9, 7.8 Hz, H-1^E, H-1^D), 5.02-4.73 (m, 9H, incl. H-2^B, H-3^E, H-1^C, H-4^F, H-5^E), 4.60 (d, 1H, J=10.9 Hz, OC*H*HPh), 4.55-4.36 (m, 5H, incl. H-1^A), 4.33-4.23 (m, 4H, incl. H-1^B), 4.23-3.88 (m, 10H, incl. H-9^F, C*H*HN$_3$), 3.88-3.61 (m, 10H, incl. H-2^C, $COOCH_3$), 3.61-3.53 (m, 3H), 3.50 (bd, 1H, J=7.8 Hz, H-3^C), 3.46-3.34 (m, 3H), 3.33-3.25 (m, 2H), 3.19-3.08 (m, 2H, OCH_2); 3.01 (dd, 1H, J=7.7, 11.6 Hz, CH*H*N$_3$), 2.41 (dd, 1H, J=4.4, 12.4, H-3$^F_{eq}$), 2.09 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 1.93 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 1.78 (s, 3H, CH_3), 1.65-1.54 (m, 4H, H-3$^F_{ax}$, $CH_2CH_2N_3$), 1.47 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.4-122.8, C-Ar), 100.5 (C-1^A), 100.3 (C-1^B), 100.3 (C-1^C), 99.3 (C-1^E, C-1^D), 83.4, 79.2, 77.7, 77.2, 77.1, 76.7, 75.4, 75.3, 75.1, 74.9, 74.7, 74.3, 73.9, 73.5, 72.9, 72.6, 72.4, 71.9 (C-3^A), 71.8 (C-2^E), 71.6, 71.5 (C-2^A) 71.1, 70.5, 69.4, 68.8, 68.5, 68.0 (C-4^E), 67.0 (C-8^F), 66.5 (C-7^F), 63.5, 62.5, 62.4, 61.5, 55.8 (C-2^C), 53.2 ($COOCH_3$), 48.8, 47.9 (OCH_2), 37.3 (C-3^F), 28.9 (*C*$H_2CH_2N_3$), 23.2, 21.3, 20.8, 20.7, 20.5, 20.4 (6x$COCH_3$).

3-Aminopropyl 3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-2-nonulopyranosyl)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)-[*β*-D-glucopyranosyl-(1→4)]-*β*-D-galactopyranosyl-(1→4)- *β*-D-glucopyranoside 5.



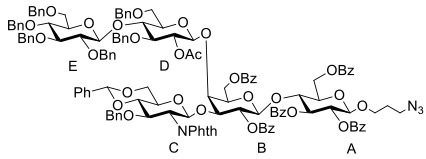
5

A mixture of protected hexasaccharide **37** (0.1 mmol) and LiI (3 mmol) in pyridine (5 ml) was heated for 24h at 120°C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 ml), and ethylenediamine (400 μL) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuum, and the residue was coevaporated with toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (5 ml), and acetic anhydride (5 ml) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH=13. After 48 h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **5** as an amorphous powder (40% yield).

^{1}H NMR (400 MHz, D_2O) δ 4.88 (d, 1H, $J_{1,2}$=8.0 Hz, H-1^{D}), 4.68 (d, 1H, $J_{1,2}$=8.2 Hz, H-1^{C}), 4.54 (d, 1H, $J_{1,2}$=7.8 Hz, H-1^{B}), 4.48 (d, 1H, $J_{1,2}$=8.0 Hz, H-1^{D}), 4.43 (d, 1H, $J_{1,2}$=7.8 Hz, H-1^{A}), 4.38 (d, 1H, $J_{1,2}$=2.7 Hz, H-4^{B}), 4.10 (dd, 1H, $J_{1,3}$=9.8 Hz, $J_{1,2}$=2.9 Hz, H-3^{E}), 4.03-3.49 (m, 33H), 3.46-3.33 (m, 2H), 3.32-3.17 (m, 4H, H-2^{D}, H-2^{A}, CH_2NH_2), 2.74 (dd, 1H, $J_{3e,4}$=12.3 Hz, $J_{3e,3a}$=4.6 Hz, H-3e^{F}), 2.01 (s, 3H, $NHCH_3$), 2.01 (s, 3H, $NHCH_3$), 2.07-1.95 (m, 2H, $CH_2CH_2NH_2$), 1.78 (t, 1H, J=12.1 Hz).

^{13}C NMR (101 MHz, $CDCl_3$) δ 175.0, 174.8, 173.8 (3xCO), 103.2 (C-1^{C}), 102.9 (C-1^{B}), 102.5 (C-1^{E}), 102.0 (C-1^{A}), 102.0 (C-1^{D}), 100.0, 99.8, 81.9 (C-3^{B}), 78.1, 75.9, 75.6, 75.5 (C-3^{E}), 75.2, 75.0 (C-4^{B}), 74.8, 74.6, 74.3, 73.6, 72.8, 72.6, 72.1, 71.7 (C-8^{F}), 70.2, 69.7, 69.3, 68.3, 68.0, 67.5 (OCH_2), 67.4 (C-4^{E}), 62.5 (C-9^{F}), 61.0, 60.8, 60.7, 60.1, 59.9, 55.6 (CH_2NH_2), 55.2 (C-2^{C}), 51.6 (C-5^{F}), 39.6 (C-3^{F}), 24.3 ($CH_2CH_2NH_2$), 22.2 ($NHCH_3$), 22.0 ($NHCH_3$).

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[(2,3,4,6-tetra-*O*-benzyl- *β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 38.



38

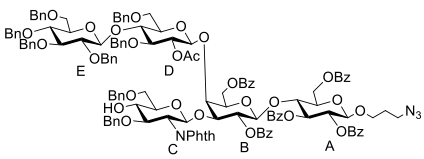
A solution of glycosyl donor **30** (0.545 g, 0.51 mmol) and glycosyl acceptor **24** (0.361 g, 0.26 mmol) with activated 4 Å molecular sieves (0.400 g) in dry DCM (5 mL) was stirred for 20 min under nitrogen. The resulting suspension was cooled down to -5°C and TMSOTf (9.4 µL, 0.052 mmol) was added. After 1 h (TLC: Toluene/EtOAc 9:1) the reaction was quenched with Et_3N, then evaporated to dryness and the crude was purified by column chromatography (gradient from 0 to 50% of EtOAc in toluene). Pure fractions were collected and evaporated, affording the target pentasaccharide **38** in 55% yield.

$[\alpha]_D^{25}$ = +17.02° (c 0.15, $CHCl_3$). ESI HR -MS ($C_{134}H_{128}N_4O_{33}$) *m/z* $[M+Na]^+$ 2343.8461; found 2343.8342.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-6.70 (m, 69H , Ar-H), 5.57 (t, 1H, $J_{1,2}$=9.2 Hz, H-3^{A}), 5.49 (s, 1H, CHPh), 5.36 (dd, 1H, $J_{1,3}$=9.7 Hz, $J_{1,2}$=7.9 Hz, H-2^{A}), 5.29 (d, 1H, $J_{1,2}$=8.2 Hz, H-1^{C}), 5.16 (d, 1H, $J_{1,2}$=11.4 Hz), 5.08 (dd, 1H, $J_{1,3}$=9.8 Hz, $J_{1,2}$=7.9 Hz, H-2^{B}), 5.00 (d, 1H, $J_{1,2}$=11.3 Hz), 4.89 (s, 2H, CH_2OPh), 4.88-4.81 (m, 2H, H-1^{D}, H-2^{D}), 4.76 (d, 2H, $J_{1,2}$=10.9 Hz), 4.73-4.66 (m, 4H), 4.60-4.49 (m, 4H, incl. H-1^{E}, H-1^{A}), 4.44 (d, 1H, $J_{1,2}$=12.1 Hz), 4.40-4.30 (m, 4H, incl. H-1^{B}), 4.30-4.21 (m, 3H), 4.21-4.11 (m, 2H, incl. H-2^{C}), 4.05-3.66 (m, 11H, incl. H-3^{B}, H-3^{D}, H-3^{C}, H-4^{A}), 3.62-3.33 (m, 9H), 3.14 (dt, 2H, $J_{1,3}$=6.4 Hz $J_{1,2}$=3.0 Hz), 3.04 (dd, 1H, $J_{1,3}$=11.9 Hz, $J_{1,2}$=7.5 Hz), 1.95 (s, 3H, $COCH_3$), 1.74-1.59 (m, 2H, C$H_2$$CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.1, 167.5, 166.8, 165.9, 165.8, 165.5, 165.3, 164.1 (8xCO), 145.7, 139.5-122.9 (C-Ar), 106.6, 102.9 (C-1^{A}), 101.3 (C-1^{E}), 101.1 (CHPh), 100.7 (C-1^{B}), 100.1 (C-1^{D}), 100.0 (C-1^{C}), 99.7, 83.0, 82.6, 81.5, 80.0, 78.9, 77.3, 75.8, 75.7, 75.3, 74.7, 74.4, 74.3, 73.8, 73.6, 73.5, 73.4, 73.2, 73.0, 72.9, 72.7, 72.4 (C-3^{B}), 72.3 (C-2^{D}), 71.5 (C-3^{A}), 71.1 (C-3^{A}), 68.6, 68.5, 68.3, 66.5, 66.0, 63.2, 62.5, 55.8 (C-2^{C}), 47.8 (OCH_2), 28.9 ($CH_2CH_2N_3$), 20.4 ($COCH_3$).

3-Azidopropyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-[(2,3,4,6-tetra-*O*-benzyl-*β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 39.

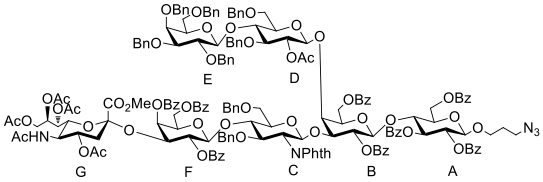


39

A solution of compound **38** (0.265 g, 0.114 mmol) in ACN (6 mL) was cooled at 0°C. Me_3NBH_3 (0.042 g, 0.57 mmol) and $BF_3{\cdot}OEt_2$ (0.070 mL, 0.57 mmol) were added and the reaction was stirred for 2 h under nitrogen. First TEA and then MeOH were added until neutral pH. The solvent was removed at reduced pressure and the crude was purified by flash

chromatography (toluene:EtOAc) to afford the target pentasaccharide acceptor **39** in 55% yield (0.140 g).
$[\alpha]_D^{25}$ = +20.41° (c 0.15, $CHCl_3$). ESI HR -MS ($C_{134}H_{130}N_4O_{33}$) *m/z* $[M+Na]^+$ 2345.8617; found 2345.8594.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-6.84 (m, 69H, Ar-H), 5.57 (t, 1H, $J_{1,2}$=9.4 Hz, H-3^A), 5.36 (dd, 1H, $J_{1,3}$=9.8 Hz, $J_{1,2}$=7.9 Hz, H-2^A), 5.22 (d, 1H, $J_{1,2}$=7.8 Hz, H-1^C), 5.16 (d, 1H, $J_{1,2}$=11.6 Hz), 5.06 (dd, 1H, $J_{1,3}$=10.0 Hz, $J_{1,2}$=7.9 Hz, H-2^B), 4.99 (d, 1H, $J_{1,2}$=11.4 Hz), 4.90-4.80 (m, 4H, incl. H-1^D, H-2^D), 4.79-4.65 (m, 3H), 4.61 (d, 1H, $J_{1,2}$=5.6 Hz), 4.59-4.54 (m, 2H, incl. H-1^E), 4.52 (d, 1H, $J_{1,2}$=7.7 Hz, H-1^A), 4.46 (d, 2H, $J_{1,2}$=12.7 Hz), 4.38-4.30 (m, 4H), 4.29-4.14 (m, 4H), 4.12-3.98 (m, 4H), 3.96-3.88 (m, 2H), 3.88-3.66 (m, 8H, incl. H-2^E, H-3^D), 3.60-3.32 (m, 10H), 3.14 (dt, 2H, $J_{1,3}$=6.5 Hz, $J_{1,2}$=3.1 Hz), 3.01-2.98 (m, 1H), 1.85 (s, 3H, $COCH_3$), 1.75-1.59 (m, 2H, C*H*$_2$$CH_2N_3$).
^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 165.9, 165.8, 165.4, 165.3, 164.1 (6xCO), 139.7-123-0 (C-Ar), 102.8 (C-1^E), 101.1 (C-1^A), 100.7 (C-1^B), 100.1 (C-1^D), 99.1 (C-1^C), 82.6, 81.4, 80.0, 78.5, 78.4, 75.6, 75.3, 75.1, 74.7, 74.6, 74.6, 74.3, 73.8, 73.6, 73.5, 73.4, 73.3, 73.2, 73.0, 72.6, 72.5 (C-2^D), 72.3 (C-3^A), 71.5 (C-2^A), 71.3 (C-2^B), 70.5, 68.4, 68.3, 66.6, 63.4, 62.5, 55.5 (C-2^C), 47.9 (OCH_2), 28.9 ($CH_2CH_2N_3$), 20.3 ($COCH_3$).

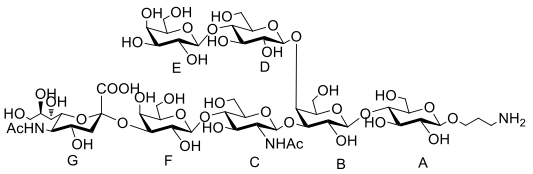
3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-O-benzyl-2-deoxy-2-N-phthalimido-β-D-glucopyranosyl-(1→3)-[2,3,4,6-tetra-O-benzyl-β-D-galactopyranosyl-(1→4)-*O*-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranosyl}-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside 40.



40

A solution of disaccharide donor **11** (0.080 g, 0.070 mmol) and pentasaccharide acceptor **39** (0.109 g, 0.047 mmol) with activated 4 Å molecular sieves (0.100 g) in dry DCM (3 mL) was stirred for 20 min under nitrogen. TMSOTf (1.7 µ L, 0.0094 mmol) was added at 0°C. After 4 h (TLC; 6:4 Toluene:acetone) the reaction was quenched with TEA, the solid filtered off and the solvent removed under pressure. The crude was purified by flash chromatography (Toluene:acetone) to afford the target heptasaccharide **40** as a yellow amorphous solid (0.090 g, 60% yield).
$[\alpha]_D^{25}$ = +20.23° (c 0.5, $CHCl_3$). ESI HR-MS ($C_{181}H_{173}N_5O_{53}$) *m/z* $[M+Na]^+$ 3293.1357; found 3295.14.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25-6.53 (m, 84H, Ar-H), 5.70, 5.62 (m, 1H, H-8^G), 5.52 (t, 1H, $J_{1,2}$=9.6 Hz, H-3^A), 5.44 (dd, 1H, $J_{1,3}$=9.8 Hz, $J_{1,2}$=7.7 Hz), 5.32 (dd, 1H, $J_{1,3}$=9.7 Hz, $J_{1,2}$=7.9 Hz, H-2^A),5.30-5.27 (m, 1H), 5.21 (dd, 1H, $J_{1,3}$=9.6 Hz, $J_{1,2}$=2.6 Hz, H-7^G), 5.15 (d, 1H, $J_{1,2}$=11.7 Hz), 5.07 (d, 1H, $J_{1,2}$=7.8 Hz), 5.03 (d, 1H, $J_{1,2}$=7.8 Hz), 5.01-4.87 (m, 3H), 4.84-4.70 (m, 6H), 4.70-4.64 (m, 2H), 4.56-4.53 (m, 2H), 4.53-4.37 (m, 5H incl. H-1^E, H-1^A), 4.34-4.29 (m, 5H), 4.20-3.90 (m, 12H), 3.90-3.77 (m, 6H), 3.76 (s, 3H, $COOCH_3$), 3.71-3.63 (m, 3H), 3.57 (dd, 1H, $J_{1,3}$=10.6 Hz, $J_{1,2}$=2.7 Hz), 3.53-3.24 (m, 9H), 3.16-3.06 (m, 2H, OCH_2), 3.00 (dd, 1H, $J_{1,3}$=12.1 Hz, $J_{1,2}$=8.1 Hz), 2.40 (dd, 1H, $J_{3e,4}$=12.7 Hz, $J_{3e,3a}$=4.6 Hz, H-3e^G), 2.11 (s, 3H, $COCH_3$), 1.94 (s, 3H, $COCH_3$), 1.89 (s, 3H, $COCH_3$), 1.77 (s, 3H, $COCH_3$), 1.69 (s, 3H, $COCH_3$), 1.68-1.57 (m, 3H, incl. $CH_2CH_2N_3$, H-3a^G), 1.48 (s, 3H, $COCH_3$).
^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.7, 170.4, 170.3, 170.1, 169.8, 168.1, 167.4, 167.0, 165.7, 165.5, 165.4, 165.3, 164.9, 164.1 (15xCO), 139.9-114.4 (C-Ar), 102.9 (C-1^E), 101.2 (C-1^A), 101.1 (C-1^B), 100.6 (C-1^D, C-1^F), 100.3 (C-1^C), 100.0, 98.6, 97.0, 96.9, 82.7, 82.4, 81.3, 79.9, 77.4, 77.2, 76.7, 76.6, 75.5, 75.4, 75.1, 74.6, 73.8, 73.4, 73.1, 72.7, 72.5, 72.2, 72.1 (C-3^A), 71.9, 71.8, 71.7, 71.5 (C-2^A), 71.4, 71.3, 70.4, 69.4, 68.3, 68.1, 67.8, 67.4 (C-8^G), 67.1, 66.6 (C-7^{1qw}), 63.3, 62.6, 62.5, 62.3, 61.5, 55.8 (C-2^C), 53.1 ($COOCH_3$), 48.9, 47.9 (OCH_2), 37.4 (C-3^G), 29.4 ($CH_2CH_2N_3$), 21.5, 21.3, 20.7, 20.4, 20.3, 20.1 (6x$COCH_3$).

3-Aminopropyl 3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-2-nonulopyranosyl)-*β*-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)-[*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→4)]-*β*-D-galactopyranosyl-(1→4)- *β*-D-glucopyranoside 6.



6

A mixture of protected heptasaccharide **40** (0.1 mmol) and LiI (3 mmol) in pyridine (5 ml) was heated for 24h at 120°C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (3 ml), and ethylenediamine (300 µL) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuum, and the residue was coevaporated with toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (5 ml), and acetic anhydride (5 ml) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH=13. After 48 h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **6** as an amorphous powder (40% yield).

^{1}H NMR (400 MHz, D_2O) δ 4.95 (s, 1H, $J_{1,2}$=7.7 Hz, H-1^D); 4.71 (d, 1H, $J_{1,2}$=8.4 Hz, H-1^C), 4.56 (d, 1H, $J_{1,2}$=7.7 Hz, H-1^F), 4.52 (d, 1H, $J_{1,2}$=7.7 Hz, H-1^B), 4.49-4.39 (m, 3H, incl. H-4^B, H-1^A, H-1^E), 4.12 (dd, 1H, $J_{1,3}$=9.9, $J_{1,2}$=3.1 Hz), 4.08-3.44 (m, 40H), 3.38-3.27 (m, 2H, H-2^A, H-2^D), 3.25-3.13 (m, 2H, CH_2NH_2), 2.77 (dd, 1H, $J_{3e,4}$=12.3 Hz, $J_{3e,3a}$=4.5 Hz, H-3e^G); 2.04 (s, 3H, $NHCH_3$), 2.03 (s, 3H, $NHCH_3$), 2.09-1.95 (m, 2H, $CH_2CH_2NH_2$), 1.81 (t, 1H, $J_{1,2}$=11.9 Hz, H-3a^G).

^{13}C NMR (101 MHz, D_2O) δ 104.1 (C-1^C), 103.6 (C-1^A, C-1^E), 103.4 (C-1^F), 103.2 (C-1^B), 102.5 (C-1^D), 82.8, 79.3, 79.0, 78.9, 78.8, 76.1, 75.8 (C-4^B), 75.7, 75.5, 74.0, 73.9, 73.6, 73.2, 73.1, 72.6, 71.5, 69.3, 69.2, 69.2, 68.6, 63.4, 61.9, 61.8, 61.5, 61.4, 61.0, 56.0, 52.5, 47.7 (CH_2NH_2), 40.2 (C-3^G), 23.2 (CH_3), 23.2 (CH_3), 21.1 ($CH_2CH_2NH_2$).

Quantitative estimation of sialic acid[29].

Yields of the synthetic oligosaccharide was calculated on the basis of the sugar quantification, which was done by quantitative estimation of the sialic acid content with a colorimetric resorcinol-hydrochloric acid method.

Standard samples of sialic acid at concentrations 5, 10, 15, 20, 25 µg/mL were prepared to build the calibration curve. GBS PSIa samples were prepared targeting the calibration curve midpoint. The reference and analytical samples were treated with a solution of Resorcinol/HCl. The reagent resorcinol/HCl was prepared by mixing 10 mL of a 2% resorcinol solution, 0.25 mL of a 0.1 M Copper (II) Sulphate solution in 20 mL of water with 37% hydrochloric acid up to a volume of 100 mL. After treatment of samples and references with the resorcinol/HCl reagent, the resulting mixtures were heated in a oil bath at 110°C for 20 min. After that, absorbance was read at 564 nm.

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Chapter 5

Synthesis of GBS CPS serotype Ib branched and linear repeating units

Part of this Chapter has been published: Del Bino, L.; Calloni, I.; Oldrini, D.; Raso, M.M.; Cuffaro, R.; Arda, A.; Codée, J.; Barbero, J.J.; Adamo, R. *Chem. Eur. J.*, **2019**, *25 (71)*, 16277-16287.

Introduction

Group B *Streptococcus* (GBS) capsular polysaccharide is, as mentioned, a main target for vaccine development[1-3] and, on the basis of its structure, ten GBS serotypes have been classified and described[4, 5], and some of them share the same composition in terms of sugar residues differing only for the linkage position. GBS type Ib structure, for instance, is structurally very similar to GBS type Ia, which has been described in Chapter 4. Rebecca Lancefield was the first to notice serologic cross reactions between strains bearing Ia and Ib antigens, although she did not know what cellular structure contained the differing epitopes. NMR analysis showed that the native CPSs of GBS types Ia and Ib are indeed structurally similar, differing only in the linkage of the side chain galactose to *N*-acetylglucosamine[6], the type Ia CPS has a β-(1→4) linkage, and the type Ib CPS has a β-(1→3) linkage in this position. Full characterization of the CPS antigen revealed that the repeating unit of Ia and Ib CPSs is a pentasaccharide with a disaccharide backbone and a trisaccharide side chain. Like all GBS CPSs, each possesses an α-(2→3)-linked sialic acid as a terminal side chain saccharide (Figure 1).

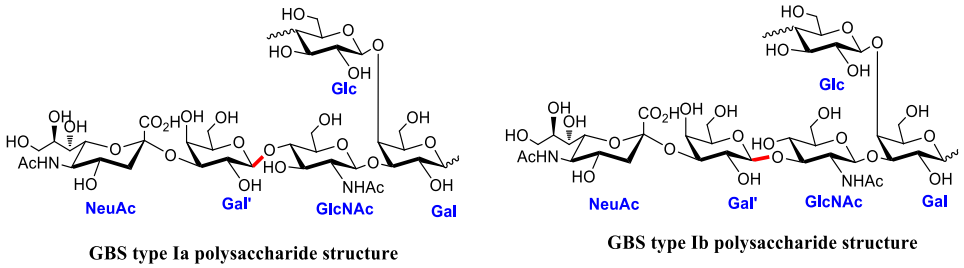


Figure 1. Structure of GBS serotypes Ia and Ib CPS repeating units

Recent European studies (2008-2010) indicated that serotype Ib is cause of 7.3% of the Early-onset and 5.4% of the Late-Onset diseases and together with serotype Ia is the most common strain colonizing the gastro-intestinal and vaginal tracts[7]. Moreover, more than 12% of GBS infections in elderly or immunocompromised patients can be related to type Ib[8].

Given these data, an effective GBS vaccine should include also serotype Ib. Indeed, monovalent conjugate vaccine prepared with Ib polysaccharide has been tested in phase I/II clinical trials, as well as a trivalent combination (Ia, Ib, III) in order to develop a maternal vaccination strategy[9-11]. Immunization with conjugated PSIa and PSIb vaccines led to isolation of serotype-specific antibodies, showing that the different linkage in the side chain is determining the immunospecificity of the polysaccharides.

As for GBS type Ia, neither chemical nor enzymatic depolymerization method are available for CPS Ib and therefore, chemical synthesis is the only viable option to obtain homogeneous oligosaccharides from the CPS, which can be used to explore interactions with serotype specific

monoclonal antibodies and to elucidate the mechanism of action of the polysaccharide conjugates[12, 13].

While a synthesis of the pentasaccharide corresponding to the branched GBS PSIa repeating unit was recently described[14], there is no synthesis currently reported for GBS type Ib repeating unit.

With the ultimate goal to identify the structural features of the CPS relevant for antibody recognition, this Chapter describes how the best Glc*p*NAc-β-D-(1→3)-Gal*p* synthons among those described in Chapter 3 were chosen for elongation to give the pentasaccharide repeating unit of GBS serotype Ib. The synthesized oligosaccharide fragments will be used to map the interaction to an anti PSIb monoclonal antibody and to highlight (thanks to NMR and Molecular Dynamics simulation) the conformational properties of the corresponding polysaccharide resulting in immunospecificity with respect to the very similar type Ia CPS, reported in the previous Chapter. All targeted synthetic structures are designed with a free amino group at the oligosaccharide reducing end in order to facilitate further manipulation and conjugation to carrier protein for immunological evaluation.

Results and discussion

Synthesis of GBS serotype Ib repeating unit

As mentioned, the repeating unit of GBS serotype Ib is a pentasaccharide with a disaccharide backbone and a trisaccharide side chain, structurally very similar to the one of GBS type Ia and III.

This structure can be described by the branched and the linear frameshifts **1** and **2** depicted in Figure 2.

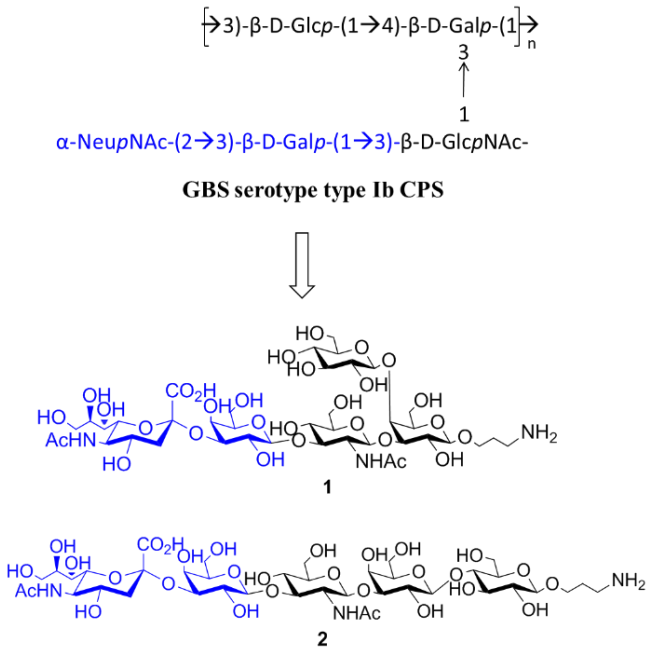
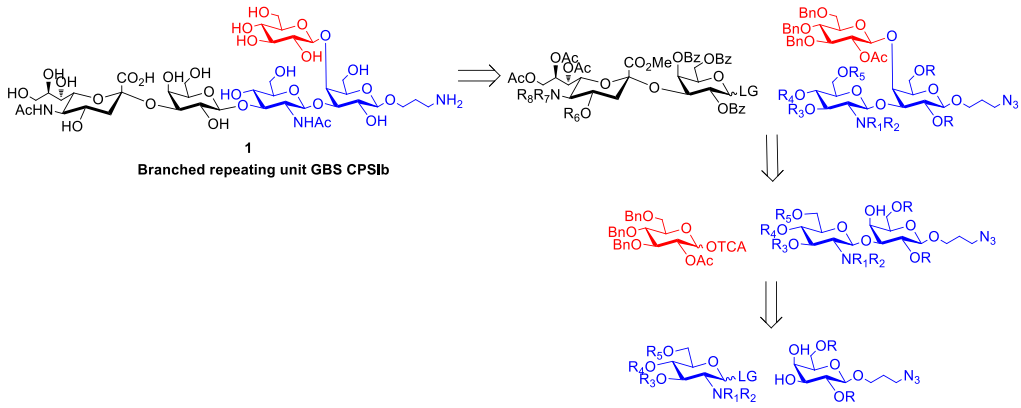


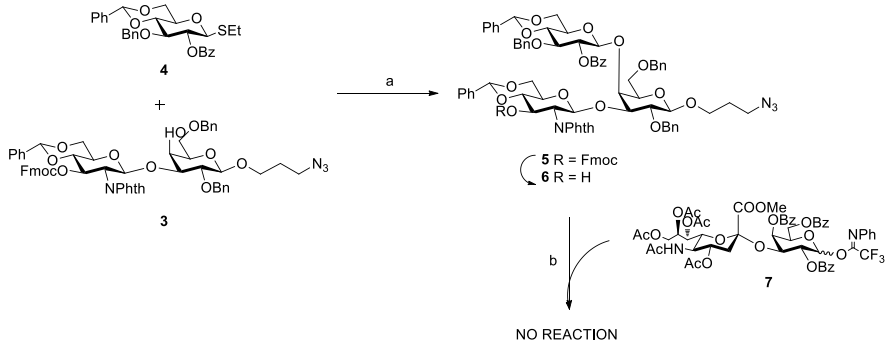
Figure 2. Structure of type Ib GBS CPS and the synthetic targets: its branched (**1**) and linear (**2**) repeating units.

As already mentioned for GBS serotype Ia oligosaccharides (Chapter 4), two main challenges in the synthesis of pentasaccharide **1** can be identified: the presence of the difficult α sialic acid connected to the upstream Gal residue and an effective approach to the branched trisaccharide Glc*p*NAc-β-D-(1→3)-Gal*p*-β-D-(1→4)-Glc*p*. The stereoselectivity of a late stage α-sialylation could be challenging and impact on the overall yield of the process and therefore, a convergent [3+2] regioselective approach employing a sialogalactoside donor and a trisaccharide acceptor was selected. The trisaccharide acceptor was deriving in turn from the disaccharide synthon Glc*p*NAc-β-D-(1→3)-Gal*p* obtained by means of a regioselective β-(1→3) galactose glycosylation (see Chapter 3). Moreover, the synthetic design to pentasaccharide **1** and **2** anticipated the installation of an azidopropyl linker (converted into aminopropyl during the deprotections) at the reducing end to make these structures suitable for further manipulation and conjugation to carrier proteins (Scheme 1).



Scheme 1. Retrosynthetic scheme to GBS serotype Ib branched repeating unit

Differently than the GBS CPS Ia pentasaccharides, the two Ib frameshifts **1**-**2** required a glucosamine building block bearing a temporary protecting group at its C3-OH and the creation of the Galβ1-3GlcNAc linkage, which had a strong impact on the synthetic design. At first, in line with the synthesis of the CPS Ia branched oligosaccharides, attempts to prepare the branched pentasaccharide **1** started from the NPhth protected trisaccharide acceptor **6** which was prepared by Fmoc removal from **5**, obtained by the coupling of disaccharide **3** and glucose donor **4**. Unfortunately, the reaction of **6** with the sialogalactoside donor **7** was unsuccessful (Scheme 2).

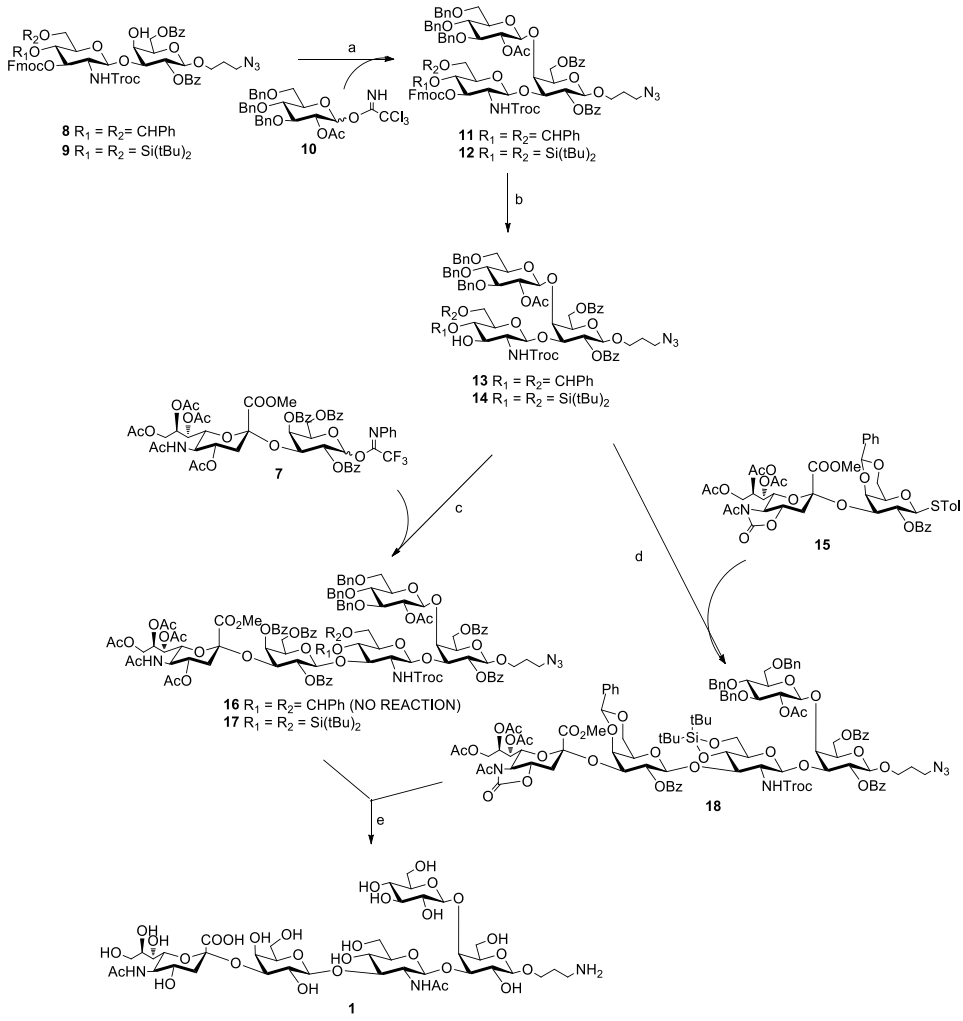


Scheme 2. *Reagents and conditions:* a) TfOH, NIS, DCM dry, 80%; b) TMSOTf, DCM dry, 0°C.

From this trial, it was realized that the C3-OH of the glucosamine was significantly less reactive than the C4-OH, likely due to the presence of the bulky NPhth group that could hinder the glycosylation reaction at the neighbouring alcohol. It was anticipated that replacement of the NPhth by the Troc group could result in a higher nucleophilicity of the vicinal hydroxyl. To test this hypothesis, disaccharides **8** and **9**, obtained with good yields by means of a regioselective glycosylation and differing only in the cyclic protecting group blocking the glucosamine C4,6-OH, were selected to be elongated to the branched pentasaccharide **1** (Scheme 3). Glycosylation of the two acceptors with the armed Glc donor **10** under TMSOTf activation at 0°C afforded the

trisaccharides **11** and **12** in 63% and 70% yield, respectively. After Fmoc removal by treatment with 10% piperidine in DCM (92%), glycosylation with the sialogalactoside donor **7** of the two acceptors **13** and **14** was undertaken. Reaction of the 4,6-*O*-benzylidene trisaccharide **13** and **7** with TMSOTf as promoter failed to afford the target pentasaccharide, leading to complete recovery of the unreacted acceptor. In contrast, reaction of acceptor **14**, bearing the more flexible 4,6-*O*-silylidene ketal[15], with **7** in the presence of TMSOTf gave the target pentasaccharide **17** in 80% yield (Scheme 3). This result suggests that the glycosylation of **13** was prevented by the steric and torsional constrain of the 4,6-*O*-benzylidene ring. Trisaccharide **14** was also efficiently glycosylated with disaccharide donor **15** by NIS/TfOH activation, affording **18** in 65% yield (Scheme 3). Despite a slightly lower yield in this step, the overall efficiency of the synthesis of GBS serotype Ib branched repeating unit was superior using the thioglycoside **15**[16, 17].

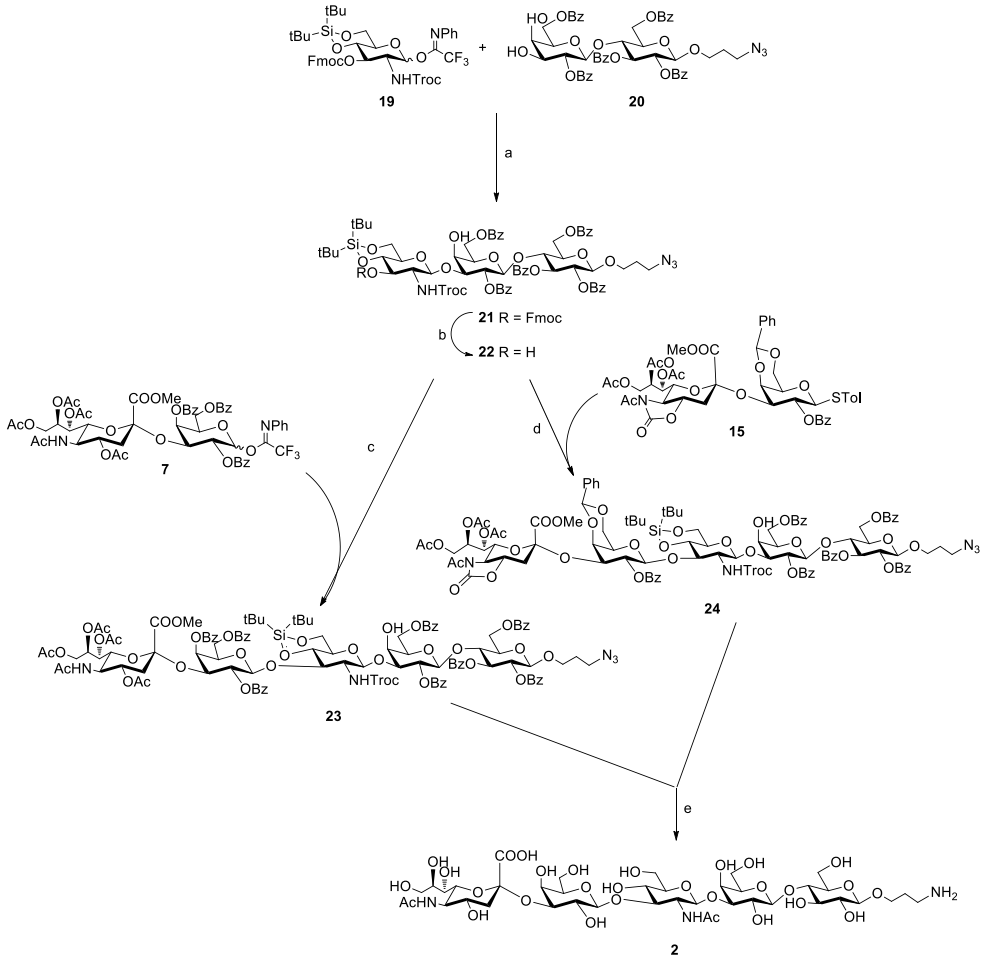
Pentasaccharides **17** and **18** were then deprotected by a four-steps protocol: (i) desilylation by treatment with HF·pyridine, (ii) saponification with NaOH in refluxing THF, for concomitant hydrolysis of the acyl esters, the Troc group and the 5-*N*,4-*O*-oxazolidinone protecting group and Neu5Ac methyl ester; (iii) reacetylation of the amines using a 2:3 acetic anhydride/methanol mixture; (iv) hydrogenation over Pd/charcoal. The target branched pentasaccharide **1** was obtained in 40% yield as estimated by spectrophotometric quantification of the sialic acid content[18].



Scheme 3 Assemble of GBS CPSIa repeating unit. *Reagent and conditions*: a) TMSOTf, DCM dry, -10°C, 75% from **8**; 68% from **9**; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 70% ; c) TMSOTf, DCM dry, 0°C, 75%; d) TfOH, NIS, DCM dry, -40°C, 73%; e) LiI, Py, 120°C; NaOH 3M, THF, reflux; Ac_2O, MeOH; H_2, Pd-C, 40% (over four steps).

At this point, the linear frameshift **2** of the GBS serotype Ib repeating unit was targeted and the feasibility of the regioselective approach was assessed again (Scheme 4). For this purpose, benzoylated lactose acceptor **20**, previously described for the synthesis of the linear repeating unit of GBS PSIa (Chapter 4), was glycosylated with the 4,6-*O*-silylidene glucosamine imidate **19** under TMSOTf activation to give the target trisaccharide **21** with full stereo- and regioselectivity (55%). Following Fmoc deprotection with piperidine in DCM, the obtained acceptor **22** was glycosylated with imidate **7** to attain the linear protected pentasaccharide **23** (66%). Reaction with thioglycoside **15** in TfOH and NIS reaction conditions provided the analogous pentasaccharide **24** (40%). The obtained pentasaccharides were deprotected and purified with the four-step

deprotection protocol described above. NMR data of the synthesized CPS Ib fragments were in excellent agreement with NMR data from samples of the bacterial polysaccharide[19] (Table 1 and Figure 3).



Scheme 4. Synthetic route to GBS type Ib linear repeating unit. *Reagents and conditions*: a) TMSOTf, DCM dry, 0°C, 55%; b) Piperidine, DCM, 90%; c) TMSOTf, DCM dry, 0°C, 66%; d) TfOH, NIS, DCM dry, -40°C, 40%; d) HF/pyridine; 3 M NaOH, THF, reflux; Ac_2O/MeOH; H_2/Pd-C, 40%.

Table 1. Chemical shift (ppm) of ^{1}H and ^{13}C NMR signals of compound **1-2** in D_2O

Residue		**Compound 1**		**Compound 2**		**PS Ib**[a]	
		^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
Gal	1	4.38/ *J* 7.6 Hz	103.3	4.48/J 8.3	103.3	4.49	101.9
	2	3.69	70.2	3.55	69.8	3.70	71.0
	3	3.80	82.0	3.76	81.9	3.80	82.7
	4	4.36	74.8	4.11	68.2	4.11	69.4
	5	3.69	64.2	3.63	74.9	3.69	75.5
	6	3.74	60.8	3.75	60.5		
	6’	3.74		3.75			
GlcNAc	1	4.72/ *J* 8.1 Hz	102.7	4.68/J 8.3 Hz	102.0	4.73	102.9
	2	3.88	54.8	3.85	54.6	3.89	55.3
	3	3.78	82.2	3.69	81.6	3.80	82.7
	4	3.56	68.6	3.53	68.5		
	5	3.48	75.4	3.43	75.2	3.45	75.9
	6	3.91	60.8	3.93	60.1		
	6’	3.91		3.93			
Glc	1	4.87/ *J* 7.6 Hz	102.0	4.48/J 8.3 Hz	102.9	4.90	103.3
	2	3.23	73.6	3.28	72.5	3.28	74.2
	3	3.51	75.9	3.59	72.8	3.55	75.2
	4	3.36	69.8	3.59	78.4	3.60	69.4
	5	3.42	75.6	3.65	74.9	3.66	75.4
	6	3.87	60.8	3.94	60.0		
	6’	3.77		3.76			
Gal’	1	4.47/ *J* 8.1 Hz	102.9	4.41/J 7.5 Hz	102.5	4.49	102.3
	2	3.51	69.0	3.50	68.9		
	3	4.07	75.5	4.04	75.5	4.06	73.4
	4	3.92	67.3	3.89	67.3	3.91	68.2
	5	3.70	74.2	3.67	74.8	3.64	75.5
	6	3.71	61.0	3.68	60.8		
	6’	3.71		3.68			
Neu5Ac	3	2.73	39.7	2.72	39.7	2.74	40.9
	3’	1.75		1.75		1.78	
	4	3.66	68.4	3.63	68.3		
	5	3.82	51.7	3.80	51.5	3.82	52.4
	6	3.61	72.8	3.59	74.5	3.59	73.5
	7	3.58	68.1	3.54	68.2		
	8	3.86	71.9	3.83	71.6	3.86	72.3
	9	3.83	62.6	3.80	62.4	3.83	63.3
	9’	3.62		3.60		3.62	

[a]. Not assigned peaks are due to overlapping in the HSQC spectrum of the polysaccharide

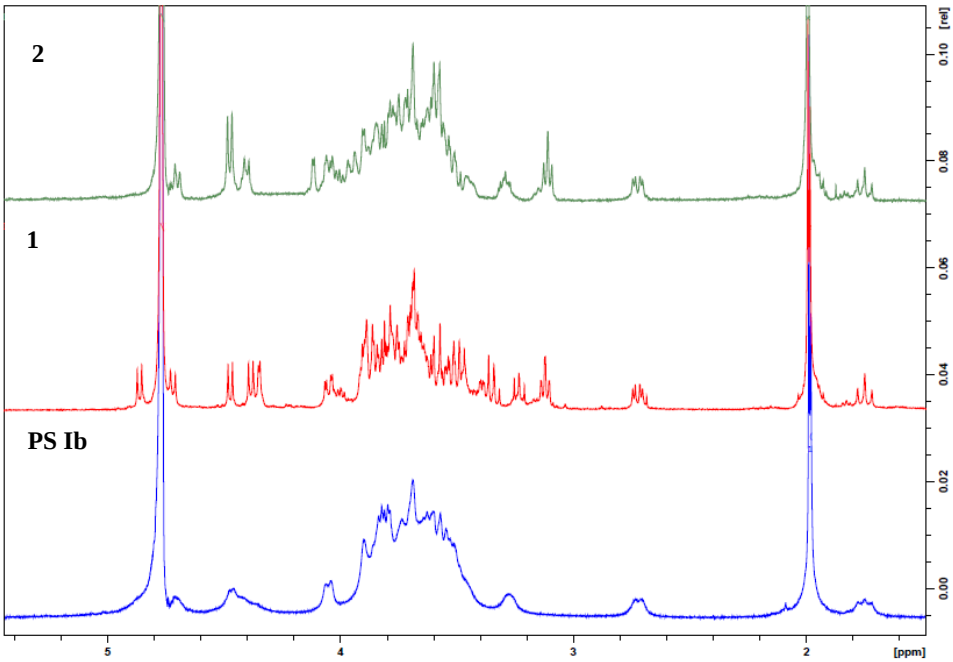


Figure 3. ^{1}H NMR spectra of pentasaccharides **1–2** in comparison to PSIb (D_2O, 400 MHz, 298 k)

Conformational studies

Having access to structurally defined GBS type Ib related glycans is a key step forward to investigate the structural-immunogenicity relationship of the capsular polysaccharide and to gain insights into its conformation and understanding how the conformational behaviour of type Ib polysaccharide could influence the exposure of different glycotopes compared to the type Ia polysaccharide. Considering that Ia and Ib CPS differ exclusively in the connection of a Gal residues to the GlcNAc-Gal, which is β-(1→4) in type Ia and β-(1→3) in type Ib, this linkage appears to be crucial for the immunospecifity[11, 19].

To elucidate the impact of this linkage on the shape of the polysaccharides, conformational properties of the branched repeating unit pentasaccharides **1** were studied by a combination of NMR and modelling tools[20], to build up a model of the natural CPS. This model was then compared to the one of GBS type Ia (see Chapter 4). These studies revealed a different preferential shape for Ib polysaccharide compared to Ia was revealed; in particular, the main difference highlighted regards the presentation of the protruding Neu5Ac-α-(2→3)-Gal moieties, with a major *exo-anti-Φ* population for Ia and *exo-syn-Φ* for Ib, which results in a higher flexibility of the Ib polymer (Figure 5 and 6). These unique structural features are expected to influence antibody recognition and immunospecificity.

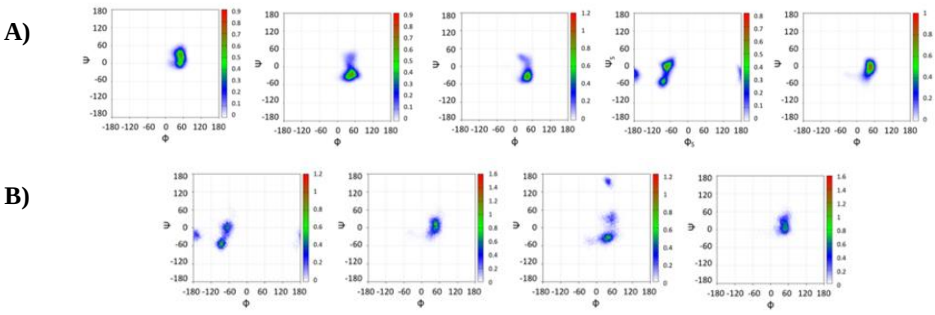


Figure 4. A) Glycosidic linkage analysis for GBS Ia polysaccharide: φ/ψ plots for representative glycosidic bonds of a 10 repeating unit model along the 2.5 μs MD simulation B) Glycosidic linkage analysis for GBS Ia pentasaccharide 1: φ/ψ plots for representative glycosidic bonds along 200 ns of MD simulation.

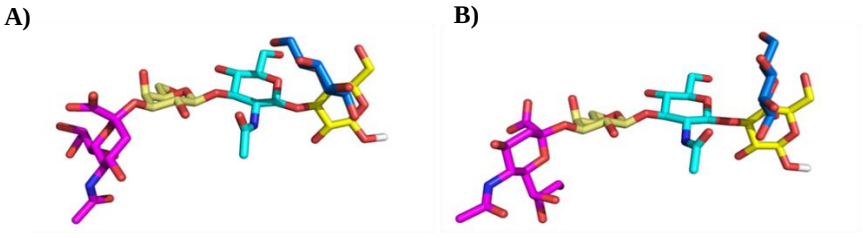


Figure 5. Perspectives of the two populations, deduced by ROESY NMR experiments, which define the conformational behaviour of the pentasaccharide repeating unit of GBS Ib. A) the φ_S torsion angle adopts the major trans (t) geometry. B) the φ_S torsion angle shows the minor -g conformation.

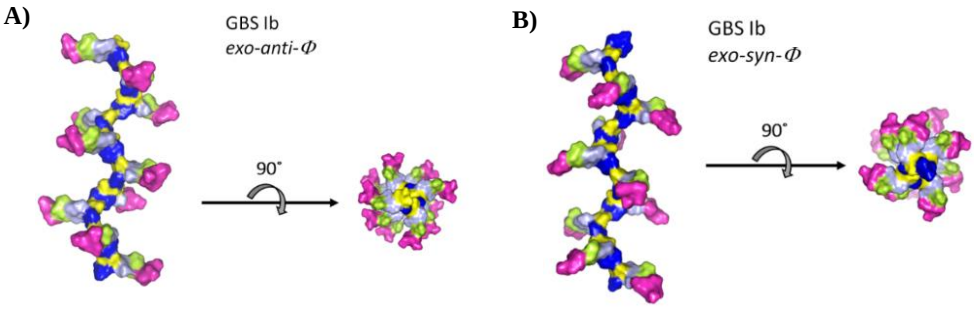


Figure 6. Model structures for the GBS Ib polysaccharide: A) with exo-anti-Φ conformation around all Neu5Acα2-3Gal linkages and B) Ib with the major exo-syn-Φ conformation around all Neu5Acα2-3Gal linkages.

Conclusion

This Chapter describes the application of regioselective strategies to the synthesis of Group B streptococcus serotype Ib repeating unit, which can be represented both by a linear and a branched pentasaccharide. The synthetic design was based on a [2+3] glycosylation strategy employing a sialogalactoside donor and a trisaccharide acceptor, which in turn was obtained by a regioselective

β-(1→3)-glycosylation of galactose and lactose for the branched and the linear repeating unit, respectively.

Pentasaccharides **1** and **2** helped to investigate the conformational behaviour of the corresponding capsular polysaccharide, especially compared to the structurally close GBS serotype Ia CPS. Indeed, Ia and Ib CPS differ exclusively in the connection of a Gal residues to the GlcNAc-Gal but despite these similarities, no immune cross reactivity was observed between the two serotypes[11, 19].

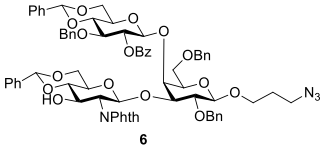
Thanks to a combination of NMR and computational tools[20], the branched repeating unit **1** was used as probe to build a model to of the natural CPS. On the basis of this model, a higher flexibility of the Ib polymer compared to the Ia was revealed, which can result in the exposure of different epitopes.

The synthetic glycans **1** and **2** will be used to map the relevant glycotopes by a combined approach including competitive ELISA, SPR and STD-NMR. Moreover, the presence of the aminopropyl handle will be exploited for conjugation to carrier proteins and immunological evaluation of the resulting glycoconjugates.

Experimental

General Methods and Procedures. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50ºC on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm) or using pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (Biotage, silica 0.050 nm). Unless otherwise specified, a gradient 0-100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) or Biotage Isolera instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{1}H values are reported in ppm, relative to internal Me_4Si (^{1}H = 0.00, $CDCl_3$); solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{13}C values are reported in ppm relative to the signal of $CDCl_3$ (^{13}C = 77.0, $CDCl_3$). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Assignment of ^{13}C NMR spectra of some compounds was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters. Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data and *m/z* values found in their mass spectra.

3-Azidopropyl 3-*O*-benzyl-4,6-O-benzylidene-2-*O*-benzoyl-*β*-D-glucopyranosyl-(1-4)-[4,6-*O*-benzylidene-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1-3)-]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 6.



6

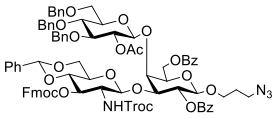
Compound **4** (41 mg, 0.08 mmol) and **3** (62 mg, 0.06 mmol) were dissolved in dry DCM (4 mL) with 4 Å activated molecular sieves and the mixture was stirred for 15 min under nitrogen. NIS (36 mg, 0.16 mmol) and TfOH (1.7 mg, 0.18 mmol) were added at -40°C, and the reaction was stirred overnight at rt, when TLC (7:3 Tol:EtOAc) showed complete reaction. The reaction was quenched with TEA, molecular sieves were filtered off and the solvent was removed at reduced pressure. The crude was purified by flash chromatography (Tol: EtOAc) to afford **5** (73 mg, 81 % yield).

Trisaccharide **5** (73 mg, 0.05 mmol) was dissolved in dry DCM (4 ml) and 10% of piperidine (0.4 ml) were added at the solution. 10 min later, TLC (Tol:EtOAc) showed complete conversion, and the reaction was concentrated under reduced pressure. Purification of the crude material by flash chromatography (Tol:EtOAc) gave **6** (57 mg) in 90% yield. $[\alpha]_D^{25}$ = -7.89° (c 0.05, $CHCl_3$). ESI HR-MS ($C_{62}H_{59}N_4O_{17}$) *m/z* $[M+H]^+$ found 1267.3391; calcd 1267.3315.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-6.63 (m, 34 H, H-Ar), 5.55 (s, 1H, C*H*Ph), 5.52 (s, 1H, C*H*Ph), 5.43 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1^B), 5.40 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1^C), 5.24 (t, J = 7.9 Hz, 1H, H-2^C), 4.87 (d, ^2J = 12.7 Hz, 1H, C*H*HPh), 4.73 (d, ^2J = 12.7 Hz, 1H, CH*H*Ph), 4.57 (t, J = 9.4 Hz, 1H H-3^B), 4.47 (s, 2H, CH_2Ph), 4.31-4.21 (m, 5H, H-2^B, H-4^A, H-6a^A, CH_2Ph), 4.10 (t, J = 8.7 Hz, 1H, H-3^C), 4.02 (d, J = 7.7 Hz, 1H, H-1^A), 3.79 (t, J = 9.2 Hz, 1H, H-4^C), 3.72-3.51 (m, 10H, H-3^A, H-4^B, H-5^B, H-5^C, H-6b^A, H-6^B, H-6^C OCH$_{2a}$), 3.44 (dd, $J_{4,5}$ = 2.5, $J_{5,6a}$ = 8.8 Hz, 1H, H-5^A), 3.22-3.16 (m, 1H, OCH$_{2b}$), 2.98-2.86 (m, 3H, H-2^A, CH2N$_3$), 1.54-1.41 (m, 2H, CH_2CH$_2$N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 165.0 (C=O), 134.1-123.4 (C-Ar), 103.3 (C-1^A), 102.02 (*C*HPh), 101.4 (*C*HPh), 100.4 (C-1^B), 99.9 (C-1^C), 82.1, 81.8, 78.9, 78.3, 74.2, 73.8, 73.7, 73.6, 72.8, 72.57, 69.0, 68.9, 68.6, 68.3, 66.4, 66.1, 65.7, 57.0 (C-2^C), 48.1 (*C*H$_2$N$_3$), 29.0 (*C*H$_2$CH$_2$N$_3$).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)-[4,6-*O*-benzylidene-3-*O*-(9H-fluoren-9-ylmethylcarbonate)-2-deoxy-2--(2,2,2-trichloroethoxycarbonyl]amino]-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 11.



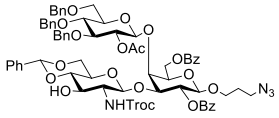
11

A solution of trichloroacetimidate donor **10** (50 mg, 0.078 mmol) and acceptor **8** (0.073 g, 0.065 mmol) with 4 Å molecular sieves (100 mg) in dry DCM (5.0 mL) was stirred for 20 min under nitrogen. TMSOTf (2.4 µL, 0.013) was added at -20°C. After 4 h (TLC; 4:1 Tol: EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:EtOAc) to afford trisaccharide **11** in 63% yield (0.130 g). $[\alpha]_D^{25}$ = +16.32° (c 0.25, $CHCl_3$). ESI HR-MS ($C_{83}H_{81}Cl_3N_4O_{22}$) *m/z* ([*M*+ Na]$^+$ found 1613.4491; calcd 1613.4306.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17-7.08 (m, 38H, H-Ar), 5.55 (s, 1H, C*H*Ph), 5.40 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.1, 1H, H-2^A), 5.34 (t, *J* = 10.1, 1H, H-2^C), 5.06 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^B), 5.00 (d, *J* = 8.2 Hz, 1H, H-1^C), 4.96 (t, *J* = 8.7 Hz, 1H, H-2^C), 4.91-4.78 (m, 3H, CH_2Ph, CH*H*Ph), 4.74 (dd, 1H, *J* = 12.2 Hz, *J* = 4.2 Hz, C*H*HCCl$_3$), 4.62 (d, *J* = 10.7 Hz, 1H, C*H*HPh), 4.59-4.48 (m, 4H, incl. H-1^A, CH*H*CCl$_3$, CH_2Ph), 4.40-4.33 (m, 4H, incl. CH_2^{Fmoc}, H-4^A, H-6a^B), 4.33-4.26 (m, 1H, H-6a^A), 4.26-4.16 (m, 1H, CH^{Fmoc}), 4.01-3.8 (m, 5H, incl. H-3^C, H-3^A, H-6b^A, H-4^C, OCH_{2a}), 3.8-3.6 (m, 5H, incl. H-6b^B, 2H-6^C, H-4^B, H-5^A, OCH_{2a}), 3.62-3.40 (m, 5H, incl. H-2^B, OCH_{2b}, H-5^C, H-5^B), 3.30-3.08 (m, 2H, CH_2N_3), 2.24 (s, 3H, CH_3CO), 1.86-1.64 (m, 2H, C$H_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 166.4, 164.9, 154.7, 153.5 (5 x CO), 143.2-120.0 (C-Ar), 102.1 (C-1^C), 101.6 (*C*HPh), 101.1 (C-1^A), 100.0 (C-1^B), 82.6 (C-3^C), 80.3 (C-3^A), 78.7 (C-5^A), 78.2 (C-4^B), 75.3 (C-5^B), 75.2, 74.9 (2 x *C*H_2Ph), 74.2 (C-4^A), 74.1 (C-3^B), 74.0 (C-4^A), 73.7 (C-2^C), 73.5 (*C*H_2Ph), 72.3 (C-4^C), 70.8 (C-2^A), 70.3 (*C*H_2^{Fmoc}), 69.2 (C-6^B), 68.4 (C-6^C), 66.2 (C-5^C), 65.6 (O*C*H_2), 64.5 (*C*H_2CCl$_3$), 57.5 (C-2^B), 48.0 (*C*H_2N$_3$), 46.5 (*C*H^{Fmoc}), 29.0 ($CH_2CH_2N_3$), 21.2 (*C*H$_3$ CO).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)-[4,6-*O*-benzylidene-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 13.



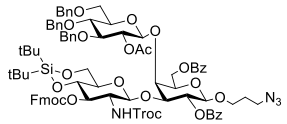
13

Trisaccharide **11** was (60 mg, 0.038 mmol) was dissolved in 2.0 mL of dry DCM and piperidine (0.2 mL) was added. After 1 h (TLC 4:1 Tol:EtOAc) the solvent was evaporated under reduced pressure and the crude was purified by flash chromatography (Tol:EtOAc) affording compound **13** (48 mg, 92% yield). $[\alpha]_D^{25}$ = -59.72° (c 0.155, $CHCl_3$). ESI HR-MS ($C_{68}H_{71}Cl_3N_4O_{20}$) *m/z* = ([*M*+ Na]$^+$ found 1391.2691; calcd 1391.2695.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-7.01 (m, 30H, Ar-H), 5.54 (s, 1H, C*H*Ph), 5.35 (dd, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.1 Hz, 1H, H-2^A), 5.00-4.86 (m, 3H, incl. H-1^C, H-2^C, H-1^B), 4.85-4.67 (m, 4H, incl. CH_2Ph, C*H*HPh, C*H*HCCl$_3$), 4.61-4.43 (m, 5H, incl. H-1^A, 3 x CH*H*Ph, CH*H*CCl$_3$), 4.43-4.34 (m, 1H, H-6a^A), 4.34-4.26 (m, 2H, incl. H-4^A, H-6a^B), 4.15 (t, 1H, J=8.99 Hz, H-3^B), 3.95-3.81 (m, 4H, incl. H-3^C, H-3^A, H-4^C, OCH_{2a}), 3.75-3.60 (m, 5H, incl. H-5^C, H-6^C, H-6b^B, H-6b^A), 3.57-3.38 (m, 4H, incl. H-5^A, H-4^B, H-5^B, OCH_{2b}), 3.25-3.07 (m, 3H, incl. H-2^B, CH_2N_3), 2.18 (s, 3H, CH_3CO), 1.80-1.57 (m, 2H, C$H_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 166.4, 165.1 (3 x CO), 138.3-126.3 (C-Ar), 101.9 (*C*HPh), 101.8 (C-1^B), 101.1 (C-1^A), 100.3 (C1-^{C}), 82.6 (C-3^C), 81.4 (C-5^A), 80.0 (C-3^A), 78.1 (C-5^C), 75.3 (C-4^B), 75.2, 75.00 (2 x *C*H_2Ph), 74.6 (C-4^A), 74.0 (C-2^C), 73.8 (*C*H_2Ph), 73.5(C-6^A), 72.3 (C-4^C), 71.0 (C-2^A), 69.7 (C-3^B), 69.1 (C-6^C), 68.5 (C-6^B), 66.1 (C-5^B), 65.6 (O*C*H_2), 64.5 (*C*H_2CCl$_3$), 59.4 (C-2^B), 47.9 (*C*H_2N$_3$), 29.0 ($CH_2CH_2N_3$), 21.1 (CO*C*H$_3$).

3-Azidopropyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl-(1→4)-[4,6-*O*-di-*tert*-butylsilylidene-3-O-(9H-fluoren-9-ylmethylcarbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino]-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 12.



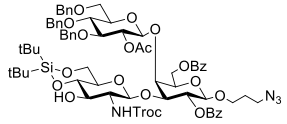
12

A solution of acceptor **9** (175 mg, 0.150 mmol) and donor **10** (190 mg, 0.300 mmol) in dry DCM (5.0 mL) with 4 Å molecular sieves was stirred for 20 min under nitrogen atmosphere. The resulting suspension was then cooled down to 0° C and TMSOTf (5.4 µL, 0.03 mmol) was added. The reaction was stirred for 2 h and left to slowly reach rt. After 2 h (TLC 9:1 Tol/EtOAc) the reaction was quenched by addition of TEA, then evaporated to dryness and the crude purified by medium pressure column chromatography using a gradient from 0 to 70% of EtOAc in toluene. Pure fractions were collected and evaporated under reduced pressure affording the target compound **12** 173 mg, 70% yield) as a clear syrup. $[\alpha]_D^{25}$ = -17.01° (c 0.4, $CHCl_3$). ESI HR-MS ($C_{84}H_{93}Cl_3N_4O_{22}Si$) *m/z* ([*M*+ Na]$^+$ found 1665.4995; calcd 1665.5009.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-7.28 (m, 33H, Ar-H); 5.36 (dt, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 7.9 Hz, 1H, H-3^A), 5.09 (t, 1H, *J* = 9.9 Hz, H-3^B), 5.01 (d, $J_{N,H}$ = 7.5 Hz, 1H, NH), 4.97-4.88 (m, 3H, H-1^B, H-1^C, H-2^C), 4.86-4.73 (m, 3H, CH_2CCl_3, C*H*HPh), 4.69 (dd, $J_{5,6a}$ = 4.2 Hz, $J_{6a,6b}$ = 12.1, Hz, 1H, H-6a^A), 4.62-4.37 (m, 7H, incl. H-6$^A{}_b$, CH_2Ph, H-1^A), 4.27 (d, $J_{3,4}$ = 2.1 Hz, 1H, H-4^A), 4.24-4.15 (m, 4H); 3.96-3.82 (m, 7H, incl. H-4^B, OCH_{2a}), 3.73-3.62 (m, 2H, incl. OCH_{2b}), 3.56-3.38 (m, 4H, incl. H-3^C, H-2^B), 3.23-3.08 (m, 2H, CH_2N_3), 2.18 (s, 3H, CH_3CO), 1.80-1.65 (m, 2H, C*H*$_2$$CH_2N_3$), 1.03 (s, 9H, tBu), 0.94 (s, 9H, tBu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 166.4, 164.9, 154.9, 153.5 (5 x CO), 143.3-120.1 (C-Ar), 101.1 (C-1^B, C-1^C), 100.2 (C-1^A), 82.5, 78.1, 75.5, 75.3, 75.2, 75.0, 74.9, 74.4 (C-4^A), 74.0, 73.7, 73.4, 72.3, 70.8 (C-3^A), 70.6, 70.3, 69.0, 66.2, 65.6 (O*C*H$_2$), 64.4 (C-6^A), 56.7 (C-2^B), 47.9 (*C*H$_2$N$_3$), 46.6 (*C*H^{Fmoc}), 29.0 (*C*H$_2$CH$_2$N$_3$) 27.4 (tBu), 26.9 (tBu), 22.6 (*C*H$_3$CO), 21.1 (*C*(CH$_3$)$_3$), 20.0 (*C*(CH$_3$)$_3$).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)-[4,6-*O*-di-*tert*-butylsilylidene-3-*O*-(9H-fluoren-9-ylmethyl carbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino]-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 14.



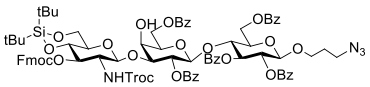
14

Compound **12** (173 mg, 0.105 mmol) was dissolved in 5.0 mL of DCM and 500 µL of piperidine were added. After 30 min, analytical TLC (toluene/EtOAc 8:2) showed full consumption of the starting material. The reaction was evaporated under reduced pressure and the crude was purified by column chromatography using a gradient from 0 to 70% of EtOAc in toluene. Pure fractions were collected and evaporated to dryness, affording compound **14** as a pale solid (137 mg, 92% yield). $[\alpha]_D^{25}$ = -5.96 (c 1.25, $CHCl_3$). ESI HR-MS ($C_{69}H_{83}Cl_3N_4O_{20}Si$) *m/z* ([*M*+ Na]$^+$ found 1443.4331; calcd 1443.4328.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-7.11 (m, 25H, Ar-H), 5.33 (dd, $J_{2,3}$ = 7.9 Hz, $J_{3,4}$ = 9.7 Hz, 1H, H-3^A), 5.05 (d, $J_{N,H}$ = 7.4 Hz, 1H, NH), 4.92-4.80 (m, 2H, H-1^C, H-2^C), 4.82 (d, $J_{1,2}$ = 8.2 Hz, 1H,H-1^B), 4.80-4.68 (m, 3H, CH_2CCl_3, C*H*HPh), 4.65 (dd, $J_{5,6a}$ = 4.2 Hz, $J_{6a,6b}$ = 12.1, 1H, H-6$^A{}_a$), 4.57-4.39 (m, 6H, incl. H-1^A, H-6b^A, CH_2Ph), 4.20 (d, $J_{3,4}$ = 2.3 Hz, 1H, H-4^A), 4.14 (dd, 1H, J = 10.0, 4.9 Hz), 3.91-3.75 (m, 7H, incl. CH*H*N$_3$, H-3^A, H-3^C, H-3^B), 3.70-3.57 (m, 4H), 3.53-3.44 (m, 1H), 3.44-3.34 (m, 2H), 3.20-3.06 (m, 3H, H-2^B, CH_2N_3), 2.14 (s, 3H, CH_3CO), 1.78-1.59 (m, 2H, C*H*$_2$$CH_2N_3$), 1.04 (s, 9H, tBu), 0.95 (s, 9H, tBu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 166.4, 165.0, 153.9 (4 x CO), 138.3-127.5 (C-Ar), 101.5 (C-1^B), 101.1 (C-1^A), 100.4 (C-1^C), 82.6, 79.7, 78.1, 77.7, 75.2, 75.0, 74.9, 74.6 (C-4^A), 73.9 (C-2^C), 73.5, 72.8, 72.3, 71.0 (C-2A), 70.4, 69.0, 66.2 (O*C*H$_2$), 65.5 (C-6^B), 64.4 (C-6A), 58.3 (C-2^B), 48.0 (*C*H$_2$N$_3$), 29.0 (*C*H$_2$CH$_2$N$_3$), 27.5 (tBu), 27.0 (tBu), 22.7 (*C*(CH$_3$)$_3$), 21.1 (CH$_3$CO), 20.0 (*C*(CH$_3$)$_3$).

3-Azidopropyl 4,6-*O*-di-*tert*-butylsilylidene-3-*O*-deoxy-(9H-fluoren-9-ylmethylcarbonate)-2-(2,2,2-trichloroethoxyarbonylamino]-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 21.



21

A solution of don trifluoroacetimidate donor **19** (0.337 g, 0.380 mmol) and acceptor **20** (200 mg, 0.211 mmol) in dry DCM (5.0 mL) in presence of 4 Å molecular sieves was stirred under nitrogen atmosphere for 20 min.

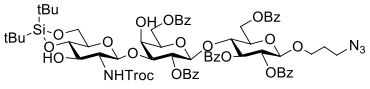
The reaction mixture was the cooled down to -10°C and TMSOTf (7.6 µL, 0.0422 mmol) was added dropwise. The resulting reaction mixture was stirred and allowed slowly to reach 5°C, when analytical TLC (Tol/EtOAc 9:1) showed formation of a new spot with intermediate. The reaction mixture was evaporated under reduced pressure and the crude was purified by medium pressure column chromatography using a gradient from 0 to 30% of EtOAc in toluene. Pure fractions were collected and evaporated under reduced pressure affording compound **21** as a pale solid (190 mg, 55% yield).

$[\alpha]_D^{25}$ = -12.99° (c 0.15, $CHCl_3$). ESI HR-MS ($C_{82}H_{85}Cl_3N_4O_{24}Si$) *m/z* ([*M*+ Na]$^+$ found 1665.4262; calcd 1665.4281.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14-7.20 (m, 33H, Ar-H), 5.68 (t, *J* = 9.2 Hz, 1H, H-3^A), 5.42-5.33 (m, 2H, H-2^A, H-2^B), 4.97-4.85 (m, 2H, incl. H-3^C), 4.77 (d, *J* = 7.8 Hz, 1H, H-1^C), 4.59 (d, $J_{1,2}$ = 7.9 Hz, 1H, H-1^B), 4.54 (d, $J_{1,2}$ =7.9 Hz, 1H, H-1^A), 4.51-4.45 (m, 1H), 4.42-4.24 (m, 3H), 4.22-4.07 (m, 4H, incl. H-4^A), 4.07-3.98 (m, 2H), 3.91-3.77 (m, 4H), 3.77-3.69 (m, 2H, incl. H-3^B), 3.64 (dd, *J* = 11.1, 7.1 Hz, 1H), 3.54-3.32 (m, 4H, incl. H-2^C), 3.21-3.08 (m, 2H, CH_2N_3), 1.79-1.56 (m, 2H, C*H*$_2$$CH_2N_3$), 0.97 (s, 9H, tBu), 0.87 (s, 9H, tBu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 165.5, 165.2, 164.6, 155.0 (5 x CO), 143.3-120.0 (C-Ar), 101.1 (C-1^C), 100.6 (C-1^B), 100.0 (C-1^A), 77.2, 75.5, 74.0, 73.8, 73.0, 72.6 (H-3^A), 72.2, 71.8, 71.0, 70.5, 70.3, 68.2, 66.6, 65.9, 62.5, 56.5 (H-2^C), 47.8 (*C*H_2N_3), 46.5 (CH^{Fmoc}), 28.9 (*C*$H_2$$CH_2N_3$), 27.3 (tBu), 26.8 (tBu), 22.6 (*C*$(CH_3)_3$), 19.9 (*C*$(CH_3)_3$).

3-Azidopropyl 4,6-*O*-di-*tert*-butylsilylidene-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside 22.



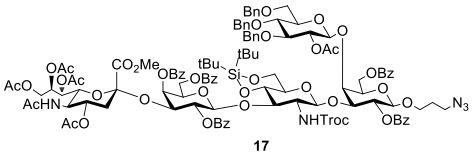
22

Compound **21** (140 mg, 0.085 mmol) was dissolved in 5.0 mL of DCM and 500 mL of piperidine were added. The resulting reaction mixture was stirred for 30 min at rt, when analytical TLC (Tol/EtOAc 85:15) showed full consumption of the starting material. The reaction mixture was evaporated under reduced pressure and the crude was purified by medium pressure column chromatography using a gradient from 0 to 80% of EtOAc in toluene. Pure fractions were collected and evaporated under reduced pressure affording compound **22** a pale solid (110 mg, 90% yield). $[\alpha]_D^{25}$ = -13.62° (c 0.2, $CHCl_3$). ESI HR-MS ($C_{82}H_{85}Cl_3N_4O_{24}Si$) *m/z* ([*M*+ Na]$^+$ found 1443.3568; calcd 1443.3600.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-7.21 (m, 25H, Ar-H); 5.68 (t, *J* = 9.3 Hz, 1H, H-3^A), 5.42-5.32 (m, 2H, H-2^A, H-2^B), 5.02 (bs, 1H, NH), 4.75 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1^C), 4.58 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1^B), 4.54 (d, $J_{1,2}$ = 7.90 Hz, 1H, H-1^A), 4.43-4.35 (m, 2H, CH*H*CCl_3), 4.17-4.06 (m, 2H, H-4^A), 4.03-3.94 (m, 2H), 3.91-3.66 (m, 6H, incl. H-3^{B,C}), 3.66-3.54 (m, 2H), 3.54-3.42 (m, 2H), 3.28 (dt, 1H, *J* = 9.7, 5.0 Hz), 3.20-3.09 (m, 3H, CH_2N_3, H-2^C), 1.79-1.62 (m, 2H, C*H*$_2$$CH_2N_3$), 0.99 (s, 9H, tBu), 0.90 (s, 9H, tBu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 165.9, 165.6, 165.3, 164.7 (5 x CO), 133.5-128.2 (C-Ar), 101.1 (C-1^B), 100.6 (C-1^C), 100.0 (C-1^A), 79.9, 77.3, 75.6, 73.9, 73.1, 72.6 (C-3A), 72.3, 71.8, 71.2, 70.3, 68.3, 66.6, 65.9, 62.5, 57.8 (C-2C), 47.8 (*C*H_2N_3), 28.9 (*C*$H_2$$CH_2N_3$), 27.4 (tBu), 26.9 (tBu), 22.6 (*C*$(CH_3)_3$), 19.9 (*C*$(CH_3)_3$).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-*β*-D-glucopyranosyl-(1→4)-[2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→3)-4,6-*O*-di-*tert*-butylsilylidene-3-*O*-(9H-fluoren-9-ylmethylcarbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino-β-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 17.



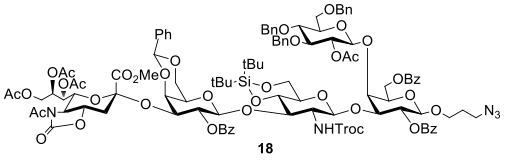
17

A solution of trisaccharide acceptor **14** (114 mg, 0.080 mmol) and disaccharide donor **7** (135 mg, 0.120 mmol) with 4Å molecular sieves in dry DCM (3.0 mL) was stirred for 20 min. TMSOTf (2.9 µL, 0.016 mmol) was added at 0°C and the reaction was stirred for 2 h and allowed to slowly reach rt. After 2 h (TLC 3:2 Tol/Acetone) the reaction was quenched by addition of TEA, and the crude purified by column chromatography using a gradient from 0 to 80% of acetone in toluene. Pure fractions were collected and evaporated affording **17** (150 mg, 80% yield). $[\alpha]_D^{25}$ = -28.20° (c 0.1, $CHCl_3$). ESI HR-MS ($C_{116}H_{132}Cl_3N_5O_{40}Si$) *m/z* $[M+Na]^+$ found 2390.7193, calcd 2390.7176.

^{1}H NMR (400 MHz, ACN-d_3) δ 8.13-7.12 (m, 40H, Ar-H), 5.97 (d, $J_{N,H}$ = 9.8 Hz, 1H, NH), 5.60-5.51 (m, 2H), 5.45-5.38 (m, 1H), 5.31-5.14 (m, 3H, H-1^C, H-2^{C,D}), 5.08 (t, J = 9.0 Hz, 1H, H-2^A), 4.85 (d, $J_{1,2}$ = 7.5 Hz, 1H, H-1^D), 4.80-4.59 (m, 6H, incl. H-1^B, H-3^B), 4.58-4.40 (m, 6H, incl. H-1^A), 4.40-4.31 (m, 3H), 4.24 (bs, 1H), 4.22-3.98 (m, 7H), 3.9 (dd, J = 9.4, 2.0 Hz, 1H), 3.91-3.81 (m, 2H), 3.81-3.79 (m, 4H, incl. H-2^B) 3.66-3.58 (m, 4H, incl. $COOCH_3$), 3.58-3.49 (m, 3H), 3.48-3.39 (m, 2H), 3.39-3.32 (m, 1H), 3.14-2.98 (m, 2H, OCH_2), 2.24 (dd, $J_{3e,4}$ = 4.5 Hz, $J_{3a,3e}$ = 12.2 Hz, 1H, H-3e^E), 2.01 (s, 3H, CH_3CO), 1.96 (s, 6H, 2 x CH_3CO), 1.78 (s, 3H, CH_3CO), 1.69-1.56 (m, 8H, 2 x CH_3CO, C$H_2CH_2N_3$), 1.35 (t, 1H, H-3a^E), 1.04 (s, 9H, tBu), 0.87 (s, 9H, tBu).

^{13}C-NMR (101 MHz, ACN-d_3) δ 170.5, 170.4, 169.9, 169.8, 169.7, 169.5, 165.9, 165.7, 165.6, 165.1, 165.0, 153.7 (13 x CO), 138.6-127.4 (C-Ar), 102.7 (C_q), 101.3 (C-1^B), 100.6 (C-1^A), 98.9 (C-1^D), 97.3 (C-1^C), 95.8 (C_q), 82.4, 80.2, 78.2, 78.0, 75.3, 74.7, 74.5, 74.4, 73.5, 73.2, 72.9, 72.0, 71.8, 71.4, 70.9, 70.8, 70.4, 69.1, 68.9, 67.5, 66.6, 66.1, 63.9, 62.4, 61.9, 58.0, 52.7 ($COOCH_3$), 48.2, 47.6 (CH_2N_3), 37.4 (C-3^E), 28.6 ($CH_2CH_2N_3$), 27.0 (tBu), 26.4 (tBu), 22.2, 22.1, 20.7 (3 x CH_3CO), 20.5 ($C(CH_3)_3$), 20.2, 20.1, 20.0 (3 x CH_3CO), 19.5 ($C(CH_3)_3$).

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-O-benzyl-*β*-D-glucopyranosyl-(1→4)-[3-*O*-(methyl 7,8,9-tri-*O*-acetyl-5-*N*-acetoamido,4-*O*-oxazolidinone-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→3)-4,6-*O*-di-*tert*-butylsilylidene-3-*O*-(9H-fluoren-9-ylmethylcarbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl-(1→3)]-2,6-di-*O*-benzoyl-*β*-D-galactopyranoside 18.



18

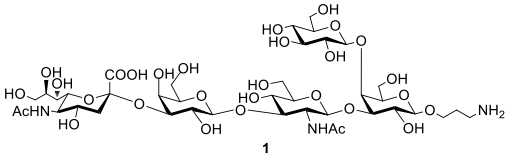
A solution of donor **15** (0.050 g, 0.053 mmol) and acceptor **14 (**0.050 g, 0.035 mmol) with 4 Å molecular sieves in dry DCM (3.0 mL) was stirred for 20 min under nitrogen. N-iodosuccinimide (0.016 g, 0.074 mmol) and TfOH (0.6 µL, 0.007 mmol) were added at -40°C. After 3 h (TLC 7:3 Tol/Acetone) the reaction was quenched with TEA, the solid was filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:Acetone) to afford pentasaccharide **47** in 65% yield (0.053 g). $[\alpha]_D^{25}$ = +9.7° (c 1.6, $CHCl_3$). ESI HR-MS **(**$C_{108}H_{124}Cl_3N_5O_{38}Si$) *m/z* $[M+Na]^+$ found 2254.6687, calcd 2254.6651.

^{1}H NMR (400 MHz, ACN-d_3) δ 8.13-7.31 (m, 35H, Ar-H), 5.90 (d, J = 9.0 Hz, 1H), 5.61 (s, 1H, C*H*Ph), 5.47-5.40 (m, 1H), 5.29 (d, $J_{N,H}$ = 8.2 Hz, 1H, NH), 5.24 (dd, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.9, Hz, 1H, H-2^D), 5.11 (dd, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.0 Hz, 1H, H-2^A), 5.01 (d, $J_{1,2}$ = 7.7 Hz, 1H, H-1^D), 4.98 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1^B), 4.92 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^C), 4.87-4.80 (m, 3H), 4.67 (t, J = 10.7 Hz, 2H), 4.62 (d, $J_{1,2}$ = 9.6 Hz, 1H), 4.58 (d, J = 5.4 Hz, 1H), 4.56-4.51 (m, 3H), 4.47 (d, J = 12.0 Hz, 1H), 4.42 (dd, J = 12.3, 2.8 Hz, 1H), 4.38 (d, J = 12.3 Hz, 1H), 4.35-4.30 (m, 2H),

4.30-4.12 (m, 5H), 4.12-4.06 (m, 2H), 4.06-3.80 (m, 6H), 3.77-3.60 (m, 6H), 3.61-3.55 (m, 4H, incl. $COOCH_3$), 3.54-3.47 (m, 2H), 3.34 (t, J = 8.0 Hz, 1H, H-2^B), 3.22-3.06 (m, 2H, CH_2N_3), 2.47-2.41 (m, 1H, H-$3e^E$), 2.43 (s, 3H, $COCH_3$), 2.17 (s, 3H, $COCH_3$), 2.05 (s, 6H, 2 x $COCH_3$), 1.97 (t, 1H, $J_{3a,3e}$ = 12.7 Hz, H-$3a^E$), 1.92 (s, 3H, $COCH_3$), 1.75-1.65 (m, 2H, C$H_2CH_2N_3$), 1.17 (s, 9H, tBu), 1,09 (s, 9H, tBu).

^{13}C-NMR (101 MHz, ACN-d_3) δ 171.2, 170.6, 170.2, 169.8, 169.7, 167.8, 165.8, 165.1, 164.8, 153.8, 153.4 (11xCO), 138.7-126.6 (C-Ar), 101.8 (C-1^B), 101.2 (C-1^A), 100.8 (*C*HPh), 100.5 (C-1^C), 100.0 (C-1^D), 98.6 (C_q), 95.9 (C_q, CCl_3), 82.2, 80.1, 79.2, 78.1, 75.6, 75.4, 74.7, 74.6, 74.5, 74.5, 74.4, 73.5, 73.0, 72.9, 71.7, 71.1, 70.9, 70.5, 70.3, 69.0, 68.6, 68.2, 66.1, 63.8, 62.9, 58.3, 58.1 (C-2^B), 52.8 ($COOCH_3$), 47.6 (CH_2N_3), 34.4 (C-3^E), 28.5 ($CH_2CH_2N_3$), 26.9 (tBu), 26.5 (tBu), 23.9, 20.5, 20.5, 20.4 (5 x CH_3CO), 20.1 ($C(CH_3)_3$), 19.5 ($C(CH_3)_3$).

2-Aminopropyl *β*-D-glucopyranosyl-(1→4)-[3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-*α*-D-*galacto*-non-2-ulopyranosyl)-*β*-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)]-*β*-D-galactopyranoside 1.



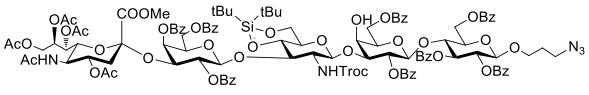
1

*Protocol followed for both compound **17** and compound **18***: the protected pentasaccharide (0.07 mmol) was dissolved in THF (5 mL) to which 3 M NaOH (0.5 mL) was added. After refluxing for 2 d, the mixture was neutralized with 0.1% HCl and concentrated. The residue was re-dissolved in 2:3 Ac_2O-MeOH (5 mL) and stirred overnight, when C18-TLC (2:3 H_2O/MeOH) showed disappearance of the starting material. After concentration, the residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **3** as an amorphous powder (40% yield). $[\alpha]_D^{25}$ = +1.95° (c 0.1, H_2O). ESI HR-MS ($C_{40}H_{69}N_3O_{29}$) *m/z* $[M-H]^-$ 1054.3971; found 1054.3847.

^{1}H NMR (400 MHz, D_2O) δ 4.87 (d, $J_{1,2}$ = 7.6 Hz, 1H, H-1^C), 4.72 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^B), 4.47 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^D), 4.38 (d, $J_{1,2}$ = 7.6 Hz, 1H, H-1^A), 4.35 (d, $J_{3,4}$ = 2.9 Hz, 1H, H-4^A), 4.05 (dd, 1H, $J_{2,3}$ = 9.9 Hz, H-3^D), 4.03-3.97 (m, 1H), 3.94-3.44 (m, 27H), 3.41-3.30 (m, 2H), 3.23 (dd, 1H, $J_{2,3}$ = 9.2 Hz, H-2^C), 3.12 (dt, J = 6.9, 8.2 Hz, 2H), 2.73 (dd, $J_{3e,4}$ = 4.7, 12.5, Hz, 1H, H-$3e^E$), 2-03-1.93 (m, 2H, C$H_2CH_2NH_2$), 1.99 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 1.75 (t, 1H, H-3^E_{ax}).

^{13}C-NMR (101 MHz, D_2O) δ 174.9, 174.8, 173.8 (3 x CO), 103.3 (C-1^A), 102.9 (C-1^D), 102.7 (C-1^C), 102.0 (C-1^B), 100.0 (C_q), 99.6 (C_q), 82.1, 81.9, 75.8, 75.6, 75.3, 75.1, 74.8, (C-4^A) 74.2, 73.6 (C-2^C), 72.8, 71.8, 70.1, 69.7, 69.0, 68.6, 68.3, 68.0, 67.9, 67.2, 62.4, 61.0, 60.8, 60.7, 60.6, 54.7, 51.6, 39.7 (C-3^E), 37.7 (CH_2N_3), 26.6 (C$H_2CH_2NH_2$), 22.3 (CH_3CO), 22.0 (CH_3CO). See Table 1 for full assignments.

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→3)-4,6-*O*-di-*tert*-butylsilylidene-3-*O*-(9H-fluoren-9-ylmethyl carbonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 23.



23

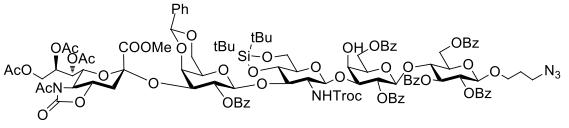
A solution of trisaccharide acceptor **22** (95 mg, 0.067 mmol) and disaccharide donor **7** (114 mg, 0.100 mmol) with 4 Å molecular sieves (0.100 g) in dry DCM (3.0 mL) was stirred for 20 min. TMSOTf (2.4 mL, 0.0134 mmol) was then added at 0°C. The reaction was stirred for 2 h and let slowly reach rt. The reaction was quenched by addition of TEA (TLC 6:4 Tol/acetone) and the crude purified by column chromatography using a

gradient from 0 to 70% of acetone in toluene. Pure fractions were collected and evaporated affording 110 mg (66% yield) of compound **23** as a pale solid. $[\alpha]_D^{25}$ = +11.27° (c 0.45, $CHCl_3$). ESI HR-MS ($C_{114}H_{124}Cl_3N_5O_{42}Si$) *m/z* $[M+Na]^+$ found 2290.6468, calcd 2290.6556.

^{1}H NMR (400 MHz, ACN-d_3) δ 8.18-7.10 (m, 40H, Ar-H), 5.95 (t, J = 9.3 Hz, 1H), 5.67-5.58 (m, 2H, incl. H-3^A), 5.57-5.32 (m, 3H), 5.27-5.07 (m, 4H, incl. H-1^D, H-2^A), 4.86-4.65 (m, 3H, incl. H-1^A, H-4^E), 4.61 (d, 2H, incl. $J_{1,2}$ = 8.2 Hz, H-1^C), 4.48-4.26 (m, 6H), 4.21 (t, *J* = 9.5 Hz, 1H), 4.17-4. 09 (m, 2H), 4.03-3.85 (m, 5H), 3.84-3.78 (m, 1H), 3.78-3.73 (m, 3H), 3.73-3.65 (m, 3H), 3.65-3.55 (m, 4H, incl. $COOCH_3$), 3.53-3.45 (m, 1H, H-2^C), 3.43-3.28 (m, 1H), 3.10 (t, *J* = 6.6 Hz, 2H, OCH_2), 2.22 (dd, 1H, $J_{3e,4}$ = 4.5 Hz, $J_{3a,3e}$ = 12.5 Hz, H-3e^E), 2.17 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 1.97 (s, 3H, CH_3CO), 1.78 (s, 3H, CH_3CO), 1.65 (s, 3H, CH_3CO), 1.67-1.59 (m, 2H, $CH_2CH_2N_3$), 1.34 (t, $J_{3a,3e}$ = 12.2 Hz, 1H, H-3a^E), 0.99 (s, 9H, tBu), 0.82 (s, 9H, tBu).

^{13}C-NMR (101 MHz, ACN-d_3) δ 170.5, 169.9, 169.8, 169.8, 169.7, 169.5, 168.0, 165.7, 165.6, 165.5, 165.4, 165.1, 165.0, 164.9, 153.9 (15 x CO), 133.8-125.3 (C-Ar), 101.5 (C-1^{A,B}), 100.8 (C-1^C), 100.2 (C-1^D), 98.7, 97.2, 79.7, 77.6, 75.6, 74.7, 73.2, 73.0, 72.8, 72.4, 72.2, 72.1, 71.9, 71.9, 71.8, 71.4, 70.9, 70.6, 70.2, 70.0, 69.8, 69.6, 69.2, 69.1, 68.9, 68.0, 68.0, 67.9, 67.5, 67.0, 66.6, 65.8, 62.9, 62.6, 62.5, 62.0, 61.9, 61.8, 57.5, 52.7 ($COOCH_3$), 47.7 (CH_2N_3), 37.3 (C-3^E), 28.5, ($CH_2CH_2N_3$), 26.9 (tBu), 26.3 (tBu), 22.2 (CH_3CO), 22.1 (CH_3CO), 20.7 (CH_3CO), 20.5 ($C(CH_3)_3$), 20.1 (CH_3CO), 20.0 (CH_3CO), 19.4 ($C(CH_3)_3$).

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-*β*-D-galactopyranosyl-(1→3)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-*β*-D-glucopyranosyl-(1→3)-2,6-di-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranoside 24.



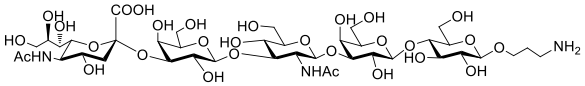
24

A solution of donor **15** (40 mg, 0.042 mmol) and acceptor **22 (**40 mg, 0.028 mmol) with 4 Å molecular sieves (0.100 g) in dry DCM (3.0 mL) was stirred for 20 min under nitrogen. N-iodosuccinimide (0.013 g, 0.056 mmol) and TfOH (0.5 μL, 0.0056 mmol) were added at -40°C. After 3 h (TLC 6:4 Tol:Acetone) the reaction was quenched with TEA, the solid was filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:Acetone) to afford pentasaccharide **51** in 40% yield (0.025 g). $[\alpha]_D^{25}$ = +20.93° (c 0.3, $CHCl_3$). ESI HR-MS ($C_{106}H_{116}Cl_3N_5O_{40}Si$) *m/z* ([*M*+ Na]$^+$ found 2253.63; calcd 2254.59.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67-7.27 (m, 35H, Ar-H), 5.80 (dd, *J* = 9.1, 1.2 Hz, 1H), 5.61 (t, 1H, *J* =9.5 Hz, H-3^A), 5.50 (s, 1H, CHPh), 5.40 (d, 1H, $J_{N,H}$ = 8.2 Hz, NH), 5.37-5.30 (m, 1H), 5.23 (dd, $J_{1,2}$ = 8.1 Hz, 1H, H-2^A), 5.11-5.05 (m, 2H, H-2^B, H-2^D), 4.91 (d, $J_{1,2}$ = 8.0 Hz, 1H, H-1^D), 4.79-4.71 (m, 2H, H-1^C, H-1^A), 4.59 (d, $J_{1,2}$ = 8.1 Hz, 1H, H-1^B), 4.56-4.49 (m, 1H), 4.45-4.40 (m, 1H), 4.40-4.29 (m, 3H), 4.26-4.20 (m, 2H), 4.19-4.09 (m, 4H), 4.08-3.97 (m, 3H), 3.97-3.89 (m, 2H), 3.84-3.79 (m, 1H), 3.79-3.71 (m, 4H), 3.58-3.50 (m, 2H), 3.49 (s, 3H, $COOCH_3$), 3.48-3.43 (m, 1H), 3.38 (dt, *J* = 4.9, 9.8 Hz, 1H, Hz), 3.19-3.09 (m, 3H, H-2^C, OCH_2), 2.34 (s, 3H, $COCH_3$), 2.33 (s, 3H, $COCH_3$), 2.35-2.30 (m, 1H, H-3$^E_{eq}$), 2.08 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.87 (t, 1H, J=12.3 Hz, H-1$^E_{ax}$), 1.68-1.58 (m, 2H, $CH_2CH_2N_3$), 1.04 (s, 9H, tBu), 0.96 (s, 9H, tBu).

^{13}C-NMR (101 MHz, ACN-d_3) δ 172.1, 171.5, 170.7, 170.6, 168.7, 166.6, 166.5, 166.3, 166.0, 165.8, 154.7, 154.6, 139.4 (13 x CO), 134.3-127.4 (C-Ar), 101.6, 101.3 (*C*HPh), 101.6 (C-1^B), 101.3 (*C*HPh), 101.1 (C-1^A), 100.9 (C-1^C), 100.7 (C-1^D), 99.5 (C_q), 80.0, 79.7, 76.5, 75.4, 75.4, 75.3, 74.1, 73.9, 73.7, 73.1, 73.0, 72.0, 71.6, 71.4, 71.0, 69.4, 69.1, 68.8, 67.5, 67.1, 66.8, 63.8, 63.4, 59.2, 58.2 (C-2^C), 53.7, 48.7 (OCH_2), 35.3 (C-3^E), 29.4 ($CH_2CH_2N_3$), 27.7 (tBu), 27.4 (tBu), 24.8 (CH_3CO), 23.0 ($C(CH_3)_3$), 21.4 ($COCH_3$), 21.3 (CH_3CO), 21.0 (CH_3CO), 20.3 ($C(CH_3)_3$).

3-Aminopropyl 3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosyl)-*β*-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside 2.



2

*Protocol followed for both compound **23** and compound **24***: the protected pentasaccharide (0.07 mmol) was dissolved in THF (5 ml) to which 3 M NaOH (0.5 ml) was added. After refluxing for 2 d, the mixture was neutralized with 0.1% HCl and concentrated. The residue was re-dissolved in 2:3 Ac_2O-MeOH (5 mL) and stirred overnight. After concentration, the residue was dissolved in *t*BuOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (5 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **4** as an amorphous powder (40% yield). $[\alpha]_D^{25}$ = -4.37° (c 0.05, H_2O). ESI HR-MS ($C_{40}H_{69}N_3O_{29}$) *m/z* $[M-H]^-$ 1054.3971; found 1054.3867.

^{1}H NMR (400 MHz, D_2O) δ 4.68 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1^C), 4.48 (d, $J_{1,2}$ = 8.3 Hz, 2H, H-1^{A,B}), 4.41 (d, $J_{1,2}$ = 7.5 Hz, 1H, H-1^D), 4.12 (d, $J_{3,4}$ = 2.9 Hz, 1H, H-4^B), 4.07-3.46 (m, 33H), 3.33-3.26 (m, 1H, H-2^A), 3.11 (t, *J* = 6.8 Hz, 2H, CH_2N_3), 2.72 (dd, $J_{3e,4}$ = 4.1 Hz, $J_{3e,ea}$ = 11.9 Hz, 1H, H-3e^E), 1.99 (s, 6H, 2 x CH_3CO), 2.03-1.91 (m, 2H, C*H*$_2$$CH_2N_3$), 1.75 (t, 1H, H-3a^E).

^{13}C-NMR (101 MHz, D_2O) δ 174.9 (CO), 173.9 (CO), 103.3 (C-1^A), 102.9 (C-1^B), 102.5 (C-1^D), 102.0 (C-1^C), 81.9, 81.7, 78.3, 75.5, 75.0, 74.8, 74.7, 74.3, 72.6, 71.8, 69.9, 69.0, 68.4, 68.3, 67.8 (O*C*H$_2$), 67.2, 62.4, 61.0, 60.9, 60.4, 59.9, 54.5, 51.6, 39.7 (C-3^E), 37.5 (*C*H$_2$N$_3$), 26.6 (*C*H$_2$CH$_2$N$_3$), 22.2 (*C*H$_3$CO), 22.0 (*C*H$_3$CO). See Table 1 for full assignments.

Quantitative estimation of sialic acid[18].

Yields of the synthetic oligosaccharide was calculated on the basis of the sugar quantification, which was done by quantitative estimation of the sialic acid content with a colorimetric resorcinol-hydrochloric acid method. Standard samples of sialic acid at concentrations 5, 10, 15, 20, 25 μg/mL were prepared to build the calibration curve. GBS PSIa samples were prepared targeting the calibration curve midpoint. The reference and analytical samples were treated with a solution of Resorcinol/HCl. The reagent resorcinol/HCl was prepared by mixing 10 mL of a 2% resorcinol solution, 0.25 mL of a 0.1 M Copper (II) Sulphate solution in 20 mL of water with 37% hydrochloric acid up to a volume of 100 mL. After treatment of samples and references with the resorcinol/HCl reagent, the resulting mixtures were heated in a oil bath at 110°C for 20 min. After that, absorbance was read at 564 nm.

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Chapter 6

Characterization of sugar-mAb interactions of GBS serotype Ia and Ib oligosaccharides

Del Bino, L.; Carboni, F.; Romano, M.R.; De Ricco, R.; Brogioni, B.; Adamo, R. were involved in the research included in this Chapter.

Introduction

Bacterial cell surface carbohydrates are at the interface of multiple host-pathogen interactions and have been targeted to develop highly efficacious glycoconjugate vaccines against severe infections. Traditional glycoconjugate vaccines consist of long polysaccharides (obtained from bacterial sources) which are chemically conjugated to the carrier protein. Conjugation is performed through random linkage along the saccharide chain, resulting in the formation of high molecular weight, cross-linked heterogeneous structures[1-3].

To understand the mechanism of action of this type of vaccines, mapping of the epitopes recognised by functional antibodies, that mediate protection from infection, is crucial. In the absence of structural information, long oligosaccharides have to be employed to fully cover all of the relevant glycotopes[4]. An understanding of the structural basis for the immune recognition of carbohydrate epitopes and the elucidation of minimal saccharide antigens responsible for an effective immune response is relevant to support the rational design of modern glycoconjugate vaccines with well-defined synthetic oligosaccharides[5, 6].

Chemically assembled oligosaccharides would result in easier characterization of the corresponding glycoconjugates, lack of bacterial contaminants, higher reproducibility and more robust correlation of the immune response to the chemical structure of the saccharide[7].

Among GBS serotypes, the most studied is type III, that is known to form a helical structure[8, 9], and this feature has an impact on epitope exposure[9]. Recently CPSIII oligosaccharides have been synthesized and used along with fragments obtained from CPS depolymerization to map a sialylated structural epitope spanning two repeating units[6, 10]. The same approach could be theoretically applied also to other GBS serotypes such as Ia and Ib, which share with GBS III the same residue composition of the repeating unit[11], in order to define the glycotopes interacting with protecting mAbs. Since neither chemical nor enzymatic depolymerization reactions are available for CPS Ia and Ib, structurally defined oligosaccharides can be obtained only by chemical and/or enzymatic synthesis.

In Chapter 4 and 5 the synthesis of a panel of oligosaccharide fragments from GBS type Ia and Ib CPS has been described. Availability of these fragments allow for the first time a study of the interactions with anti CPS monoclonal antibodies to gain information on the structure of the minimal glyco epitope. Moreover, NMR studies can help to understand whether the specificity of the immune response observed for the two serotypes can be correlated to a different conformation.

As described in Chapter 4 and 5, NMR studies and molecular dynamics simulations highlighted a different conformational preference of GBS Ia polysaccharide compared to type Ib, with a particular effect in the orientation of the Neu5Acα2-3Gal branching (*exo-anti-Φ* for Ia and *exo-syn-Φ* for Ib). This reflects in a more repetitive and regular structure for PSIa, with the Neu5Ac exposed according to a well-defined spatial orientation, while PSIb has a more irregular, helix-shape backbone.

This different conformation is expected to influence antibody recognition.

In this Chapter, the study of the interactions of the library of synthesized oligosaccharides with serotype specific functional monoclonal antibodies is described. Competitive SPR, competitive ELISA and STD-NMR were performed with the aim to shed lights on the structural motif interacting with the mAb.

Finally, conjugates of the saccharides to the carrier protein CRM_{197} were prepared and characterized for future *in vivo* study and immunological evaluation.

Results

Competitive SPR of GBS type Ia and Ib oligosaccharides

The antigenicity of the synthesized GBS fragments was first assessed by competitive Surface Plasmon Resonance. The fragments included in the experiments are represented in Figure 1: four fragments from GBS serotype Ia CPS (the linear and branched repeating unit, **1** and **2** the Y-shape and the branched hexasaccharides **3** and **4**) and the two repeating unit frameshifts **5** and **6** from type Ib CPS. The capsular polysaccharides of both serotypes were included in each experiment as controls.

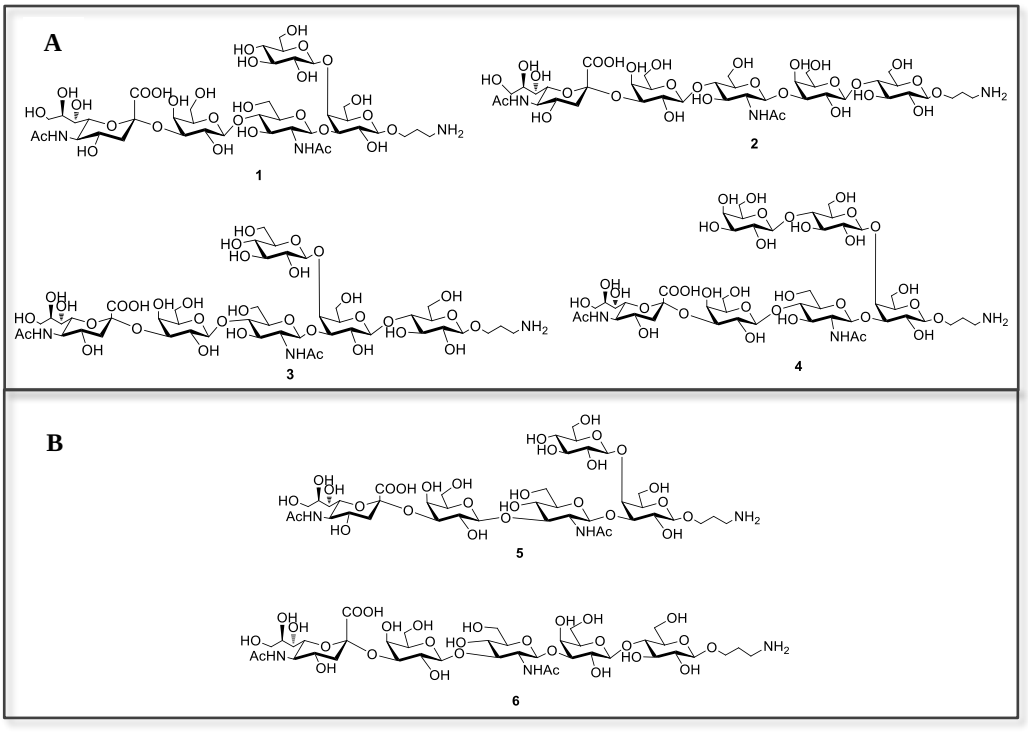


Figure 1. A) Oligosaccharide structures from GBS type Ia tested in competitive SPR. B) GBS type Ib repeating units tested in competitive SPR.

The structural features of the oligosaccharide determining antibody recognition were studied using two protective monoclonal antibodies raised in mice to PSIa and PSIb conjugates to CRM.
In the first experiment, the compounds **1**-**4** were used as inhibitors of the binding of the soluble murine anti PSIa mAb to a PSIa-Human Serum Albumin coniugate immobilized on a CM5 chip (Figure 2). PSIa was used as positive control, while PSIb and the branched Ib repeating unit **5** were the negative controls and were used to confirm that the mAb was non cross-reactive with serotype Ib.

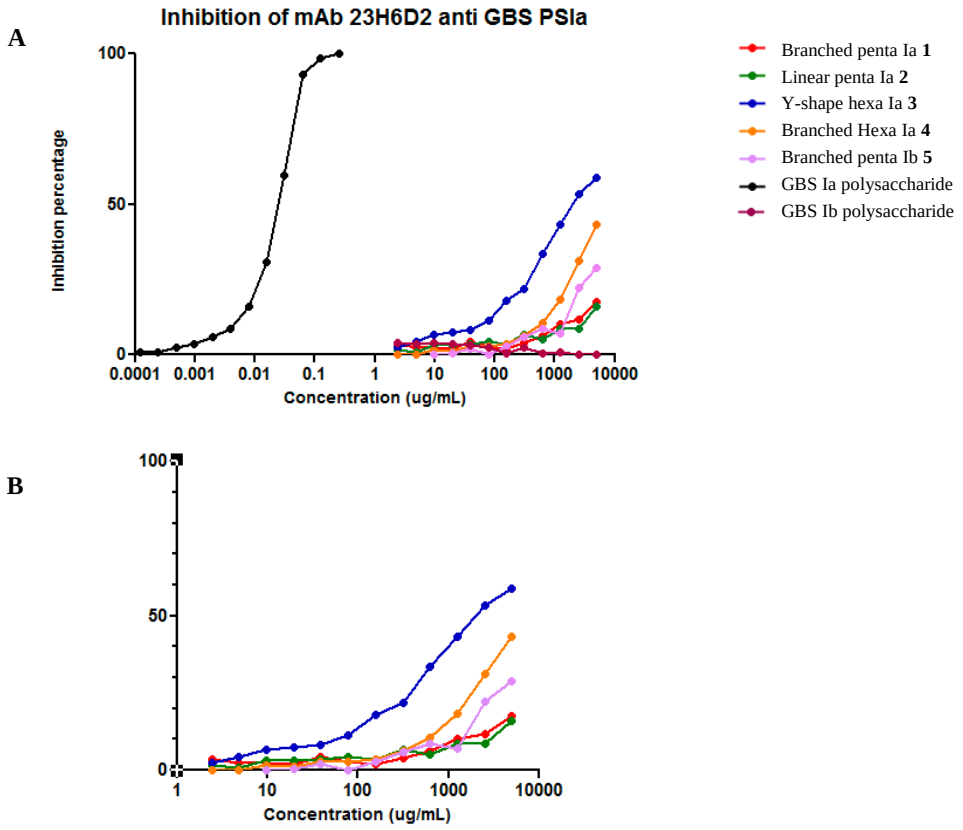


Figure 2. A) Competitive SPR vs anti PSIa murine monoclonal antibody 23H6D2. In black the Ia CPS used as positive control in the experiment. B) Enlargement on the synthetic fragments, showing the two hexasaccharides partially inhibiting the binding of the mAb to the immobilized PS.

Starting from a concentration of 5 mg/mL of each oligosaccharide, 2-fold serial dilution were made. GBS Ia pentasaccharide repeating units **1** and **2** failed completely to inhibit the binding. Only the hexasaccharides **3** and **4** were recognized by the murine protective mAb. Inhibition of binding was only partial even at the highest concentration, with the Y-shape hexasaccharide **3** being a slightly better inhibitor than the branched one (60% inhibition of **3** versus 45% of the branched structure **4**). As expected, the type Ib polysaccharide was not competing for binding to

the mAb with the PSIa. This confirmed that despite the high similarity between type Ia and Ib polysaccharide, the anti PSIa monoclonal antibody used was very specific and showed no cross reactivity. Interestingly, the branched repeating unit of GBS PSIb (**5**) was very weakly recognized by the mAb, better than the PSIb.

From these data it can be concluded that the synthesized GBS Ia fragments are not able to deplete the binding of type Ia polysaccharide to a monoclonal antibody anti PSIa. This indicates that they are not adequately covering the antigenic determinants required for antibody recognition. However, some differences can be observed in the affinity of the oligosaccharides to the mAb, with increasing binding affinity with increasing oligosaccharide size: in particular, the hexasaccharides **3** and **4** showed better inhibition activity compared to the pentasaccharides **1** and **2**. Additionally, when comparing short glycans and long polysaccharides, it has to be taken into consideration the higher avididy (re-binding) effect of the natural polymer, because of its exposure of many epitopes and this may explain the big affinity difference between the oligosaccharide fragments and the PSIa.

Next, the interactions of oligosaccharides **5** and **6** with an anti-PSIb mAb were investigated. To this aim, the two oligosaccharides, together with PSIb were used as inhibitors of the binding of the soluble anti PSIb mAb to PSIb conjugated to Human Serum Albumin and immobilized on a CM5 chip. PSIa and the branched hexasaccharide **4** were used as negative controls. The results, expressed as inhibition percentage of binding, indicate that both fragment **5** and **6** is recognized by the anti-PSIb mAb, with the branched repeating unit able to deplete the mAb recognition with a 2-log higher concentration compared to the positive control PSIb. A weaker inhibition (1-log difference) was detected with the linear structure **6**, suggesting that the arrangement of sugars in the RU impacts antibody recognition and the ramification point is relevant for binding (Figure 3). Finally, confirming the serotype-specificity of this mAb, PSIa was only very weakly recognized while the hexasaccharide **4** was not able to inhibit the binding.

IC_{50} values were calculated for PSIb and for both pentasaccharides **5** and **6** (Table 1). The branched pentasaccharide **5**, with an IC_{50} of 85.47 ug/mL, showed an affinity for the anti-PSIb mAb about 2-log lower with respect to the polysaccharide. As mentioned, the avidity effect due to the multivalent epitopes exposure can impact the binding of the polysaccharide. The calculated IC50 of the linear pentasaccharide **6** was 850.7 ug/mL, 1-log higher compared to the branched frameshift of the repeating unit, and this confirmed the importance of the ramification for mAb recognition and binding.

Figure 3. Competitive SPR vs anti PSIb murine monoclonal antibody

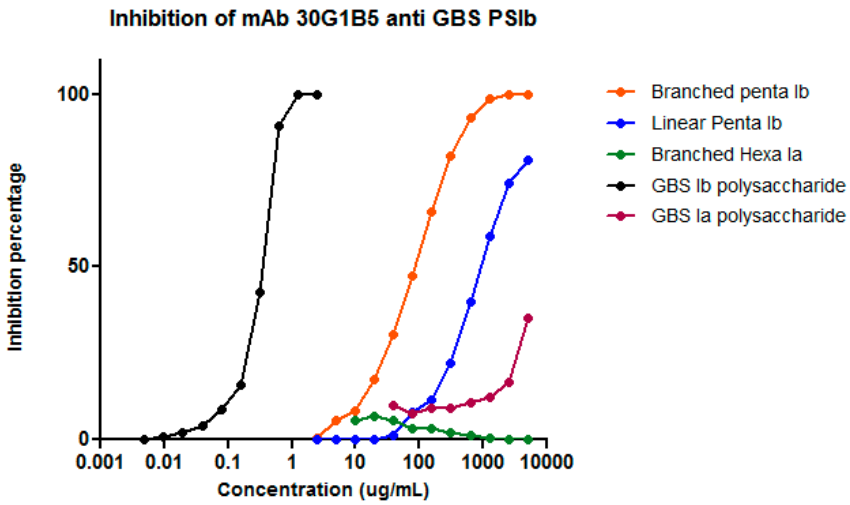


Table 1. IC50 for the interaction of mouse mAb with different glycans

Glycan	IC_{50} (µg/ml)
DP1 branched **5**	85.47
DP1 linear **6**	850.7
PSIb	0.58

STD-NMR of pentasaccharide 5

Considering the results obtained by competitive SPR, mapping of the interaction of the fragment with the protective mAb starting with the most promising compound **5** was commenced. To characterize the sugar/mAb interactions at the atomic level, Saturation Transfer Difference (STD-NMR) studies were undertaken[12, 13]. Indeed, STD experiments permit the deduction of interactions between a ligand (the sugar in this case) and a target receptor (the mAb) and, in favourable cases, the identification of the ligand epitope. STD NMR spectra of the pentasaccharide-mAb complex (ligand/protein 100:1 molar ratio) were derived by subtracting two different ^{1}H NMR spectra performed on the same sugar-mAb sample: the on-resonance, in which selective irradiation of protein protons is performed for a few seconds, and the off-resonance, which is recorded as a reference or blank spectrum. The obtained STD NMR spectrum only contains the ligand resonance whose intensities have been affected by the protein saturation. The experiment with a 1:100 mixture of mAb:pentasaccharide provided strong STD signals for the oligosaccharide (Figure 4).

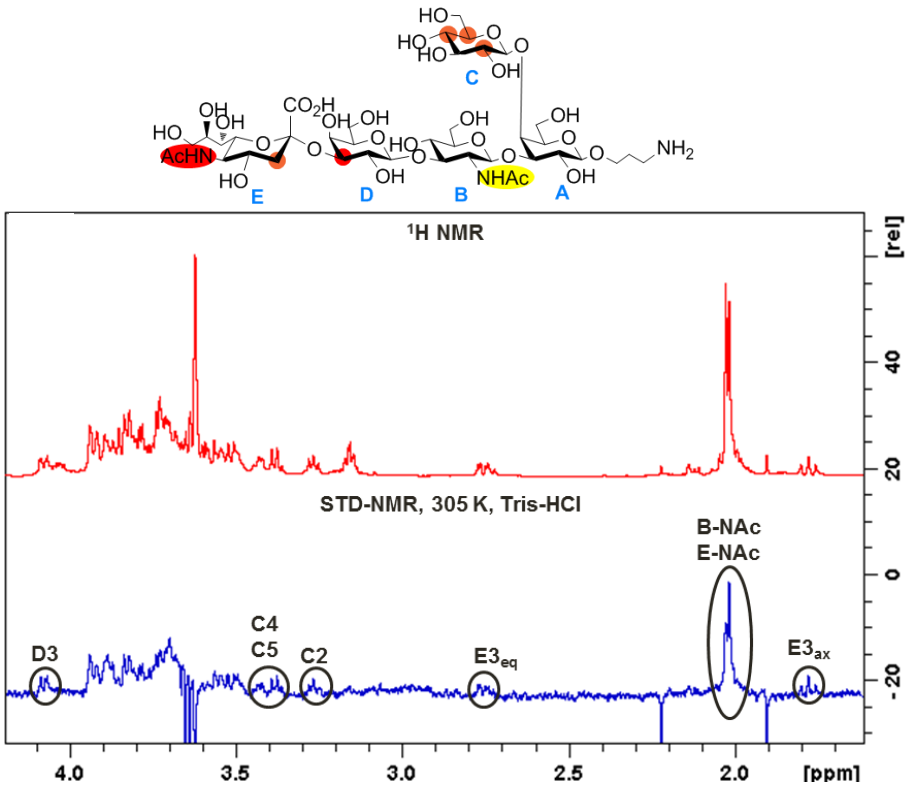


Figure 4. A) STD-NMR interpretation: mapping of the pentasaccharide protons closely interacting with the mAb; B) Off-resonance; C) STD-NMR of the complex pentasaccharide: anti PSIb mAb at 305 K, recorded at 600 MHz. Proton positions receiving strongest saturation after protein irradiation are circled in black.

Notably, most of the ^{1}H STD-NMR resonances that could unequivocally be assigned to the pentasaccharide **5**-mAb complex were related to the Glc residue of the backbone and the α-Neu5Ac-(2→3)-β-Gal-(1→4) residues, indicating a direct involvement of this branch.

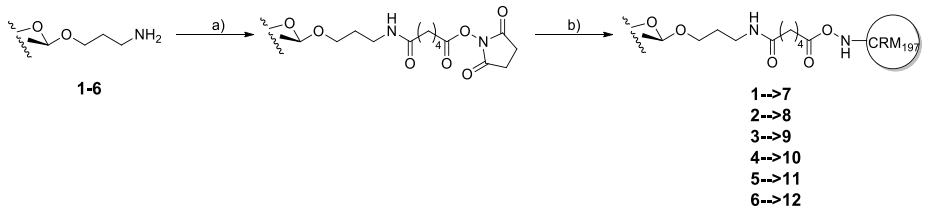
Transfer of saturation was received especially by H_3-E equatorial and axial, H_3-D, H_2-C, H_4-C and H_5-C. Interestingly, only the acetamide on the sialic acid showed a high STD signal (76% of recovery versus 45% for the acetamide on the glucosamine), confirming the engagement of the Neu5Ac moiety in the binding to the mAb, while the backbone glucosamine seems to be involved to a lower extent. Notably, strong involvement of the side chain Neu5Ac and Gal sugars in the interaction with the corresponding anti PS mAb has been observed previously for GBS type III. In the GBS type Ib case, the STD-NMR pointed out that the Glc residue also plays a key role in the interaction with the mAb, indicating a direct involvement also of the backbone.

GBS glycans conjugates to CRM$_{197}$

In order to evaluate the immunogenicity of the synthetic oligosaccharides, selected fragments were conjugated to the classical carrier protein CRM$_{197}$. When it comes to the immunogenicity of

glycoconjugate vaccines, there are several parameters that can influence the immune response, among which, the type of protein carrier, the length of the saccharide antigen, the presence of charges, and the degree of carbohydrate loading[14]. In particular, for short glycans the saccharide/protein ratio has been shown to play a key role. Carboni at al. recently demonstrated that CRM conjugates of GBS type III oligosaccharides elicited higher antibody titers at increasing saccharide/protein ratio, with the influence of glycosylation degree being crucial for the shortest oligosaccharides[15]. For this reason, the aim was to obtain glycoconjugates with an average number of sugar chains per protein above 10 to understand whether multivalent exposure of sub-optimal epitopes could still result in an immune response in vivo.

Glycoconjugates of compounds **1**-**6** were conjugated to CRM_{197} taking advantage of the aminopropyl linker, which was activated by treatment with an excess of di-*N*-hydroxysuccinimidyl adipate[10]. The percentage of activation was monitored by NMR and the resulting activated ester was incubated with the carrier protein CRM_{197} in sodium phosphate buffer at pH 7.2. 75-100 equivalents of the active ester were used to obtain conjugates with a high glycosylation degree.



Scheme 1. *Reagents and conditions*: a) Di-*N*-hydroxysuccinimidyl adipate, triethylamine, DMSO; b) 0.8 M sodium phosphate 7.2.

The glycoconjugates were purified by filtration against sodium phosphate buffer and characterized by 3-8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the carbohydrate/protein molar ratio and by microBCA to quantify the protein content. The degree of carbohydrate incorporation was then corroborated by quantification of the amount of galactose moieties present in the conjugated saccharide through high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Apart from the conjugate CRM197-hexa Y-shape **9**, all the others reached the target of at least 10 saccharide chains per mol of protein as summarized in Table 1.

The reason why the CRM-hexa-Y shape conjugate **9** was obtained with a very low glycosylation degree was investigated retrospectively by analysis of the proton NMR spectra, which showed a very low activation percentage of the saccharide after treatment with di-*N*-hydroxysuccinimidyl adipate. This problem was caused by a reduced availability of the primary amine on the linker,

which was alkylated in the deprotection procedure, as confirmed after careful anaysis of the NMR and ESI-MS characterization of compound **3**.

Table 1. Attributes of the prepared glycoconjugates

Glycoconjugate	**mol NHS/ mol protein**	**Protein conc. (µg/mL)**	**Sacch. conc. (µg/mL)**	**Saccharide/protein ratio**
CRM_{197}-branched penta **7**	1:100	524	178.6	17:1
CRM_{197}-linear penta **8**	1:75	1245	342.1	14:1
CRM_{197}-hexa Y-shape **9**	1:75	257	5.9	1:1
CRM_{197}-hexa branched **10**	1:75	247	65.8	12:1
CRM_{197}-branched penta **11**	1:100	466	191.8	20:1
CRM_{197}-linear penta **12**	1:75	992	200.2	10:1

Western blot analysis with anti PSIa and PSIb monoclonal antibodies was performed on all conjugates (Figure 5 and 6). Unfortunately, none of the conjugates obtained with type Ia oligosaccharides were recognized by the mAb, confirming the results obtained by competitive SPR.

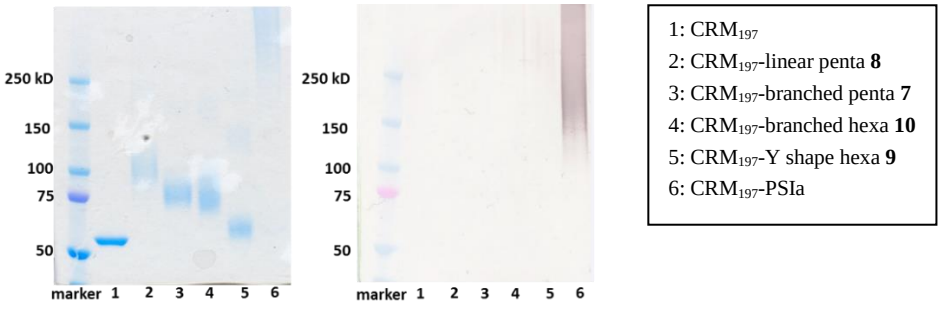


Figure 5. SDS-Page on GBS serotype Ia glycoconjugates and corresponding Western Blot

The Western Blot of GBS Ib oligosaccharide conjugates showed recognition only of the branched pentasaccharide repeating unit (compound **11**), confirming that the arrangement of the sugar residues in the repeating unit impacts antibody recognition.

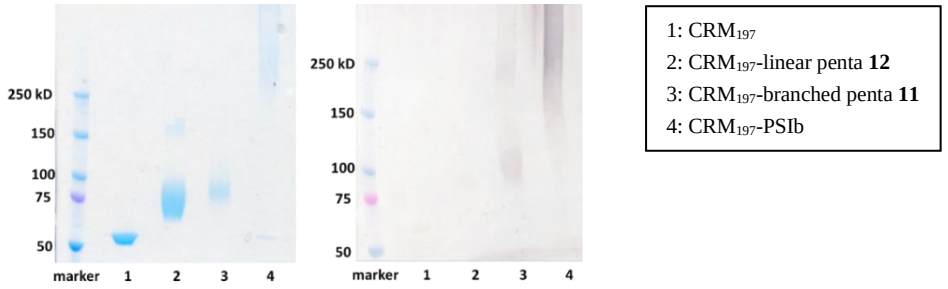


Figure 6. SDS-Page on GBS serotype Ib glycoconjugates and corresponding Western Blot

Overall, the data confirmed that GBS Ia and Ib capsular polysaccharides, despite a very similar chemical structure, present different carbohydrate epitopes. Monoclonal antibodies indeed show no cross-reactivity, not only with short fragments but also for the long polysaccharides. Moreover, while for GBS Ib a branched pentasaccharide is able to interact with an anti PSIb monoclonal antibody, longer structures from GBS Ia seem to be required for having a similar interaction.

Conclusion

The availability of structurally well-defined oligosaccharides is an essential requirement to gain information on structure-immunogenicity correlations of bacterial capsular polysaccharides and consequently to design better glycoconjugate vaccines[7]. When methods for the enzymatic and/or chemical depolymerization of the polysaccharide are not available, chemical synthesis is an essential alternative to obtain short oligosaccharides. GBS serotype Ia and Ib polysaccharides are typical examples of structures for which depolymerization is not applicable. For this reason, oligosaccharides **1**-**4** from GBS Ia and **5**-**6** from GBS Ib capsular polysaccharides were prepared and used to study their interactions with functional serotype specific murine mAbs. Competitive SPR confirmed that both monoclonal antibodies used were not cross-reactive towards the other GBS serotype. Unfortunately, GBS Ia oligosaccharides **1**-**4** were not able to deplete the binding of the anti PSIa mAb to the PSIa-HSA immobilized on a CM5 chip. This indicated that such short fragments are not covering the antigen involved in recognition by the anti-PSIa mAb used for the experiments. Most likely, longer oligosaccharides presenting more than one glucosamine and/or Neu5Ac moiety, are needed for full inhibition.

On the other hand, GBS Ib branched repeating unit **5** could fully deplete the binding of a soluble anti PSIb mAb to a PSIb-HSA conjugate immobilized on the chip. Recognition occurred also for the linear repeating unit **6**, with an IC50 1-log higher compared to compound **5**. The different recognition of the two frameshifts confirmed, as previously observed for GBS type III repeating unit[10], that the arrangement of the monosaccharide residues in the repeating unit is crucial to antibody recognition. To better map the interactions between the pentasaccharide and the mAb,

STD-NMR experiments were performed, highlighting that the α-Neu5Ac-(2→3)-β-Gal-(1→4) part is the most involved in the binding, as well as the Glc and the Gal of the backbone.
All the saccharides were then conjugated to CRM_{197} in order to evaluate their immunogenicity *in vivo*. The glycoconjugates were characterized in terms of protein and sugar content and a high carbohydrate loading was achieved for almost all compounds with the aim to compensate the poor immunogenicity of sub-optimal epitopes with their multivalent exposure. The conjugates will be tested in mice for their capability of eliciting specific anti-PSIa and anti-PSIb antibodies able to mediate opsonophagocytic killing (OPKA) of strains expressing type Ia and Ib PS respectively.

Experimental

SPR mAb binding inhibition

Inhibition assays were performed by SPR with a BIACORE X100 system using a CM5 sensor chip (Biacore) with immobilized HSA-PSIa or HSA-PSIb. Immobilizations were performed using the amine coupling kit supplied by the manufacturer (Biacore) and were conducted in 10-20 mM sodium acetate (pH 4-5) at sugar concentrations of 2-4 µg/mL. The immobilized surface density was ~50 resonance units in each instance. Measurements were conducted in 10 mM HEPES (pH 7.2), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 at 25°C and at a flow rate of 45 µL/min. Following mAb binding, conjugate surfaces were regenerated with 3.5 M $MgCl_2$ and a contact time of 120 s. Binding analysis was performed with samples of mAb at a fixed concentration pre-incubated with PSIa/PSIb or their fragments serially diluted (2x) starting from a concentration of 5 mg/mL. Sensorgram data were analyzed using BIAevaluation software (Biacore).

Competitive ELISA

Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 µL of 1 µg/mL of PSIa and PSIb capsular polysaccharide conjugated to Human Serum Albumin in PBS 1x. Plates were incubated overnight at 2–8°C, washed three times with PBST (0.05% Tween-20 in PBS pH 7.4) and saturated with 250 µL/well of PBST-B (2% Bovine Serum Albumin-BSA in PBST) for 90 min at 37 °C.

Two-fold serial dilutions of the oligosaccharide fragments and polysaccharide controls were incubated at rt for 30 min with anti PSIa and PSIb mice sera in PBST-B, then were added to each well and tested in duplicate. Plates were incubated at 37 °C for 1 h, washed with PBST, and then incubated for 1:30 h at 37 °C with anti-mouse IgG-alkaline phosphatase (Sigma) diluted 1:2000. After washing, the plates were developed with a 4 mg/mL solution of *p*-Nitrophenyl Phosphate (pNPP) in 1 M diethanolamine (DEA) pH 9.8, at rt for 30 min. After blocking with 7% EDTA, the absorbance was measured using a SPECTRAmax plate reader with wavelength set at 405 nm. Results were expressed as inhibition percentage of the anti PSIa and PSIb IgGs.

STD NMR experiments

NMR experiments were carried out on a Bruker 600 MHz NMR instrument equipped with a TBI cooled probe at controlled temperature (± 0.1 K). Data acquisition and processing were performed using TOPSPIN 1.3 and 3.1 software, respectively. Suppression of water signal was achieved by excitation sculpting (2 msec selective square pulse). Proton-Carbon Saccharides resonances were assigned collecting both 1D (^{1}H and ^{13}C) and 2D (TOCSY, NOESY, HSQC, HSQC-TOCSY) experiments, using standard pulse sequences. STD-NMR experiments were acquired with 96 scans over 96 accumulations and spectral width of 8000 Hz (12 ppm) at 305 K; a saturation transfer of 2/4 sec. was applied to enhance the saturation transfer effect, irradiating at a frequency of 7.5 and -0.5 ppm. To avoid pitfalls in the interpretation of STD-NMR spectra, a negative control spectrum was always recorded in absence of mAb (ligand and buffer) at the very same condition (concentration and pH) of the mAb-saccharide samples. The STD negative controls were always subtracted to the relative mAb-saccharide STD spectrum, obtaining the STDD experiments.

NMR sample preparation

mAb was exchanged in the working buffer (Tris d-11 50 mM in D_2O at pH 8.0 +/- 0.1) through 2 mL Zeba Spin desalting column saturated with 3 cycles of working buffer. mAb was finally eluted at the same starting concentration of 0.3 mg/ml. The 100% of recovery was assessed through microBCA spectrophotometric assay. The saccharides used was desalted, quantified, dried and then dissolved in the working buffer too. mAb final concentration in the NMR tube was of 3 µM. Ligand (saccharide):mAb ratio was set 100:1 mol/mol.

CRM_{197} conjugation of synthetic DPs

A solution of di-N-hydroxysuccinimidyl adipate (12 eq) and triethylamine (5 eq) in DMSO was added to compounds **1**-**6** to have a final concentration of 40 umol/mL of saccharide. The reaction was stirred for 3h, then the product was precipitate at 0°C by adding ethyl acetate (9 volumes). The solid was washed 10 times with ethyl acetate (5 volumes each) and lyophilized. The activated sugar was conjugated to CRM_{197} in sodium phosphate 0.8 M at a protein concentration from 23 to 42.6 mg/ml (depending on the sample), using a molar ratio of 75-100:1 saccharide/protein.

SDS-PAGE analysis

Sodium Dodecyl Sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 3-8% pre-casted polyacrylamide gel (NuPAGE®Invitrogen) using tris acetate as running buffer (NuPAGE®Invitrogen). 5µg of protein were loaded for each sample. After electrophoretic running with a voltage of 150V for about 45 min, the gel was stained with blue Coomassie.

Western Blot analysis

SDS-Page was run as described before. Gel was transferred on cellulose with iBlot® gel transfer stacks nitrocellulose kit and blocked with PBS 1x pH 7.2+BSA 3%. Anti PSIa and PSIb mAbs were used as primary antibody (dilution 1:1000, 1 h) followed by 5 washes with PBS+tween 0,05% buffer. Anti-mouse IgG alkaline phosphatase was used as secondary antibody (dilution 1:2000, 30min) followed by 5 washes with PBS+tween 0,05% buffer. Western-Blot was finally developed with AP conjugate substrate kit (BIORAD®).

HPAEC-PAD analysis for revealing Gal, Glc and GlcNAc

Standard samples of GBS PSIa and PSIb at five increasing concentrations ranging between 0.5 and 10.3 µg/mL were prepared to build the calibration curve. GBS PSIa and Ib samples were prepared targeting the calibration curve midpoint. The reference and analytical samples were prepared in 4 M trifluoracetic acid, incubated at 100°C for 3h, dried under vacuum (SpeedVac Thermo), and suspended in water. All analytical samples were filtered with 0.45 µm Phenex-NY (Phenomenex) filters before analysis. HPAEC-PAD analysis was performed with a Dionex ICS-3000 equipped with a CarboPac PA1 column (4 × 250 mm; Dionex) coupled with a PA1 guard to column (4 × 50 mm; Dionex). Samples (50 µL injection volume) were run at 1 mL/min, using isocratic elution with 14 mM NaOH, followed by a washing step with 0.5 M NaOH.

HPAEC-PAD data acquisition and analysis

In HPAEC-PAD analysis, the effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8 (Dionex). Gal, Glc, GlcNAc and Neu5Ac concentration were determined and converted in µmol/mL to estimate the relative mol/mol ratio.

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Chapter 7

Structure guided design of a fully synthetic GBS type III glycoconjugate vaccine

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Introduction

Among the 10 different serotypes of GBS, type III is the most prevalent serotype causing infections[1]. As mentioned, GBS infections in infants can occur within six days from the delivery, and in this case they are called Early-Onset Diseases (EOD), or after the first week of life up to three months of age (Late-OnSet Diseases, LOD)[2].

More than 90% of EOD are caused by serotypes Ia, Ib, II, III, and V, while LODs are caused predominantly by serotype III[3]. GBS infections can also occur in elderly or immunocompromised patients. In adults, serotype V (27.5%) was shown to be a predominant serotype, followed by Ia (24.3%) and III (16.5%)[4, 5].

GBS capsular polysaccharides are the main targets for vaccine development. However, purified GBS PSs are only variably immunogenic in adults, therefore PS–protein conjugate vaccines have been developed to enhance their immunogenicity[6, 7].

PSIII repeating unit is composed of pentasaccharide shown on Figure 1[8]:

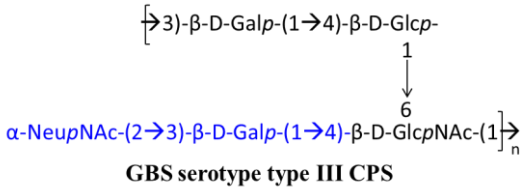


Figure 1. Repeating unit of GBS serotype III capsular polysaccharide

GBS PSIII glycoconjugates have been tested, alone and in combination with PS Ia and Ib conjugates in a trivalent formulation, in Phase I/II clinical trials and proven to be safe, well-tolerated and to elicit functionally active antibodies which are transferred to the baby through the placenta[9].

Although bacterial surface polysaccharides are often optimal target for vaccine discovery, structural details of the antigenic determinants responsible for their binding to specific functional antibodies are unknown[10].

GBS type III PS has been exceptionally studied (also compared to other GBS serotypes) and has been proposed as the prototype of a unique length-dependent conformational epitope. Molecular dynamics simulations and NMR studies[11, 12] showed that the GBS PSIII is flexible in solution and predominantly exists as a random coil. However, more than four repeating units (RUs) are able to form extended helical structures stabilized by the presence of the charged sialic acid residues[13, 14]. On the other hand, the structurally related *Streptococcus pneumoniae* type 14 polysaccharide, which differs from PSIII by the absence of the Neu5Ac, results in a more disordered structure[15]. Furthermore, a high affinity monoclonal antibody (mAb) specific to PSIII was found, and PS fragments of different size were recognized by the anti-PSIII mAb in a length dependent manner. A dimer of 2RUs (Figure 3) was shown to be sufficient to bind to a monoclonal IgG, although

with much lower affinity as compared to fragments composed of a larger number of RUs[14]. These properties have been rationalized in terms of the presence of a conformational epitope situated on an extended segment of the GBS PSIII[16-18].

Recently, three different sequences, one linear glycan (**1**) and the two branched structures **2** and **3**, corresponding to GBS PSIII repeating units, were prepared (Figure 2)[19].

The three synthesized structures where conjugated to Cross Reacting Material 197 (CRM_{197}), and competitive ELISA with anti GBS PSIII serum showed the importance of the branching formed motif Glc*p*-(1→6)-Glc*p*NAc for antibody recognition. These structures, together with fragments obtained from PS depolymerization, were also used to define the molecular details of the interactions with anti-PSIII mAbs by STD-NMR and X-ray crystallography[10]. Carboni et al. demonstrated the existence of a sialic acid-dependent functional epitope in GBS III where most of the contact area with the Fab involves a single repeating unit; the minimal binding structure contains four consecutive sugars composing the PSIII backbone and one disaccharide present in the branching (Figure 3). The dimer obtained by polysaccharide depolymerization was shown immunogenic after conjugation to the carrier protein[20].

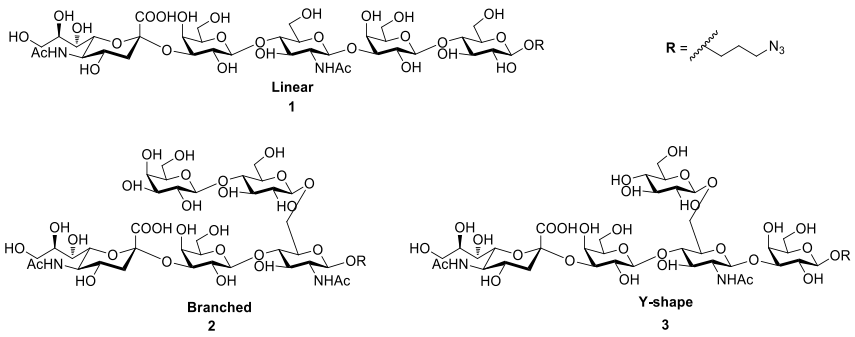


Figure 2. Linear, branched and Y-shape frameshifts of GBS serotype III repeating unit

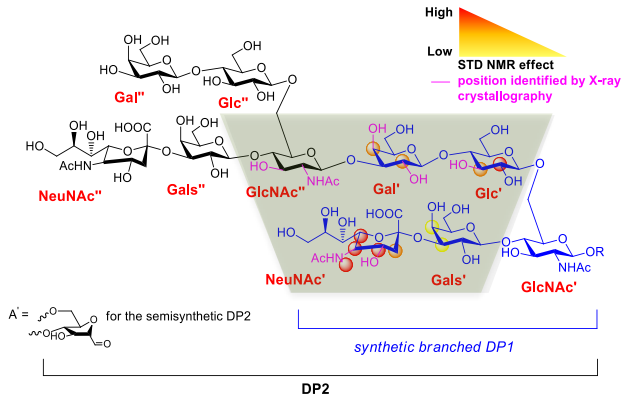
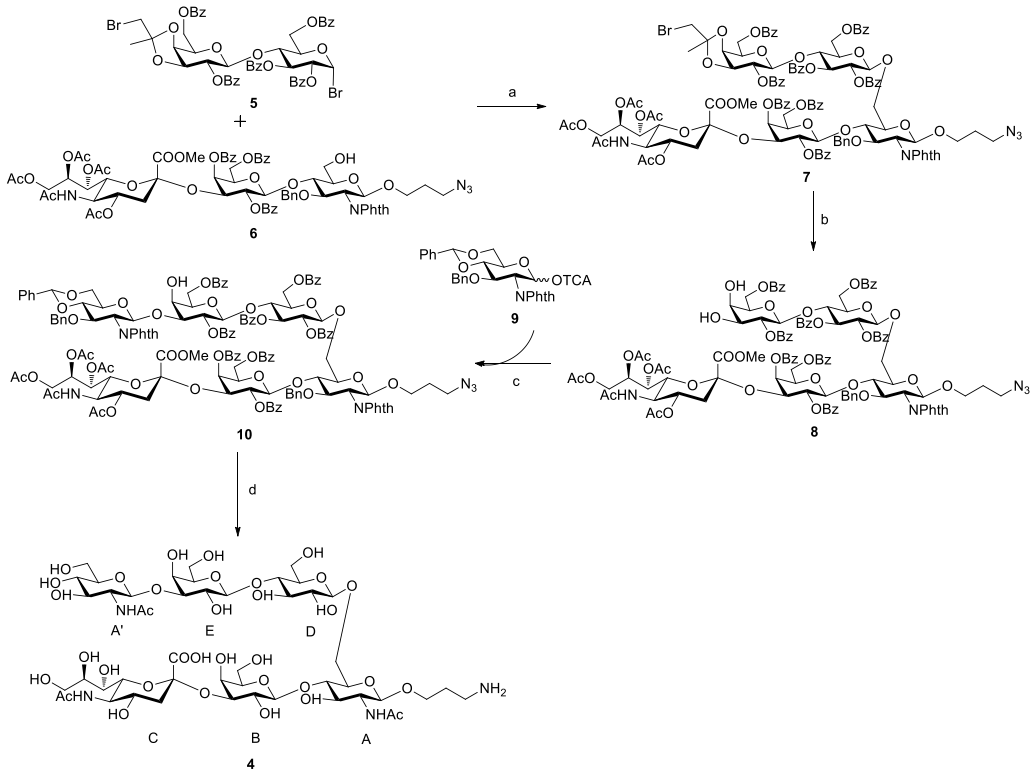


Figure 3. GBS PSIII epitope mapping with 2 RU semi-synthetic fragment. Protons interacting with the mAb identified by STD-NMR and X-ray crystallography.

This Chapter describes on the basis of the X-Ray crystallographic structure of the dimer-Fab complex, the design of a hexasaccharide comprising the residues involved in the binding of the polysaccharide with the protective mAb. Next to this, the synthesis of this hexasaccharide is outlined, followed by a study of the interactions between the hexasaccharide and a protective anti PSIII rabbit mAb by competitive Surface Plasmon Resonance and Saturation Transfer Difference NMR (SDT-NMR). The obtained results were corroborated by Molecular Dynamics simulation and NMR experiments comparing the conformation of the hexasaccharide in the binding pocket of the anti PSIII Fab to the one of the semisynthetic DP2 of the published X-Ray crystal structure (pdb 5m6).

Results

Starting from the previously described structural results, the use of a structure-guided approach was envisaged to design the small hexasaccharide **4** comprising all sugar moieties directly interacting with mAb binding pocket as a minimal immunogenic epitope (Scheme 1).
Compound **4**, bearing a linker at the reducing end for conjugation to a carrier protein, was chemically synthesized by conventional carbohydrate chemistry procedures. To do so, known trisaccharide **6**[19] was elongated with lactose donor **5** bearing a participating protecting group at position 2 to give pentasaccharide **7**, that was subjected to acid hydrolysis. The free C3 and C4 hydroxyl groups in the galactose residue of obtained **8** set the stage for a regioselective glycosylation at Gal C-3 (see Chapter 3) with donor **9** to give the fully protected hexamer **10**. The final stage, in which all protecting groups in **10** were removed, consists of the following five step: (i) removal of the methyl ester of Neu5Ac with lithium iodide in pyridine; (ii) reaction with ethylenediamine in refluxing ethanol for concomitant removal of the *O*-acetates and the *N*-Phth protecting group; (iii) reacetylation with acetic anhydride/pyridine to install the acetamide group of the GlcNAc residues along with acetyl esters; (iv) methanolysis and v) final catalytic hydrogenation over Pd/charcoal to provide the target branched hexasaccharide **4**, which was desalted and characterized.



Scheme 1. *Reagents and conditions*: a) AgOTf, dry DCM, -10°C, 65%; b) 9:1 TFA-H_2O, 80%; c) TMSOTf, dry DCM, -30°C, 69%; d) LiI, Py, 120°C; $H_2NCH_2CH_2NH_2$, EtOH, 90°C; Ac_2O, Py; MeONa, MeOH; H_2, Pd-C, 40% (over five steps).

With hexasaccharide **4** in hand, it was investigated whether this structure corresponds to a minimal immunogenic epitope of GBS III.

The capacity of the hexasaccharide **4** to cover the determined epitope was evaluated by competitive SPR by which its ability as an inhibitor of the binding of a soluble Fab fragment to PSIII conjugated to Human Serum Albumin (HSA) immobilized on a chip was assessed.

The hexasaccharide showed an affinity 2 orders of magnitude higher than the branched structure and fully mimicking the semisynthetic PSIII dimer DP2. This result confirmed that only the upstream GlcNAc **A'** is engaged in interactions with the mAb, while the downstream end GlcNAc **A**, which was replaced by a mannofuranoside in the crystallized semisynthetic glycan, is not accommodated in the binding pocket (Figure 4A).

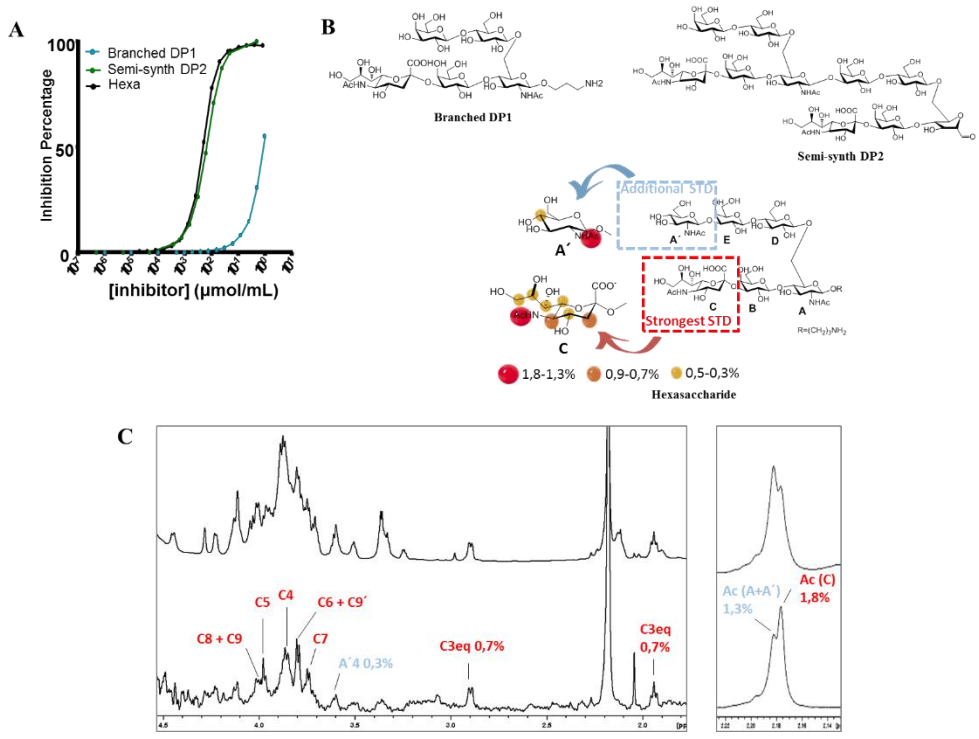


Figure 4. A) Competitive SPR against an anti PSIII rabbit Fab of the branched DP1, the semisynthetic DP2 and the hexasaccharide. B) Structure of the tested oligosaccharides and STD-NMR interpretation: mapping of the hexasaccharide protons more closely interacting with the mAb. C) ^{1}H-STD-NMR experiment: off-resonance (top) and STD (below) spectra for the hexasaccharide **4** interacting with mAb, with a zoom on N-acetyl signals on the right. Proton positions receiving strongest saturation after protein irradiation (STD effect) are indicated in red, and isolated proton signals with weaker STD are in blue.

A more detailed ligand-mAb interaction analysis was carried out by STD-NMR (Figure 4B/C)[21, 22]. Most of the peaks in the STD-NMR correspond to signals related to Neu5NAc and to Gal' in the branch, confirming that Neu5Ac is a major anchoring site for mAb interaction. Notably, the peak at 2.03 ppm, corresponding to the NAc moiety of sialic acid, received the highest transfer of saturation, as compared to the two NAc signals of GlcNAc overlapping at 2.04 ppm. In the ^{1}H NMR, these two NAc moieties showed a ratio of 2:1 with respect to the sialic acid NAc, while in the STD-NMR analysis, the intensity of the peaks at 2.04 and 2.03 ppm was comparable.

These results show that the identified hexasaccharide was interacting with Fab similarly to the dimer obtained by depolymerization previously used for crystallography. To further corroborate this conclusion, a Molecular Dynamics simulation of the hexasaccharide-Fab complex was performed to provide insights on how the synthetic fragment is accommodated in the binding pocket of the mAb.

First, an NMR experiment was carried out to compare the conformational preferences of the hexasaccharide in its free and bound forms. For the free state, the NOESY spectrum was analyzed

and all NOE cross peaks were negative. With respect to interglycosidic flexibility, special attention was payed to the linkage Neu5Acα2-3Gal. A conformational ensemble involving three conformations is present for this flexible linkage (Fig. 5A, left): POP1, where φ/ψ values are ca. 180^0/-20^0, and POP2 and POP3, in a close region of the conformational map, with φ/ψ values ca. -90^0/-50^0, and -65^0/-10^0, respectively.

Inter-residue distances were estimated form the intensities of the NOE cross peaks. Experimentally (Fig. 5B, right), the strong H3ax Neu5Ac(C) – H3 Gal(B) NOE yielded an estimated distance of 2.9Å suggesting the existence of a conformational equilibrium in solution, with the participation of more than one conformation. Indeed, for POP1 conformation, this distance is below 2.5Å, while for the two other energy minima, POP2 and POP3, this distance is 3.6 Å and 4.2 Å respectively. A similar evaluation was done for the interaction of H8 Neu5Ac(C) - H3 Gal(B), which yields an estimated distance of 3.4 Å. This distance is around 4.1 Å in POP1 and 2.6-2.7 Å for POP2 and POP3, corroborating the flexibility around the Neu5Acα2-3Gal linkage.

Interestingly, the situation is different in the trNOESY spectrum (Fig. 5B, left). In this case, the only observable NOEs for H3axNeu5Ac(C) are the intraresidual ones with H4 and H5, while the inter-residue contacts are absent. On the contrary, the NOE between H3eqNeu5Ac(C)–H4Gal(B) is more intense in the trNOESY than in the NOESY, yielding an estimated distance for the bound conformation of 2.7 Å.

These data clearly indicate that there is a conformational selection process upon binding. In particular, in the bound conformation, H3-Gal is oriented towards the glycerol chain of the Neu5Ac residue, such as in POP2 and POP3, while the POP1 conformation has disappeared.

However, the trNOESY analysis do not permit to quantitatively define the torsion angles.

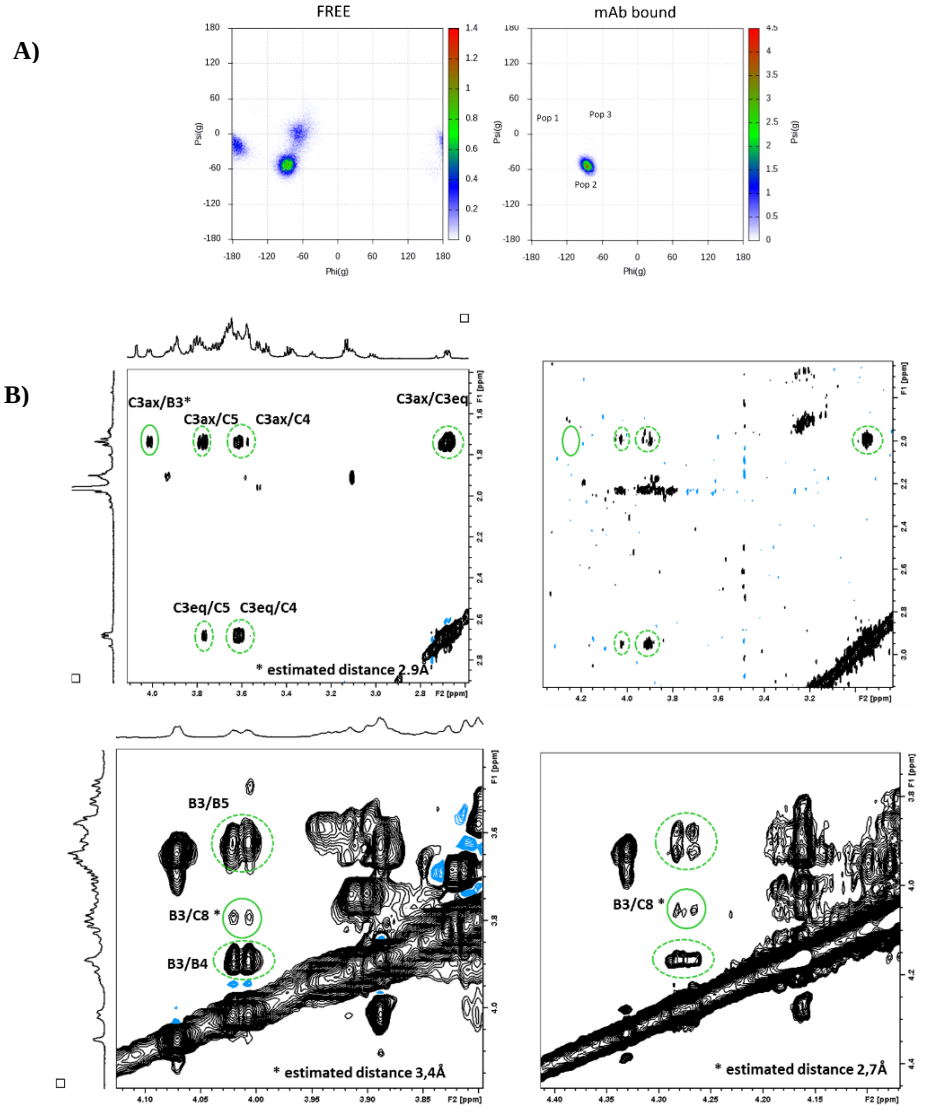


Figure 5. A) MD simulations of Neu5Acα2→3Gal linkage in the hexasaccharide in the free and bound state B) Comparison of the same regions in the NOESY spectrum of the hexasaccahride free (left) (800MHz) and trNOESY of the hexasaccharide in the presence of the mAb (right) (600MHz).

Thus, a Molecular Dynamics (MD) simulation was performed using this geometry as starting structure for the Neu5Acα2-3Gal fragment. The result of the MD simulation is shown in Fig. 6A, which also shows the superimposition of different frames of the ligand bound to the mAb. A single frame for the complex is shown in Fig. 6B.

The hexasaccharide complexed with the mAb shows that the ligand exclusively populates the POP2 region (Fig. 6B right), with a minimum centered at $-90^0/-50^0$. Interestingly, this value is in

perfect agreement with the X-ray crystallographic structure of the fragment DP2 complexed with the mAb (pdb 5m63) for which the φ/ψ values around Neu5Acα2-3Gal are $-80^0/-16^0$.
The Neu5Ac residue establishes different intermolecular hydrogen bonds with polar amino acid chains of the protein. In particular, the carboxylate group at C1, the carbonyl moiety of NHCOMe at C5, and the hydroxyl group at C4 are involved in interactions. On the other hand, the Me group of the NHCOMe is buried into a mAb groove and stacked against the aromatic rings of Y52 and Y115 of the light chain. Interestingly, the carbonyl group of the NHCOMe of the GlcNAc A´ residue also establishes hydrogen bonds with N55 and R66 of the light chain, while the Me group is stacked against Y126 of the heavy chain.

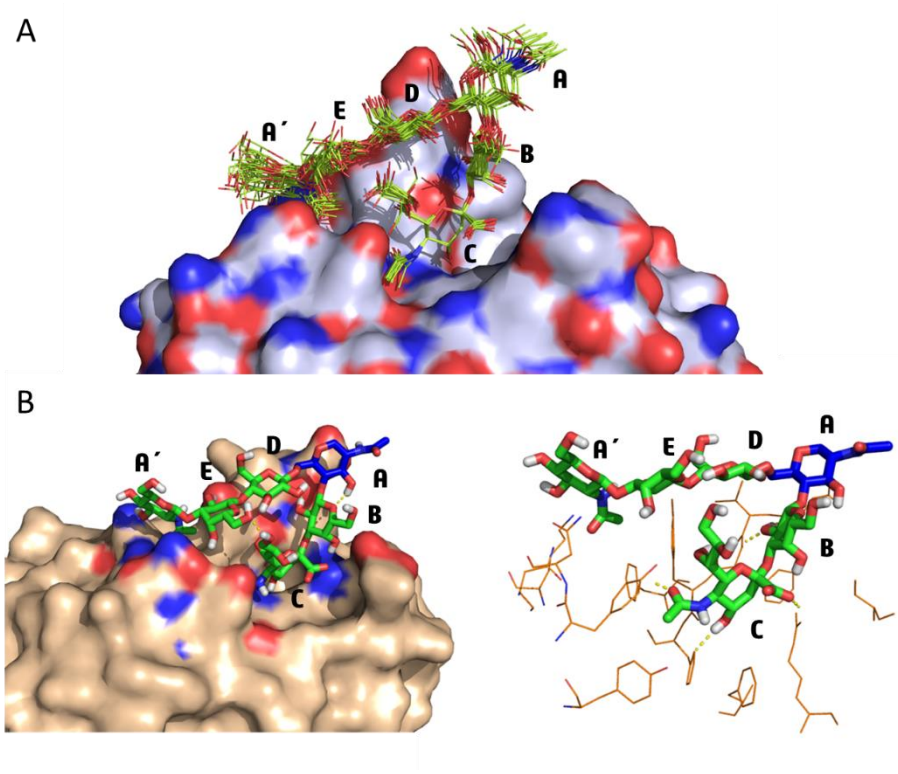


Figure 6. A) Result of the MD simulation: superimposition of different frames of the ligand bound to the mAb is shown. B) A single frame for the complex is shown.

The complex between the hexasaccharide and the mAb obtained by NMR and MD was then compared to the X-ray crystallography structure complex of DP2 with the mAb previously reported (pdb 5m63). Superimposition (Figure 7) shows that both complexes are very similar and share the main interaction points described above. Major differences involve the orientation of residue D (highlighted in red in Figure 7). In the DP2 complex, the ring O5 oxygen and hydroxymethyl group of residue D are pointing towards residue C, while in the hexasaccharide

complex described herein, the pyranose ring is turned 180^0 with respect to that conformation. Consequently, the glycosidic linkages involving this residue are different for both complexes. For the linkage E-D the DP2 complex is in an unusual non-exoanomeric ($-16^0/-118^0$) conformation conformation, while in the hexasaccharide complex, standard exo-anomeric/syn values are obtained($40^0/20^0$). Moreover, for the D-A linkage, the DP2 complex displays a non-exo-anomeric $-47^0/78^0$ conformation, while the hexasaccharide shows a regular $30^0/-160^0$ geometry.

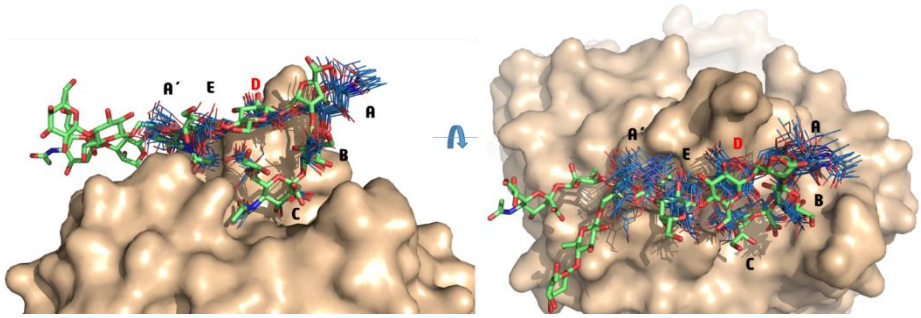
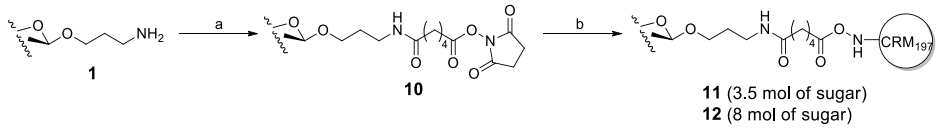


Figure 7. Superimposition of the complex between the hexasaccharide and the mAb and the X-ray crystallography-based complex of DP2 with the mAb already reported (pdb 5m63)

Having established that hexasaccharide **4** contains the moieties related to the minimal antigenic determinant, immunization study with the hexasaccharide conjugated to the well-known carrier protein CRM_{197} was planned to understand whether this small glycan was also behaving as minimal polysaccharide epitope.

It is well-know that multivalence is a crucial attribute in obtaining strong immunogenicity with such small fragments[23, 24]. To this aim, two hexasaccharide conjugates differing in their glycosylation degree were generated. The amino-propyl linker of the hexasaccharide was therefore activated by treatment with an excess of di-N-hydroxysuccinimidyl adipate (Scheme 2)[25]. The isolated activated ester was incubated with the carrier protein CRM_{197} in sodium phosphate buffer at pH 7.2. The amount of coupled glycan was proportional to the active ester used in conjugation (Table 1). Thus, the use of 15 equivalents led to incorporation of an average of 3.9 glycan moieties; when 50 equivalents were used the level of carbohydrate incorporation was increased to 7.8 mol/mol of protein. Glycoconjugates were purified by filtration against sodium phosphate buffer and characterized by 4–12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the carbohydrate/protein molar ratio and by microBCA for the protein content quantification. The degree of carbohydrate incorporation was then corroborated by quantification of the galactose moieties present in the conjugated saccharide through high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)[26].



Scheme 2. *Reagents and conditions*: a) Di-N-hydroxysuccinimidyl adipate, triethylamine, DMSO; b) 100mM sodium phosphate 7.2.

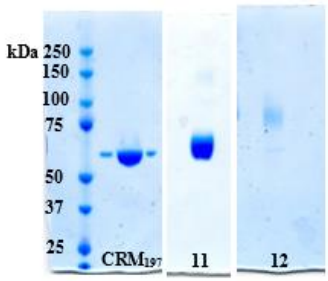


Figure 8. SDS-PAGE on GBS type III hexasaccharide glycoconjugate. From left to right: marker, CRM_{197}, CRM_{197}-hexa low glycosylation degree **11**, CRM_{197}-hexa high glycosylation degree **12**.

Table 1. Attributes of the synthesized glycoconjugates

Glycoconjugate	mol NHS/ mol protein	Protein conc. (μg/mL)	Sacch. conc. (μg/mL)	Saccharide/protein ratio
CRM_{197}-hexa **11**	1:15	502	41.3	3.9
CRM_{197}-hexa **12**	1:50	270	44.9	7.8

The conjugates of the previously synthesized pentasaccharide units and the novel hexasaccharide were tested in mice[27] for their capability of eliciting specific anti-PSIII antibodies able to mediate opsonophagocytic killing (OPKA) of strains expressing type III PS. Two separated immunizations of BALB/c female mice were conducted by three intraperitoneal injections of the pentasaccharides (0.5 μg carbohydrate dose) and hexasaccharide (1.0 μg carbohydrate dose), respectively. All the glycoconjugates were formulated with Alum hydroxide (AlumOH) and the PSIII-CRM_{197} conjugate was used as a control for both immunizations. The animals received booster doses of the same vaccines 21 and 35 days after the first immunization. Immunogenicity of the glycoconjugates was evaluated by measurement of IgG titers by ELISA after the second and third immunization.

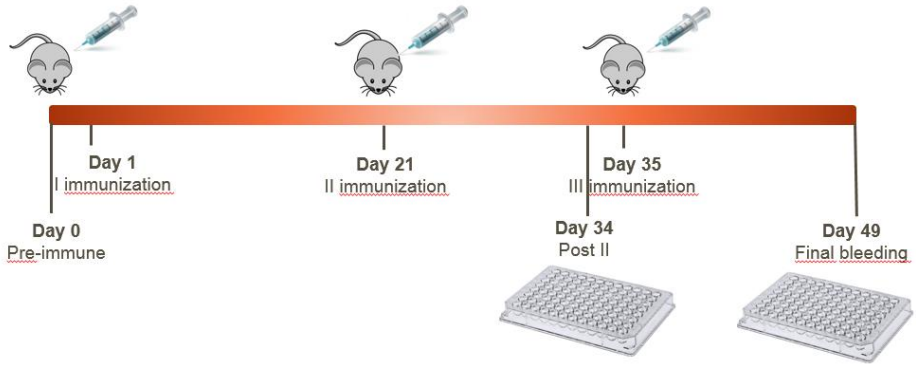


Figure 9. Immunization scheme for the CRM-oligosaccharides conjugates

As shown in Figure 10, in the first immunization with the CRM-conjugates of the GBS type III repeating unit **1**, **2** and **3** only the highly glycosylated (~23 sugar chains per mol of protein) CRM_{197} conjugate of the branched pentasaccharide **2** could elicit some anti-PS antibodies. These antibodies were also functional in the OPKA assay. No immune response was observed upon immunization with the low glycosylated conjugate of compound **2** or glycoconjugates of the linear and Y-shape repeating unit **1** and **3**. In contrast, a strong immune response was observed after 2 doses and 3 doses using the hexasaccharide-CRM_{197} conjugate having the higher saccharide/protein ratio, while no antibody titers was detected for the hexasaccharide conjugate with the lower saccharide/protein ratio. This proved that the hexasaccharide not only represents an antigenic determinant but also the minimal immunogenic epitope. The upstream glucosamine A' of the hexasaccharide proved to be crucial both for antibody recognition and for immunogenicity.

In addition, the glycosylation degree of the conjugate appeared to be a crucial parameter to elicit an immune response in mice with these relatively short synthetic oligosaccharide.

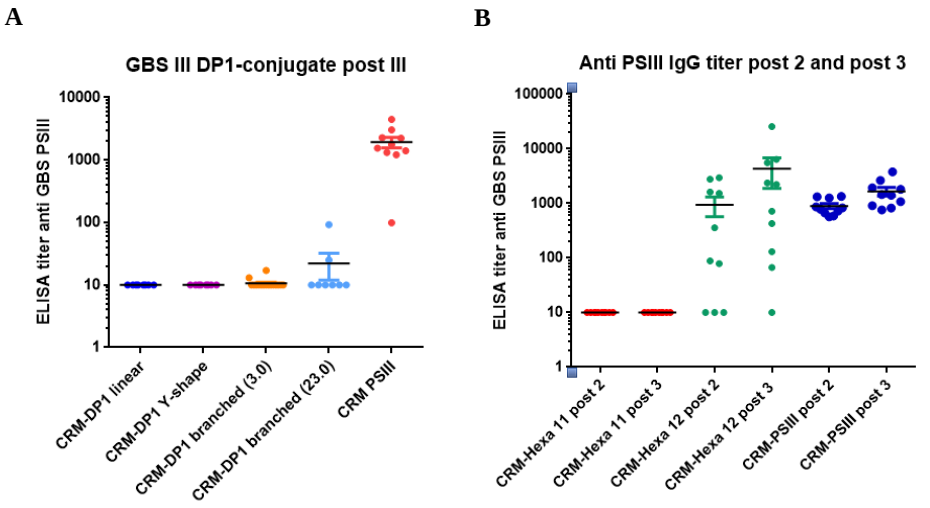


Figure 10. A) Elisa titers post III elicited by CRM_{197}-DP1 linear (in blue), CRM_{197}-DP1 Y-shape (in purple), CRM_{197}-DP1 branched 3.0 (in orange), CRM197-DP1 branched 23.0 (in light blue). CRM_{197}-PSIII (in red) was used as positive control. B) Elisa titers post II and post III elicited by CRM_{197}-hexa **11** (in red), CRM_{197}-hexa **12** (in green) and CRM_{197}-PSIII (in blue).

Antibody functional activity was assessed by an *in vitro* OPK assay which is a well-established technique to mimic the *in vivo* process of GBS killing after bacterial opsonization by effector cells in the presence of complement and specific antibodies. In this assay, sera are tested at different serial dilutions and the reported OPKA titers, expressed as the reciprocal of the serum dilution leading to 50% bacterial killing, have been shown to correlate with mouse protection levels in a passive immunization/GBS challenge models.

In agreement with the ELISA analysis, OPKA of the pooled sera after 3 doses of the high density hexa-CRM vaccine indicated good functionality of the elicited antibodies (See Table 2).

Table 2. OPKA titers post second and third immunization with glycoconjugates Hexa-CRM_{197} **12** and PSIII-CRM_{197}. Pre-immune sera were added as negative control.

Post	Vaccine	OPKA titer
Pre-immune		<30
2	Hexa-CRM_{197} **12**	<100
3	Hexa-CRM_{197} **12**	369
2	PSIII-CRM_{197}	<100
3	PSIII-CRM_{197}	420

Moreover, the level of IgM antibodies was also measured for both conjugate **12** and CRM-PSIII in mice having received two immunizations. While CRM-PSIII elicited high levels of IgMs, the

conjugated hexasaccharide induced primarily IgGs. Apparently, the long polysaccharide seems to be recognized with higher avidity by B-cell receptors, driving IgM production.

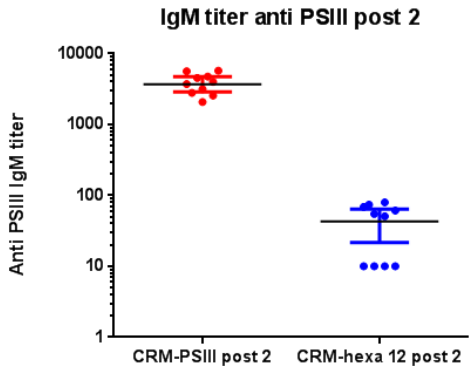


Figure 11. Elisa IgM titers after second immunization with CRM-PSIII and CRM-hexa **12**

Overall, these data suggest that the hexasaccharide fragment which is comprised within two PSIII RUs is an immunogenic epitope, in line with the minimal structure demonstrated to be necessary for antibody recognition. Furthermore, it is confirmed that the glycosylation degree plays a key role in the immune response to small carbohydrate epitopes. Indeed, only the glycoconjugate **12** with higher carbohydrate loading is able to elicit GBSIII specific functional antibodies.

Conclusion

Group B streptococcus type III is one of the serotypes which causes the highest number of GBS related diseases. Its capsular polysaccharide has always been considered a main virulence factor and target of vaccine development program. For many years, it has been considered the prototype of a length-dependent conformational glyco epitope but a recent study has shown how a fragment as long as two repeating units is able to interact with a functional anti PSIII rabbit monoclonal antibody. In particular, X-ray crystallography combined with STD-NMR of a semisynthetic PS dimer complexed with a protective mAb demonstrated the existence of a sialic acid-dependent antigenic determinant that is fully contained within six sugars (four deriving from the PSIII backbone and one from the disaccharide arm). This structure has recently been proven to be immunogenic after conjugation to carrier protein, demonstrating to contain the minimal polysaccharide epitope[10]. These findings led to design and synthesis of a hexasaccharide structure to be tested as an immunogenic mimick of the full polysaccharide.

First, the antigenic nature of the assembled hexasaccharide was confirmed by competitive SPR, where the short glycan proved to be as good an inhibitor of the binding of an anti PSIII mAb to a chip coated with PSIII-HSA as the full DP2. Furthermore, STD-NMR showed how the acetamides of the sialic acid and of the glucosamine A' were in close proximity to the mAb binding site, and

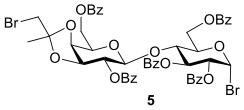
therefore likely involved in the binding. Molecular modelling simulations corroborated these observations by showing the conformational preferences of the hexasaccharide in the binding pocket of the mAb. Superimposition of the complex Fab-DP2 and the simulated mAb-hexasaccharide showed that both are very similar and share most of the contact points.

Overall, these data confirmed that the hexasaccharide is the minimal antigenic determinants recognized by a protective anti PSIII rabbit mAb. Conjugation of the hexasaccharide to the well-known carrier protein CRM_{197} allowed the evaluation of its immunogenicity *in vivo*. To this aim, two glycoconjugates to CRM_{197} were prepared to understand the impact of the glycosylation degree, that is the number of sugar chains per mol of protein, on the immune response *in vivo*. Confirming a trend that has been previously observed for short glycan antigens, only the glycoconjugate at higher glycosylation degree (about 8 chains of sugar per mol of protein) elicited a significative titer of functional antibodies comparable to the titer elicited by the positive control PSIII-CRM_{197}. An opsonophagocytic killing assay showed that these antibodies were functional. Interestingly, the immune response was not homogeneous in all animals, with some mice showing a very low antibody titer. While previous literature reports suggested GBSIII as an extremely complex epitope and pointed out the necessity of using long polysaccharide portions to contain functional epitopes, this structure guided approach counteracts this paradigm and shows that a small hexasaccharide can be designed to contain all the carbohydrate moieties needed for immunogenicity. This opens new possibilities for further improvement of synthetic vaccines based on small synthetic antigens, for instance by using a larger carrier (i.e. nanoparticles) to maximize the multivalent exposure of the epitope.

Experimental

General Methods and procedures. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50ºC on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm) or using pre-packed silica cartridges Reside (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (Biotage, silica 0.050 nm). Unless otherwise specified, a gradient 0-100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) or Biotage Isolera instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{1}H values are reported in ppm, relative to internal Me_4Si (^{1}H = 0.00, $CDCl_3$); solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{13}C values are reported in ppm relative to the signal of $CDCl_3$ (^{13}C = 77.0, $CDCl_3$). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Assignment of ^{13}C NMR spectra of some compounds was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters. Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data and *m/z* values found in their mass spectra.

[2,6-Di-O-benzoyl-3,4-O-(1-bromomethylethylidene)-β-D-galactopyranosyl)]-(1-4)-2,3,6-tri-O-benzoyl-α-D-glucopyranosyl bromide 5.



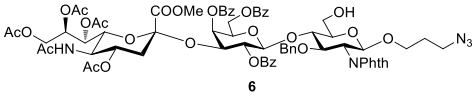
5

Ethyl thiol lactose starting material (850 mg, 0.90 mmol) was dissolved in CH_2Cl_2 (10 mL) and bromine (167 µL, 3.14 mmol) was added at 0°C. The reaction was stirred for 80 min and then quenched by the addition of triethylamine (2 mL). The solution was washed with Na_2SO_3 and water, organic phase was dried with Na_aSO_4 and evaporated under reduced pressure. The crude was finally purified by flash chromatography (toluene +3% TEA:Acetone) to afford **5** as a white solid in 75% yield (704 mg, 0.68 mmol). ESI MS m/z $[M-Br]^+$ found 964.6761; calcd 963.7834

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02-7.20 (m, 25H, H-Ar), 6.64 (d, J=3.68, 1H, H-1^A), 5.99 (t, 1H, H-3^A), 5.15-5.09 (m, 2H, H-2^A, H-2^B), 4.66 (d, J=7.06, 1H, H-1^B), 4.51 (dd, 2H, H-6$_{a,b}$), 4.39-4.34 (m, 2H, H-4^B), 4.28 (d, 1H), 4.21 (t, 1H), 4.14 (m, 1H, H-6$_a$), 3.78 (m, 2H, H-6$_b$), 3.22 (q, 2H, (C(CH_3)(CH_2Br)), 1.51 (s, 3H, (C(CH_3)(CH_2Br)).

^{13}C NMR (101 MHz, $CDCl_3$): d 128.4–133.8 (C-Ar), 109.4 (*C*(CH3)(CH2Br)), 100.5 (C -1$_B$), 86.9 (C-1$_A$), 78.0, 74.6, 74.5, 73.5, 73.2, 71.6, 71.2, 70.3, 62.7, 61.8, 37.2 (C(CH3)(*C*H2Br)), 24.5 (C(*C*H3)(CH2Br)).

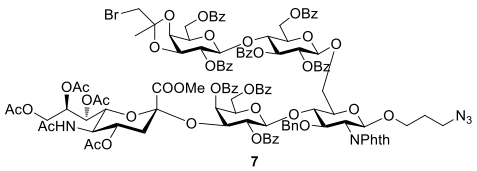
3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl)-(1→4)-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 6



6

Compound **6** was synthesized as previously reported[20]; ^{1}H NMR and ^{13}C NMR are in line with ones reported in literature[20].

3-Azidopropyl [2,6-di-O-benzoyl-3,4-O-(1-bromomethylethylidene)-β-D-galactopyranosyl-(1-4)-2,3,6-tri-O-benzoyl-β-D-glucopyranosyl-(1-6)]-[2,4,6-tri-O-benzoyl-3-O-(methyl 4,7,8,9-tetra-O-acetyl-5-N-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl-(1-4)]-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 7.



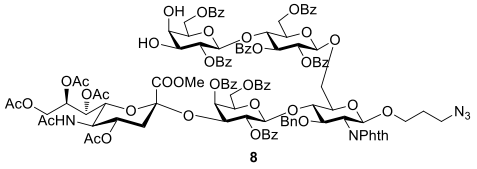
7

A solution of trisaccharide acceptor **6** (330 mg, 0.24 mmol) and donor **5** (420 mg, 0.60 mmol) with activated molecular sieves (4 A, 800 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. AgOTf (77 mg, 0.30 mmol) was added at -40 °C. The reaction mixture was stirred for 10h at rt, when TLC (7:3 Toluene:acetone) showed complete reaction. TEA was added, the solid filtered off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Toluene:acetone 8:2) to afford **7** (370 mg, 0.16 mmol) in 65% yield. yield. $[\alpha]_D^{25} = +42.73^\circ$ (c 1.4, CHCl3). ESI MS m/z $[M+H]^+$ found 2395.1274; calcd 2395.1439.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-6.58 (m, 49H, H-Ar), 5.71 (t, J = 8.90 Hz, 1H, H-8^C), 5.45-5.37 (m, 2H, H-2^B, H-3^D), 5.22-5.14 (m, 3H, H-2^D, H-4^B, H-2^E), 5.07 (dd, J = 2.84, 9.86 Hz, 1H, H-7^C), 4.92 (d, J=10.2, 1H, NH), 4.82-4.68 (m, 5H, H-1^A, H-1^B, H-3^B, H-4^C, C*H*HPh^A), 4.42 (d, J=7.22, 1H, H-1^E), 4.39-4.24 (m, 5H), 4.20- 4.16 (m, 2H, H-1^D, CH*H*Ph^A), 4.05 (dd, J = 5.35, 11.22 Hz, 1H, H-9^C), 3.97-3.86 (m, 6H), 3.73 (m, 6H), 3.63-3.42 (m, 7H, incl. OCH_{2a}), 3.25 (q, J=8.69, 2H, C*H*$_2$Br), 3.10-3.05 (m, 1H, OCH_{2b}), 2.91-2.84 (m, 2H, C*H*$_2$N$_3$), 2.77-2.73 (m, 1H, H-5^E), 2.38 (dd, 1H, H-3^C_e), 2.03, 1.75, 1.71, 1.66, 1,53 (5 x s, 3H each, 5 x C*H*$_3$CO), 1.60 [s, 3H, C(C*H*$_3$)], 1.54 (m, 1H, H-3^C_a), 1.37-1.28 (m, 2H, OCH_2CH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.80-164.89 (13x C=O esters) 134.20-122.37 (m, 49, C-Ar), 101.88 (C-1^D), 100.99 (C-1^B), 100.54 (C-1^E), 97.93 (C-1^A), 96.87, 80.25, 78.18, 78.03, 75.68, 74.97, 74.62, 74.56, 73.39, 72.70, 72.45, 72.72, 72.58, 72.52, 72.43, 71.06, 70.58, 70.41, 69.46, 68.16, 67.54, 67.02, 66.85, 66.18, 63.21, 62.21, 62.76, 62.33, 61.55, 55.76, 53.23, 48.62, 48.01, 37.31 (C-3^C), 37.07, 28.62, 24.58, 23.14, 21.33, 20.75, 20.73, 20.43.

3-Azidopropyl [2,6-di-*O*-benzoyl-β-D-galactopyranosyl-(1-4)-2,3,6-tri-*O*-benzoyl-β-Dglucopyranosyl-(1-6)]-[2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate)-β-D-Galactopyranosyl-(1-4)]-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 8.



8

Pentasaccharide **7** (370 mg, 0.16 mmol) was dissolved in a 90% solution of TFA. After 1h at rt, TLC (Toluene:Acetone 6:4) showed complete reaction. Reaction was concentrated under reduced pressure and purified via flash chromatography (Toluene:acetone 7:3) giving **8** (302 mg, 0.13 mmol) in 83% yield as a white solid.

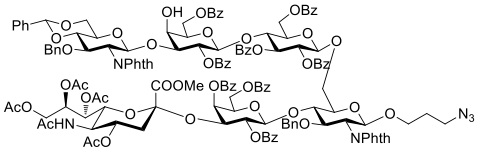
$[\alpha]_D^{25} = +38.78^\circ$ (c 1.5, CHCl3). ESI MS m/z $[M+H]^+$ found 2276.9871; calcd 2277.1840.

^{1}H NMR (400 MHz, CDCl3) δ 8.08-6.58 (m, 49H, H-Ar), 5.69 (t, J = 8.90 Hz, 1H, H-8^C), 5.46-5.38 (m, 2H, H-2^B, H-3^D), 5.26-5.17 (m, 3H, H-2^D, H-2^E, H-4^B), 5.08 (dd, J = 2.36, 9.81 Hz, 1H, H-7^C), 4.98 (d, J = 10.07, 1H, NH), 4.80-4.68 (m, 5H, H-1^B, H-1^A, H-4^C, H-3^B, C*H*HPh), 4.45 (d, J = 7.81, 1H, H-1^E), 4.39-4.32 (m, 2H, H-9^C_a, H-6^E_a), 4.26 (d, J = 7.64, 1H), 4.13 (d, J = 12.25, 1H, CH*H*Ph), 3.97-3.85 (m, 9H, incl. H-2^A, H-4^E), 3.72 (m, 6H, incl. H-5^C, H-9^C_b, $COOCH_3$), 3.64-3.59 (m, 3H), 3.56-3.44 (m, 5H, incl. OCH_{2a}), 3.16-3.10 (m, 1H, OCH_{2b}), 2.91 (q, J = 6.04, 2H), 2.83-2.79 (m, 1H), 2.37 (dd, J = 4.62, 12.79, 1H, H-3^C), 2.01, 1.83, 1.70, 1.67, 1.52, (5 x s, 3H each, 5 x CH_3CO), 1.46 (m, 1H, H-3^C), 1.36 (m, 2H, OCH_2CH_2)

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0-164.9 (13x C=O esters), 134.0-125.1 (C-Ar), 101.66 (C-1^D), 101.04 (C-1^B), 100.98 (C-1^E), 97.93 (C-1^A), 96.89, 80.15, 74.95, 74.57, 73.69, 72.99, 72.51, 72.39, 71.68, 71.58, 71.45, 70.58,

70.09, 69.48, 68.17, 67.00, 66.86, 66.19, 63.14, 62.91, 61.70, 61.53, 55.74, 53.24, 48.61, 48.02, 46.71, 37.31 (C-3^C), 28.64, 23.13, 21.33, 20.75, 20.72, 20.45.

3-Azidopropyl [3-*O*-benzyl-4,6-O-benzyliden-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1-3)-2,6-di-*O*-benzoyl-β-D-galactopyranosyl-(1-4)-2,3,6-tri-*O*-benzoyl-β-Dglucopyranosyl-(1-6)]-[2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl-(1-4)]-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 10.



10

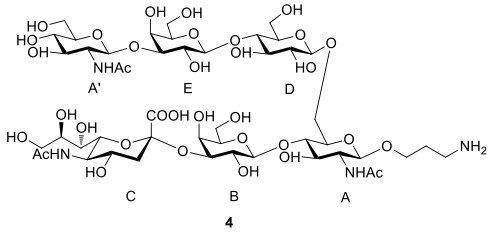
A solution of Glucosamine donor **9** (68 mg, 0.11 mmol) and acceptor **7** (190 mg, 0.08 mmol) was stirred for 20 min in dry DCM with activated molecular sieves 4Å under nitrogen. TfOH was then added at -25°C and the reaction was stirred for 1h at 0°C. After that, TLC (Toluene:Acetone 6:4) showed complete reaction, so the mixture was quenched with TEA, the solid was filtered off and the crude was purified with flash chromatography. Hexasaccharide **10** (164 mg, 0.06 mmol) was obtained has a white solid in 69% yield.

$[\alpha]_D^{25}$ = + 36.31° (c 0.32, CHCl3). ESI MS m/z $[M+H]^+$ found 2745.8362; calcd 2745.6693.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04-6.61 (m, 63H, H-Ar), 5.73 (t, J = 9.09, 1H, H-8^C), 5.55 (s, 1H, C*H*Ph), 5.46 (t, J = 8.46, 1H, H-2^B), 5.30-5.10 (m, 6H, H-1^F, H-3^D, H-2^D, H-2^E, H-4^B, H-7C), 4.91 (d, J = 10.04, 1H, NH), 4.79-4.68 (m, 6H, H-1^B, H-1^A, C*H*HPh^A, C*H*HPh^F), 4.36-4.22 (m, 6H, incl. H-1^E), 4.12-4.03 (3H), 3.98-3.87 (m, 7H, incl. H-1^D), 3.83-3.67 (m, 11H), 3.62-3.47 (m, 8H, OCH_{2a}), 3.14-3.08 (m, 1H, OCH_{2b}), 2.94-2.90 (m, 2H, C*H*$_2$N$_3$), 2.49-2.40 (m, 2H, H-3^C, H-5^E), 2.06, 1.91, 1.77, 1.67, 1.59 (5 x s, 3H each, 5 x C*H*$_3$CO), 1.58 (m, 1H, H-3^C), 1.37 (m, 2H, C*H*$_2$CH$_2$N$_3$)

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.80-164.24 (13 x C=O esters), 138.00-125.11 (C-Ar), 102.04 (C-1^D), 101.36 (*C*HPh), 101.01 (C-1^B), 100.88 (C-1^E), 99.98 (C-1^F), 97.88 (C-1^A), 82.66, 80.59, 80.53, 78.12, 75.57. 75.01, 74.43, 74.18, 74.03, 72.45, 72.33, 72.06, 71.67, 71.54, 71.42, 71.33, 70.82, 70.64, 70.47, 69.53, 98.53, 68.40, 68.14, 67.07, 66.80, 66.23, 66.11, 63.25, 62.71, 62.46, 61.50, 55.73, 55.50, 53.25, 48.57, 48.03, 37.36 (C-3^C), 28.59, 23.13, 21.49, 21.32, 20.77, 20.34.

3-Aminopropyl 3-O-(5-N-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl)-(1→4)-O-[(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→6)]-O-2-acetamido-2-deoxy-β-D-glucopyranoside 4.



4

A mixture of protected hexasaccharide **10** (0.1 mmol) and LiI (3 mmol) in pyridine (5 mL) was heated for 24h at 120 °C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2% MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 mL), and ethylenediamine (400 µL) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuo, and the residue was coevaporated with Toluene (2 x 10 mL) and EtOH (2 x 5 mL). The crude mixture was re-dissolved in pyridine (5 mL), and acetic anhydride (5 mL) was added. After being stirred for 16 h at rt, the reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (gradient 10 % of MeOH in DCM). The residue was dissolved in MeOH and MeONa was added until pH=13.

After 48 h the reaction was neutralized and the solvent removed under vacuum and the crude was purified with C18 5g column (gradient 20% MeOH in H_2O). The residue was finally dissolved in MeOH and Pd/C (1:1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (3 bar) for 72 h. Then, the catalyst

was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution.

Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **4** as an amorphous powder (31 % yield).

$[\alpha]_D^{25}$ = +1.56° (c 0.81, H_2O). ESI MS m/z $[M+Na]^+$ found 1281,4932; calcd 1281,4610.

^{1}H NMR (400 MHz, D_2O) δ 4.68 (d, J = 8.4 Hz, 1H, H.1^F), 4.60 (d, J= 7.9 Hz, 1H, H-1^B), 4.53 (d, J= 8.49 Hz, 1H, H-1^D), 4.50 (d, J= 8.63 Hz, H1, H-1^A), 4.43 (d, J= 7.77 Hz, 1H, H-1^E), 4.30, (d, J= 10.5 Hz, 1H, H-6$^A{}_a$), 4.14 (d, J= 2.7 Hz, 1H, H-4^E), 4.08, (dd, J= 2.7, 9.8 Hz, 1H, H-3^B), 3.99-3.80 (8H), 3.79-3.54 (22H), 3.46-3.44 (2H), 3.34 (t, J= 8.2 Hz, 1H, H-2^E), 3.20 (t, J= 8.6 Hz, 2H, C*H*$_2$N$_3$), 2.75, (dd, J= 4.6, 12.4 Hz, 1H, H-3$^C{}_{eq}$), 2.03 (s, 6H, 2 x CH_3CO), 2.02 (s, 3H, CH_3CO), 1.97 (m, 2H, C*H*$_2$CH$_2$N$_3$), 1.80 (t, J= 12.4 Hz, 1H, H-3$^C{}_{ax}$).

^{13}C NMR (101 MHz, D_2O) δ 102.91 (C-1^E), 102.81 (C-1^F), 102.42 (C-1^D), 102.12 (C-1^B), 101.33 (C-1^A), 81.87, 78.20, 77.25, 75.60, 75.00, 74.85, 74.58, 74.24, 73.50, 75.44, 72.91, 72.62, 72.08, 71.75, 69.98, 69.93, 69.33, 68.33, 67.98, 67.51, 67.30, 62.56, 61.05, 60.93, 60.42, 59.99, 55.60, 55.01, 51.63, 49.09, 47.37, 39.59 (C-3^C), 23.52, 22.11, 22.01.

SPR Fab binding inhibition

Inhibition assay were performed by SPR using a BIACORE X100 system. HSA-PSIII Glycoconjugate was immobilized on CM5 sensor chips (Biacore) using the amine coupling kit supplied by the manufacturer (Biacore). Immobilizations were conducted in 10-20 mM sodium acetate (pH 4-5) at sugar concentrations of 2-4 μg/mL. The immobilized surface density was ~50 resonance units in each instance. Binding analysis was performed with samples of Fab at a fixed concentration pre-incubated with PSIII or its fragments serially diluted (2x) starting from a concentration of 2 mg/mL. Measurements were conducted in 10 mM HEPES (pH 7.2), 150mM NaCl, 3 mM EDTA, 0.005% Tween 20 at 25°C and at a flow rate of 45μL/min. Following mAb or Fab binding, conjugate surfaces were regenerated with 3.5M $MgCl_2$ and a contact time of 120 s. Sensorgram data were analyzed using BIAevaluation software (Biacore).

NMR Sample preparation

mAb were exchanged in the working buffer (Tris d-11 50 mM in D_2O at pH 8.0 +/- 0.1) through 2 mL Zeba Spin desalting column saturated with 3 cycles of working buffer, mAb were finally eluted at the same starting concentration of 1 mg/ml. The 100% of recovery was assessed through microBCA spectrophotometric assay. Hexasaccharide fragment was desalted, quantified, dried and then dissolved in the working buffer too. mAb final concentration in the NMR tube was of 4 μM. mAb:ligand (saccharide) ratio was set at 1:50 mol/mol.

STD NMR experiments

NMR experiments were carried out on a Bruker 600 MHz NMR instrument equipped with a QCI cooled probe at controlled temperature (± 0.1 K). Data acquisition and processing were performed using TOPSPIN 3.5 software, respectively. Suppression of water signal was achieved by excitation sculpting (2 msec selective square pulse). STD-NMR experiments were acquired with 72 scans over 72 accumulations and spectral width of 9600 Hz (16 ppm) at 303 K; a saturation transfer of 0,5/1/1,5/2/4 sec. was applied to enhance the saturation transfer effect, irradiating at a frequency of 8.0 and -1.0 ppm (2 different spectra recorded for each sample). No differences were observed in the STD spectra irradiating at 8.0 or -1.0 ppm (4800 and -600 Hz respectively) for all samples, confirming that the saturation transfer effect was not irradiation dependent. To avoid pitfalls in the interpretation of STD-NMR spectra, a negative control spectrum was always recorded in absence of mAb (ligand and buffer) at the very same condition (concentration and pH) of the mAb-saccharide samples. Increasing saturation times from 0.5 up to 4.0 sec were applied to avoid overseeing of possible bias in the calculation of STD effects due to the different proton longitudinal relaxation times (T1), as well as the intramolecular spin diffusion within the bound state.

Conjugation to CRM_{197}

Triethylamine (3.0 eq) was added to a 9:1 DMSO/water solution of hexasaccharide, followed by di-N-hydroxysuccinimidyl adipate (12 eq). The reaction was stirred for 3 h, then the product was precipitate at 0°C by adding ethyl acetate (9 volumes). The solid was washed 10 times with ethyl acetate (5 volumes each) and lyophilized. The activated sugar was conjugated to CRM197 in sodium phosphate 100 mM at a protein concentration of 20 mg/mL, using the mol saccharide/mol protein ratio reported in Table 1.

After incubating overnight, the glycoconjugates **11** and **12** were purified by dialysis against 10 mM sodium phosphate buffer pH 7.2 (30 washings) in 30 kDa Vivaspin Turbo (Sartorius) centrifugal concentrators and reconstituted in the same buffer.

SDS-PAGE was performed on 4–12 % pre-casted polyacrylamide gel (NuPAGE®Invitrogen) using MOPS 1x as running buffer (NuPAGE®Invitrogen). 5 μg of protein were loaded for each sample. After electrophoretic running with a voltage of 150 V for about 45 min, the gel was stained with blue Coomassie.

HPAEC-PAD analysis for revealing Gal, Glc and GlcNAc

Standard samples of GBS PSIII at five increasing concentrations ranging between 0.5 and 10.3 μg/mL were prepared to build the calibration curve. GBS PSIII samples were prepared targeting the calibration curve midpoint. The reference and analytical samples were prepared in 4 M trifluoracetic acid, incubated at 100°C for 3h, dried under vacuum (SpeedVac Thermo), and suspended in water. All analytical samples were filtered with 0.45 μm Phenex-NY (Phenomenex) filters before analysis. HPAEC-PAD analysis was performed with a Dionex ICS-3000 equipped with a CarboPac PA1 column (4 × 250 mm; Dionex) coupled with a PA1 guard to column (4 × 50 mm; Dionex). Samples (50 μL injection volume) were run at 1 mL/min, using isocratic elution with 14 mM NaOH, followed by a washing step with 0.5 M NaOH.

HPAEC-PAD data acquisition and analysis

In HPAEC-PAD analysis, the effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8 (Dionex). Gal, Glc, GlcNAc and Neu5Ac concentration were determined and converted in μmol/mL to estimate the relative mol/mol ratio.

Immunogenicity of conjugates in mice

Two groups of ten female BALB/c mice were immunized by intraperitoneal injection of 1 μg in saccharide content of each produced glycoconjugate using alum hydroxide as an adjuvant. CRM-PSIII were used as controls. Mice received the vaccines at days 1, 21 and 35. Sera were bled at days 1, 35 and 49.

ELISA analysis using unconjugated PSIII as coating reagent

Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 μL of unconjugated PS III in PBS pH 7.4 (35μg/mL), followed by overnight incubation at 2-8°C. After washing three times with PBST, a PBS solution containing 2% BSA and sucrose (51 mg/mL) was added (250 μL/well), and plates were incubated 90 min at 37°C, and then aspirated to remove the solution. Two-fold serial dilutions of test and standard sera in PBST-B were added to each well. Plates were then incubated at 37°C for 2 hours, washed with PBST, and incubated for 2 additional hours at 37°C with either anti-mouse IgG-alkaline phosphatase (Sigma) diluted 1:2000 in PBST-B. After washing, the plates were developed with a 4 mg/mL solution of *p*-Nitrophenyl Phosphate (pNPP) in 1 M diethanolamine (DEA) pH 9.8, at rt for 30 min. After blocking with 7 % EDTA, the absorbance values measured with a SPECTRAmax plate reader with wavelength set at 405 nm. IgG concentrations were calculated as reciprocal serum dilution giving OD of 1.0.

ELISA analysis using GBS PS III conjugated to human serum albumin (HSA) as coating reagent

Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 µL of 1µg/mL of GBS PSIII conjugated to HSA *via* the spacer adipic acid dihydrazide in Phosphate Buffered Saline (PBS) pH 7.4. Plates were incubated overnight at 2-8°C, washed three times with PBST (0.05% Tween-20 in PBS pH 7.4) and saturated with 250 µL/well of PBST-B (2% Bovine Serum Albumin-BSA in PBST) for 90 min at 37°C. The plates were then aspirated to remove the solution. Two-fold serial dilutions of test and standard sera in PBST-B were added to each well. Plates were then incubated at 37°C for 1h, washed with PBST, and then incubated for 90 min at 37°C with anti-mouse IgG or IgM - alkaline phosphatase (Sigma) diluted 1:2000 or anti-rabbit IgG-alkaline phosphatase diluted 1:1000 in PBST-B. After washing, the plates were developed with a 4 mg/mL solution of p-Nitrophenyl Phosphate (pNPP) in 1 M diethanolamine (DEA) pH 9.8, at rt for 30 min. After blocking with 7% EDTA, the absorbance was measured using a SPECTRAmax plate reader with wavelength set at 405 nm. IgG concentrations were expressed as relative ELISA Units/mL (EU/mL) and were calculated by interpolating the absorbance values of serial sample dilutions on the standard calibration curve (Reference line method). The murine standard consists of a pool of hyperimmune sera obtained from animals immunized with 3 doses of GBS PS III-CRM conjugate vaccines adjuvanted with Alum.

Opsonophagocytic Killing Assay (OPKA)

Functional activity of anti-GBS antibodies was estimated by Opsono Phagocytic Killing Assay (OPKA) using differentiated HL-60 cells and strains COH1-III[2]. The percent of killing was calculated as (mean Colony Forming Units at T0 - mean CFU at T60)/(mean CFU at T0). OPK titers were expressed as the reciprocal serum dilution mediating 50% bacterial killing, estimated through piecewise linear interpolation of the dilution-killing OPK data. The Lower Limit of Detection was 1:30 and the assay coefficient of variation was approximately 30%.

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Chapter 8

Summary and future perspectives

The design of innovative glycoconjugate vaccines, for which a robust correlation between epitope structure and immunogenicity can be established, implies availability of structurally defined oligosaccharide fragments[1]. In some cases, such oligosaccharides can be obtained by depolymerization of the capsular polysaccharide[2] and subsequent careful selection of the shortest DPs (DP=Degree of Polymerization). In other cases, this approach is not efficient since neither chemical nor enzymatic depolymerization methods of the capsular polysaccharide are available. This is the challenge, for example, with GBS serogroup Ia and Ib, for which chemical synthesis is the only tool to prepare defined short oligosaccharides to be used for epitope mapping studies. In Chapter 1, an overview of glycoconjugate vaccines is provided, including their efficacy and mechanism of action compared to polysaccharide vaccines. Traditional approaches to the preparation of glycoconjugate vaccines are also described. Finally, the focus shifts to enzymatic and synthetic methods currently available for antigen preparation.
The epidemiology of Group B streptococcus (GBS) is described in Chapter 2: clinical symptoms, differences between Early-Onset Disease and Late-Onset Disease, main prevention strategies and serotype distribution according to the age of the patient are described. It is underlined how a multivalent vaccine composed of the most representative serotypes (Ia, Ib, II, III, IV, V) is needed to prevent the disease in an efficacious way both in infants and in elderly and immunocompromised patients[3, 4]. Finally, a literature review of the syntheses of the repeating units of GBS Ia and III is provided: chemo-enzymatic methods, one-pot reactions, pre-activation and regioselective strategies are synthetic approaches that have all been used to obtain the target complex oligosaccharides of GBS, showing how progress in carbohydrate chemistry can give access to biologically relevant structures for implementation of existing vaccines or development of new ones.

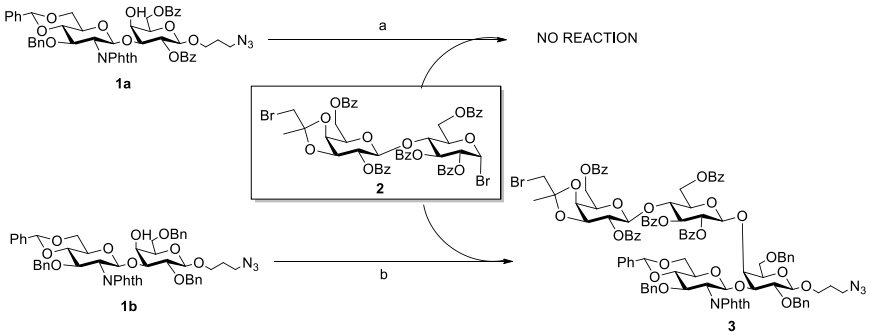
In Chapter 3, a regioselective glycosylation strategy to the synthesis of the biologically relevant GlcNAcβ1-3Gal disaccharide synthon is presented. Efforts to optimize the regioselective glycosylation of galactose 3-OH were pursued with the aim to obtain the disaccharide with high yield and complete regioselectivity to have faster access to branched oligosaccharide structures from GBS type Ia and Ib. Indeed, PSIa and Ib share a very similar structure, since the repeating unit is a pentasaccharide composed by the same sugar residues with, as the only difference, the linkage of the side chain galactose to the glucosamine, which is β-(1→4) in type Ia and β-(1→3) in type Ib[5]. Given this structural analogy, with a limited number of building blocks and a common synthetic design, glycans from both serotypes can be prepared. Optimization of the regioselective glycosylation involved the screening of glycosylation conditions using several glucosamine donors with different protecting group patterns allowing the possibility to elongate the glycosylation products at either the glucosamine C4 or C3. Benzoyl esters and benzyl ethers were installed as protecting groups on the galactose diol acceptor to investigate their arming/disarming effect. The results showed that when 2,6-benzoyl galactose was used as the glycosyl acceptor only the target β(1→3) disaccharide was isolated, indicating that the electron withdrawing effect of the

2,6-benzoyl groups has a beneficial effect on the regioselectivity. In addition, the outcome of the glycosylation reaction seems to benefit from the torsional disarming effect of the 4,6-benzylidene ring, locking the glucosamine 4,6-hydroxyls thereby disarming the donor glycoside. Disaccharide synthons that could be elongated at the galactose C-4 and either at the glucosamine C-3 or C-4 were obtained and their use as intermediates for GBS related glycans is discussed in the following Chapters of this Thesis.

The disaccharide bearing a temporary protecting group at C-4, that was obtained with the highest yield and full regioselectivity, was selected as key intermediate for the synthesis of a panel of fragments from GBS type Ia capsular polysaccharide as outlined in Chapter 4. This small library comprises the pentasaccharide repeating unit (both in the linear and a branched frameshift form), a non-sialylated tetrasaccharide, two hexasaccharides and one heptasaccharide. All compounds were synthesized using a convergent approach and an aminopropyl linker was installed at the non-reducing end to be exploited for further manipulation of the final oligosaccharide or conjugation to protein. Thanks to the developed regioselective glycosylation strategy, the synthesis of GBS PSIa repeating unit was optimized in terms of the number of reaction steps and protecting groups employed. Furthermore, fragments longer than one single repeating unit could be prepared with high efficiency, using a limited number of building blocks.
With the aim to translate the same approach to the synthesis of GBS type Ib glycans a similar GlcNAcβ(1→3)Gal disaccharide bearing a temporary protecting group at glucosamine C-3 was selected for elongation as described in Chapter 5. The β(1→3) connection between the Neu5Acα2-3Gal disaccharide and glucosamine 3-OH proved to be particularly challenging. For this reason, the reactivity of this position needed to be modulated by installation of different protecting groups on the nearby amino and hydroxyl groups. The *N*-phthaloyl group was replaced with the less electron withdrawing *N*-trichloroethoxy carbonyl group and a silylidene ketal (which is more flexible than its benzylidene counterpart) was installed to lock glucosamine 4,6-OH. These glucosamine modifications gave access to the branched and linear frameshifts of GBS type Ib repeating unit.
The branched repeating units of GBS type Ia and Ib were used for structural studies employing NMR and Molecular Dynamics to give insight in the conformational preferences of the corresponding polysaccharides. Conformational analysis, using the individual repeating units, highlighted as main difference the presentation of the protruding Neu5Ac-α-(2→3)-Gal moieties, with a major *exo-anti-Φ* population for Ia and *exo-syn-Φ* for Ib. Both polysaccharides have a relaxed helix-shaped backbone, with a more rigid and repetitive structure for PSIa. PSIb has a less defined preferred conformation and a larger solvent exposure. Potentially, these features also allow the exposure of different epitopes for the two polysaccharides, which would justify the serotype-specifity of the immune response.
To investigate this hypothesis in more detail, a study of the interactions between anti PS Ia and Ib mAbs and the prepared synthetic oligosaccharides is presented in Chapter 6. Competitive SPR

experiments were set up to verify the affinity of the fragments for the serotype specific functional mAbs. While for GBS type Ia none of the synthetic glycans showed high affinity for the mAb and the hexasaccharides showed a better inhibition activity than the single repeating unit, the branched pentasaccharide from type Ib was able to deplete the binding of the anti PSIb mAb to PSIb-HSA conjugate attached on the chip. To map the interactions of this fragment to the mAb, STD-NMR experiments were undertaken, underlining a high saturation transfer to the protons of the Neu5Ac-Gal branching and of the glucose moiety in the backbone. These positions therefore appear to be involved in the binding with the mAb. This also holds true for the protons of the sialic acid acetamide group which, when compared to the GlcNAc acetamide present in the molecule, shows a higher STD-signal.

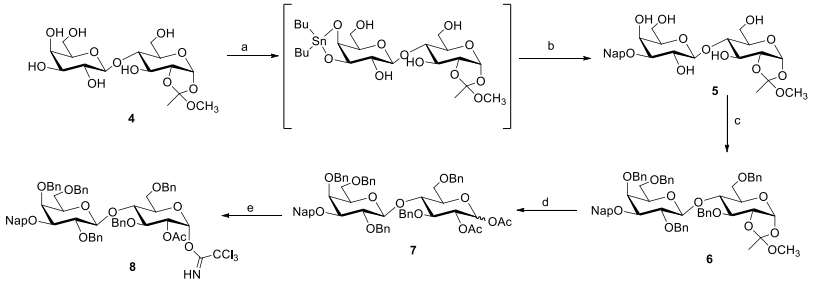
While for PSIb the repeating unit already represents a promising antigen, the type Ia synthetic glycans prepared so far probably do not cover the minimal epitope able to interact with a protective mAb. For this reason, a synthetic route giving access to longer oligosaccharide structures by elongation at the galactose 3-OH with a second glucosamine moiety or even with a trisaccharide to obtain a DP2 structure was designed and its feasibility tested. The key step of the synthesis was to identify a lactose donor which could glycosylate the poorly reactive galactose 4-OH of the previously described GlcNAcβ(1→3)Gal **1a** while carrying a temporary protecting group at C-3 of galactose moiety. To this aim, the lactose bromide donor **2**[6], described in Chapter 7, was tested but unfortunately, glycosylation reaction under AgOTf activation was unsuccessful and the target tetrasaccharide was not obtained (Scheme 1). In contrast, the benzylated disaccharide counterpart **1b** was successfully glycosylated by lactose bromide **2**, confirming again how the presence of electron withdrawing benzoyl groups plays a key role in reducing the nucleophilicity of the galactose 4-OH.



Scheme 1. Glycosylation trial with lactose bromide. *Reagents and conditions*: a) AgOTf, DCM, MS 3Å, -40°C to rt, no reaction; b) AgOTf, DCM, MS 3Å, -40°C to rt, 65%.

As discussed in Chapter 3, disaccharide **1b** was prepared in only 40% yield due to poor regioselectivity in the glycosylation reaction between the glucosamine donor and the galactose diol acceptor. To avoid using a route commencing with a non-selective reaction, the research of a more reactive lactose donor was undertaken.

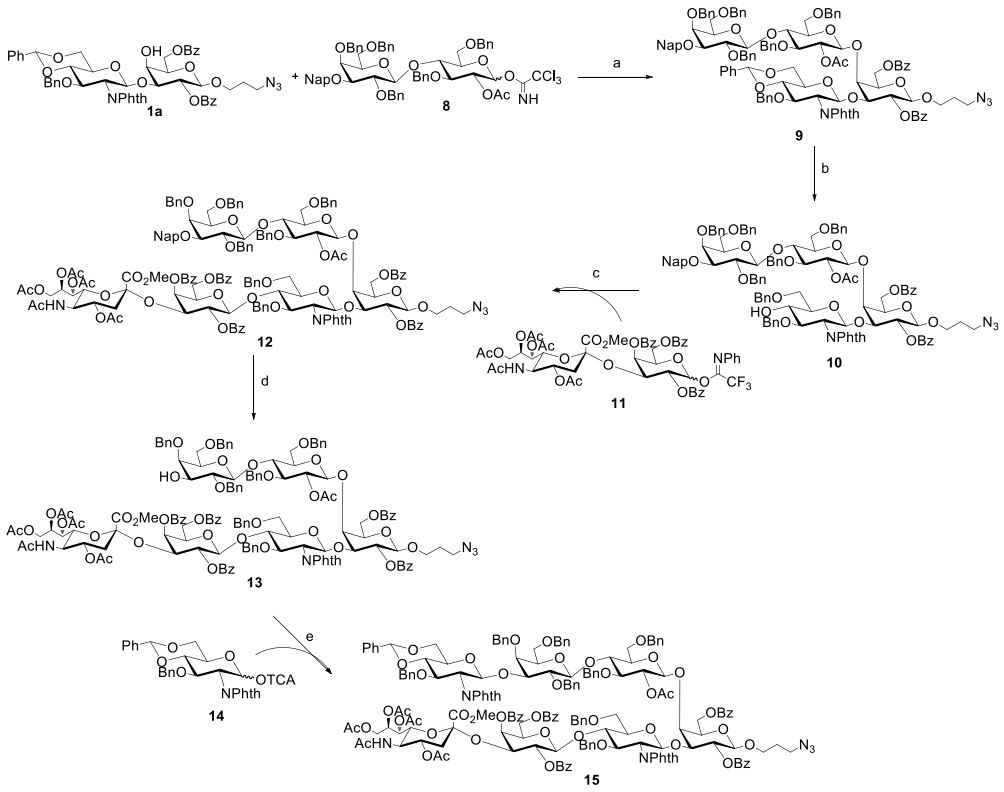
Therefore, an armed lactose analogue of the benzylated compound used for the synthesis of the branched GBS Ia hexasaccharide and heptasaccharide (Chapter 4) bearing an orthogonal protective group on galactose 3-OH was designed (Scheme 2). The synthesis started from lactose orthoester **4**, which was treated with dibutyl tin oxide to give the C3'-C4'-stannylene acetal. Subsequent treatment with an electrophile such as NapBr gave the regioselective alkylation of the 3-OH of the galactose moiety. The resulting compound **5** was then benzylated to afford lactose **6**, which was subjected to ortho ester hydrolysis followed by acetylation with Ac_2O and pyridine. The anomeric was selectively removed and the resulting lactol was directly converted into trichloroacetimidate **8**, which was used as glycosyl donor with disaccharide **1a** acceptor.



Scheme 2. Synthetic pathway to lactose **8**. *Reagents and conditions*: a) 3Å MS, Bu_2SnO, toluene, reflux; b) NapBr, TBAI, THF, 50% c) NaH, BnBr, TBAI, DMF; 80%; d) TFA/H_2O 1:1, DCM; Ac_2O/Py, 0°C to rt, 86%; e) H_2NNH_2, AcOH, THF; CCl_3CN; DBU, DCM, 70%.

When lactose donor **8** was coupled with disaccharide **1a** under influence of TMSOTf activation, the target branched tetrasaccharide **9** was obtained in satisfactory 60% yield (Scheme 3). After regioselective benzylidene opening, the obtained tetrasaccharide **10** was glycosylated with the already described Neu5Ac-Gal disaccharide donor **11**, leading to the formation of hexasaccharide **12** in 72% yield. Oxidative cleavage of Nap protecting group with DDQ in a DCM/MeOH mixture yielded the hexasaccharide acceptor **13**, key intermediate for the synthesis of longer and more complex GBS Ia glycans.

To test whether glycosylation of this intermediate could be effective, heptasaccharide **15** was targeted. To this aim, trichloroacetimidate glucosamine donor **14** reacted with hexasaccharide **13** under TMSOTf activation, providing the desired oligosaccharide **15** in 68% yield.



Scheme 3. Synthetic pathway to heptasaccharide **15**. *Reagents and conditions*: a) TMSOTf, 4Å MS, DCM, 0°C to rt, 60%; b) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 0°C, 55%; c) TMSOTf, DCM dry, 72%; d) DDQ, DCM/MeOH 4:1, 75%; e) TMSOTf, DCM dry, 68%.

The successful assembly of heptasaccharide **15** indicates that the route is also suitable to acquire even more complex structures such as oligomers encompassing multiple repeating units. The heptasaccharide that can be obtained after global deprotection of **15** will be used to assess the impact of the additional upstream glucosamine on antibody recognition.
Moreover, key building block **8** can be used to approach the synthesis of PSIb glycans longer than one single repeating unit, in case the structures synthesized so far prove not immunogenic *in vivo*.

To test the immunogenicity of synthetic oligosaccharides, conjugation to a carrier protein through covalent linkage is needed. Indeed, the protein provides the T-cell epitopes required for the development of the immunological memory[7]. To evaluate whether the GBS type Ia and Ib synthetic fragments could elicit an immune response *in vivo*, conjugation to the classical carrier CRM_{197} was performed as described in Chapter 6. A saccharide/protein ration of at least 10:1 was targeted because a high glycodensity is pivotal to achieve a robust immune response with short OS-conjugates, as was recently shown for GBS type III. Characterization of the conjugates by SDS-Page, Western Blot, microBCA to measure the protein content and HPAEC-PAD for the

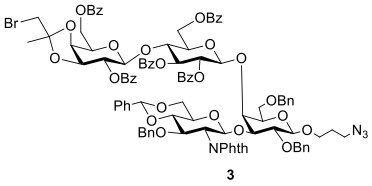
total saccharide amount calculation was performed and the target saccharide/protein ratio was reached for almost all glycoconjugates. Ongoing immunological studies will tell whether the multivalent display of these short oligosaccharides (corresponding to optimal or suboptimal epitopes) allows to achieve antigenicity characteristics of larger PS glycans.
Moreover, the Ia and Ib glycoconjugates will be used to complete the epitope mapping, by studying their affinity (in terms of K_d) with serotype specific functional mAbs or Fab fragments.

With the aim to demonstrate that identification of antigenic determinants from highly complex polysaccharide is propaedeutic for the rational design of conjugates based on synthetic carbohydrates[8, 9], Chapter 7 reports a structure-guided approach to obtain an innovative anti GBS type III glycoconjugate vaccine. A hexasaccharide was designed starting from the X-ray crystal structure of a semisynthetic GBS III DP2 fragment with a protective Fab[10]. This oligosaccharide comprises all moieties interacting with the Fab and consists of a full branched repeating unit and a second glucosamine. Structural and conformational studies confirmed that the hexasaccharide is recognized by a functional anti-PSIII rabbit mAb similarly to the dimer previously obtained by PS depolymerization. Conjugation to the carrier protein led to obtainment of two glycoconjugates with different glycodensity. When tested for immunogenicity, only the conjugate with a saccharide/protein ratio of about 8 elicited a robust and functional immune response, non inferior to the polysaccharide conjugate, indicating that this structure is the minimal epitope of GBSIII.
Of note, the synthetic glycan based conjugate showed a predominant IgG vs IgM production, which is relevant for a vaccine intended to elicit primarily IgG antibodies crossing the placenta.
While literature reports have suggested GBSIII to be an extremely complex epitope and while it has been pointed that long polysaccharide portions are required to present immunogenic epitopes, the described structure guided approach changes this paradigm, demonstrating that a small synthetic glycan can be designed to contain all the structural features required for a robust immune response.

Experimental

General Methods and procedures. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50ºC on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm) or using pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (Biotage, silica 0.050 nm). Unless otherwise specified, a gradient 0-100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) or Biotage Isolera instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{1}H values are reported in ppm, relative to internal Me_4Si (^{1}H = 0.00, $CDCl_3$); solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker AvanceIII 400 spectrometer; ^{13}C values are reported in ppm relative to the signal of $CDCl_3$ (^{13}C = 77.0, $CDCl_3$). Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Assignment of ^{13}C NMR spectra of some compounds was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters. Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data and *m/z* values found in their mass spectra.

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[2,6-di-*O*-benzoyl-3,4-*O*-(1-bromomethylethylidene)-β-D-galactopyranosyl-(1-4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl-(1-4)]-2,6-di-*O*-benzyl-β-D-galactopyranoside 3.



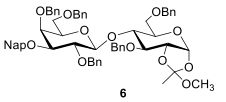
3

Lactose bromide donor **2** (0.137 g, 0.131 mmol) and disaccharide acceptor **1b** (0.080 g, 0.088 mmol) with 4Å MS were dissolved in 3.0 mL of dry DCM under nitrogen atmosphere. The resulting suspension was cooled down to -10°C and AgOTf (0.045 g, 0.176 mmol) was added. The resulting reaction mixture was stirred in the darkness for 2 hours, then (TLC: Tol/EtOAc 8:2) it was quenched by addition of TEA. The solvent was removed under reduced pressure and the crude was purified by column chromatography. Pure fractions were collected and evaporated to dryness, affording the target compound **3** as a colourless oil (0.107 g, 65% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22-6.88 (m, 49H, Ar-H), 6.16 (t, 1H, J=9.5 Hz), 5.61 (s, 1H, CHPh), 5.55 (d, 1H, J=8.2 Hz), 5.48 (d, 1H, J=8.3 Hz), 5.46-5.36 (m, 1H), 5.26-5.18 (m, 1H), 4.92 (d, 1H, J=7.4 Hz), 4.78 (d, 1H, J=12.1 Hz), 4.70 (d, 1H, J=12.1 Hz), 4.61-4.53 (m, 2H), 4.52-4.46 (m, 2H), 4.43 (t, 1H, J=6.1 Hz), 4.38-4.13 (m, 7H), 4.08 (d, 1H, J=7.5 Hz), 3.92-3.80 (m, 4H), 3.76-3.62 (m, 3H), 3.62-3.45 (m, 7H), 3.36 (dd, 2H, $J_{1,2}$=10.9 Hz, $J_{1,3}$=17.2 Hz, CH_2Br), 3.10-2.96 (m, 3H), 1.61 (s, 3H, CH_3), 1.67-1.51 (m, 2H, C*H*$_2$$CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.9, 166.0, 165.8, 165.6, 165.5, 165.1, 165.0, 138.3, 1398.0, 137.2, 134.4-126.1 (m, Ar-H), 109.5, 103.3, 101.2, 100.0, 99.0, 98.8, 82.9, 79.0, 78.5, 78.4, 76.3, 74.6, 74.5, 74.2, 74.0, 73.8, 73.7, 73.5, 73.1, 72.6, 72.5, 72.3, 71.1, 70.9, 69.3, 68.5, 66.1, 65.8, 62.5, 56.7, 48.1, 46.1, 37.3, 29.7, 29.1, 24.5, 22.9,

2,4,6-tri-*O*-benzyl-3-*O*-(2-naphtyl methyl)-*β*-D-galactopyranosyl-(1→4)-1,2-(methyl orthoacetate)-3,6-di-*O*-benzyl-*α*-D-glucopyranose 6.



6

3Å molecular sieves (5.0 g) were activated under nitrogen and then poured into a flask containing 5.5 g (13.8 mmol) of lactose orthoester **4** suspended in toluene. Bu_2SnO (5.1 g, 20.7 mmol) was added and the reaction mixture was stirred in refluxing toluene for 2 h. After 2 hr toluene was evaporated under reduced pressure, the crude was redissolved in 100.0 mL of THF, Nap-Br (4.3 g, 20.7 mmol) and TBAI (700 mg) were added. The resulting reaction mixture was stirred at 50°C overnight. After 16 hr (TLC: EtOAc/MeOH 85:15) the solvent was evaporated under reduced pressure and the crude was purified by column chromatography using a gradient from 0 to 50% of MeOH in EtOAc (containing 3% of TEA). Pure fractions were collected and evaporated under reduced pressure affording compound **5** (3.0 g, 50% yield).

Lactose 3-Nap orthoester **5** (0.600 g, 1.11 mmol) was dissolved in 10.0 mL of DMF and the resulting solution was cooled down to 0°C in an ice-water bath. NaH (0.445 g, 11.14 mmol) was added under nitrogen atmosphere and the resulting suspension was stirred for 20 minutes. After 20 minutes the ice-water bath was removed and BnBr (2.0 mL, 16.7 mmol) was added dropwise under nitrogen atmosphere, followed by TBAI (100 mg). The reaction mixture was stirred overnight at rt.

After 16 h (TLC: Hexane/EtOAc) the reaction was quenched with MeOH, then it was diluted with DCM and washed with $NaHCO_3$ and H_2O. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure affording a crude product, which was purified by column chromatography. The column was eluted with a gradient from 0 to 100% of EtOAc in Cyclohexane (containing 3% of TEA).

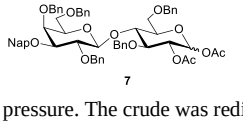
Pure fractions were collected and evaporated affording compound **6** (857 mg, 80% yield) as a colorless oil.

$[\alpha]_D^{25} = +23.98^\circ$ (c 0.185, $CHCl_3$). ESI MS ($C_{61}H_{64}O_{12}$) *m/z* $[M+H]^+$ found 992.46; calcd 988.4398.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.11 (m, 32H, Ar-H), 5.69 (d, 1H, J=5.2 Hz, H-1^A), 4.96 (d, 1H, J=11.8 Hz), 4.84 (s, 2H, OC*H*$_2$Ph), 4.77 (s, 2H, OC*H*$_2$Ph), 4.75-4.67 (m, 2H, OC*H*$_2$Ph), 4.66-4.49 (m, 3H, OC*H*$_2$Ph), 4.37 (d, 1H, J=12.2 Hz), 4.34-4.30 (m, 2H), 4.28 (s, 1H, J=7.8 Hz, H-1^B), 4.15 (t, 1H, J=2.0 Hz), 3.98 (d, 1H, J=9.3 Hz), 3.89 (d, 1H, J=2.7 Hz, H-4^B), 3.70-3.58 (m, 2H), 3.54 (dd, 1H, $J_{1,2}$=7.2 Hz, $J_{1,3}$=8.5 Hz), 3.50-3.40 (m, 3H), 3.24 (s, 3H, OCH_3), 1.62 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 169.6, 139.2-125.6 (C-Ar), 103.1 (C-1^B), 102.7 (C-1^A), 91.3, 90.4, 82.3, 80.3, 80.0, 79.9, 77.5, 75.5, 75.4, 75.3, 75.1, 74.7, 73.6, 73.5, 75.5, 73.2, 73.2, 73.1, 73.0, 72.9, 72.5, 70.6, 70.4, 68.1, 67.5, 21.2, 20.9.

2,4,6-tri-*O*-benzyl-3-*O*-(2-naphtyl methyl)-*β*-D-galactopyranosyl-(1→4)-1,2-di-*O*-acetyl-3,6-di-*O*-benzyl-*α*-D-glucopyranose 7.



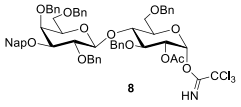
7

To a solution of compound **6** (567 mg, 0.57 mmol) in CH_2Cl_2 (5.0 ml), TFA (0.025 ml) and water (0.025 ml) were added and the mixture was stirred 3 hr at rt. After 16 h (TLC: Cyclohexane/EtOAc 7:3) the reaction mixture was concentrated under reduced pressure. The crude was redissolved in 3.0 mL of pyridine, then cooled to 0°C and Ac_2O (3.0 mL) was added. After 2 hr, analytical TLC (Cyclohexane/EtOAc) the reaction was quenched by addition of MeOH at 0°C, co-evaporated with toluene and then purified by medium pressure column chromatography. The column was eluted with a gradient from 0 to 100% of EtOAc in cyclohexane. Pure fractions were collected and evaporated to dryness affording compound **7** as a clear syrup (500 mg, 86% yield, only α anomer). $[\alpha]_D^{25} = +21.40^\circ$ (c 0.145 $CHCl_3$). ESI MS ($C_{62}H_{64}O_{13}$) m/z $[M+NH_4]^+$ found 1034.47; calcd 1034.4685.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7-15 (m, 32H, Ar-H), 6.30 (d, 1H, J=3.7 Hz, H-1^A), 5.07-4.97 (m 3H), 4.91-4.79 (m, 4H, incl. H-2^A), 4.60 (d, 1H, J=11.4 Hz), 4.55 (d, 1H, J=12.0 Hz), 4.46-4.21 (m, 4H, incl. H-1^B), 4.08 (t, 1H, J=9.5 Hz), 3.97 (d, 1H, J=2.9 Hz), 3.91-3.73 (m, 4H), 3.62-3.43 (m, 4H), 3.42-3.32 (m, 2H), 2.17 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.9 (CO), 169.2 (CO), 139.1, 139.0, 138.8, 138.0, 137.9, 135.9, 133.3, 132.9, 128.4-125.6 (C-Ar), 102.9 (C-1^B), 89.8 (C-1^A), 82.4, 79.9, 77.7, 76.1, 75.3, 74.9, 74.7, 73.5, 73.4, 73.3, 73.2, 73.0, 72.5, 71.1, 68.1, 67.5, 56.6, 21.1 (CH_3), 20.7 (CH_3).

2,4,6-tri-*O*-benzyl-3-*O*-(2-naphtyl methyl)-*β*-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,6-di-*O*-benzyl-*α*-D-glucopyranosyl trichloroacetimidate 8.



8

Compound **7** (0.633 g, 0.622 mmol) was dissolved in dry THF (6.0 mL). Ethylenediamine (0.062 mL, 0.933 mmol) was added, followed by acetic acid (0.053 mL, 0.933 mmol).

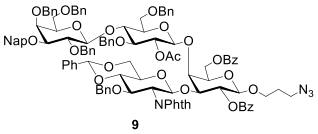
The reaction was stirred overnight at rt. After 16 hr (TLC: Cyclohexane/EtOAc) the reaction mixture was evaporated under reduced pressure, then the crude was purified by medium pressure column chromatography using a gradient from 0 to 100% of EtOAc in Cyclohexane. Pure fractions were collected and evaporated to dryness affording the lactose 1-OH, which was directly progressed to the next step.

Lactose 1-OH (0.5 g, 0.512 mmol) was dissolved in dry DCM (5 mL) under nitrogen and trichloroacetonitrile (0.257 mL, 2.56 mmol) and 1,8-diazobicyclo[5.4.0]undec-7-ene (0.023 mL, 0.15 mmol) were added. After stirring for 2h at rt, TLC (7:3 cyclohexane:EtOAc) showed complete reaction. The solvent was removed at reduced pressure and the crude was purified by flash chromatography (cyclohexane:EtOAc) to afford **8** (0.487 g) in 70% yield over two steps. ESI MS ($C_{62}H_{62}Cl_3NO_{12}$) *m/z* $[M\text{-}CNHCCl_3+NH_4]^+$ found 992.46; calcd 992.4580.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.6 (s, 1H, NH), 7.89-7.14 (m, 32H, Ar-H), 6.5 (d, 1H, J=3.4 Hz, H-1^A), 5.01-4.99 (m, 3H), 4.92-4.85 (m, 3H), 4.81 (t, 1H, J=12.6 Hz), 4.67 (d, 1H, J=11.2 Hz), 4.62 (d, 1H, J=11.3 Hz), 4.55 (d, 1H, J=12.0 Hz), 4.42 (d, 1H, J=7.4 Hz), 4.36 (t, 2 H, J=11.7 Hz), 4.28 (d, 1H, J=11.6 Hz), 4.21-4.11 (m, 1H), 4.04-3.87 (m, 4H), 3.83 (t, 1H, J=8.5 Hz), 3.60-3.52 (m, 2H), 3.49-3.36 (m, 3H), 1.99 (s, 3H, $COCH_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0 (CO), 160.9 (CO), 139.1-125.6 (C-Ar), 102.9 (C-1^A), 100.0, 93.9 (C-1^B), 82.4, 79.8, 75.8, 75.3, 75.0, 74.7, 73.6, 73.5, 73.4, 73.1, 73.0, 72.5, 71.6, 68.1, 67.3, 20.6 (CH_3).

3-Azidopropyl 4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[(2,4,6-tetra-*O*-benzyl-3-*O*-(2-naphtyl methyl)-β-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 9.



9

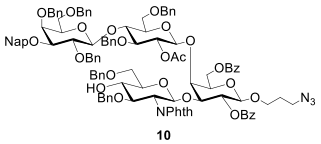
A solution of donor **8** (0.200 g, 0.191 mmol) and disaccharide acceptor **1a** (0.116 g, 0.127 mmol) with activated 4 Å molecular sieves (0.200 g) in dry DCM (3 mL) was stirred for 20 min under nitrogen. TMSOTf (4.6 µL, 0.025 mmol) was added at 0°C. After 4 h (TLC: 8:1 Tol: EtOAc) the reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol:EtOAc) to afford the tetrasaccharide **9** in 60% yield (0.146 g) as a white amorphous solid.

$[\alpha]_D^{25} = +9.15^\circ$ (c 0.185, $CHCl_3$). ESI MS ($C_{111}H_{108}N_4O_{25}$) *m/z* $[M+NH_4]^+$ found 1915.77; calcd 1914.7641.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13-6.77 (m, 56H, Ar-H), 5.55 (s, 1H, CHPh), 5.40 (d, 1H, J=8.2 Hz), 5.19-5.12 (m, 2H), 5.05-4.80 (m, 7H), 4.75 (d, 1H, J=11.5 Hz), 4.73 (d, 1H, J=12.1 Hz), 4.69-4.59 (m, 2H), 4.53 (d, 1H, J=7.6 Hz), 4.51-4.29 (m, 10H), 4.24 (d, 1H, J=11.3 Hz), 4.02-3.70 (m, 11H), 3.67-3.58 (m, 1H), 3.57-3.48 (m, 3H), 3.46-3.32 (m, 3H), 3.11-2.90 (m, 2H, CH_2N_3), 1.91 (s, 3H, $COCH_3$), 1.70-1.50 (m, 2H, $CH_2CH_2N_3$)

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 167.6, 166.9, 166.4, 164.5, 139.6, 139.1, 138.9, 138.4, 138.1, 137.6, 137.1, 136.0, 133.5-122.9 (m, C-Ar), 103.1, 101.4, 100.9, 100.3, 99.9, 83.0, 82.5, 81.3, 80.0, 79.2, 75.7, 75.3, 74.7, 74.6, 74.4, 74.3, 73.8, 73.6, 73.5, 73.2, 73.1, 73.0, 72.6, 72.6, 72.3, 70.7, 68.7, 68.5, 68.2, 66.1, 65.1, 64.6, 55.9, 47.9, 29.7, 28.9, 20.5.

3-Azidopropyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[(2,4,6-tetra-*O*-benzyl-3-*O*-(2-naphtyl methyl)-β-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 10.



10

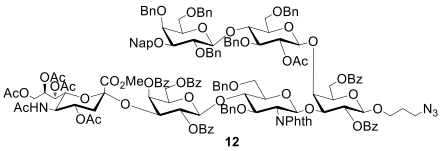
A solution of compound **9** (0.146 g, 0.076 mmol) in ACN (3 mL) was cooled at 0°C. Me_3NBH_3 (0.028 g, 0.38 mmol) and $BF_3{\cdot}OEt_2$ (0.047 mL, 0.38 mmol) were added and the reaction was stirred for 2 h under nitrogen. First TEA and then MeOH were added until neutral pH. The solvent was removed at reduced pressure and the crude was purified by flash chromatography (toluene:EtOAc) to afford the target tetrasaccharide acceptor **10** in 55% yield (0.080 g).

$[\alpha]_D^{25}$ = +3.95° (c 0.7, $CHCl_3$). ESI MS ($C_{111}H_{110}N_4O_{25}$) *m/z* $[M+NH_4]^+$ found 1917.78; calcd 1916.7797.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-6.81 (m, 56H, Ar-H), 5.32 (d, 1H, J=7.9 Hz), 5.19-5.10 (m, 2H), 5.04-4.80 (m, 7H), 4.71 (d, 1H, J=11.7 Hz), 4.66-4.58 (m, 3H), 4.55-4.44 (m, 4H), 4.41 (d, 1H, J=4.2 Hz), 4.38 (d, 1H, J=4.0 Hz), 4.34 (t, 1H, J=4.5 Hz), 4.32-4.28 (m, 2H), 4.22 (d, 1H, J=11.7 Hz), 4.14 (d, 1H, J=7.8 Hz), 4.12 (d, 1H, 7.8 Hz), 4.01 (d, 1H, J=9.4 Hz), 3.99-3.96 (m, 1H), 3.93 (dd, 1H, $J_{1,2}$=2.6 Hz, $J_{1,3}$=9.8 Hz), 3.89-3.71 (m, 10H), 3.67 (d, 1H, J= 11.1 Hz), 3.64-3.58 (m, 1H), 3.55-3.47 (m, 3H), 3.44-3.38 (m, 2H), 3.37-3.30 (m, 1H), 3.09-2.93 (m, 2H, CH_2N_3), 1.78 (s, 3H, $COCH_3$), 1.70-1.50 (m, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 166.3, 164.4, 139.7, 139.2, 139.0, 138.5, 138.1, 137.9, 137.4, 136.0, 133.5-123.0 (C-Ar), 102.9, 100.9, 100.3, 99.1, 82.5, 81.3, 80.0, 78.7, 78.5, 76.9, 75.5, 75.2, 74.7, 74.6, 74.5, 74.3, 74.0, 73.7, 73.6, 73.5, 73.4, 73.2, 73.0, 72.6, 72.5, 72.4, 70.8, 70.6, 68.5, 68.2, 64.9, 64.8, 55.5, 47.9, 29.7, 28.9, 20.4.

3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[(2,4,6-tetra-*O*-benzyl-3-*O*-(2-naphtyl methyl)-β-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 12.



12

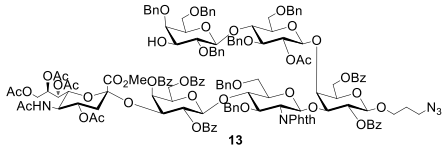
A solution of disaccharide donor **11** (0.071 g, 0.063 mmol) and tetrasaccharide acceptor **10** (0.080 g, 0.042 mmol) with 4 Å molecular sieves (0.100 g) in dry DCM (5.0 mL) was stirred for 20 min under nitrogen. TMSOTf (1.5 μL, 0.0084 mmol) was added at -10°C. After 4 h (TLC; 6:4 Toluene: Acetone) the reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Toluene:Acetone) to afford hexasaccharide **12** in 72% yield (86 mg).

$[\alpha]_D^{25}$ = -1.26° (c 0.7, $CHCl_3$). ESI MS ($C_{158}H_{159}N_5O_{45}$) *m/z* $[M+NH_4]^+$ found 2865.07; calcd. 2864.0645.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33-6.57 (m, 71H, Ar-H), 5.73-5.65 (m, 1H), 5.48 (dd, 1H, $J_{1,2}$=7.8 Hz, $J_{1,3}$=9.9 Hz), 5.33 (d, 1H, J=3.2 Hz), 5.22 (dd, 1H, $J_{1,2}$=2.6 Hz, $J_{1,3}$=9.7 Hz), 5.18-5.05 (m, 3H), 5.01-4.91 (m, 3H), 4.91-4.75 (m, 7H), 4.66 (d, 1H, J=11.7 Hz), 4.61-4.54 (m, 3H), 4.54-4.39 (m, 5H), 4.35-4.27 (m, 3H), 4.25 (d, 1H, J=2.9 Hz), 4.24-4.09 (m, 6H), 4.08-3.91 (m, 5H), 3.90-3.75 (m, 9H, incl. $COOCH_3$), 3.75-3.64 (m, 3H), 3.59 (dd, 1H, $J_{1,2}$=2.7 Hz, $J_{1,3}$=10.7 Hz), 3.55 (d, 1H, J=10.6 Hz), 3.52-3.46 (m, 2H), 3.44 (d, 1H, J=8.4 Hz), 3.42-3.32 (m, 3H), 3.32-3.26 (m, 1H), 3.08-2.90 (m, 2H, CH_2N_3), 2.43 (dd, 1H, $J_{1,2}$=4.7 Hz, $J_{1,3}$=12.7 Hz, H-3^F_{eq}), 2.14 (s, 3H, $COCH_3$), 1.98 (s, 3H, $COCH_3$), 1.91 (s, 3H, $COCH_3$), 1.79 (s, 3H, $COCH_3$), 1.66 (t, 1H, J=12.4 Hz, H-3^F_{ax}), 1.62 (s, 3H, $COCH_3$), 1.50 (s, 3H, $COCH_3$), 1.54-1.42 (m, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.3-123.0 (C-Ar), 103.0, 100.8, 100.5, 100.4, 98.5, 82.6, 81.2, 79.9, 77.6, 77.5, 77.2, 76.8, 75.3, 75.1, 74.7, 74.5, 74.3, 73.7, 73.4, 73.1, 72.9, 72.7, 72.5, 72.4, 72.0, 71.8, 71.7, 70.5, 69.4, 68.2, 68.0, 67.1, 66.5, 64.7, 62.4, 61.5, 55.9, 53.2, 48.8, 48.0, 37.4, 29.7, 28.9, 23.2, 21.4, 20.8, 20.4, 20.2.

3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[(2,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 13.



13

Hexasaccharide **12** (0.080 g, 0.028 mmol) was dissolved in a 4:1 DCM/MeOH mixture (5.0 mL). Then, DDQ (0.032 g, 0.140 mmol) was added and the resulting reaction mixture was stirred for 2 hr at rt. After two hours (TLC: Toluene/Acetone 6:4) the reaction was quenched by addition of aqueous $NaHCO_3$, then washed with $NaHCO_3$ (20 mL) for three times.

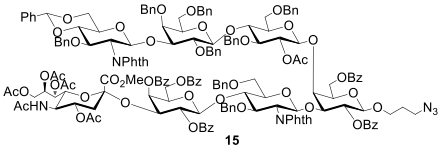
The organic phase was dried over Na_2SO_4, then evaporated to dryness. The crude was purified by column chromatography (Toluene:Acetone). Pure fractions were collected and evaporated under reduced pressure affording heptasaccharide **13** in 75% yield (50 mg).

$[\alpha]_D^{25}$ = -1.26° (c 0.7, $CHCl_3$). ESI MS ($C_{147}H_{151}N_5O_{45}$) *m/z* $[M+NH_4]^+$ found 2725.01; calcd. 2724.0019

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33-6.58 (m, 64H, Ar-H), 5.72-5.65 (m, 1H), 5.48 (dd, 1H, $J_{1,2}$= 7.7 Hz, $J_{1,3}$=9.9 Hz), 5.33 (d, 1H, J=3.2 Hz), 5.22 (dd, 1H, $J_{1,2}$=2.6 Hz, $J_{1,3}$=9.9 Hz), 5.16 (d, 1H, J=7.9 Hz), 5.13-5.05 (m, 2H), 4.99-4.78 (m, 7H), 4.76 (d, 1H, J=8.6 Hz), 4.71 (d, 1H, J=11.3 Hz), 4.65 (d, 1H, J=10.6 Hz), 4.62-4.56 (m, 3H), 4.55-4.52 (m, 1H), 4.51-4.39 (m, 4H), 4.36-4.26 (m, 4H), 4.25-4.08 (m, 6H), 4.07-3.93 (m, 4H), 3.90-3.75 (m, 9H, incl. $COOCH_3$), 3.75-3.66 (m, 3H), 3.60 (d, 1H, J=2.7 Hz), 3.59-3.53 (m, 2H), 3.52-3.44 (m, 3H), 3.44-3.34 (m, 3H), 3.32 (dd, 1H, $J_{1,2}$=2.6 Hz, $J_{1,3}$=6.5 Hz), 3.08-2.90 (m, 2H, CH_2N_3), 2.43 (dd, 1H, $J_{1,2}$=4.3 Hz, $J_{1,3}$=12.7 Hz, H-3^F_{eq}), 2.13 (s, 3H), 1.98 (s, 3H), 1.91 (s, 3H), 1.94-1.88 (m, 2H, $CH_2CH_2N_3$), 1.78 (s, 3H), 1.65 (t, 1H, J=12.7 Hz, H-3^F_{ax}), 1.62 (s, 3H). 1.50 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.9-127.6 8, C-Ar), 103.5, 101.6, 101.2, 101.0, 99.3, 81.9, 81.3, 78.4, 78.0, 77.8, 77.6, 77.5, 76.7, 75.9, 75.8, 75.7, 75.7, 75.6, 75.5, 75.4, 75.3, 75.2, 75.1, 75.0, 74.6, 72.8, 72.6, 72.4, 71.6, 71.2, 70.1, 69.0, 68.9, 68.8, 68.7, 68.7, 68.3, 67.7, 67.3, 65.9, 65.5, 63.1, 62.2, 56.6, 54.1, 53.8, 48.5, 38.2, 29.7, 23.9, 22.0, 21.7, 21.6, 21.2, 20.9.

3-Azidopropyl 3-*O*-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-[4,6-*O*-benzilidene-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-(2,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→4)]-2,6-di-*O*-benzoyl-β-D-galactopyranoside 15.



15

A solution of glucosamine donor **14** (0.018 g, 0.028 mmol) and acceptor **10** (0.050 g, 0.018 mmol) with 4 Å molecular sieves (0.070 g) in dry DCM (3.0 mL) was stirred for 20 min under nitrogen. TMSOTf (0.7 μL, 0.0036 mmol) was added at -10°C. After 4 h (TLC; 6:4 Toluene:Acetone) the reaction was quenched with TEA, the solid filtered off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Toluene:Acetone) to afford heptasaccharide **15** in 60% yield (35 mg).

$[\alpha]_D^{25}$ = +14.95° (c 0.125, $CHCl_3$). ESI MS ($C_{175}H_{174}N_6O_{51}$) *m/z* $[M+Na]^+$ found 3199.13; calcd. 3198.1099

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30-6.56 (m, 78H, Ar-H), 5.76-5.68 (m, 1H), 5.63 (s, 1H, CHPh), 5.54-5.43 (m, 2H), 5.38 (d, 1H, J=2.9 Hz), 5.13 (d, 1H, J=7.7 Hz), 5.08 (d, 1H, J=8.2 Hz), 5.06-4.92 (m, 3H), 4.92-4.69 (m, 7H), 4.66-4.40 (m, 9H), 4.40-4.23 (m, 7H), 4.23-4.12 (m, 5H), 4.12-3.99 (m, 5H), 3.96 (d, 1H, J=12.2 Hz), 3.93-3.74 (m, 11H, incl. $COCH_3$), 3.73-3.56 (m, 7H), 3.55-3.25 (m, 7H), 3.18-3.07 (m, 1H), 3.06-2.91 (m, 2H, CH_2N_3), 2.45 (dd, 1H, $J_{1,2}$=4.9 Hz, $J_{1,3}$=12.6 Hz, H-3^E_{eq}), 2.20 (s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$), 1,92 (s, 3H, $COCH_3$), 1.80 (s, 3H, $COCH_3$), 1.78 (s, 3H, $COCH_3$), 1,68 (t, 1H, J=12.6 Hz, H-3^E_{ax}), 1.64-1.56 (m, 2H, $CH_2CH_2N_3$), 1.53 (s, 3H, $COCH_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.5-123.2 (C-Ar), 102.6, 101.4, 101.2, 100.8, 100.7, 100.5, 100.2, 83.1, 81.6, 81.0, 77.6, 77.2, 76.7, 76.6, 75.9, 75.6, 74.9, 74.7, 74.3, 74.2, 73.3, 73.0, 72.9, 72.7, 72.4, 72.0, 71.8, 71.3, 71.0, 70.6, 70.5, 69.4, 68.8, 68.3, 67.9, 67.6, 67.2, 66.5, 65.8, 64.7, 62.4, 61.5, 56.3, 56.0, 55.9, 55.0, 53.2, 48.9, 47.9, 37.4, 28.9, 23.2, 21.4, 20.8, 20.7, 20.4, 20.0.

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Italian Summary (Riassunto)

Il design di nuovi vaccini glicoconiugati, per i quali sia possibile stabilire una correlazione fra immunogenicità e struttura dell'epitopo, richiede la disponibilità di frammenti oligosaccaridici con una struttura ben definita. In alcuni casi, questi oligosaccaridi possono essere ottenuti per depolimerizzazione del polisaccaride caspulare (CPS) e successiva selezione dei frammenti più corti. In altri casi invece, questo approccio non può essere applicato per la mancanza di metodi chimica e/o enzimatici per depolimerizzare il CPS. Questo problema viene riscontrato, per esempio, con i serotipi Ia e Ib di Group B streptococcus (GBS o *Streptococcus agalactiae*), per i quali la sintesi chimica è l'unico modo per ottenere oligosaccaridi corti e definiti da impiegare per mappare l'epitopo minimo.
Nel Capitolo 1 di questa tesi viene presentata una panoramica sui vaccini glicoconiugati, sulla loro efficacia e sul loro meccanismo d'azione in confronto con i vaccini polisaccaridici, e vengono descritti gli approcci tradizionali per la loro preparazione. Infine si fa riferimento a metodi chimici ed enzimatici che sono stati applicati con successo per accelerare la sintesi di antigeni oligosaccaridici.

Nel Capitolo 2 viene descritta l'epidemiologia di GBS, con particolare riferimento ai sintomi, alle differenze fra le manifestazioni cliniche che si presentano entro la prima settimana di vita del neonato (Early On-Set Diseases, EOD) e quelle che compaiono invece successivamente (Late On-Set Diseases, LOD), alle principali strategie di prevenzione e alla diffusione dei vari serotipi sulla base dell'età del paziente. Si sottolinea come un vaccino multivalente che comprenda i serotipi maggiormente diffusi (Ia, Ib, II, III, IV, V) sia necessario per prevenire la comparsa di patologie causate da GBS sia in neonati che in pazienti anziani o immunocompromessi. Il capitolo si conclude con una revisione delle sintesi pubblicate delle unità ripetenti di GBS Ia e III: metodi chemo-enzimatici, reazioni one-pot e strategie regioselettive sono approcci impiegati per ottenere gli oligosaccaridi complessi di GBS e ciò sta a sottolineare come i progressi nella chimica dei carboidrati possono dare accesso a strutture biologicamente rilevanti per migliorare vaccini glicoconiugati che sono già in commercio oppure per svilupparne di nuovi.

Nel capitolo 3 si descrive una strategia di glicosilazione regioselettiva per la sintesi del sintone GlcNAcβ1-3Gal, che è presente in molti saccaridi di interesse biologico, fra cui i polisaccaridi di GBS Ia e Ib. Vari tentativi sono stati fatti per ottimizzare la glicosilazione regioselettiva del 3-OH del galattosio allo scopo di ottenere il disaccaride desiderato con alte rese e completà regioselettività e avere di conseguenza una sintesi più rapida delle strutture ramificate di GBS Ia e Ib. Infatti, i polisaccaridi capsulari di GBS Ia e Ib presentano una struttura molto simile, dato che l'unità ripetente di entrambi è un pentasaccaride composto dagli stessi residui monosaccaridici con l'unica differenza del legame fra il galattosio della catena laterale e la glucosammina che è di

tipo β-(1→4) nell'Ia e β-(1→3) nell'Ib. Data questa analogia strutturale, con un numero limitato di building block e un design sintetico comune si possono preparare glicani di entrambi i serotipi. Per ottimizzare la glicosilazione regioselettiva è stato effettuato uno screening su diverse condizioni di reazione usando come donatori glucosammine con vari gruppi protettivi ortogonali al C-3 e al C-4. Esteri benzoilici e eteri benzilici sono stati invece installati come gruppi protettivi sul galattosio accettore per studiare il loro effetto stereo-elettronico. I risultati hanno mostrato che, quando il 2,6-di-benzoil galattosio è stato usato come glicosil accettore, solo il disaccaride β(1→3) desiderato è stato isolato, indicando che l'effetto elettron-attrattore del gruppo benzoile (OBz) ha un effetto positivo sulla regioselettività della reazione. Inoltre, il risultato della glicosilazione sembra beneficiare anche della tensione torsionale del anello benzilidenico, che, bloccando gli ossidrili in posizione 4 e 6 della glucosammina, ha un effetto disattivante. Sintoni disaccaridici che possono essere allungati alla posizione C-4 del galattosio e al C-3 o C-4 della glucosammina sono stati così ottenuti e il loro uso come intermedi per la sintesi di glicani derivanti dalla capsula di GBS è discusso nei successivi capitoli.

Il disaccaride GlcNAcβ1-3Gal recante un gruppo protettivo temporaneo al C-4 della glucosammina e che è stato ottenuto con la resa più alta e con completa regioselettività, è stato quindi selezionato come intermedio per la sintesi di vari frammenti derivanti dal CPS del serotipo Ia, come riportato nel Capitolo 4. La libreria di oligosaccaridi sintetizzata comprende l'unità ripetente pentasaccaridica (sia nella forma lineare che ramificata), un tetrasaccaride non-sialilato, due esasaccaridi e un eptasaccaride. Tutti i composti sono stati sintetizzati usando un approccio convergente e un linker amminopropilico è stato installato all'estremità non riducente in modo da poterlo sfruttare per ulteriori manipolazioni del saccaride finale o per la coniugazione alle proteine.
Grazie all'approccio regioselettivo che è stato sviluppato, la sintesi dell'unità ripetente di GBS Ia è stata ottimizzata in termini di numero di reazioni e gruppi protettivi usati. Inoltre, frammenti più lunghi di una singola unità ripetente sono stati preparati con elevata efficienza e usando un numero limitato di building block.

La stessa strategia sintetica è stata poi applicata alla sintesi di glicani derivanti dal GBS Ib e, a tale scopo, un disaccaride GlcNAcβ(1→3)Gal recante un gruppo protettivo temporaneo al C-3 della glucosammina è stato selezionato per essere allungato come descritto nel Capitolo 5. Formare il legame β(1→3) fra il disaccaride Neu5Acα2-3Gal e il 3-OH della glucosammina si è dimostrato particolarmente impegnativo. Per questo motivo, la reattività di questo ossidrile è stata modulata tramite l'utilizzo di gruppi protettivi differenti sull'ammina vicinale e sugli altri gruppi -OH della glucosammina. In particolare, il gruppo *N*-ftaloil, precedentemente utilizzato per i saccaridi di GBS Ia, è stato sostituito con l'*N*-tricloroetossi carbonil, avente minor carattere elettron attrattore, mentre il sililidene, più flessibile del corrispondente benzilidene, è stato

installato per bloccare i 4,6-OH della glucosammina. Queste modifiche hanno permesso quindi di sintetizzare l'unità ripetente, sia nella forma lineare che ramificata, del serotipo Ib di GBS.
Le unità ripetenti ramificate di GBS Ia e Ib sono state usate per studi strutturali tramite un approccio combinato di NMR e Dinamica Molecolare per investigare le preferenze conformazionali dei polisaccaridi corrispondenti. L'analisi conformazionale, intrapresa a partire dalle singole unità ripetenti, ha evidenziato come differenza principale fra i due serotipi la presentazione del disaccaride Neu5Ac-α-(2→3)-Gal, con una popolazione prevalente *exo-anti-Φ* per l'Ia e *exo-syn-Φ* per l'Ib.
Entrambi i polisaccaridi hanno uno scheletro a forma di elica rilassata, con una struttura più rigida e ripetitiva per il polisaccaride Ia. Il polisaccaride Ib ha invece una conformazione preferita meno definita e un'area esposta al solvente più ampia. Queste differenze potrebbero determinare l'esposizione di epitopi differenti da parte dei due polisaccaridi e ciò giustificherebbe la risposta immunitaria serotipo-specifica che è stata osservata per GBS Ia e Ib.

Per verificare quest'ipotesi, è stato intrapreso uno studio delle interazioni fra due anticorpi monoclonali (uno anti-PS Ia e l'altro anti-PS Ib) e gli oligosaccaridi sintetici preparati, i cui risultati sono descritti nel Capitolo 6. Esperimenti di competizione tramite SPR hanno mostrato l'affinità dei frammenti saccaridici per gli anticorpi funzionali serotipo-specifici. Mentre per il serotipo Ia i glicani sintetizzati non hanno mostrato un'alta affinità per il corrispondente monoclonale (anche se gli esasaccaridi presentano una migliore interazione rispetto alla singola unità ripetente), il pentasaccaride ramificato di GBS Ib ha inibito completamente il legame fra il coniugato HSA-PS Ib immobilizzato sul chip e il monoclonale anti PS Ib. Partendo da questo risultato, esperimenti di STD-NMR hanno permesso di mappare a livello atomico le interazioni di questo pentasaccaride con l'anticorpo, evidenziando in particolare un'alta saturazione per i protoni del disaccaride sialico-galattosio e del glucosio. Tali posizioni sembrano pertanto essere coinvolte nel legame con il monoclonale. Lo stesso può essere detto anche per i protoni dell'acetammide dell'acido sialico che, in confronto con l'acetammide della glucosammina, presenta un segnale STD più intenso.
Per testare l'immunogenicità degli oligosaccaridi sintetici e confermare i risultati delle analisi strutturali, è necessario coniugare i glicani a una proteina carrier, la quale fornisce gli epitopi che, attivando le cellule T, sono necessari per lo sviluppo della memoria immunologica. I frammenti sintetici di GBS Ia e Ib sono stati quindi coniugati al classico carrier CRM_{197} per vedere la loro capacità di indurre una risposta immunitaria *in vivo*. I coniugati sono stati preparati in modo da avere un rapporto molare saccaride/proteina di almeno 10:1, in quanto un maggior grado di glicosilazione è spesso fondamentale per ottenere un risposta immunitaria con oligosaccaridi corti, come è stato recentemente dimostrato per GBS III. I glicoconiugati sono stati caratterizzati con SDS-Page, Western Blot e microBCA per misurare il contenuto in proteina, mentre la quantifica del saccaride, e di conseguenza il rapporto saccaride/proteina, è stata effettuata tramite HPAEC-PAD. Lo studio animale in corso dirà se l'esposizione multivalente di questi saccaridi corti

permette loro di assumere caratteristiche antigeniche tipiche dei polisaccaridi. Inoltre i glicoconiugati così preparati saranno usati per completare la mappatura dell'epitopo minimo, studiando la loro affinità in termini di cinetica con gli anticorpi monoclonali serotipo-specifici.

Allo scopo di dimostrare che identificare l'epitopo minimo di polisaccaridi molto complessi è propedeutico al design razionale di glicoconiugati basati su carboidrati sintetici, il Capitolo 7 riporta un approccio strutturale per ottenere un vaccino glicoconiugato contro il serotipo III di GBS. Un esasaccaride di GBS III è stato progettato e sintetizzato a partire dalla struttura cristallografica di un frammento semisintetico di GBS III (due unità ripetenti, DP2, ottenuto per idrolisi del PSIII) con il Fab. Questo esasaccaride comprende tutte le porzioni coinvolte nell'interazione con il Fab e consiste di una unità ripetente ramificata più una seconda glucosammina. Studi strutturali e conformazionali hanno confermato che l'esasaccaride è riconosciuto da un anticorpo monoclonale funzionale anti PS III in modo simile al dimero ottenuto per depolimerizzazione. La coniugazione dell'esasaccaride alla proteina carrier CRM_{197} ha portato ad ottenere due glicoconiugati con grado di glicosilazione diverso. Quando sono stati testati in vivo, solo il coniugato con un rapporto saccaride /proteina di circa 8 ha indotto una risposta immunitaria robusta e funzionale, non inferiore a quella del coniugato del PS III, confermando che questa struttura è l'epitopo minimo di GBS III.
E' inoltre da notare che il coniugato dell'esasaccaride ha mostrato una produzione predominante di IgG rispetto alle IgM, cosa importante per un vaccino che ha lo scopo di indurre la formazione soprattutto di IgG in grado di attraversare la placenta. Mentre in letteratura è suggerito che GBS III sia un esempio di epitopo conformazionale molto complesso, per il quale strutture lunghe sono richieste per presentare epitopi immunogenici, l'approccio descritto nel Capitolo 7 cambia questa visione, dimostrando che un saccaride sintetico corto può essere progettato per contenere tutte le caratteristiche strutturali necessarie a indurre una risposta immunitaria.

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- **Synthesis and biological evaluation of non-glucose glycoconjugated N-hydroxyindole class LDH inhibitors as anticancer agents.** Di Bussolo, V.; Calvaresi, E.C.; Granchi, C.; Del Bino, L.; Frau, I.; Dasso Lang, M.C.; Tuccinardi, T.; Macchia, M.; Martinelli, A.; Hergenrother, P.J.; Minutolo, F. *RCS Advances*, **2015**, *5 (26)*, 19944-19954.

- **The conformation of the mannopyranosyl phosphate repeating unit of the capsular polysaccharide of Neisseria meningitidis serogroup A and its carba mimetic.** Calloni, I.; Unione, L.; Jiménez-Osés, G.; Corzana, F.; Del Bino, L.; Corrado, A.; Pitirollo, O.; Colombo, C.; Lay, L.; Adamo, R.; Jiménez-Barbero, J. *Eur. J. Org. Chem.* **2018**, *33*, 4548-4555.

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- **Regioselective glycosylation strategies for the synthesis of Group Ia and Ib Streptococcus related glycans enable elucidating unique conformations of the capsular polysaccharides.** Del Bino, L.; Calloni, I.; Oldrini, D.; Raso, M.M.; Cuffaro, R.; Ardà, A.; Codée, J.D.C:, Jiménez-Barbero, J.; Adamo, R. *Chem. Eur. J*., **2019**, *25 (71)*, 16277-16287.

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- **Role of carbohydrate antigens in antifungal glycoconjugate vaccine and immunotherapy.** Del Bino, L.; Romano, M.R. *Submitted to Drug Discovery Today: Technology***.**

- **Defining GBS Ia and Ib antigenic determinants using synthetic glycans.** Del Bino, L.; Carboni, F.; De Ricco, R.; Brogioni, B.; Margarit Y Ros; I.; Romano, M.R.; Adamo, R. *In preparation.*

Cover features

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Curriculum vitae

The author was born in Empoli on February 26th 1989. In 2008, she graduated from "Liceo Linguistico Virgilio" in Empoli and then started to study Chemical and Pharmaceutical Technologies (combined Bachelor and Master's degree, 5 years) at the University of Pisa.

She obtained the Master's degree in 2014 after completing her Master research project titled "Stereoselective synthesis of a Glc-NAc conjugated as potential modulator of carbohydrate metabolism and regio- and stereoselective behaviour of carbapyranose 1,2-epoxides with α-gluco and β-manno configuration" under the supervision of Prof. Valeria Di Bussolo. After three monts spent in the same group as Research Fellow working on "Synthesis of carba-analogs of natural disaccharides as potential antiviral agents", the author moved to Mölndal, Sweden, where she was enrolled in the IMED Graduate Programme at AstraZeneca in 2014. During the two years of the programme, she worked in three different groups: RIA (Respiratory, Inflammation and Autoimmune Diseases) Medicinal Chemistry, RIA Drug Metabolism and Pharmacokinetics and Discovery Sciences iLab. In each group she took part in company projects and the results of each placement were presented in internal Poster Session. Along with the scientific training, she received also an extensive support to develop her soft skills (time and project management, communication, presentation, teamworking) thanks to the Graduate Development Programme Modules held by the Oxford Group.

In 2016 the author started her PhD project in the Glycoconjugates Synthesis and Analytics Group (GSAL) in GSK Vaccines in Siena, Italy, under the co-supervision of Dr. Roberto Adamo and Prof. Jeroen Codèe at Leiden University. The PhD research was performed in the frameshift of the Marie Curie ITN Glycovax, which aimed to rationally design innovative glycoconjugate vaccines. Part of this research was orally presented at ICS Lisbon (2018), EuroCarb Leiden (2019), GSK Global PhD and PostDoc Workshop (2018 and 2019), GSK Science Hive and at the Glycovax Workshops in Lisbon (2017), Bilbao (2018) and Milan (2019). Posters were presented at ICS Lisbon (2018), GSK PhD Workshop (2017) and GSK R&D Days (2019). Presentation titled "Synthesis of oligosaccharide libraries from Group B streptococcus capsular polysaccharide for structure-based selection of vaccine candidates" was awarded with the Best Presentation Prize at the 11th GSK PhD Students Workshop in 2018.

The author is currently working as Scientist at GSK Vaccines in Siena.

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